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GAS CHROMATOGRAPHY OF AMINO ACIDS

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1. INTRODUCTION

In general, amino acid analysis cannot be carried out without employing chromatographic techniques. Of the various possibilities such as paper chromatography (PC), thin-layer chromatography (TLC), gas chromatography (GC) and ion-exchange chromatography (IEC), the last technique has probably been the most widely used since the classical work of Moore and co-workers[244–246]. This was obviously due to the construction of fully automated devices for the IEC of amino acids. With slight modification, automated amino acid analyzers are applicable to the separation of amines, carbohydrates and a number of other compounds. In comparison with GC, however, the main drawback of these techniques is their low sensitivity and relatively long operating time. Both of these drawbacks remain in spite of the application of fluorescamine[357], pyridoxal[197] or radioactive reagents[209] instead of ninhydrin for detection, and spherical ion exchangers for the column packing.

The advantages of using a gas chromatograph are obviously the low cost and the much greater versatility of the instrument compared with specialized amino acid analyzers, despite the above possibilities. Moreover, GC offers a relatively simple means of combining the analytical system with mass spectrometry, which in many situations is the only way of identifying an unknown substance. Mass spectrometry

itself can also serve for very sensitive detection (down to 10^{-15} g). The capability of GC to serve as the ultimate means for identifying PTH-amino acids (see 2.2.2 and 3.3.4.2) renders it suitable for wide application in the sequence analysis of proteins (Sequence Analyzer, *e.g.*, Beckman 890c).

The use of an electron capture detector (ECD) increases the sensitivity to the picomole level; the use of capillary columns affords a precise and complete separation of individual components. Thus, in comparison with IEC, the initial amount of material to be analyzed that is required can be considerably decreased. An evident disadvantage, however, is the necessity to derivatize the starting material in order to convert the amino acids into more volatile, less polar compounds that are suitable for GC separation. With the exception of pyrolysis (see 3.1.3) this step cannot be avoided. Therefore, not only the development of GC but also the concomitant development of suitable derivatization reactions were factors that led to successful separations. While the classical methods reached their peak at the end of the 1950s, the full development of the GC of amino acids did not occur until 10 years later when the technique for quantitative analysis of the 20 common amino acids was established[102]. Most of the present work carried out in this field makes use of the principle of derivatization developed by Gehrke's group; this is discussed in more detail in section 3.2.1.5. Other derivatization techniques have not escaped attention and have been surveyed several times since 1962[24,37,54,84,226,260,299,391]. Other reviews[12,14,95,149,155,178,203,204,206,218,273,348,394,415] refer to one or several methods of greater importance and they may serve to give the basic principles of the subject.

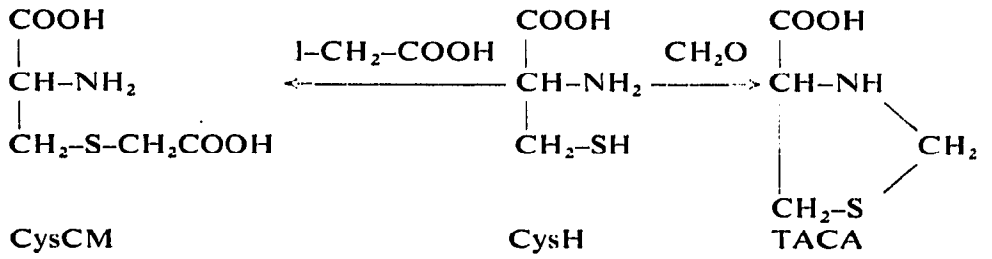
The aim of this review is to survey all the papers published that deal with the GC of amino acids, including the separation of their enantiomers and applications. We have tried to cover the period 1956–mid-1974 (with two exceptions[2,177]) and it appears for the 20th anniversary of the introduction of the GC of amino acids.

2. GENERAL

2.1. Classification of amino acids and abbreviations used

For the purposes of this review it would seem useful to divide the main protein amino acids into two groups: the simple amino acids, which contain only the characteristic functional groups, and those amino acids, which possess one or more additional reactive groups in the side-chain. The amino acids of the latter group, which cause no problems when analyzed by IEC, are generally more difficult to determine successfully by GC. To the group of simple amino acids belong those with an aliphatic chain, *i.e.*, glycine (Gly), alanine (Ala), valine (Val), leucine (Leu), isoleucine (Ile), and three with a non-reactive grouping, *i.e.*, proline (Pro), methionine (Met) and phenylalanine (Phe). The amino acids with additional groups comprise those which bear a hydroxyl group, *i.e.*, serine (Ser), threonine (Thr) and tyrosine (Tyr); those with a second carboxyl group, *i.e.*, aspartic acid (Asp) and glutamic acid (Glu) together with their amides asparagine (Asn) and glutamine (Gln); and, finally, the basic amino acids, which possess a second amino (or imino) group in the molecule, *i.e.*, lysine (Lys), arginine (Arg), tryptophan (Trp) and histidine (His). The remaining protein amino acid, cystine (Cys), is composed of two molecules of cysteine (CysH) so containing a disulphide bond in addition to the characteristic groups. CysH, the reduced form of

Cys, does not in fact belong to the structural protein amino acids; however, as the conversion of one form into the other proceeds easily, it often used to be analyzed together with the other amino acids. Because of the lability of the thiol group, some other modified forms are employed in the derivatization process instead of CysH, *e.g.*, cysteic acid ($CysO_3H$), S-methylcysteine (CysM), S-carboxymethylcysteine (CysCM) and thiazolidine-4-carboxylic acid (TACA). The last two forms can easily be prepared by reaction of CysH with iodoacetic acid[69] or formaldehyde[303], respectively:



In addition to the structural protein amino acids, there are some other rarely occurring, biologically important amino acids that usually have a particular function. Many of them also have trivial names and they were determined together with the protein amino acids. To this group belong hydroxyproline (Hypro), ornithine (Orn), β-alanine (β-Ala), hydroxylysine (Hylys), 3,4-dihydroxyphenylalanine (DOPA), γ-aminobutyric acid (γ-ABA) and some others. The following simple aliphatic amino acids often used to be analyzed together with the above amino acids: α-aminobutyric acid (ABA), α-aminoisobutyric acid (AIBA), norvaline (Nval), norleucine (Nleu) and sarcosine (Sar). To the iodinated amino acids belong the iodinated homologues of tyrosine and thyronine, *viz.*, monoiodotyrosine (MIT), diiodotyrosine (DIT), monoiodothyronine (T_1), diiodothyronine (T_2), triiodothyronine (T_3) and thyroxine (T_4). However, only the analysis of the last two iodoamino acids, the thyroid hormones, is of practical importance.

In order to distinguish the optical antipodes (enantiomers) and to indicate absolute steric configuration, the designations L and D, and *S* and *R*, respectively, are used. As the amino acids Ile and Thr possess two asymmetric centres in the molecule, the second pair of isomers was assigned the terms *allo*-Ile and *allo*-Thr.

2.2. Analysis of amino acids in peptides and proteins

2.2.1. Total hydrolysis

Most amino acids can be determined accurately by IEC or GC analysis of protein hydrolyzates. The choice of the method used for the hydrolysis of proteins prior to amino acid analysis is of considerable importance as some amino acids are preferentially destroyed in part and the hydrolysis of others is incomplete. In view of the high precision attained in GC analysis of amino acids, the nature of the hydrolytic conditions plays an increasingly important role and can easily be evaluated. The particular hydrolysis agent used must be able to cleave all peptide bonds in a protein. Some problems are encountered with aliphatic amino acids because there is steric hindrance due to the bulky side-chains. Some difficulties were found especially during

the determination of Ile owing to the well known stability of the peptide bonds in which this amino acid is involved. Because of the different stabilities of the various functional groups of amino acids, a compromise between several experimental conditions must be made in order to achieve the optimal hydrolysis of a protein.

Acid hydrolysis is the preferred method to convert proteins into the substituent amino acids. During acid hydrolysis, however, Trp is completely destroyed even under conditions that are optimal for other amino acids. Satisfactory alternative procedures have been sought for many years, including acid hydrolysis with some protective reagents, alkaline hydrolysis and enzymatic assays. Alkaline hydrolysis of proteins results in much less destruction of Trp than hydrolysis under acidic conditions; however, it cannot be recommended for routine analyses because CysH, Cys, Ser, Thr and Arg are destroyed. Moreover, alkaline hydrolysis causes racemization of the optical antipodes of amino acids and should never be used if the enantiomers are to be determined.

Most frequently used[247] is acid hydrolysis with 6 *N* HCl at 110° for 20–24 h. More recently, however, a faster modification has been suggested[310], which permits hydrolysis with 6 *N* HCl to be completed within 4 h at 145° with minimal decomposition of the amino acids. At this temperature, however, Pro, Thr, Ser, Met, Hypro and Arg are the most sensitive to heat. In general, acid hydrolysis will give recoveries of over 95% for most protein amino acids, except for destroyed Trp, partially destroyed Ser and Thr (typical losses are 5–15%), and, when a higher hydrolysis temperature is used, also Arg (partially degraded to Orn). The amide groups of both Asn and Gln are completely hydrolyzed to give the corresponding acids, Asp and Glu. All of the peptide bonds in Val, Ile and Leu require a longer hydrolysis time in order to obtain maximum yields for these three amino acids. The technique of hydrolysis is also very important because, in the presence of oxygen, other amino acids are lost: CysH and Cys are oxidized to $CysO_3H$ and Met is partially converted into Met-sulphone. Hence, hydrolysis is usually performed in a sealed glass ampoule under a nitrogen atmosphere or *in vacuo*. The replacement of the ampoule with a modified culture tube with a PTFE-lined screw-cap has also been suggested[49]. On the other hand, an experimental study of amino acid degradation under open-flask hydrolytic conditions, in constantly boiling HCl and in an oxygen-free atmosphere, was reported to be both successful and useful[242]. Also, any previous treatment of protein, *e.g.*, with dimethyl sulphoxide (DMSO), should be avoided as oxidative loss of Met, Cys and Tyr, resulting in production of Met-sulphoxide, $CysO_3H$ and chloro-Tyr, respectively, was observed during the hydrolysis of protein with 6 *N* HCl in the presence of trace amounts of DMSO[205].

Of the other procedures for acid hydrolysis, one more should be mentioned, the so-called "dry hydrolysis"[214] with crystallized oxalic acid (1.5 g) and 6 *N* HCl (0.5 ml) at 115° for 24 h. This hydrolytic agent was reported to be more effective than the usual use of 6 *N* HCl alone. Higher yields were obtained for all amino acids except Arg, Thr and Ser. However, with regard to the subsequent derivatization and GC analysis, this technique is limited by the presence of non-volatile oxalic acid, which cannot be removed by evaporation.

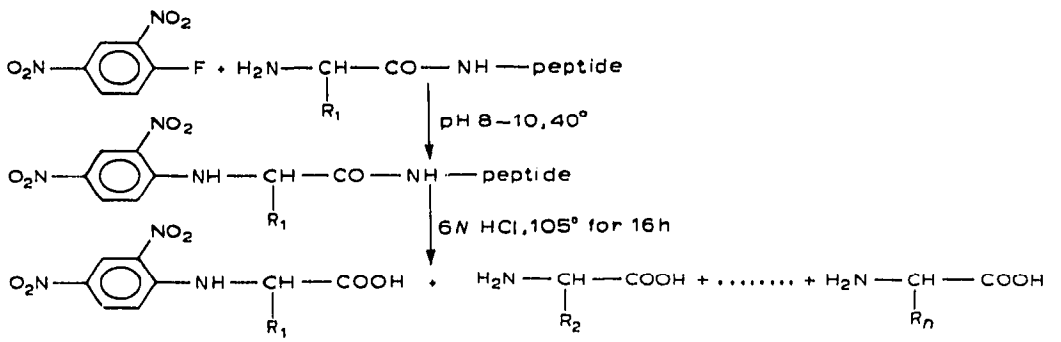
The determination of protein-bound Trp still remains difficult. This problem was dealt with in one valuable report in 1971[90], in which many useful methodological findings were summarized. Except for some alkaline and enzymatic methods, which

are not usually employed, a protective reagent must be used in conjunction with an acidic agent, for example thioglycolic acid (TGA), together with 6 *N* HCl[221] or 3-(2-aminoethyl)indole added in an amount of 0.2% to *p*-toluenesulphonic acid[207] or, more recently, to methanesulphonic acid[243]. However, only the first system mentioned was investigated for its usefulness in the GC analysis of amino acids[106]. The hydrolysis with 5% TGA in 6 *N* HCl was performed at 110° for 20 h and the TGA with some other interfering material was subsequently removed by IEC with Bio-Rad AG 50W-X8 resin. This clearing step, necessary prior to the GC analysis, led to the loss of Trp on the resin column (30–35%). Even Cys and Arg showed unexpected losses (30–50%) on the chromatogram; however, no explanation was found for this phenomenon.

2.2.2. Successive hydrolysis

Several methods have been developed for the elucidation of the primary structures of peptides and proteins. These methods are based on successive, controlled chemical degradation of the peptide chain, mostly from the amino-group end, followed by identification of the cleaved-off terminal amino acid. The technique requires reaction of the protein or peptide with a convenient reagent under the mildest possible conditions (to avoid hydrolysis of the peptide chain that would lead to the formation of new end-groups). Further, the bond between the reagent and the amino end-group must be resistant to vigorous hydrolysis.

The numerous reagents used for the identification of N-terminal amino acids were well reviewed in two recent papers[314,315]. Therefore, in this review, only two of the methods will be mentioned, which are closely related to the GC analysis of amino acids. The first, the dinitrophenylation method, which was used by Sanger[326] with great success in the determination of the structure of insulin, is based on the reaction of 2,4-dinitrofluorobenzene with an N-terminal amino acid:

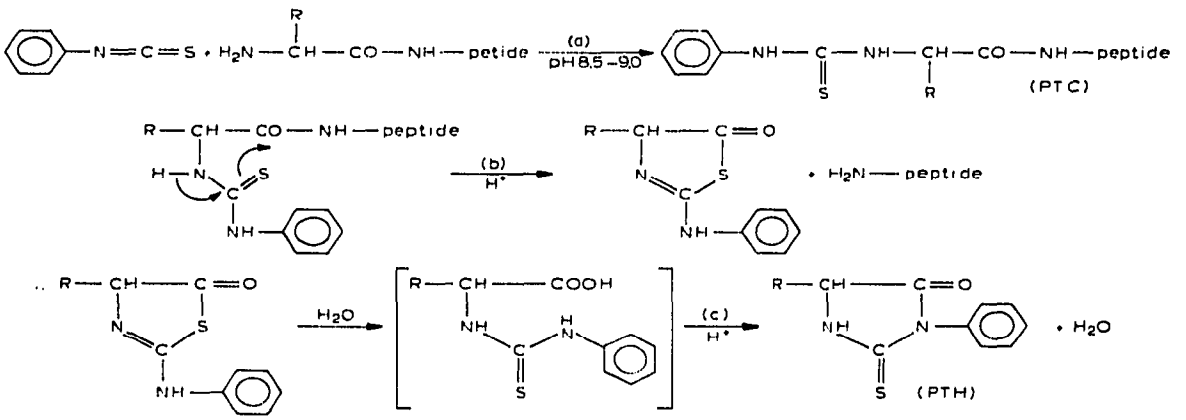


The coupling of the reagent with the terminal amino group proceeds under mild conditions, *e.g.*, in aqueous alcoholic hydrogen carbonate medium, within several hours. This step is followed by total hydrolysis in which the stable C–N bond, formed between the reagent and the N-terminal group, remains intact so that the N-DNP-amino acid (DNP = 2,4-dinitrophenyl) can be isolated. However, this type of hydrolysis may cause considerable degradation of DNP-Pro (-Hypro) and DNP-Gly and also of other DNP-amino acids[298]. The DNP-amino acids were subjected to GC analysis after methylation of the carboxyl group (see 3.2.3.2). Moreover, the N-DNP-

amino acid methyl esters exhibit a strong electron attachment, so that they can be detected with an ECD at very low concentrations[195,196].

The second method of sequence analysis is nowadays generally known as the Edman degradation[75]. In comparison with the DNP method, it is a more sophisticated procedure as, after coupling of the N-terminal group with the reagent, phenyl or methyl isothiocyanate (PITC or MITC), total hydrolysis is avoided and instead the derivative of the N-terminal amino acid is released under mild hydrolytic conditions leaving the $(n-1)$ peptide untouched. Based on this procedure, a protein sequenator[76], a device that allows the largely automatic analysis of the amino acid sequences in proteins and peptides, was described.

The reaction of the peptide with PITC or MITC proceeds in three stages: (a) coupling procedure (in a pyridine–aqueous NaOH system at pH 8.5–9.0) leading to a phenylthiocarbamyl (PTC) intermediate; (b) cyclizing cleavage (by treatment of PTC with CF_3COOH or $CH_3COOH \cdot HCl$ at 40°) leading to 2-anilino-5-thiazolinone; and (c) conversion of the latter (pH 1, 80°) into the 3-phenyl-2-thioxoimidazolidin-4-one (3-phenyl-2-thiohydantoin, PTH) derivative:



As with the DNP procedure, some thiohydantoins of amino acids are not formed quantitatively, losses occurring especially with Ser, Thr, Gln and CysH. Prolonged reflux of the thiocarbamyl intermediate with 1 *N* HCl led to undefined products (*e.g.*, a release of H_2S from CysH and H_2O from Ser). Therefore, a milder procedure for the cyclization step was suggested[159] and the PTHs of Ser and Thr were successfully prepared. This procedure was later adopted[77] for preparing the PTHs of Glu, CysCM and $CysO_3H$ and it was also successfully used for the preparation of Ser and Thr methylthiohydantoins (MTHs)[191]. Moreover, the addition of a very small amount ($5 \cdot 10^{-4}$ *M*) of dithioerythritol to the extracting solvent during automated Edman degradation was found to increase the yield of PTH of Ser markedly[146].

The analysis of PTHs by GC has been the subject of a relatively large number of papers. They can be analyzed either in their native form or after acylation or silylation of the reactive proton of the thiohydantoin ring (see 3.3.4.2). The same is valid for MTHs, which were produced by the reaction of peptides with MITC as the degradative reagent[358,388]. They are more volatile than the corresponding PTHs and

can therefore be chromatographed more easily (see 3.3.4.3). Also, the use of pentafluorophenyl isothiocyanate (PFPITC)[201a] instead of the conventional PITC led to enhanced volatility of the resulting derivatives (PFPTHs), which then possessed more desirable GC features and, moreover, the coupling procedure with PFPITC was found to proceed more easily and rapidly. However, the use of this reagent is not frequent because it is expensive and not readily available commercially.

2.3. Derivatization requirements and choice of technique

The development of a particular derivatization method is not possible without a good knowledge of organic chemistry, and with a variety of specific reactions and reagents available. In view of the multifunctional nature of the protein amino acids, it was not easy to devise reaction schemes that would deal successfully with all of the possible groups, with their many chemical differences and varying reactivities. However, for satisfactory GC analysis, a substantially complete derivatization is necessary. The yield, if not 100%, must at least be constant. The derivative chosen must be sufficiently volatile to elute within the time and temperature limits dictated by the liquid phase. On the other hand, it should not be so volatile as to make evaporation of the derivatization reagent impossible owing to its own evaporation losses. Moreover, as the preparation of derivatives is usually the most time-consuming part of the procedure, it is highly desirable that it should be carried out as rapidly and precisely as possible with a minimum number of reaction steps. If we optimize the requirements, it would be desirable: (1) to use one reagent in only one reaction step; (2) to prepare stable derivatives that do not decompose with time and on any column packing; and (3) to separate the derivatives completely in one column in one programmed operation. None of the methods developed so far fulfills these requirements absolutely. Despite the fact that some compromises must be made, there are several methods that enable the quantitative GC analysis of all the desired amino acids to be performed successfully.

The adoption of a particular derivatization procedure for GC analysis is dependent on the purpose of the chemical treatment. If the main task is the quantitative analysis of the 20 protein (or some non-protein) amino acids, the choice of a convenient organic reaction is virtually unrestricted as a review of the methods applied shows: in total, nearly 100 chemical treatments have been tried. There is still no general agreement on the volatile derivatives of amino acids that are most suitable for GC analysis, despite the apparent advantages of some procedures. Among the proposed methods, the most preferable are those which require at least two derivatization steps, *e.g.*, esterification of the carboxyl group followed by acylation of the α-amino and remaining functional groups. Further, the single-step procedure called "silylation" seems to be attractive owing to the simplicity of dealing with the sample. However, some problems arise with the stability of the resulting derivatives and double-derivative formation in the case of some amino acids. From the present state of our knowledge, it can be stated that any publication on the formation of a new amino acid derivative that does not permit the good and reproducible determination of Trp, His, Arg and Cys no longer makes an effective contribution to the subject unless it has other advantages.

For the analysis of thyroid hormones, which are known to have the highest

molecular weights of all amino acids, the choice of a convenient derivatization procedure is narrowed considerably. The use of pivalic anhydride, an acylation reagent that is not used in any procedures applied to the derivatization of the protein amino acids, gives the derivatives the desirable features that are necessary for the high operating temperatures of the column.

Finally, other esterification or even acylation reagents are required if the aim is to separate the amino acid enantiomers. Except for the use of the optically active stationary phases (see 5.2), the derivatization agents must be optically active in order to give diastereomeric amino acids, which can then be resolved on the usual column packings. With the present techniques, however, the resolution of His and Cys into their enantiomers has not yet been achieved, although it should be mentioned that the analysis of amino acid enantiomers was developed more recently (the first published paper appeared in 1963[45]). The 7-year difference cannot be immediately overcome even though our knowledge has made rapid progress. It is believed, however, that a specific method involving these two amino acids will soon be reported.

2.4. Chromatographic column and its filling

During the last 20 years of GC amino acid analysis the GC technique, and especially the key element in the separation process, the column and its filling, has undergone rapid development. Most studies have been carried out on packed columns. The column lengths usually used are 1–3 m (with 2000–4000 theoretical plates) with an inner diameter (I.D.) of 2–4 mm. The use of an I.D. of 2 mm is highly recommended because of the inherently higher separation efficiency. If more than 5000–6000 theoretical plates are required for a separation (mostly for the resolution of amino acid enantiomers), capillary columns 15–150 m long, usually with 20,000–150,000 theoretical plates, are utilized. Although no specific instances have been reported in which metal columns have been used in the cleavage of amino acid derivatives, it is probably preferable to use the all-glass system that is now becoming standard for biochemical applications in GC. Breakdown of N-TFA-butyl esters (TFA = trifluoroacetyl) occurred in a heated metal injection port[190,352] and therefore direct on-column injection, which eliminates the effects due to metal ports, might be employed.

2.4.1. Support

The support plays a critical role in the performance of the column in several ways. The ideal solid support for GC should have a large surface area with a strong affinity for the liquid phase but remaining inert to the components of the sample. However, in practice interaction does occur; *e.g.*, increasing the loading of the ethylene glycol adipate (EGA) polyester phase alters the order of elution of some amino acid derivatives[356], whereas if interaction with the liquid phase only were involved, a proportionately longer retention of all solutes would be expected. In order to eliminate or reduce the adsorption properties of the support, two treatments are usually necessary: acid washing (AW) with strong mineral acids in order to remove metallic ions, and "deactivation" of the surface with silanizing agents, which convert the polar silanol groups into the more inert silyl ether groups. Dimethylchlorosilane (DMCS) is the best reagent for this purpose. An additional special treatment of the acid-washed silanized supports, *e.g.*, by prolonged heating, gives the supports the

most desirable feature of inertness. Such a support is identified commercially as HP (high-performance grade).

Prior to 1960, much GC work was carried out with Celite 545 (firebrick). Since that time, superior supports obtained by the calcining of diatomaceous earths have largely supplanted Celite. When non-silanized Celite was compared with a number of silanized supports, the greater efficiency of the latter was clearly demonstrated[162]. The order of support efficiency Chromosorb W AW < Chromosorb W AW DMCS < Gas-Chrom Q < Chromosorb W HP or G HP was found in a study on the use of Carbowax 6M as stationary phase[54]. Smith *et al.*[343] tested some supports loaded with the same liquid phase for interaction with silylated Lys, a highly sensitive derivative. The best results were achieved with Gas-Chrom Q and Chromosorb W and G, both HP. The other supports tested caused decomposition of the derivative to a lesser or greater extent. The above-mentioned supports, together with, for example, Supelcoport (which was not tested) and even other supports with similar outstanding features, are highly recommended for amino acid analysis, particularly when silicones are used as the stationary phases.

The silicone stationary phases, especially when coatings of 1–5% are employed, are not effective in deactivation of the support and require a silane-treated support. When a polar phase is used (polyester, polyglycol), the active sites of the support are partially blocked and the use of AW supports is usually sufficient. A comparison between Chromosorb W AW and HP grade (coated with 0.65% EGA polyester) has shown, contrary to what might have been expected, that the least deactivated support, the AW grade, gave a more reproducible resolution of the 17 protein amino acids than the HP grade, which gave a poorer resolution[370]. Nevertheless, the interaction of the sample component with the column filling (support plus liquid phase as a whole) must not cause a loss in quantity. Even the best HP-grade supports may behave unexpectedly in this respect. It was found, for example, that triacetylated Arg-propyl ester was eluted from Chromosorb W HP, but not from Chromosorb G HP, when both were coated with the same liquid phase (OV-17)[54]. A conclusion can be drawn that at least two supports of equally high quality should be tested if new derivatives of amino acids are to be analyzed. For analytical purposes mesh sizes of 80–100 (177–149 μm) or 100–120 (149–125 μm) are currently recommended.

2.4.2. Stationary phase

The number of stationary phases evaluated in GC analysis has reached several hundreds, from the obsolete and technical grades to the up-to-date selective and chromatographic grades. More than 100 have been tested for their usefulness in separating amino acid derivatives. Some new phases, such as the trifluoroacetylated dipeptide esters (see 5.2), have been developed for the resolution of amino acid enantiomers. An interesting report appeared on the use of an amino acid (artificial leather, poly-γ-alkyl glutamate) as the stationary phase in the GC of amino acids and other compounds[165]. The large number of available phases is very helpful when choosing an appropriate phase; however, it is difficult to make comparisons among them. Rohrschneider[313], and later McReynolds[232], recommended a method for characterizing the polarity and selectivity of liquid phases and compared many of them from this point of view. Using their constants, one can see that there are a number of stationary phases that are essentially the same. Thus, the numerous phases used in amino acid

analysis can be evaluated by this method and the search for a convenient phase can be narrowed considerably.

The stationary phase should be able to separate all 20 protein amino acids and it ought to possess a high temperature stability because in most instances a temperature programme above 200° is necessary. Only a few phases fulfil the stability demands required for the high-temperature analysis of thyroid hormones with temperatures ranging from 250° to 300°. In general, the resolution improves as the polarity of the liquid phase increases. A comparison between the three predominantly used classes of liquid phases, namely the silicones, polyglycols and polyesters, shows that the polarity increases and the thermal stability decreases in that order.

The linear polyorganosiloxanes (silicones) generally possess high chemical and thermal stabilities, not exceptionally up to 350°. The organic groups currently commercially available are methyl, phenyl, cyanoethyl, fluoroalkyl and chlorophenyl. This offers a wide range of polarities, from the essentially non-polar methyl to the polar cyanoalkyl group; in comparison with the polarity of polyglycols and polyesters, the cyanoalkyl silicones are, however, of only intermediate polarity. The various commercial names, *e.g.*, OV (Ohio Valley), DC (Dow Corning), GE (General Electric), UC (Union Carbide), MS (Midland Silicones) and SP (Supelco), very often include the same chemical compounds. Some silicones often applied to the GC analysis of amino acids, with the following alkyl groups and with essentially the same separation characteristics within each group, are as follows: (a) methyl (fluids): MS-200, GE-SF-96, DC-200, DC-500, OV-101, SP-2100; (b) methyl (gums): JXR, UC-W-98, GE-SE-30, DC-410, OV-1; (c) methylvinyl (1%): UC-W-982, GE-SE-31, GE-SE-33, GE-SE-54, DC-430; (d) methylphenyl (25%): DC-550, DC-703, OV-7 (20%), OV-11 (35%); methylphenyl (50%): DC-710, OV-17, SP-2250; methylphenyl (75%): DC-705, OV-25; (e) methylchlorophenyl (5%): DC-560 (F-60), SP-400; (f) methyltrifluoropropyl (50%): DC-QF-1 (FS-1265), OV-210, SP-2401; (g) methylcyanoalkyl: GE-XF-1105 (5% cyanoethyl), GE-XE-60 (25% cyanoethyl), OV-225 (25% cyanopropyl + 25% phenyl).

The chromatographic grade OV and SP silicones enjoy great popularity nowadays and should replace some of the obsolete stationary phases. Currently, silicone loadings in the range 1–5% are used; loadings of more than 20% were not rare previously, however.

The polyesters are usually prepared from dicarboxylic acids and diols. Comparing the polarity of the reactants, the following sequence of decreasing polarity is obtained: ethylene glycol (EG) > diethylene glycol (DEG) > butanediol (BD) > cyclohexanedimethanol (CHDM) > neopentylglycol (NPG); succinate (S) > adipate (A) > isophthalate (I) > sebacate (Sb).

Thus, the most polar polyester phases are EGS, EGA, DEGS (LAC-3-R-728), DEGA (LAC-1-R-296, LAC-2-R-446, Reoplex 400), etc., the temperature stability of which is not high (max. 200°). EGA polyester was utilized for the separation of 17 protein amino acids as N-TFA-butyl esters. It may be programmed beyond 200° for a short time. Under the commercial name TABSORB (Regis Chemical Co., Morton Grove, Ill., U.S.A.), an effective, pre-tested stabilized EGA-coated support is supplied, which was used as a column filling in some work. The mixed polymers (*e.g.*, EGSP-Z and EGSS-X) employ siloxane monomers together with the diols. They have the same polarity as polyesters but a higher thermal stability. The most stable polyesters are

those which are the least polar, derived from highly sterically hindered alcohols (NPGI, NPGSb). The amount of polyester coated on the support is usually low (up to 1%).

The third important group of stationary phases is the polyglycols. The polyethylene glycols (PEGs) were very frequently used for their ability to separate polar compounds. A survey of liquid phases in use[213] showed that this material has been the third most frequently used phase for many years. Evaluation of the properties of PEGs as stationary phases in GC was reported recently[44]. A series of PEGs of different molecular weights is known under the trade name Carbowax (the name is usually followed by the molecular weight, *e.g.*, Carbowax 1540, 4000, 20M). Carbowax 20M has the highest temperature stability (225–250°). In the early work on amino acid analysis, the PEGs occupied a significant position, together with the silicones, but owing to their relatively low temperature stability, they are used only rarely today. The loadings used are rather low, as with the polyesters.

Owing to the instability of some amino acid derivatives, the relatively nonpolar or slightly polar silicones are the most desirable for amino acid analysis. Unfortunately, the separation of all of the amino acid derivatives desired can hardly be achieved on these phases. Such separations can more readily be achieved on a polyester or polyglycol phase. However, many of these otherwise excellent stationary phases have been found to cause decomposition of some amino acid trifluoroacetylated esters[54,63]. Thus, the choice of an appropriate liquid phase must be made with great care. For the quantitative analysis of His, Arg and Cys, the use of silicone phases appears to be essential.

2.5. Detectors and quantitative analysis

The most widely used detector is the flame ionization detector (FID), which can be used in temperature-programmed operations and possesses a high sensitivity (10^{-10} moles and lower). Its response is roughly a function of the number of carbon atoms, the linearity of the relationship between the response and the carbon number having been shown for amino acids that possess the same reactive groups[163]. The response factor cannot be predicted for any compound of known structure and must be determined separately by the injection of known amounts of pure compounds. This is also the case for other detectors, except for the gas-density balance detector (GDBD), which is the only detector for which the area of a peak produced by any compound can be predicted and related directly to the weight applied (provided that the molecular weight is known). It has been used for determining N-TFA-amino acid amyl esters[60]; however, it lacks sensitivity and cannot be used in temperature-programmed systems. The same is true to a lesser extent for the thermal conductivity and argon ionization detectors (TCD and AID), the latter being used only rarely today. The two other selective detectors, the alkali flame ionization detector (AFID) and the ECD showed themselves to be useful for determining picomole amounts of derivatized amino acids containing, respectively, a phosphorus atom[79] and iodine atoms (see 4.1), for example. With the latter type of detector, the range of linearity is not wide, unless the newly developed linear ECD[210] is employed. If some problems associated with its temperature programming are solved[229], this type of detector can meet all requirements in the analysis of amino acids in small amounts of biological materials. Another

selective detector, the flame photometric sulphur detector (FPSD), was used successfully for the analysis of MTH-amino acids[191]. Also, the usefulness of a mass spectrometer to function as a selective and very sensitive identifier–detector of the GC effluent has been demonstrated in the last few years and the combined GC–mass spectrometric (MS) system has become more familiar in many laboratories. The use of this combination gives a high specificity to the analysis, in which two or more (multiple ion detection, MID) mass fragment ions are monitored in the MS system. It was demonstrated that such a method, called mass fragmentography (MF), is capable of detecting femtomole (10^{-15} mole) levels of certain substances[140]. MF with MID has been used for analysis of amino acids in biological material[1,225,363].

Before 1968, a complete general GC procedure for the quantitative analysis of the 20 protein amino acids has not been reported (even when some workers believed the problem to have been solved). In that year, an excellent monograph by Gehrke and his co-workers appeared[102] which dealt with the development of a GC technique for the quantitative analysis of the 20 protein amino acids as their trifluoroacetylated butyl (N-TFA-butyl) esters. The reaction conditions for the quantitative preparation of the derivatives together with their GC resolution were established. Since that time, several valuable reports on the same problem have appeared in which some alternative successful procedures with other derivatization techniques are offered. Nevertheless, the greater part of the published papers have presented only preliminary attempts at the main task, *i.e.*, to analyze all the protein amino acids quantitatively.

Some precautions should be taken and some criteria met if quantitative determinations are to be carried out. Firstly, the complete derivatization of all amino acids must be achieved. Secondly, a complete or at least high and reproducible elution of the derivatives from the column is necessary. In this respect, the behaviour of the derivatized amino acids Arg, His and Cys should be followed particularly carefully. Thirdly, the use of an all-glass system is highly recommended, and prolonged and efficient conditioning of the column filling prior to analysis is of basic importance. A dual column system (with one reference column) should be used in order to compensate for column bleeding when temperature-sensitive stationary phases are used and the temperature is programmed over a wide range. Fourthly, moisture in the GC system should always be completely excluded, not only in the analysis of moisture-sensitive solutes such as trifluoroacetylated or trimethylsilylated amino acids. Even a freshly installed septum can, because of its moisture content, cause a breakdown of a moisture-sensitive derivative, as was demonstrated in the case of trimethylsilylated His[346]. The loss of the derivative was very pronounced immediately after the installation of a new septum, but it was not noticeable after 2–3 h use. Fifthly, the use of an internal standard (I.S.), a compound that is added in a known amount together with the other amino acids before derivatization and whose derivative gives a peak on an otherwise vacant area of the chromatogram, is to be highly recommended. The I.S. is very often a suitable non-protein amino acid, *e.g.*, Nleu, but it need not be an amino acid. Alternatively, an external standard may be added after derivatization has been completed and before the sample is injected into the column. On the basis of the added standard, a calculation can be made where each peak is calculated as a percentage of the sum of the peak areas, after corrections for individual response factors have been made. The correction is made by determining the relative molar response (*RMR*) of

each amino acid with respect to the I.S. according to the equation

$$RMR_{a.a./I.S.} = (A_{a.a.}/M_{a.a.})/(A_{I.S.}/M_{I.S.})$$

where $A_{a.a.}$ and $A_{I.S.}$ are the areas of the peaks of the amino acid and internal standard, respectively, and $M_{a.a.}$ and $M_{I.S.}$ are their corresponding masses (number of moles injected).

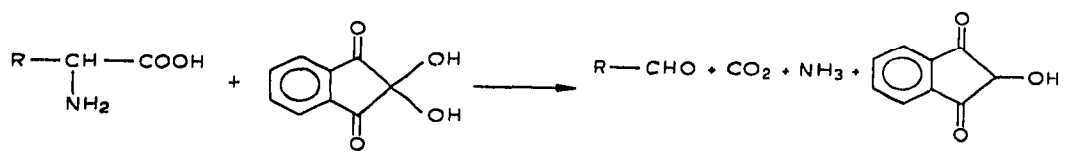
3. ANALYSIS OF PROTEIN AND SOME NON-PROTEIN AMINO ACIDS

3.1. Methods based on degradation of amino acids, conversion into other compounds or simple esterification of carboxyl groups

Conversion of an amino acid into a more simple, less polar compound such as an aldehyde, amine or nitrile, a process that can easily be performed, for example, by the well known ninhydrin oxidation or pyrolysis technique, was one of the first methods used in analysis by GC. Even the alternative approach, *i.e.*, substitution of the amino group by chloride or hydroxyl, as well as the reduction of carboxyl, was assumed to be less tedious than any other method. Such attempts, together with efforts to analyse the amino acid alkyl esters as free bases or even hydrochloride salts, underlined the lack of convenient derivatization reagents and procedures. Consequently, none of these early methods touched on the problem of polarity of the additional reactive group present, so that only simple amino acids could be chromatographed successfully while the others gave no or very poor results. Thus, most of these early procedures are of very limited practical use.

3.1.1. Decarboxylation and deamination to C_{n-1} aldehydes and/or C_{n-1} nitriles or to C_{n-1} acids

The first published paper on the GC of amino acids dealt with their determination as aldehydes after treatment with ninhydrin[151]:

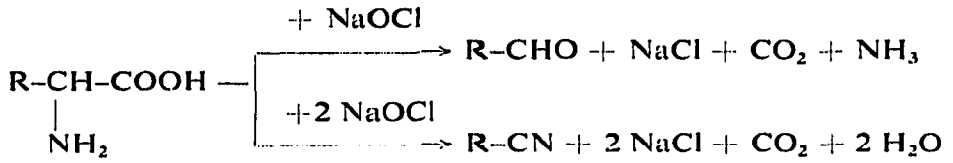


The corresponding aldehydes of Ala, Val, Leu and Ile were removed from an aqueous solution with a stream of nitrogen and after a cool trapping injected into a silicone-coated column at 69°. An improvement on the procedure was effected by using chloroform together with the aqueous ninhydrin solution[20,21]. Only about eight simple amino acids yield volatile aldehydes that can be chromatographed. A technique that permitted the direct injection of aqueous amino acid solutions into the gas chromatograph was described in another series of papers[252,406,407]. In the pre-column, with 30% ninhydrin deposited on firebrick, the amino acids were converted into their corresponding aldehydes at 125–140°, and in the second column they were sepa-

rated on a glycerine–silicone phase[252] at 65°, or on a phase consisting of an equimolar mixture of ethylene and propylene carbonates[406,407] on firebrick at 25°. In addition, an attempt was made in the latter case to automate the ninhydrin analytical technique in conjunction with catalytic hydrocracking. As each aldehyde emerged from the column it was hydrocracked in a microreactor, containing a nickel–Kieselguhr catalyst at 425°, to produce methane and water. The water was selectively removed and the methane passed over a TCD cell. Quantitative results were obtained for a limited number of samples and 1 μg of amino acid could be detected. Gly could not be detected owing to the polymerization of formaldehyde in the oxidation reaction.

For another proposed method[10], based on the ninhydrin oxidation of amino acids to aldehydes followed by $KMnO_4$ oxidation of the aldehydes to acids and their chromatography in the unesterified form on a heavily loaded polyester (PPGA) column at 150°, no reasonable explanation was given.

In a further series of papers, other oxidative reagents were used. The use of sodium hypochlorite solution[13] was at first reported to give quantitative conversion of the aliphatic amino acids into aldehydes, which were then successfully separated on a column with dinonyl phthalate at 92°. The use of this reaction for the acidic and sulphur-containing amino acids was inconvenient, as it led to a complex mixture of unspecified volatile products. Some amino acids gave identical aldehydes. In a later report[332], however, strikingly different results were found. If the oxidation with sodium hypochlorite was carried out at a fixed pH (10.8), then nitriles rather than aldehydes were formed in relatively good yields:



The nitriles of seven amino acids were analyzed on a DC-550 silicone-coated column at 60°. The products obtained after oxidation with sodium hypobromite were studied[231]. It was suggested that at pH 9.4 this oxidation may give unique and volatile products from each amino acid. The simple amino acids were obtained as nitriles in good yields (however, no tests for aldehydes were made) and chromatographed on Carbowax, DEGA or SF-96 silicone at 27–75°. Other workers[359] used N-bromosuccinimide for oxidation and obtained an accurately defined mixture of aldehyde and nitrile for each amino acid investigated. The mixtures were chromatographed on THEED at 110° or on silicone DC-710 at 220°.

In one report, various aliphatic amino acids were converted into the corresponding nitriles by treatment with iodosobenzene[402]. Simple mixtures were analyzed at 100° on either polynitriloether or polyglycol columns.

The use of the aldehydes of one less carbon atom was noted again in 1973 when silver(II) picolinate was employed as an oxidative reagent[47,48]. The single-step reaction in aqueous media with the possibility of direct injection of the reaction mixture was still sufficiently attractive. Volatile aldehydes corresponding to Ala, Val, Leu, Ile, Nleu, Pro and Gly were analyzed on a Carbowax 4000 column[48].

3.1.2. Decarboxylation to C_{n-1} amines

The "active carbonyl group" involved in the enzymatic decarboxylation of amino acids was introduced into the reaction vessel as part of a convenient aldehyde reagent such as *p*-dimethylaminobenzaldehyde[20,21]. An aromatic hydrocarbon (diphenylmethane) was also examined for this purpose. The simple amino acids, together with Ser, Thr and Lys, were heated with one of these reagents at 180–210° in a sealed ampoule. The use of the aldehyde was superior, the yields of amines varying from 36% (Phe) to 80% (Lys and Val). GC analysis of the products was not so successful owing to the lack of a suitable stationary phase.

3.1.3. Decomposition by pyrolysis and analysis of diketopiperazines

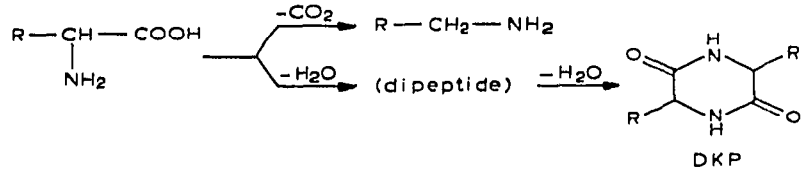
In the pyrolysis technique, each amino acid or protein produces a unique profile of volatile fragments, which can be used for qualitative or even quantitative purposes. The amino acid need not be derivatized, peptides and proteins being subjected to pyrolysis without hydrolysis. For the structural determination of the pyrolysis fragments, the GS–MS combination is the most useful method[238,302,340,341,383].

The earlier studies were undertaken in order to find out if each of the protein amino acids would yield an inherent reproducible cleavage pattern. Pyrolysis using a hot platinum spiral heated to 90° (ref. 168) or ignited to red heat[372], when analyzing the pyrolysis products on a squalane-coated column, showed that, for example, Phe gave four peaks[168]; the cleavage pattern typical for Ala was also observed with other amino acids. His, Trp and Tyr apparently yielded no decomposition products; about half of all protein amino acids gave non-specific cleavage material[372]. In two other reports, other pyrolysis fragments were demonstrated after pyrolysis had been performed in a special pyrolysis unit[238,403] at about 300°. Eighteen amino acids and, simultaneously, bovine and egg albumin and haemoglobin were reported to give unique and reproducible amino-acid profiles on a 15% Quadrol–5% KOH phase[403]. Using an unusual temperature-programmed range of −180 to +150° and 5% 1,2,3-tris(2-cyanoethoxy)propane on untreated firebrick as the column filling, Merritt and Robertson[238] succeeded in separating many of the fragments and identifying them by MS. For each amino acid there was a characteristic fragment such as acetone for Gly, toluene for Tyr, benzene for Phe and pyrrole for Pro, which was useful for the identification of single or peptide-bound amino acids. Other investigators[383] employed a ferromagnetic conductor (iron wire coated with zinc), which, after dipping into a 10% aqueous solution of an amino acid or peptide, was heated by high-frequency induction to about 700° during 20–30 msec and the fragments were applied continuously to a GC–MS system. α-Amino acids with alkyl or aryl residues were reported to give the corresponding nitriles in high yields.

Several investigations have been carried out to determine whether the pyrolysis of a mixture of amino acids yields the same results as when the amino acids are pyrolyzed individually. High-temperature treatment of equimolar mixtures of amino acids (with at least one aromatic amino acid) at 850° under a nitrogen atmosphere revealed, however, a large decrease in the aromatic hydrocarbons produced in the mixture[274–276]. Thus, the presence of a second amino acid was found to influence the yields of certain components in the pyrolyzate. An identical pyrolysis technique was employed in order to compare the pyrolysis profiles obtained by pyrolysis of protein and amino acid mixtures that possessed the same amino acid composition. The pyrol-

ysis studies at 850° showed that pyrolysis of amino acids gave the same results qualitatively (and, with a few exceptions, quantitatively) as those obtained by pyrolysis of protein with the equivalent amino acid composition[347]. Similar findings were observed when the pyrolyzate profile of collagen was compared with that of Gly and Pro obtained under the same conditions (at 840° in nitrogen)[147]. Likewise, Stack[350] pyrolyzed a mixture of Gly, Hypro and Pro in the injection port of a gas chromatograph at 300° and obtained two major peaks, as in collagen pyrolysis. The results could be applied to dental research problems and evaluated semi-quantitatively[351]. Several other amino acids yielded relatively simple pyrolysis profiles on polyester and polyglycol columns, but Arg gave a complex series of peaks[350].

In recent years, several comprehensive reports have appeared that were valuable in elucidating the general pyrolysis pathways. Pyrolysis at about 500° of a number of structurally different amino acids has been studied in order to determine the effects on mechanisms and product distribution caused by geometrical isomerism[302,340]. Most of the experiments were performed using the Tenax-GC (Applied Science Labs., State College, Pa., U.S.A.) column, which proved to be particularly useful in resolving the wide range of products in the 25–285° range; a detailed description of the pyrolysis GC–MS apparatus used in these experiments was reported elsewhere[341]. Aliphatic protein amino acids decompose predominantly by decarboxylation to produce the corresponding amines as the major organic products. A second process, also considered as a primary decomposition process, involves a condensation reaction yielding first a dipeptide and subsequently the cyclic 3,6-dialkyl-2,5-piperazinedione (diketopiperazine, DKP):



β-Amino acids lose ammonia to give unsaturated acids, while γ- and δ-amino acids give 2-pyrrolidone and 2-piperidone, respectively, as the major pyrolysis product. This is in accordance with another systematic study[175] in which unusually high yields of hydrogen cyanide were obtained in those instances in which suitable intramolecular cyclization was possible, for example, during pyrolysis of γ-aminobutyric or aminodicarboxylic acids (*e.g.*, Glu, Gln):

In compounds in which intramolecular cyclization could lead to the more sterically strained four-membered rings (Asp), only slightly higher yields of HCN were formed.

As pyrolysis products, the DKPs proved to be suitable for GC analysis. However, their analysis is of very limited practical value. Even the DKPs of the simplest

amino acids such as Gly[261], Val and Sar[398] or those derived from peptone[365] are relatively large molecules and operating temperatures above 200° had to be used in order to elute them, as rather asymmetrical peaks[398], from silicone-coated columns. However, a successful analysis of several DKPs prepared from simple protein amino acids, including Sar and Val methyl ester, by heating in molten phenol at about 150°, was reported by Mauger[223]. The peaks were symmetrical and a good separation of even the *cis*- and *trans*-isomers was achieved on SE-30 or EGSP-Z copolymer columns. Moreover, a short silylation procedure involving treatment with N,O-bistrimethylsilylacetamide at 80° for 10 min resulted in production of N,N'-bis(trimethylsilyl)-DKPs, of which the retention in the column was drastically reduced. The experiences with DKP analysis were then utilized in sequence studies on four actinomycins[224], which were pyrolyzed at 400° and the resulting DKPs analyzed on an EGSP-Z column. The assumption was confirmed that DKPs are formed only from neighbouring pairs of amino acids in the peptide.

3.1.4. Conversion into α-hydroxy acids (and their methyl esters)

Amino acids were reported to give the corresponding α-hydroxy acids when treated with sodium nitrite in acetic acid solution[202] or with 60% sodium nitrite in 1 *N* H_2SO_4 at 0° (ref. 384). In the former procedure, several simple amino acids together with Ser, Asp and Glu were chromatographed successfully after methylation with diazomethane on DC-550 silicone columns. In the latter procedure, the basic amino acids (Arg, His and Lys) were also detected, together with the others. Remarkably constant yields were found for the hydroxy acids, varying from 20% (Val) to 80% (Thr). An attempt to chromatograph them in the unesterified form failed and therefore the diazomethane treatment was chosen. Methyl esters of the α-hydroxy acids were then separated at 100° or 160° on a silicone phase. Met showed two peaks of equal size and symmetry under these conditions. In another study, five simple amino acids converted into α-hydroxy acid methyl esters were completely separated at 130–140° on a capillary column coated with Apiezon L[380].

3.1.5. Conversion into α-chloro acids (and their methyl esters)

Almost quantitative yields of the α-chloro acids were obtained by treatment of amino acids with a mixture of concentrated HCl and HNO_3 for 1 h[236]. After treatment with diazomethane, the α-chloro acid methyl esters were chromatographed with an excellent separation on either silicone or PEG stationary phases at 130°. However, no peaks were obtained from Phe and Met; Ser produced two peaks and derivatization of the basic and acidic amino acids was not attempted. Using this method, some amino acids were determined in several peptides and proteins[220].

3.1.6. Conversion into α-amino alcohols

Lithium aluminium hydride was suggested as a reagent for the reduction of amino acids to amino alcohols[385]. However, no details of this procedure have been given. Nine amino alcohols derived from the simple amino acids Ser and Trp were chromatographed at 155°, 170° or 200°, respectively, on Chromosorb W coated with a variety of liquid phases (20% each)[390]. The SF-96 and SE-52 silicones were found to be the best phases. Tryptophanol was not chromatographed successfully on any of the stationary phases examined; the aminoethanol (derived from Gly) was always

obscured by the solvent peak. However, the main problem of this approach is the direct reduction of amino acids to the corresponding amino alcohols, as such a reaction is known to proceed with only fair to good yields.

3.1.7. Esterification of carboxyl groups, leading to alkyl esters of amino acids

Methyl esters of the simple amino acids, prepared by treatment with methanolic HCl, were converted into the free base form with aqueous NaOH and, after extraction with diethyl ether, they were chromatographed on a high-loaded silicone-grease column to which 10% sodium caproate had been added in order to prevent excessive tailing[13,16]. A surprisingly successful separation of methyl esters of all protein amino acids except Tyr, Trp, His and Cys was reported when using 2% NPGS on Fluoropak 80 at 120–195° (refs. 179 and 261). An attempt was also made to chromatograph the acetate and hydrochloride salts of the methyl esters[261]. Whereas the acetates provided the same good results as the free bases at above 120°, only a few of the hydrochlorides could be chromatographed satisfactorily after dissociation to the free bases at a column temperature above 170°. The NPGS polyester column was useful in the analysis of several isomeric hydroxyprolines with a methylated carboxyl group[248]. The higher alcohols give amino esters of decreasing volatility[211]. Tailing of some simple amino acid ethyl esters was observed on a capillary column coated with polypropylene glycol[5]. An interesting approach to the analysis of the hydrochlorides of amino acid ethyl or butyl esters was the addition of ammonia (8 ml/min) to nitrogen carrier gas (50 ml/min)[330], which not only converted the salts into the free bases but also helped to prevent tailing of the peaks on a column packed with 22% PEGA on Chromosorb W. Fourteen butyl ester hydrochlorides were analyzed individually on this column (0.15 m long). To separate them in a mixture, a column 1.8 m long had to be used.

Another promising method for the esterification of the carboxyl group was described by Rühlmann and co-workers[318,319,323,324]. From the persilylated product (all protonic groups are silylated), the amino acid can yield an ester with a free amino group. The reaction conditions must therefore be carefully controlled and only a mild silylating reagent such as hexamethyldisilazane (HMDS) can be used. The amino acid trimethylsilyl (TMS) esters were discovered to be artifacts in the GC analysis of N-TMS-TMS esters (see 3.3.2). They were synthesized for analytical purposes by refluxing an amino acid with HMDS in toluene. The ammonia liberated from the reagent prevented further silylation of the amino group:

$$2\,R\text{–}CH(NH_2)\text{–}COOH + (CH_3)_3Si\text{–}NH\text{–}Si(CH_3)_3 \xrightarrow[\text{toluene}]{\text{boiling}} 2\,R\text{–}CH(NH_2)\text{–}COO\text{–}Si(CH_3)_3 + NH_3$$

The hydroxy groups of Ser, Thr and Tyr were also esterified but the thiol group of CysH was unchanged. When chromatographed on a methyl silicone column, the amino acid silyl esters showed excellent peak symmetry and lower retention times than the corresponding persilyl derivatives. This could be explained by the assumption that the free α-amino group of all amino acid silyl esters was made less polar by hydrogen bonding to the silylated carboxyl group[282]. Even when most protein amino acids af-

forded good and quantitative results, the procedure could not be accepted generally as no peaks were obtained from the basic amino acids, and with others a more complicated profile was found.

3.2. Methods based upon derivatization of the α-amino group and esterification of the carboxyl group in at least two steps

In all of the derivatives covered in this section, the carboxyl group is esterified (from methyl to isoamyl). The esterification is performed in a separate reaction step either before (more usually) or after the derivatization of the α-amino group and other reactive groups. After the completion of the first reaction step, evaporation of the preceding reaction medium is necessary.

The most general means of esterifying the carboxyl group in an amino acid is by treatment with acidified anhydrous alcohol. Dry hydrogen chloride gas was preferably used as an acid catalyst because of the ease with which it can be removed. Hydrogen bromide[174,338] and sulphuric acid[212] have enjoyed less popularity. No figure for the optimal concentration of HCl gas in alcohol can be given from the literature as this has ranged from about 1.25 *N*[104,408] to saturation[404]. Experiments on propylation have shown that a higher concentration of gas is desirable[54]. All common amino acids including Lys and His could be esterified within 20 min at 100° using propanol that is 8 *N* in HCl[53]. However, this more drastic esterification destroyed about 75% of Trp. Methylation takes place readily within 30 min at room temperature[354] when using methanol that is 1.25 *N* in HCl, but increasing time, temperature and concentration of the HCl gas are required through the series from methanol to pentanol. With the higher alcohols, further difficulties occur because of the lower solubility of both the amino acids and their hydrochlorides, particularly with Cys, Lys and His. This problem was circumvented by first esterifying with methanolic HCl (30 min at 20°) followed by transesterification with butanol that was 1.25 *N* in HCl at 100° for 150 min[354]. Temperature rather than HCl concentration was the more important factor in the transesterification reaction. An alternative approach was to dissolve the amino acids in trifluoroacetic acid; Lys·HCl could then be esterified at 108° with *n*-amyl alcohol[28,64] through which dry HCl gas was bubbled continuously.

Despite these earlier findings, a method for the direct esterification of the protein amino acids with *n*-butanol that is 3 *N* in HCl at 100° has been published[309]. The problems of solubility associated with Cys and some other amino acids have been circumvented by ultrasonic mixing in this medium for at least 15 sec at 20°. Nineteen of the amino acids were said to be quantitatively esterified within 15 min, but 35 min were required for the esterification of Ile. With this longer esterification time, Trp underwent some decomposition (approximately 15%). This technique, however, does not seem to be without problems. The latest studies[41] with nanomole amounts of amino acids revealed that in the recommended esterification system with butanol that is 3 *N* in HCl only about 50% of each amino acid was converted into the butyl ester with prior sonication for 30 sec. Addition of 10% of dichloromethane to the acidified butanol system with a simultaneous increase in HCl concentration to 3.5 *N* (to compensate for the dilution) was found to be efficient for good solubilization of the amino acids, provided that they were sonicated in such a system for at least 5 min. If the amino acids were to be esterified on a large scale, a modified procedure

was recommended[73] in which water of reaction was removed azeotropically with benzene[116]. Other water scavengers, dimethoxypropane[2] and dibutoxypropane[408], were also used to increase ester yields.

In several procedures, additional or other acid catalysts were used for the esterification step, *e.g.*, acetyl chloride (instead of HCl) together with methanol[35] or orthoformate with methanolic HCl[125]. In some instances, the amino acid esters were prepared by refluxing with the appropriate alcohol in the presence of Dowex 50 or other strong cation exchangers[216,253,329]. Eleven butyl esters were produced in this manner[216] but those difficult to esterify by other means were not included. Another procedure for methylation, which employs dimethyl sulphite together with methanolic HCl under reflux, has not been widely used despite the fact that esterification is both rapid and complete[57,366]. More popular was the thionyl chloride–methanol procedure[33] which has often been used[94,123,124,132,133,361]. The reaction proceeds via the formation of an intermediate dimethyl sulphite, which is the active donor of the methyl groups.

Treatment of amino acids with diazomethane was adopted in general, especially in procedures where the amino groups had already been converted into a derivative. The yields of methyl esters are quantitative and the reaction is very rapid. The procedure is not convenient for the esterification of native amino acids because mere evaporation of the ethereal diazomethane solution at room temperature leads to losses of Ala, Gly, Val and Leu. Alternatively, esterification can be carried out with higher diazoalkanes, *e.g.*, diazopropane and diazobutane[215,254].

Recently, a new esterification procedure was described[119] which should replace the tedious transesterification procedures. The reaction between an alkyl iodide (*e.g.*, iodobutane) and the carboxyl group is catalyzed by phenyltrimethyl- or -tetramethyl-ammonium hydroxide (0.1 *M* in methanol) and proceeds very rapidly (about 10 min) and under mild conditions (room temperature) in a highly polar solvent such as N,N-dimethylacetamide. The usefulness of this procedure for the esterification of amino acids remains to be evaluated.

3.2.1. Acylation of amino acid alkyl esters, leading to N-acylalkyl esters

The most popular means of dealing with the polarity of the amino group and the other reactive groups in the side-chain is to acylate them. Several methods utilizing the acyl ester have been shown to be quantitative and reproducible and applicable to a wide range of amino acids. The great number of publications attests to the popularity and potential of this class of derivative for the determination of amino acids. The correct nomenclature for the acylated esters should be N(O,S)-acylalkyl esters because all of the reactive groups are acylated; however, the shortened form N-acylalkyl is commonly used (if only the α-amino group is acylated it should be distinguished as N^{α}-acylalkyl).

Acylation reactions are generally carried out with acid anhydrides, exceptionally also with other acyl transferers such as the methyl or phenyl esters of trifluoroacetic acid[154,329]. Although the second rather unusual method was successfully applied to the analysis of most of the protein amino acids as N-TFA-methyl esters, the amino acids Arg, His and Cys were not included[154], and in another report incomplete acylation of –OH and ε-NH_2 groups was also observed[329]. Hence, the only recommended method is acylation with anhydrides either alone or in combination with an appropriate

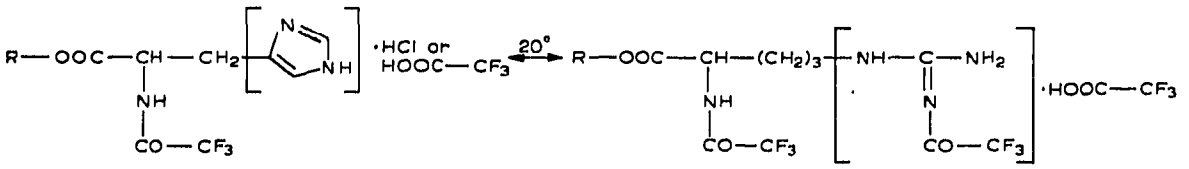
solvent. Even though, for example, formyl[208] or propionyl[67] derivatives together with some others (see 3.2.1.8) were examined and used for routine analysis in a single paper[67], from the present point of view and with regard to published techniques, the only useful anhydrides that permit quantitative GC amino acid determinations are trifluoroacetic (TFAA) and heptafluorobutyric (HFBA) anhydrides and, with some restrictions, also acetic anhydride.

TFAA is a widely used, powerful acylating agent that is very effective in the derivatization of all protonic groups except carboxyl. The acylation medium, mostly a mixture of TFAA with methylene chloride, can be injected into the GC column without prior evaporation. Direct injection is very practical, if not necessary, for two reasons. Firstly, the TFA derivatives are very sensitive to moisture and mere evaporation of the acylation medium causes a partial breakdown of some esterified groups, especially hydroxy and mercapto groups. In this way, the bis-TFA esters of Tyr, Ser, Thr, CysH and Hypro are easily hydrolyzed to the N^{α}-TFA compounds (the acylated α-NH_2 group is relatively very stable and resistant to hydrolysis) with methanol, water and even in some carefully dried aprotic solvents[61]. The second reason for "no evaporation after acylation" is the possible evaporation losses of the most volatile amino acid esters, *i.e.*, Ala, Gly, Val, etc. Only by evaporating off the excess of the reagent at low temperature (ice-bath) can the losses of N-TFA-methyl esters be avoided[65]. With regard to these findings, Gehrke and co-workers[190] selected the butyl esters in spite of the conclusion that only the amyl (or isoamyl) derivatives could be handled with any certainty[62,405].

Some of the problems of the TFAA treatment led to a search for another acylating agent. The best results were obtained with HFBA, which combines the desirable features of both TFAA and acetic anhydride. It is a strong acylating agent similar to TFAA but it forms more stable derivatives than, for example, acetic anhydride. The HFB derivatives can be exposed to water (in order to remove the HFB acid) during extraction with organic solvents without any degradation[249]. They are less volatile than the corresponding N-TFA esters so that the reaction medium, in most instances HFBA with acetonitrile or ethyl acetate, can be evaporated prior to GC analysis; no losses of N-HFB-propyl esters were observed after evaporation of the reagent[249,250] even though some workers recommend the isoamyl esters again[405]. Contrary to these findings, the HFB derivatives exert a higher volatility than the TFA analogues on the GC column (35% lower retention time)[289] and, unlike the latter, their separation and quantitative elution on a single GC column is possible[176,249,250,405]. Moreover, these derivatives possess one of the highest responses in the ECD and subpicomole amounts were readily detected[413]. On the other hand, the advantages of the use of HFBA instead of TFAA should be considered against its higher price, higher toxicity and its strong, unpleasant, penetrating odour.

Acylation of the hydrochloride salts of amino acid alkyl esters with either TFAA or HFBA requires a high-temperature treatment (150° for 5 min with TFAA or 10 min with HFBA), otherwise Arg gives no peak and His gives two peaks or poor results. This is caused by the dihydrochloride salts of both compounds, the guanidino group of Arg and the imidazolyl group of His, which are formed during the esterification of the carboxyl group in the first reaction step. The salt formation reduces volatility and, with Arg, prevents complete acylation. Thus, if the alkyl esters of these two amino acids are treated with TFAA at room temperature, for ex-

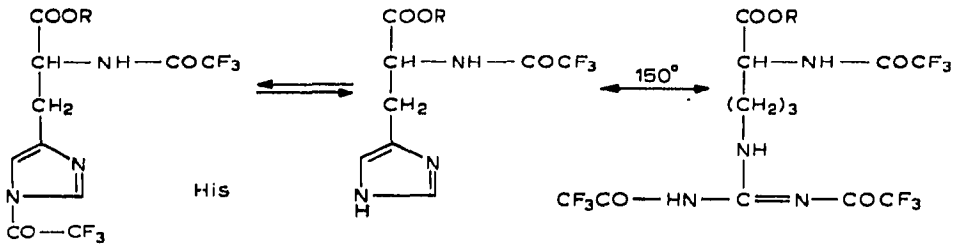
ample, the resulting insufficiently volatile guanidino salt of Arg cannot be analyzed, whereas the imidazolyl salt of His, which is dissociated by heat in the GC injection block, gives only a poor peak[212]:



N^{α}-TFA-His alkyl ester (salt)

diacyl-Arg alkyl ester (salt)

Thus, in spite of the fact that all of the other protein amino acids can be converted quantitatively into their N-TFA esters at room temperature[352], for the quantitative analysis of Arg and His high-temperature acylation is necessary. Arg is the only triacylated derivative among the other esterified amino acids. The esterified proton of the imidazolyl group of His is very sensitive to hydrolysis. If diacyl-His emerges as a peak, the anhydride should be co-injected[102,176,250]. Mere evaporation of the acylating agent was found to cause a breakdown of diacyl-His to the monoacylated compound[405] (there is only one report in which this phenomenon was not observed, in the case of diacetylated His)[2], which is then chromatographed with a longer retention time (more polar compound):



His

diacyl (shorter r_t) monoacyl (longer r_t) triacyl-Arg

The acylating ability of acetic anhydride is insufficient to acylate the dihydrochloride salt of Arg-alkyl ester. The less potent acylating properties of acetic anhydride do not permit acylation of the guanidino hydrochloride group under similar conditions as with TFAA or HFBA. The production of a free base, either by desalting with an anion-exchange resin or by neutralization with an alkali metal carbonate, was necessary at first in order to achieve complete derivatization of Arg by the subsequent acylation step[54]. An alternative procedure, suggested by the same authors, was based on enzymatic conversion of Arg into Orn and by ozonolysis of His to Asp[53]. However, the above-mentioned techniques are too tedious and not very convenient for routine use. Recently, a modified technique was published[2], which allows Arg-propyl ester to be derivatized with approximately 78% efficiency by treatment with a strongly basic aprotic acylating agent. Provided that such a small loss in the amount of Arg is acceptable, the procedure is otherwise highly reproducible and convenient for the

determination of all protein amino acids in the form of N-acetylpropyl esters on a single column[2,81,230]. Moreover, the acetylation step was reported to proceed extremely rapidly[2]. Hence, the use of acetic anhydride as a cheap, readily available derivatization reagent brings an additional advantage, the relatively good stability of the N-, O- and S-acetylated groups. This should be weighed, however, against the lower volatility of the derivatives, which makes the choice of an appropriate liquid phase more difficult. Moreover, a mixture of liquid phases coated on a support seems to be necessary for an efficient separation[2,81,230].

3.2.1.1. N-Formylmethyl to isobutyl esters. Formylation of amino acids with formic acid in acetic anhydride was reported to be effective in converting the N-amino group into the acylated form[208]; however, no examination was made of other reactive groups. Therefore, only the simple amino acids, together with Asp and Glu, were chromatographed after subsequent treatment with diazomethane at 194° on a highly loaded silicone column. Under these conditions, the N-formyldimethyl ester of Glu was converted into the cyclic methylpyrrolidone carboxylate. In a second study[336], the methyl, ethyl, propyl and isobutyl esters of amino acids were formylated or acylated, respectively, and the derivatives were compared by chromatography on some polar solvents as stationary phases. The N-formylated esters were found to be less convenient, exhibiting longer retention times and poorer resolution than the corresponding N-acetyl esters. As a result of these findings, no other investigations on this derivatization technique were carried out.

3.2.1.2. N-Acetylmethyl to isoamyl esters. The following amino acid alkyl esters were subjected to acylation with acetic anhydride: methyl (ethyl)[35,62,190,211,248,336–338,404], propyl (isopropyl)[2,53,54,81,116,211,230,336–338], butyl (isobutyl)[164,174,190,211,336–338,379,404] and amyl (isoamyl)[173,174,251,259,338,368,387].

The first use of GC for separating N-acylated amino acid esters appeared in 1959 when Youngs[404] successfully analyzed six simple amino acids as N-acetylethyl and -butyl esters on hydrogenated vegetable oil. A comparison of the retention times of N-acetylalkyl esters showed that, for example, the methyl, ethyl and isopropyl esters exerted almost the same retentions[62,337], whereas the butyl analogues exhibited a 2–3 times higher retention[211,337] when operated under the same isothermal conditions. Johnson and co-workers[173,174] compared the usefulness of several acetylated amino acid esters (butyl, isobutyl, amyl and isoamyl) on columns packed with Carbowax 1540 on Chromosorb W. Various column lengths and percentages of coating were evaluated in the separation of 17 naturally occurring amino acids as N-acetylamyl esters. His and Arg were esterified in low yields, while Trp and Cys were not eluted under any of the conditions studied. Using the same Carbowax phase, other workers[337] resolved 14 protein amino acids in the form of acetylated butyl esters; the isobutyl esters were less efficient in this respect, whereas the isopropyl esters showed better separation than the propylated analogues.

Despite the fact that a complete and rapid separation of 13 acetylated propyl esters was observed in experiments using a non-polar Apiezon L-coated column (10% on Celite 545)[338], further studies by the same workers revealed that the polyesters (0.5% PEGA on Chromosorb W AW)[336] and polyglycols (0.5% Carbowax 1540 on the same support)[337] exhibited the most desirable separation characteristics. Similarly, although the use of a silicone of intermediate polarity (QF-1) led to a pronounced decrease in retention time[62], the silicone phases were said to be less valuable

as insufficient resolution of some pairs of protein amino acids was achieved[379]. The use of a relatively long column (2.5 m) filled with 10% DC-200 silicone on Gas-Chrom Z also gave very poor results in the analysis of the N-TFA-butyl esters of the protein amino acids; some amino acids were not resolved, others were eluted non-quantitatively (His, Trp, Arg) and several (Cys, CysH, Hypro) could not be eluted[379]. On the other hand, Lamkin and Gehrke[190] succeeded in analyzing N-acetylbutyl esters on a 2.5-m column coated with 0.25% Carbowax 1500. Peaks were obtained for all protein amino acids except Arg, Cys and His. This polyethylene glycol phase was found to be superior to the NPGS polyester, the use of which caused excessive tailing of, for example, the N-acetylbutyl ester of Lys. Also, in a further series of papers, the polyester[62,190,211,248,336,368] or polyglycol[164,190,259,337] phases were found to be very convenient for analysis of a few amino acids in the form of N-acetylated alkyl esters. The percentage of coating was rather low, usually ranging from 0.25 to 2.0%. A mixed phase consisting of silicone F-60 (7%) and EGSS-Z copolymer (1%) was examined together with the analysis of acetylated methyl esters of Phe, Tyr, Trp and His[35]. An unusual column filling, 10% monosodium glutamate on sodium chloride as a support, was used for the analysis of some acetylated amino acid amyl esters[251].

As earlier studies had shown that the separation of all acetylated protein amino acid alkyl esters could hardly be achieved on a single phase, the use of mixed stationary phases was later adopted in order to solve the separation problems. The first very useful combined stationary phase, consisting of Versamid 900–NPGS–Carbowax 1540 (1:1:1), was introduced by Ward *et al.*[387] for the analysis of several amino acids occurring in the actinomycins. Using a 0.6% coating on Chromosorb W, the peptide amino acids could be readily separated as N-acetylamyl esters at 125–200°. However, the acetylated propyl esters were finally evaluated as the most convenient derivatives for the determination of the protein amino acids. These derivatives were first selected by Graff *et al.*[116] and a high degree of quantitative conversion was reported. Over 30 different highly pure N-acetylpropyl esters were prepared as reference standards and a good separation of most of them on a Carbowax-coated column was achieved. Coulter and Hann[53,54] succeeded in separating the N-acetylpropyl esters of 19 amino acids (Arg and His were modified before to Orn and Asp, respectively) using a column containing equal amounts of 0.7% Carbowax 6000 and 0.7% Carbowax plus 0.05% TCEPE. A column packing consisting of 0.82% of XE-60–Polyamide A-103–Carbowax 4M (10:11:20) on Anakrom ABS permitted the separation and quantitative elution of 17 protein amino acids except Arg, His and Cys[230]. Finally, the two most useful column packings, which were very effective in separating 19 protein amino acids in a short (15 min) programmed operation, consisted of Carbowax 4000–NPGS–Versamid 900 (2:1:1)[81], 3% on Chromosorb W AW, and of Carbowax 20M–Silar 5CP–Lexan (31:28:6)[2], 0.65% on the same support. GC analyses on both types of column are shown in Figs. 1 and 2.

Based on the work of Graff *et al.*[116] and on the analytical system presented in Fig. 1, Packard Instrument Company (Downers Grove, Ill., U.S.A.) announced, in 1971, the production of an automatic instrument (amino acid derivatizer), and the Gallard-Schlesinger Corporation (New York, N.Y., U.S.A.) later introduced a Reagent Kit containing the reagents and column filling necessary for the derivatization of amino acids to the N-acetylpropyl esters. The major obstacle with this technique, however, has been the inherent derivatization procedure for the determination of

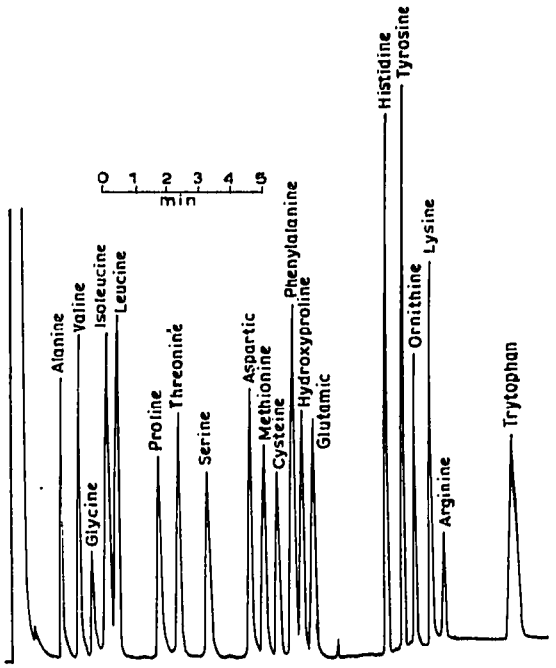


Fig. 1. Analysis of N-acetylpropyl esters of the protein amino acids on a mixed stationary phase, Carbowax 4000–NPGS–Versamid 900 (2:1:1), 3% on Chromosorb W AW. Total run, 16 min. Column dimensions and programme range unspecified. Reproduced from an information leaflet of Gallard-Schlesinger Corp. (U.S.A.).

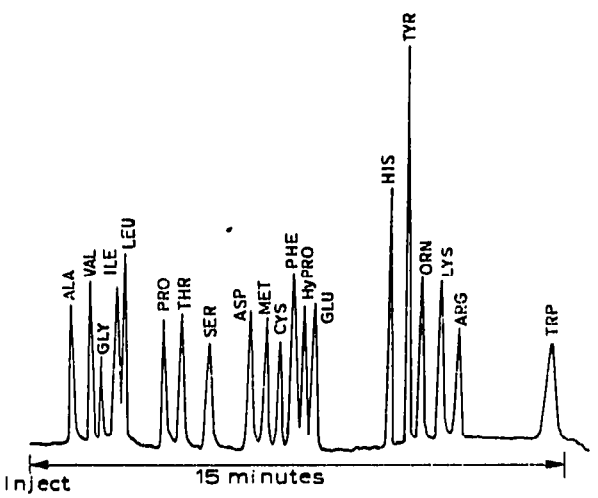


Fig. 2. Analysis of N-acetylpropyl esters prepared according to the method described by Adams[2]. Column: 90 cm × 3 mm O.D. stainless steel, filled with 0.65% Carbowax 20M–Silar 5CP–Lexan (31:28:6) on 120–140 mesh Chromosorb W AW (pre-heated to 400° for 1 h). Temperature programme: 125–180° at 8°/min and to 250° at 32°/min. Detector: FID. Carrier gas (He) flow-rate: 12 ml/min. Reproduced from *J. Chromatogr.*, 95 (1974) 189, by kind permission of the author.

Arg. In accordance with the findings of Coulter and Hann[53,54], the acylation with acetic anhydride–pyridine reagent is not efficient enough to acylate fully the hydrochloride salt of the guanidino group of Arg (see also 3.2.1). Its poor replication is seen in Fig. 1. The difficulties associated with the quantitation of Arg were probably the reason why the Packard derivatizer was not made commercially available. The possibilities of the acetylation technique were fully evaluated by Adams[2] in the latest study on this topic. Even a strong basic acylating agent, consisting of acetone–triethylamine–acetic anhydride (5:2:1) and permitting the quantitative conversion of all other protonic groups within 30 sec at 60°, did not give complete esterification of the guanidine hydrochloride but only 78%. This level of conversion was said, however, to be highly reproducible. Together with the esterification step, being accomplished within 15 min at 100° using propanol that was 8 *N* in HCl, the whole derivatization time could thus be shortened to less than 30 min. The short analysis time in together with satisfactory resolution of the derivatives (Fig. 2) are the further advantages of the procedure. However, none of the useful mixed stationary phases enabled Cys to be determined together with the other amino acids.

The N-acetylpropyl esters were used for the analysis of amino acids in biological material[2,215,217,230].

3.2.1.3. N-Trifluoroacetylmethyl esters. After being introduced into the amino acid field by Weygand and Csendes in 1952[396], the trifluoroacetylating agents were found to be very effective in conversion of the protonic functional groups into their esterified forms. However, it was 8 years before this chemical treatment was applied to the GC analysis of amino acids[397]. A comparison between the N-TFA-methyl and the corresponding N-acetylmethyl esters revealed that the former had about a 6-fold shorter retention time on the polyester and about a 3-fold shorter retention on the silicone phases, respectively[62].

The first attempts to analyze the trifluoroacetylated methyl esters brought some problems associated with the derivatization technique, stability of the derivatives and decomposition of some of them on some types of column fillings. The studies of Darbre and Blau[25,26,61–63,65] were particularly valuable in explaining the behaviour of the TFA esters and the findings were later applied generally to all trifluoroacetylated amino acid alkyl esters. Based on their investigations, it can be summarized that: (1) methylation with diazomethane should be avoided because mere evaporation of its ethereal solution causes considerable losses of Ala, Val, Gly and Leu[25,62]; (2) in order to avoid evaporation losses on N-TFA-methyl esters, the acylating agent (mostly TFAA–CH_2Cl_2) should be evaporated at 0° using a 1333 Pa vacuum applied for no longer than 4 min[65,162]; (3) any treatment of the TFA esters with alcohol (*e.g.*, CH_3OH–CH_2N_2 ethereal solution) is inadvisable because of the immediate breakdown of O- and S-TFA esters[26,61]; thus, the bis-TFA derivatives of Tyr, Ser, Thr, Hypro and CysH are decomposed to the mono-N^{α}-TFA esters (with free –OH or –SH groups), which are still volatile and may be eluted, however, non-quantitatively, with greater retention times[124,212,329]; (4) additionally, the O-TFA and S-TFA esters are also prone to decomposition on some types of column packings[63], the list of stationary phases that cause breakdown of the TFA esters being large and including most of the polyesters (but not the EGA phase), the polyethylene glycols and also the polar cyanoethylsilicone XE-60; (5) the recommended derivatization technique includes esterification with methanolic HCl at 70° for 30 min followed by acylation

with TFAA at 20° for 30 min; however, for Arg the high-temperature TFAA treatment (140° for 10 min) is obligatory, His still giving low yields (about 5%) under these conditions[65]. In a recent study, acylation at 120° for 20 min was generally recommended[48]. An apparatus for preparing amino acid N-TFA-methyl esters based on evaporation of aqueous solutions of amino acids on platinum wire followed by subsequent derivatization has also been described[367].

Having recapitulated the conclusions of Darbre and Blau, let us review the earlier reports concerning the preparation and GC analysis of the trifluoroacetylated amino acid methyl esters. It can be seen that many of the pioneer works very often suggest approaches that should be avoided. TFAA was usually used for acylation either before (acylation of amino acids in TFAA–TFA medium)[57,339,385] or after the preceding esterification step[56,94,123,124,179,212]. In some instances, methyl trifluoroacetate, either alone[154] or together with a basic methanol–triethylamine system[329,397], was employed. The use of trifluoroacetylimidazole was reported in one instance[125]. The medium from the final reaction step was evaporated at room temperature without the recommended cooling in ice-water[123,124,212,339,385]. The TFA esters were dissolved in methanol either before the subsequent esterification with diazomethane[339] or during the acylation step[329,397], or even after the acylation had been completed[57,123,124], so that decomposition of the O(S)-TFA esters occurred. Copper[123,124,385] or aluminium[94] columns were not excluded. With one exception (Apiezon L phase)[385], in all other instances polyester (mostly NPGS) or Carbowax (1540, 20M) liquid phases were employed, the use of which results in decomposition or at least non-quantitative elution of some derivatives[63].

It is therefore not surprising that the reported analytical data are rather confusing. Saroff and co-workers[179,212,329] investigated extensively the nature of the decomposition of the N,O(S)-diacyl derivatives to the N^{α}-monoacyl compounds and established the retention times of both forms on a column coated with 2% NPGS on Chromosorb W. The retention times corresponding to the monoacylated forms were 5–15-fold higher, and even N-TFA-His could be eluted from the column. Trp was not converted into the monoacylated compound after the treatment with methanol (the esterified indolyl nitrogen was not hydrolyzed) and the monoacyl CysH exhibited an unexpectedly shorter retention time than the fully acylated parent compound. A surprisingly successful analysis of all protein amino acids except Cys, after dissolving their N-TFA-methyl esters in methanol, was reported by Hagen and Black[123,124], who used very unusual chromatographic conditions, *i.e.*, injection at 20° with subsequent increase in temperature to 270° within 2–3 min and isothermal operation at 270° using Carbowax 20M as the liquid phase. Lys, Arg, Trp and His were also detected on the chromatogram. Doubts remain as to whether the identification of the peaks was correct, as no other workers were able to elute His, and in some instances also Arg, from similar types of column packing (Carbowax 4000[125], NPGS[154], BDS[339]). A relatively successful analysis of N,O(S)-TFA-methyl esters (except His and Cys) was reported using 5% NPGS on Gas-Chrom P[57] or on silanized Chromosorb W[94]; however, the separation of some pairs was not complete. Better resolution was achieved when two columns were employed, the first coated with NPGS and the second with a mixture of DEGS, EGSS-X and EGSP-Z[56]. The EGSS-X copolymer was also used for analysis of the N^{α}-monomethylated analogues of 14 protein amino acids in the form of N-TFA-N-methyl amino acid methyl esters[74]. Homocystine was

analyzed at 140° on a column filled with 5% QF-1 on Gas-Chrom P[120]. A study of the analysis of Met, Met sulphoxide and Met sulphone on a Carbowax 20M column at 180° revealed that the Met sulphoxide was converted into Met during the derivatization procedure and, therefore, no additional peak could be seen on the chromatogram. Met sulphone gave no volatile derivative[360].

Finally, the best separation of N-TFA-methyl esters (except His) was achieved on a 3.25-m glass column packed with 2.5% of a mixed silicone stationary phase consisting of XE-60–QF-1–MS-200 (46:27:27), 100 cS, on Diatoport S using combinations of both temperature programming and isothermal operation[65,162]. The procedure was improved by using a pre-column and a smaller percentage of coating (1.5%)[48]. The GC analysis is shown in Fig. 3.

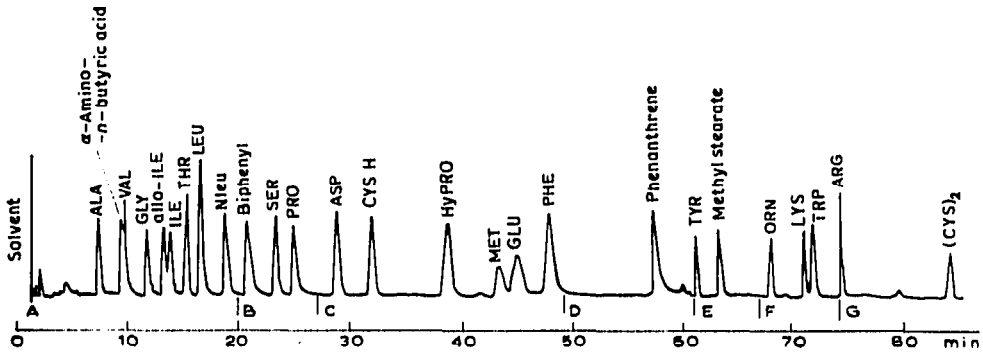


Fig. 3. Analysis of the protein amino acids in the form of N-TFA-methyl esters. Column: 3.25 m × 2.5 mm I.D. glass, filled with 1.5% of the mixed stationary phase XE-60–QF-1–MS 200 (46:27:27) on 80–100 mesh Diatoport S. Temperature programme: (A) 90° initial temperature followed by 1°/min for 20 min; (B) 3°/min for 7 min; (C) hold for 22 min; (D) 6°/min for 12 min; (E) hold for 6 min; (F) 4°/min for 7 min; (G) final hold at 231°. Reproduced from *J. Chromatogr.*, 78 (1973) 333, by courtesy of A. J. Cliffe.

Using this column packing, radioactive amino acids were employed in an investigation of the synthesis of N-TFA-methyl esters and the possible breakdown of these derivatives during GC[68]. It was found that not all of the radioactivity was associated with a single derivative peak. All derivatives showed considerable amounts of post-peak radioactivity (5–7%) whereas, with Phe, 13.1% of the activity appeared before the main peak. The methyl ester of Phe (free base) is more volatile than the corresponding N-TFA ester, thus making these observations consistent with slow, continuous degradation on the column. The poor replication of several amino acids (Tyr, Arg, Cys) is in a good agreement with a previous report[63] that the polar XE-60 phase might be responsible for the partial decomposition of the trifluoroacetylated esters. Darbre and Islam (personal communication) recommended either 2.5% MS-710 or a mixed phase consisting of equal amounts of OV-17 and SE-30 for the quantitation of this difficult group of amino acids. In another study, the same workers established the FID molar responses of N-TFA-methyl esters. The linearity of the response in relation to the carbon number was demonstrated for amino acids that possess the same reactive groups; non-linearity of the response could be used in order to show either incomplete derivatization or breakdown of the derivative during GC.

The derivatization technique was applied to analyses of amino acids in various kinds of material[38,94,120].

3.2.1.4. N-Trifluoroacetylethyl to isopropyl esters. There has only been a single mention of the elution of the vinyl esters of Tyr and O-methyl-Tyr from a silicone column[398]. The TFA-ethyl and -propyl esters of four simple amino acids were analyzed only for comparison purposes[62,211]; the latter exerted an approximately 1.5-fold higher retention time[211].

The N-TFA-isopropyl esters (usually used in the analysis of amino acid enantiomers, see 5.2) of all 20 protein amino acids were prepared by the transesterification procedure (the methyl esters were treated with isopropanol that was 1.25 *N* in HCl at 100° for 3 h), subsequent acylation with TFAA–CH_2Cl_2 at 150° and thereafter analyzed on a 150-m steel capillary column coated with SF-96 silicone[182]. In the temperature range 100–210°, all amino acids except His were eluted and well separated. However, partial decomposition due to the metal injection port occurred. Co-injection of 1 μl of TFAA was suggested in order to diminish this effect. Unfortunately, no studies have appeared on the analysis of such derivatives on packed glass columns.

The trifluoroacetylated amino acid ethyl esters were selected in one instance in order to demonstrate the possibility of the continuous detection of radioactive GC effluents by liquid scintillation[331a]. Sixteen ^{14}C-labeled amino acids were analyzed on QF-1 silicone phase (10% on Diatoport S) and the peaks of radioactivity appeared on the chromatogram simultaneously with the peaks of masses.

3.2.1.5. N-Trifluoroacetylbutyl esters. In 1962, Zomzely *et al.*[408] studied the N-TFA-butyl esters as possible derivatives for the GC determination of amino acids. The direct treatment of amino acids with acidified *n*-butanol was found to be effective when using dimethylformamide as a solvent and dibutoxypropane as a water scavenger. The acylation was performed with TFAA at 28° for 30 min. Lamkin and Gehrke[190] gave a more detailed description of the derivatization technique. As the direct esterification of amino acids with *n*-butanol gave rise to problems owing to the insolubility of Cys and the basic amino acids and acylation at room temperature did not lead to formation of a volatile derivative of Arg, the transesterification procedure (see 3.2) was developed in order to obviate the first problem[97,354] and sealed-tube acylation with CH_2Cl_2–TFFA (4:1) at 150° for 5 min was introduced[352] in order to solve the second problem (see 3.2.1). Additionally, as the N-TFA-butyl esters were found to decompose in metallic injection ports[190], direct on-column injection was said to be obligatory[352]. Finally, direct esterification of amino acids[309] after treatment with *n*-butanol that was 3 *N* in HCl at 110° for 15 min (Ile required 35 min) was suggested as a substitute for the earlier transesterification procedure[354]. The ultrasonic mixing of amino acids in this medium for at least 15 sec (at 20°) should solve the solubility problems. However, Cancalon and Klingman[41] could not achieve satisfactory results with this technique when applied to nanomole amounts of amino acids and they proposed the use of dichloromethane together with acidified butanol (CH_2Cl_2–butanol 3.5 *N* in HCl, 1:9) and simultaneous sonication for at least 5 min. As Gehrke and co-workers did not mention any difficulties in using the former esterification system in either the introductory paper to this technique[309] or in their last summarizing monograph[177], the important findings of Cancalon and Klingman should still be verified by additional experiments. The effect of salts on the derivatization technique and subsequent GC analysis was also evaluated[98]; the presence of an

amount of inorganic salts equal in weight to the total weight of amino acids was not found to be serious for qualitative work, but in certain instances it could interfere in quantitation.

From the beginning of the GC analysis of N-TFA-butyl esters, the polyester phases occupied a predominant position among the other phases. In a series of studies many of them were evaluated for their efficiency to separate derivatized amino acids. In the first report[408], almost complete separation of all protein amino acids with only one unresolved pair (Asp–Phe) was achieved by using 1% NPGS on Gas-Chrom A. The use of Chromosorb G AW together with a 0.5% coating of NPGS brought a further improvement in the separation characteristics[104]. A mixed polyester phase consisting of DEGS and EGSS-X in the ratio 3:1 (1% coating) was reported later to be superior[190], and packed into a 1 m × 4 mm column it was used successfully for the separation of 20 protein amino acids[97,103,104]. McBride and Klingman[227] succeeded in separating 17 amino acids (except Arg, His and Cys) when using 1.2% PDEAS (phenyldiethanolamine succinate) on Gas-Chrom A in a 1.1 m × 4 mm column. Stefanovic and Walker[356] examined the use of EGA as a 0.5–2.0% coating on 80–100 mesh Chromosorb W AW, together with the analysis of N-TFA-butyl esters of the protein amino acids. In the programmed temperature range 80–230°, the elution pattern obtained was a function of the amount of liquid phase on the support for certain amino acids (Gly, Met, Phe, Pro). A 0.65% coating appeared to be the most suitable for the separation of the 17 derivatized amino acids. Poly-γ-methyl-D- and poly-γ-ethyl-L-glutamate (D-PMG and L-PEG) proved to be effective liquid phases with properties similar to those of PEGA but with a higher temperature stability (up to 230°); they were used in the analysis of several amino acid N-TFA-butyl esters[165].

Stationary phases of other types (polyglycols, silicones, etc.) were employed much less in separation studies because their separation ability was lower. Eleven protein amino acids[216] and several "imino" acids[253] (derivatives of Pro and Hypro) were satisfactorily resolved on 1% Carbowax 20M. In some instances capillary columns, coated with silicone[80,199], Apiezon[380] or Polysev[110] liquid phases, were used in separation studies. Despite their high resolution power, the separations were poor and the least volatile amino acid derivatives were not eluted[110]. DC-550 silicone was applied successfully to the determination of some non-protein amino acids[104]; the cyanoethyl silicone XE-60 (5% on Aeropak 30) was evaluated, together with an analysis of the protein amino acids[189]. Metz *et al.*[239] were unable to separate completely the derivatized protein amino acids on a column filled with 0.6–1.0% OV-225 on 100–120 mesh Chromosorb G HP; moreover, Cys was not eluted from the column. Gehrke and Takeda[105] examined nine silicone phases together with some Apiezons (selected hydrocarbon fractions) for the same purpose. It was found that none of the OV silicone phases (singly or in combinations) would give complete and reproducible separations of the amino acids. The best separation was achieved using a 2.5 m × 2 mm column filled with 10% Apiezon M on 80–100 mesh Chromosorb W HP (Fig. 4). However, several amino acids showed unexpected losses on the column (Met, 14%; His, 37%; Cys, 54%).

Gehrke and co-workers recognized later the necessity for using a dual-column system with two types of stationary phase: one a polyester with the ability to separate 17 amino acids, the second a silicone, the use of which is obligatory for the quantitative elution of His, Arg and Cys. In an important series of papers, which have no equal

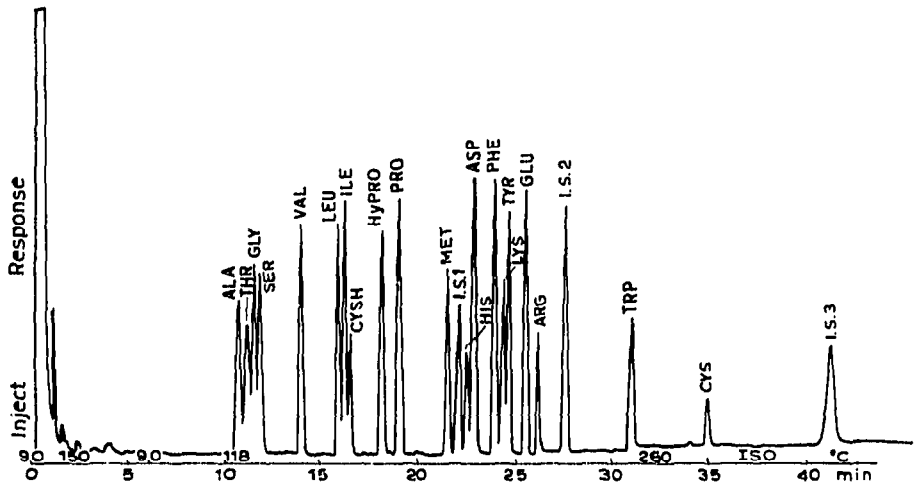


Fig. 4. Single-column GC separation of N-TFA-butyl esters of the protein amino acids. Column: 2.5 m × 2 mm I.D. glass, with 10% Apiezon M on 80–100 mesh Chromosorb W HP. Temperature programme: 90° for 6 min, 6°/min to 260°. Internal standards: (1) Orn, (2) tranexamic acid and (3) *n*-butyl stearate. Reproduced from *J. Chromatogr.*, 76 (1973) 63, by courtesy of C. W. Gehrke.

in the field of amino acid analysis, the conditions for quantitative derivatization, GC analysis and application of the technique to various types of material were presented. The studies on the dual-column system were first described in 1968[102,109] and were completed in 1974[177]. In a comprehensive study on the separation characteristics of various polyesters[109], the separation ability of EGA was found to be superior to that of NPGS. A significant improvement in resolution was achieved when columns were prepared with Chromosorb G, which was heated (heat treatment, H.T.) at 550 ± 50° for 15 h prior to coating with the stabilized EGA phase. Thus, one of the two columns (1.5 m × 4 mm) was filled with 0.325% EGA on 80–100 mesh H.T. Chromosorb G AW (Column I). For the analysis of Arg, His and Cys, a 1 m × 4 mm column with 1.5% OV-17 on 80–100 mesh Chromosorb G HP was used (Column II)[109]. In the subsequent monograph[102], the details of macro, semimicro and micro methods, reagents, samples, preparation, instrumental and chromatographic requirements, and ion-exchange clean-up of the sample for the quantitative GC analysis of the protein amino acids as their N-TFA-butyl esters were presented. Refinements of the GC method have been reported with regard to the quantitative analysis of His[311] and to improved performance and reliability of the EGA column[308]. The conversion of monoacyl-His into the diacyl derivative on the chromatographic column by injection of TFAA has obviated the need for the previously reported injection of *n*-butanol[109]. It was shown that diacylated His can be completely separated from Asp and Phe on the silicone-coated column[311]. Moreover, the investigators reported that columns containing 0.65% of stabilized EGA on 80–100 mesh Chromosorb W AW, dried at 140° for 12 h (Ia), was generally superior to 80–100 mesh H.T. Chromosorb G AW in terms of resolution, reliability, and ease of preparation[308]. The separation of the 17 protein amino acids on this column packing is shown in Fig. 5.

In later studies[177,412] Gehrke and co-workers suggested that pre-heating the Chromosorb W AW support at 140° for 24 h seems to be unnecessary (it was again

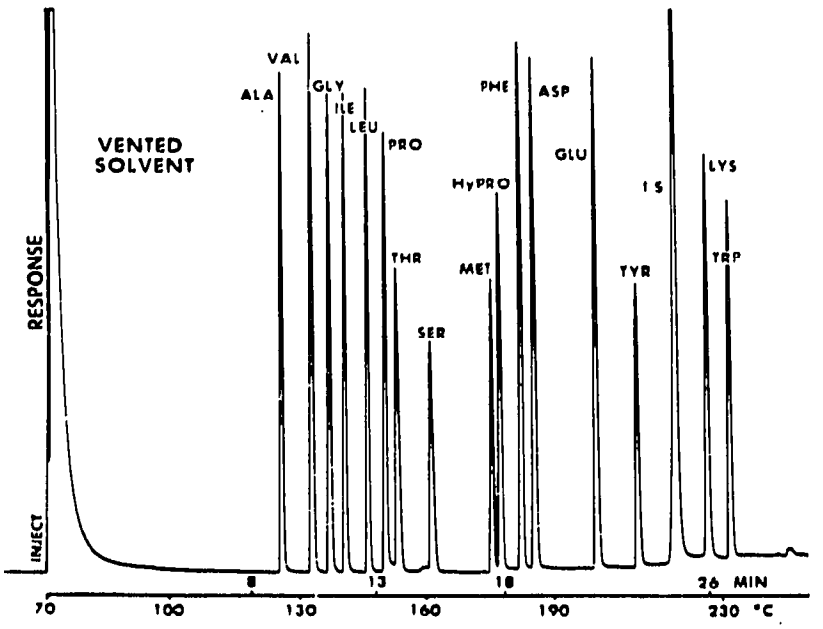


Fig. 5. GC analysis of 17 protein amino acids as N-TFA-butyl esters on 0.65% stabilized grade EGA on 80–100 mesh Chromosorb W AW. Column: 1.5 m × 4 mm I.D. glass. Temperature programme: 70–230° at 6°/min. Internal standards (I.S.): *n*-butyl stearate. Solvent vent time: 15 sec. Pre-column (10 cm × 4 mm): 1% OV-17 on 80–100 mesh Chromosorb G HP. Injection port temperature: 180°. Reproduced from *J. Ass. Offic. Anal. Chem.*, 55 (1972) 449, by courtesy of C. W. Gehrke.

stated, however, that certain batches of this support might still require heat pre-treatment for the removal of surface-adsorbed water[108]). Thom and Parsons[370] made a comparison between Chromosorb W AW and Chromosorb W HP, both coated with 0.65% EGA. The seemingly more desirable, more deactivated silanized HP support gave poorer resolution of the first six amino acids and no separation of Met and Asp, so that the AW support was clearly superior.

A general and reliable method for the analysis of samples containing nanogram to picogram amounts of amino acids was developed[412,413]. At the nanogram level of amino acid analysis, the level of general contamination of amino acids becomes a most important problem[301]. It was found that significant contamination originated from several of the following sources: laboratory supplies (water, butanol, methylene chloride, hydrochloric acid, eluate from the ion-exchange column, etc.), the human body (fingerprints, skin fragments, hair, dandruff, saliva), dust and cigarette smoke. The GC analysis of micro amounts was permitted by the invention of an injection port solvent-vent chromatographic device, which permitted the injection of the total derivatized sample (up to 100 μl) on a standard packed analytical column, simultaneously eliminating the tailing effects (caused, for example, by TFA) while still retaining the amino acids on the column (Fig. 6). In practice, a small pre-column of a mixed OV silicone packing is placed in front of the normal analytical EGA column. As the sample is injected, a timer is activated that opens a solenoid valve for a prescribed time period, allowing the solvent and other volatile substances to vent to the atmosphere. At the end of the chosen venting time, the valve is closed, the temperature

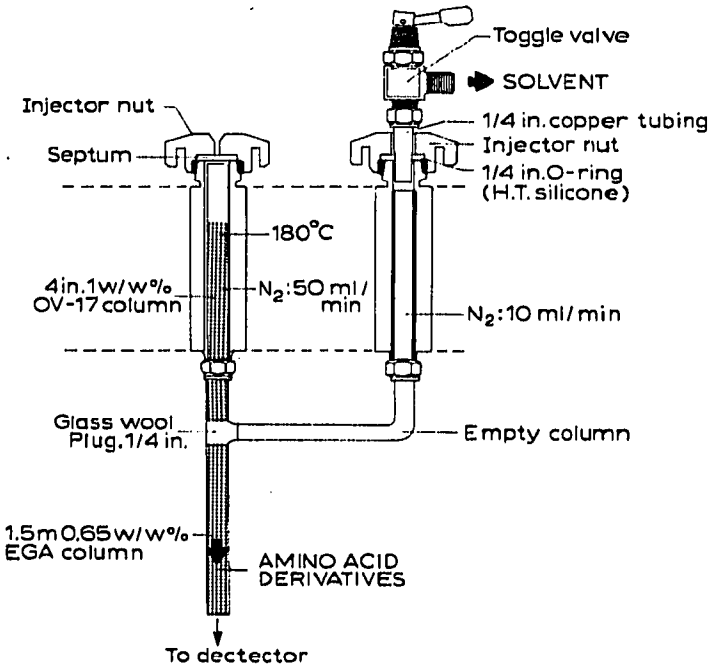


Fig. 6. Injection port solvent vent device. Reproduced from *J. Chromatogr.*, 57 (1971) 193, by kind permission of the authors.

programme is started, and the analysis performed in the usual manner. Retention of the amino acids is based on the selection of the pre-column packing and its length, initial temperature, venting time and carrier gas flow-rate. The derivatization and GC analysis (FID) of 5 ng of each amino acid[413], which was possible only by using this device, is presented in Fig. 7.

In an attempt to achieve higher sensitivities, the detection of the N-TFA- and N-HFB-butyl esters of selected amino acids using an ECD was studied[413]. The minimal detectable amounts of various amino acid derivatives were assessed by demonstrating that 1–50 pg can be clearly observed by this method, as shown in Fig. 8.

Recently, Gehrke and co-workers[95,96,108,177,412] presented a superior column packing that permitted highly efficient separations of the basic amino acids and Cys. The new column packing (IIa), 2% OV-17 plus 1% OV-210 (or the equivalent 1% SP-2401 instead of OV-210)[108] on Supelcoport 100–120 (or 80–100) mesh[95] or also Gas-Chrom Q (80–100 mesh)[177] should replace the OV-17 Chromosorb G packing. On this packing it is no longer necessary to make a calculation for His, as reported previously[311]. Thus, the complete GC separation and quantitative determination of the 20 protein amino acids can be achieved by using the dual-column system with a simultaneous programmed operation for both columns. The pattern of analysis is presented in Fig. 9.

All of the techniques developed by Gehrke and co-workers, including hydrolysis, ion-exchange clean-up (see also section 6), derivatization and quantitative GC determination of protein amino acids in the form of N-TFA-butyl esters, can be obtained from their recent paper[177]. A special technique[106] was developed for the analysis of protein-bound Trp (see 2.2.1).

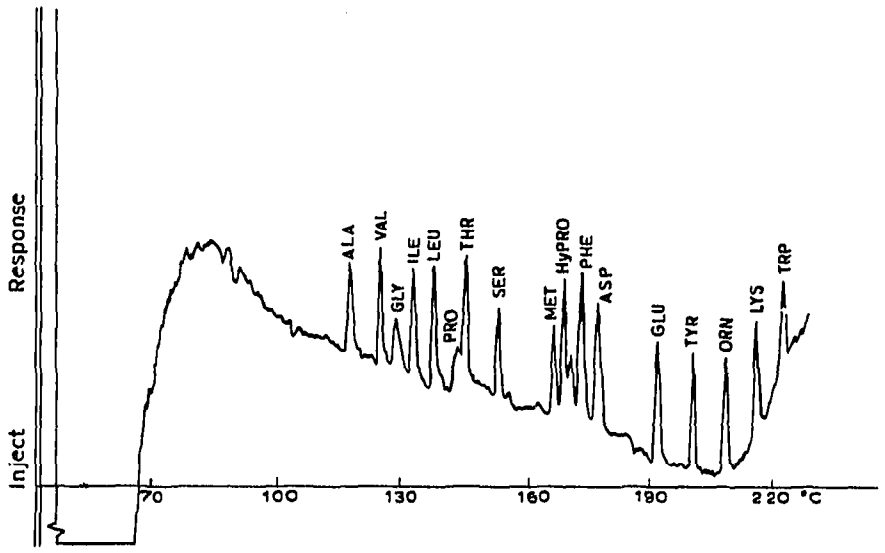


Fig. 7. Derivatization and GC analysis of 5 ng of each amino acid. Column and pre-column as in Fig. 5. Esterification was carried out with 25 μl of *n*-butanol that was 3 *N* in HCl at 100° for 70 min; acylation with 25 μl TFAA–CH_2Cl_2 (1:9) at 100° for 20 min. Amount injected: 25 μl. Solvent vent time: 30 sec. Temperature programme: 70° for 4 min, then 6°/min to 230°. Attenuation: $8 \cdot 10^{-12}$ a.f.s. Detector: FID. Reproduced from *J. Chromatogr.*, 57 (1971) 193, by kind permission of the authors.

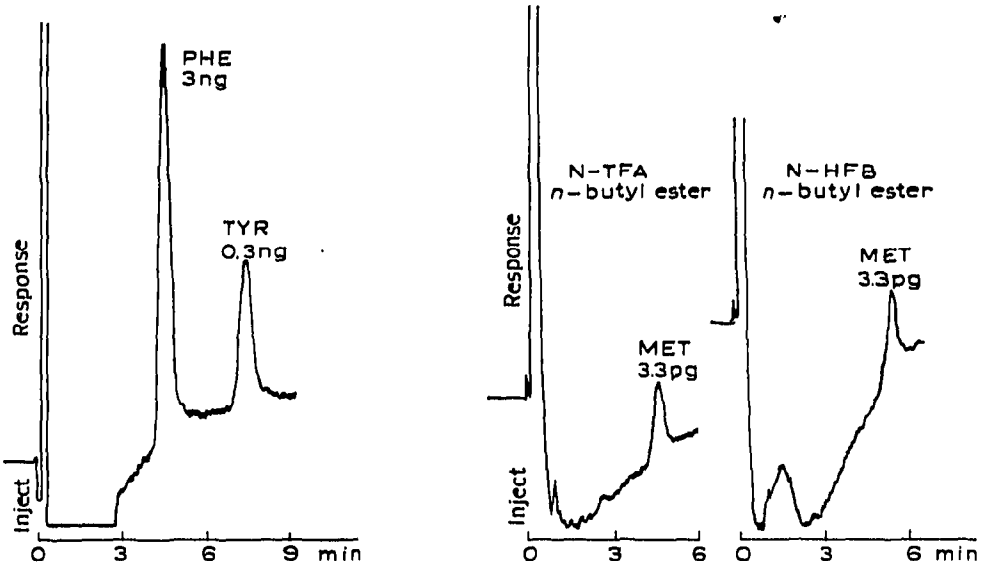


Fig. 8. GC analysis of nanogram and picogram amounts of N-TFA-butyl esters of Phe and Tyr and N-TFA- and N-HFB-butyl esters of Met. Column: 1 m × 4 mm I.D. glass, with 3% OV-101 on 80–100 mesh Chromosorb G HP. Solvent: diethyl ether. Detector: ^{63}Ni ECD maintained at 2.5 V d.c. Attenuation: $1 \cdot 10^{-10}$ or $3 \cdot 10^{-11}$ a.f.s. Reproduced from *J. Chromatogr.*, 57 (1971) 193, by kind permission of the authors.

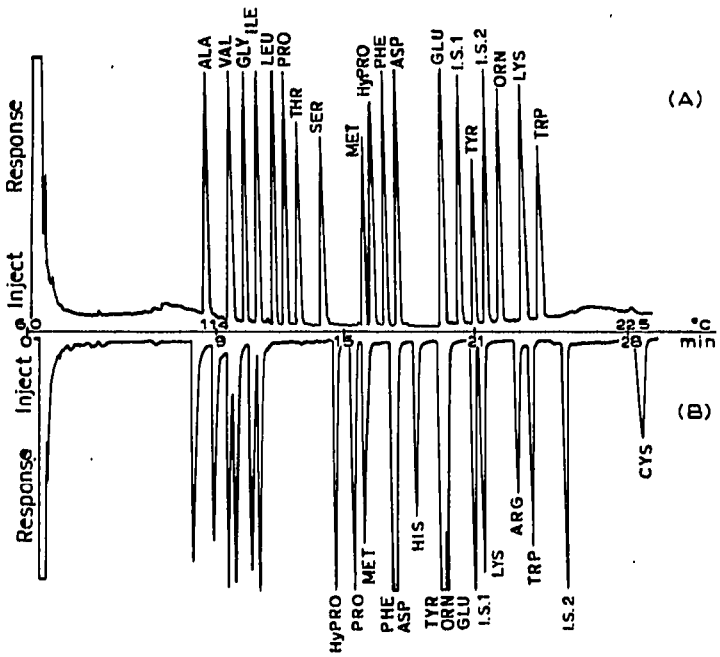


Fig. 9. Simultaneous GC separation of N-TFA-butyl esters of the protein amino acids in the dual-column system. Column I: 0.65% stabilized grade EGA on Chromosorb W AW, 1.5 m × 4 mm I.D. Column II: 3% OV-17 and OV-210 (2:1) on 100–120 mesh Supelcoport, 1 m × 4 mm I.D. Temperature programme: 60° initial temperature, programme rate 6°/min, final temperature 225°. I.S.1 = tranexamic acid; I.S.2 = *n*-butyl stearate. Reproduced from *J. Chromatogr.*, 57 (1971) 209, by courtesy of C. W. Gehrke.

The stabilized EGA polyester phase proved to be convenient not only for the separation and quantitative determination of 17 protein amino acids but also for several other derivatized compounds which could be analyzed simultaneously. Thus, together with the 17 protein amino acids, 20 other non-protein amino acids were quantitatively analyzed (Fig. 10)[305]. In another report, 35 amino acids together with 2 amino sugars (glucosamine and galactosamine) could be seen on the chromatogram[43]. After butanol–TFAA treatment, glutathione also yielded a product that could be chromatographed in the presence of the protein amino acids[234]. It is of interest that the oxidized (GSSG) and reduced (GSH) forms of glutathione gave an identical derivative, the relative molar response corresponding to GSSG being twice as high as that found for GSH. A modified esterification technique, based on variation of the esterification time during the direct butanol treatment[309], enabled the quantitative determination of Asn, Gln and pyrrolidone carboxylic acid to be achieved[144]. A simple apparatus for the derivatization of amino acids to their N-TFA-butyl esters, particularly suitable for the analysis of amino acids in biological material, was proposed in one report[235]. A study on the purification of N-TFA-butyl esters, *i.e.* removal of the incompletely acylated ^{14}C-labelled amino acid esters, by means of column chromatography with silica gel and diethyl ether–light petroleum (1–50%) as the eluent, has also been described[387a].

The analysis of amino acids in the form of N-TFA-butyl esters was the most

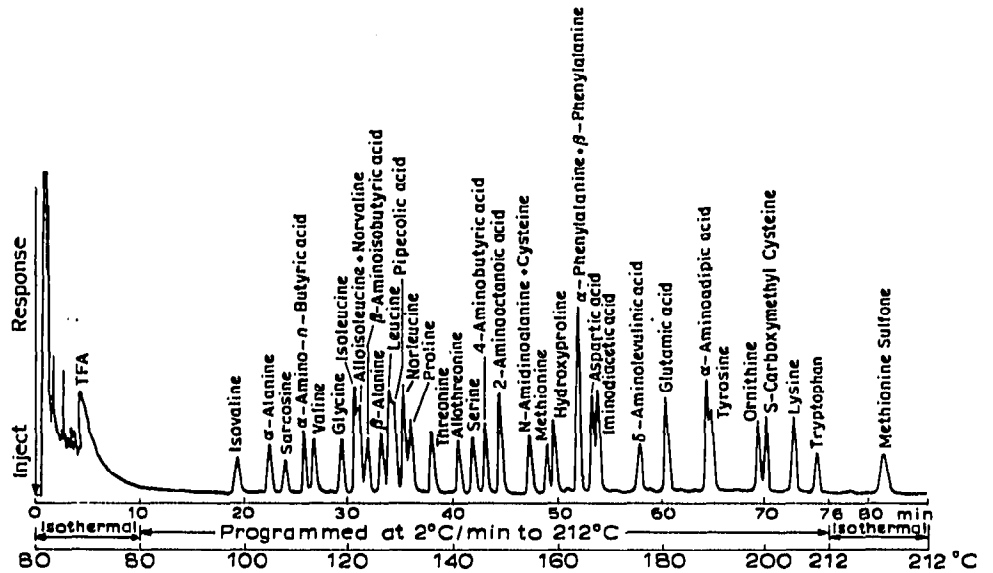


Fig. 10. GC analysis of an equimolar solution of 17 protein and 20 non-protein amino acids on a 1.83 m × 4 mm column packed with 0.325% EGA on 80–100 mesh H.T. Chromosorb G AW. Programme: 80° for 10 min, then 2°/min to 212°, held for 20 min. Reproduced from *J. Chromatogr.*, 73 (1972) 35, by courtesy of F. Raulin.

widely used procedure in application studies[36,41,42,71,89,95,102,107,157,169–171,177,199,225,228,233,277–279,301,363,371,373,412,414].

3.2.1.6. N-Trifluoroacetylamyl esters. Investigation of this derivatization technique, used during 1962–1967, already belongs to the past. These studies were carried out exclusively by Blau and Darbre[26–28,60–64] with only one exception[368]. The authors stated that only the amyl esters could be handled with any certainty of obviating the evaporation losses. Experiments carried out with Ala revealed that serious losses occurred with its N-TFA-methyl, -ethyl, -propyl and even -butyl esters when subjected to a stream of argon at 250 ml/min[62]. No loss was observed with the N-TFA-amyl ester under the same conditions. However, Lamkin and Gehrke[190] did not find any losses with the N-TFA-butyl esters of Ala, Gly and Val.

Esterification of the carboxyl group was accomplished by passing HCl continuously through amyl alcohol heated at 108° for 25 min with the addition of TFA acid as a solvent. The subsequent acylation was performed with TFAA at room temperature. It is interesting that only isothermal operations were evaluated together with the GC analysis of the N-TFA-amyl esters. The main difficulties were encountered in the search for an appropriate liquid phase. The use of DEGS (25%) led to the elution of only 8 of the original 13 derivatized amino acids[368]. In a series of studies[27,28,62], more than 100 stationary phases were evaluated for their usefulness in separating the N-TFA-amyl esters. Many of them were rejected because of the breakdown of their esterified hydroxyl (and sulphydryl) groups[63]; regardless of the alkyl group of the esterified carboxyl, the trifluoroacetylated derivatives of Tyr, Ser, Hypro, Thr and CysH undergo rapid hydrolysis on the O- or S-esterified groups[26,61] (see also 3.2.1.3).

At first, the PEGA polyester and QF-1 silicone[28,60,62], together with the MS-710 silicone phase[27], were found to be the best liquid phases. Finally, two column fillings were evaluated in connection with the analysis of the 20 protein amino acids as N-TFA-amyl esters[64]. The 9 more-volatile amino acid derivatives were well resolved at 135° on a 3–5-m glass column filled with a mixture (3:2) of 5% XE-60 and 5% MS-550, both phases coated on Anakrom ABS. For the analysis of the 11 remaining less-volatile derivatives at 170°, a 5% coating of QF-1 and MS-710 (mixed in the ratio 53:47) was found to be the best. Arg and Trp, however, were incompletely resolved; His gave no identical volatile product and Cys was eluted after about 6 h. In addition, the retention times of the N-TFA-dialkylated esters (methyl to amyl) of Asp, Glu and Cys were reviewed for comparison purposes[64].

3.2.1.7. N-Heptafluorobutyryl(pentafluoropropionyl)methyl to isoamyl esters. For the esterification of the carboxyl group either methanol[183], propanol[176,249,250], butanol[289,413] or isopentanol[405] was used. The propylation technique of Coulter and Hann[53,54], based on direct treatment of amino acids with propanol that was 8 *N* in HCl, was generally adopted for preparing the propyl ester; the transesterification procedure[354] was found to be the best for producing the isoamyl esters. Acylation with HFBA alone[176,183], or in a mixture with ethyl acetate[249,250] or acetonitrile (1:5)[405], was usually performed at 150° for 10 min, and in one instance also at 100° for 4 h (for quantitation of His and Arg)[183]. Evaporation of the acylating agent resulted in immediate decomposition of diacylated His to the monoacyl form. Thus, in agreement with the study of Roach *et al.*[311], the on-column conversion of the monoacyl back into the diacyl derivative of His by co-injection of HFBA[176] or acetic anhydride[250] was the preferred way of analyzing this amino acid. In the latter instance the 3- or 5-N-acetyl-N$^{\alpha}$-HFB-propyl ester of His was probably formed; the retention times of the other amino acid derivatives remained unchanged. Unlike the TFA moiety, the O-HFB esters were found to be much stable and resistant to hydrolysis even when subjected to treatment with water during the esterification procedure[249]. Moreover, all of the amino acids that commonly occur in proteins have been separated on single columns with a stable silicone stationary phase[176,250,405]. Acylation with pentafluoropropionic anhydride (PFPA) was also found to be very convenient; however, the cost and not always easy availability of the reagent detracted from its wider use.

Pollock[289] investigated the time saving when N-HFB- and N-PFP-butyl esters instead of N-TFA-butyl derivatives of 14 protein amino acids were analyzed on a capillary column coated with Carbowax 20M. During three isothermal operations (100°, 140° and 170°), it was found that the HFB esters exhibit about a 35% shorter retention time and the PFP esters about 30% shorter. The use of an ECD in conjunction with these derivatives was also proposed in order to increase the sensitivity. The minimum detectable amount using an ECD was found to be 1 pg for the N-HFB-butyl ester of Met and 2 pg for that of CysH (the sensitivity of the corresponding N-TFA esters was about 3–10 times lower)[413].

The first successful analysis of the HFB esters of all protein amino acids was reported with the use of N-HFB-propyl esters[250]. The derivatized compounds were resolved on a 3.66-m column filled with 3% OV-1 on 80–100 mesh Chromosorb W HP (Fig. 11). This derivatization technique was further extended by the same workers in order to determine Hypro and Hylys (in collagen, which is the only mammalian protein that contains appreciable amounts of these hydroxylated amino acids)[249], which

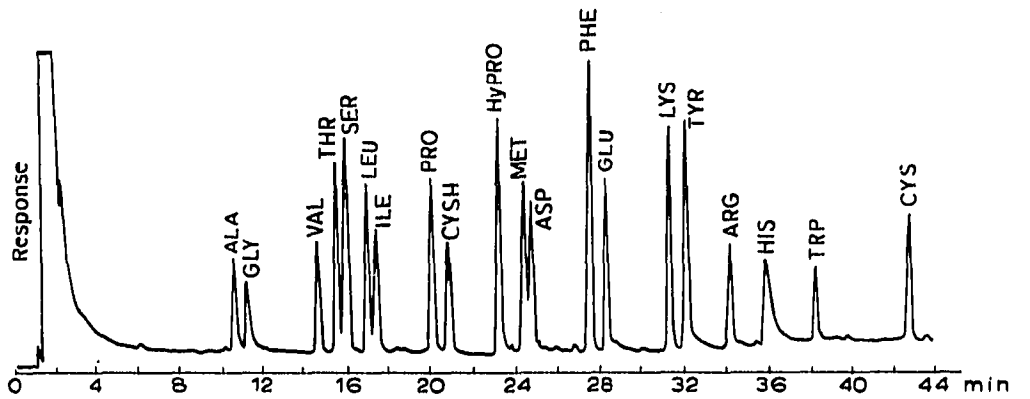


Fig. 11. GC analysis of N-HFB-propyl esters of the 20 protein amino acids. Column: 3.66 m × 6 mm O.D. with 3% OV-1 on 80–100 mesh Chromosorb W HP. Programme: 100° for 5 min, then at 4°/min to 250°. Reproduced from *J. Chromatogr.*, 60 (1971) 134, by courtesy of C. W. Moss.

were analyzed together with the other protein amino acids on a 2.44-m column coated with 15% Dexsil on Chromosorb W AW DMCS at 140–270°. The success of the technique prompted other investigators[176] also to choose N-HFB-propyl esters for further studies. A 6-m glass capillary with chemically bound dimethylsiloxane polymer allowed the resolution of all protein amino acids within 35 min.

Zanetta and Vincendon[405] preferred the N-HFB-isoamyl esters, which were said to be the only ones which could be concentrated without any loss of material. After having accomplished the acylation the reagent was evaporated and the derivatives, dissolved in ethyl acetate, were injected into a 3.5-m column filled with 3% SE-30 on Gas-Chrom Q (Fig. 12). His was thus analyzed in its monoacylated form.

The OV-1 column filling, recommended for analysis of N-HFB-propyl esters

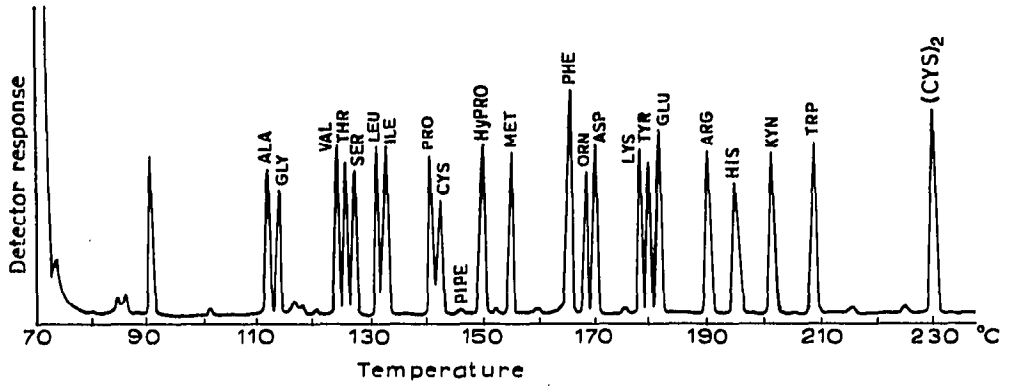


Fig. 12. Analysis of N-HFB-isoamyl esters of the protein amino acids. Column: 3.5 m × 2 mm I.D. with 3% SE-30 on Gas-Chrom Q. Temperature range 70°–240° at 4°/min. Injector temperature: 265°. Detector temperature: 280°. Carrier gas (N_2) flow-rate: 20 ml/min. Pipe = pipecolic acid; Kyn = kynurenine. Reproduced from *J. Chromatogr.*, 76 (1973) 91, by courtesy of J. P. Zanetta.

by Moss *et al.*[250], was also used in the analysis of N-HFB-methyl esters[183]. Their preparation was achieved by using an open aluminium or gold capsule (20 μl volume), which functioned as a micro-reactor in an automatic capsule-dosage system (MS 41). On the specified column, however, not all of the derivatives could be resolved.

Both the N-HFB-propyl and -isoamyl esters were applied successfully for analysis of the amino acids of insulin[176,405] and collagen[249].

3.2.1.8. Other N-acylalkyl esters. The N-carbobenzyloxy(CBO)-methyl esters of Hypro and some of its analogues were analyzed (with free OH groups) on the fluoroalkylsilicone QF-1; in the esterified form, *i.e.*, as O-TFA-N-CBO-methyl esters, they were separated on NPGS polyester[160,248]. The QF-1 liquid phase was also employed for the analysis of the N-benzoylmethyl ester of Gly[82] and the N-propionyl-isoamyl derivatives of 14 amino acids, which were prepared by treatment of amino acid isoamyl esters with propionyl chloride[67]. This technique was used to study the amino acid pool of *Candida utilis* in various types of culture. The N-CBO-methyl and -ethyl esters of several amino acids and the N-palmitoylethyl ester of Leu were also analyzed on SE-30 silicone[288].

In a comprehensive study of the GC of the N-acyl-(acetyl to enanthyl) amino acid alkyl esters (methyl to hexyl), Fu and Mak[91,92] established the conditions for preparing such derivatives and evaluated their retention times on both Carbowax 20M and GE-XE-60. The amino acids were first acylated with an anhydride or acid chloride at 20–30° for 10 min with vigorous stirring, and subsequently esterified with alcohol in dry benzene in the presence of Amberlite IR-120 (H^+). Symmetrical substitutions on the N-(X_1) and C-(X_2) terminals of the amino acid were made (*e.g.*, CH_3: N-acetylmethyl, C_2H_5: N-propionylethyl, etc., up to C_6H_{13}: N-enanthylhexyl ester) and the retention times of the derivatized compounds were evaluated. When the logarithm of the retention time was plotted against the number of carbon atoms in the substituting chains, a linear dependence was obtained. Thus, the effects of the substituents X_1 and X_2 were shown to be additive.

3.2.2. Silylation of amino acid alkyl esters, leading to N-trimethylsilylbutyl (-methyl, -ethyl) esters

Rühlmann and Michael[322,323] studied the properties of a number of silylated derivatives. Apart from the N-TMS-TMS esters (see 3.3.2), the silylated amino acid alkyl esters have been also evaluated[323]. The N-TMS-methyl and -ethyl esters of most of the protein amino acids were prepared by treatment of the amino acid alkyl esters with TMSDEA (see 3.3 for abbreviations) and chromatographed on methylsilicone phases. Their retention times were found to be about 15–20% lower than those of the corresponding N-TMS-TMS esters.

In spite of the fact that this derivatization technique requires an additional step, which is unneccessary in the case of direct silylation of amino acids, it was applied successfully to the GC analysis of 20 protein amino acids, including Hypro and CysH, by Hardy and Kerrin[142]. The amino acids were esterified with *n*-butanol that was 3 *N* in HCl at 150° for 15 min following silylation with BSTFA at the same temperature for 90 min. This long silylation time is obviously a disadvantage of this procedure if it is compared with the acylation technique, which is accomplished in a few minutes. Silylation with BSTFA was performed with either CH_3CN or CH_2Cl_2 as silylating solvents. In the former case, double derivatives of Gly and Lys (the

-NH-TMS and -N-$(TMS)_2$ compounds) were formed, whereas in the latter only the less silylated forms appeared on the chromatogram. As Arg also afforded a peak, unlike the direct silylation of the native amino acid with BSTFA–CH_2Cl_2 reagent[99], the use of CH_2Cl_2 as a silylating solvent was recommended. The use of a single column, filled with 0.2% OV-7 on 100–120 mesh GLC-110 textured glass beads, for the separation of the 20 derivatized amino acids is advantageous (Fig. 13).

Silylation of the methyl ester of urinary Phe was employed in a study aimed at the elucidation of Phe–Tyr metabolism in human beings[58].

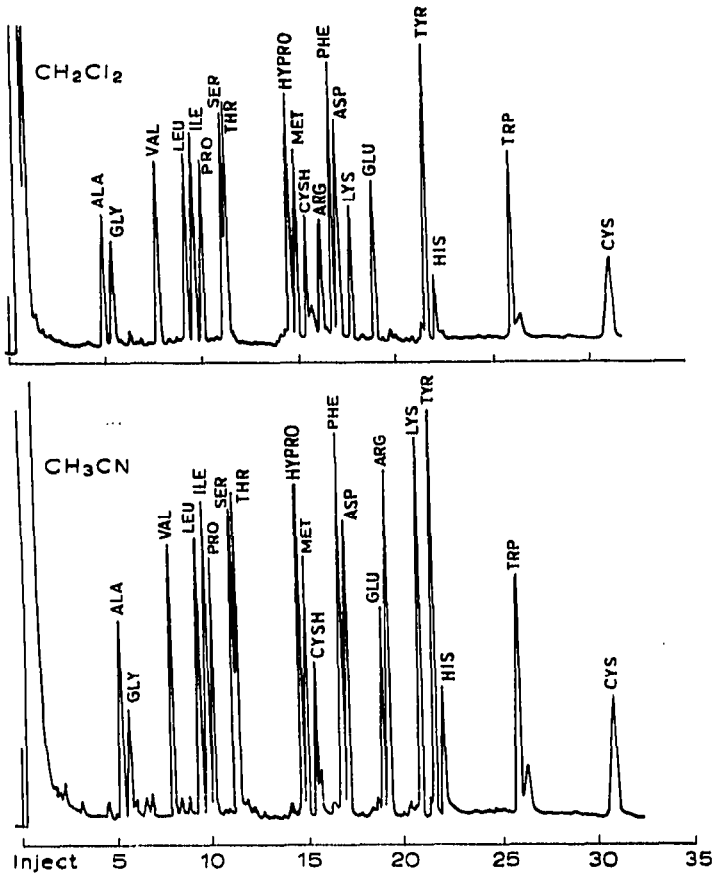


Fig. 13. GC separation of N-TMS-butyl esters of 20 amino acids. Silylation performed with BSTFA with either CH_3CN or CH_2Cl_2 as a solvent. Column: 2.44 m × 2 mm I.D. filled with 0.2% OV-7 on 100–120 mesh GLC-110 textured glass beads (Corning). Programme: 90° for 4 min, 4°/min to 225°. Reproduced from *Anal. Chem.*, 44 (1972) 1497 by kind permission of the publisher.

3.2.3. "Alkylation" of amino acids or their alkyl esters, leading to N-"alkyl"alkyl esters

It should be stated that all of the reagents treated in this section are not the real alkyl compounds derived from alkanes (for example, derivatization with DECP*[79]

* DECP = Diethyl chlorophosphate.

or CS_2[127] could be treated equally together with the acylation techniques in the former case or with the Schiff base formation in the latter case), but from a general point of view we have designated them as "alkyl" compounds and the techniques are assembled in this section. The use of these rather special derivatization agents proposed mainly in order to utilize some of the advantages connected with their application. The N-DNP esters were utilized, for example, in order to take advantage of the strong electron capturing property of the 2,4-DNP moiety in the case of electron capture detection, which, with this method of derivatization, was initially applied to the amino acid field[195,196]. The use of a phosphorus-containing reagent[79] is unique among the various derivatization techniques and allows the detection of extremely small amounts of amino acids in the phosphorus-sensitive AFID. A higher reactivity of unsaturated compounds[164] and, for example, carbon disulphide[127], which resembles the easy formation of a Schiff base (see section 3.2.4.), seemed worth investigating as a useful derivatization method. However, the methods are rather restricted and, except for the DNP procedure, have not been further investigated. This does not mean that they would be inconvenient but the most successful of them[127] is too tedious and it was developed at a time when the trifluoroacetylation of amino acid butyl esters[102] proved to be a more promising approach. Moreover, as the derivatization techniques presented affect only the α-amino group, two additional chemical treatments are necessary if the main task is to analyze all 20 protein amino acids. Silylation, the only method that could convert all of the remaining reactive groups into an esterified form simultaneously, was unfortunately not evaluated as the second reaction step.

3.2.3.1. N-Cyanoethylethyl or -butyl esters. Iwamoto and Morimoto[164] treated the ethyl esters of Ala, Gly and Leu with acrylonitrile and obtained the corresponding N-cyanoethylethyl esters:

$$\begin{array}{l} R\text{–}CH\text{–}COOC_2H_5 \\ \quad | \\ \quad NH_2 \end{array} + CH_2{=}CH\text{–}CN \rightarrow \begin{array}{l} R\text{–}CH\text{–}COOC_2H_5 \\ \quad | \\ \quad NH\text{–}CH_2CH_2CN \end{array}$$

The derivatives were separated on a 0.75-m column with DC-550 silicone on firebrick at 200°. The retention times ranged from 2.6 min (Ala) to 5.4 min (Leu). The compounds were further acylated with acetic anhydride to give the corresponding N-acetyl-N-cyanoethylethyl esters:

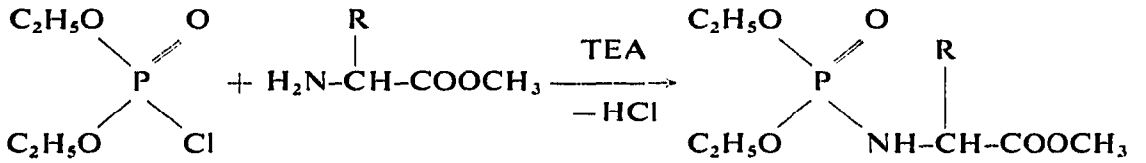
$$\begin{array}{l} \quad R\text{–}CH\text{–}COOC_2H_5 \\ \quad\quad | \\ H_3C\text{–}OC\text{–}N\text{–}CH_2CH_2CN \end{array}$$

which, on the same column packing at 200°, gave *ca.* 3-fold higher retention times. On a PEG phase (Carbowax 1540), the retention was significantly lower; the corresponding times at 148° were 3.0 min for Ala and 4.3 min for Leu.

3.2.3.2. N-Dinitrophenylmethyl esters. The N-DNP-methyl esters have been prepared by esterification of the DNP-amino acids (see 2.2.2) with diazomethane or boron trifluoride in methanol[288]. They were separated mostly on silicones (SE-30, XE-60, XE-61, QF-1) and also on polyesters[11,195,196]. Because of the relatively high molecular weights, operating temperatures above 200° were usually required. Satisfactory results were obtained for the simple alkyl and acidic amino acids, whereas

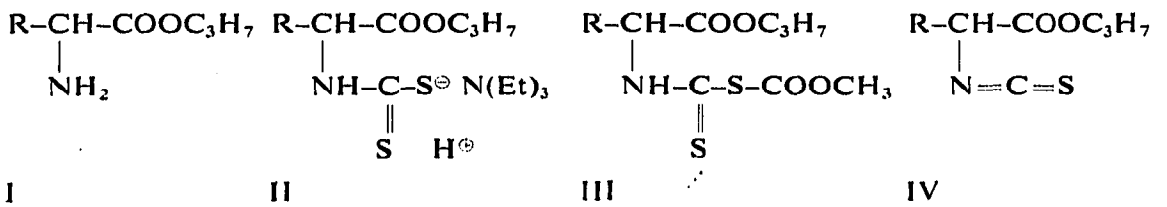
His, Ser, Thr, Tyr and the basic amino acids were not analyzed successfully because they decomposed in the column or owing to their low volatility[156,288]. Despite this, Landowne and Lipsky reported a successful analysis of di-DNP-Lys, -Orn and -Cys on a column coated with 3% NPGA (or NPGS) or with XE-60 nitrile-silicone[195,196]. These amino acids were chromatographed together with 15 other simple amino acids (including Asp and Glu) at 220–240° using an ECD. Sensitivities of the order of $3 \cdot 10^{-16}$ moles/sec were obtained for all common DNP-amino acids, indicating that the response was strictly a function of the DNP group alone and was independent of the remainder of the molecule. Thus, calibration for individual amino acids was unnecessary. The di- and the mono-DNP compounds of Lys, Orn and Cys exhibited the same response (as soon as one of the groups has captured an electron, a negatively charged molecular ion is formed which does not capture further electrons). Ikekawa *et al.*[156] succeeded in separating 13 amino acids on a 4-m column coated with 1.5% SE-30 operated in the temperature range 170–230° (at 1.7°/min). Only the Thr and Ser pair remained unresolved (these two amino acids, together with Hypro, were additionally silylated with HMDS–TMCS in pyridine at 20°) on this column, but their resolution was achieved using a 1.5% XE-61 (35% phenylmethylsilicone) phase. The method was applied to the analysis of amino acids in serum. The DNP-amino acid methyl esters were also chosen for the analysis of several amino acids in the peptide hydrolyzate malformin A (a metabolic product of a fungus which induces growth malformations in several plants)[6] and gramicidin A[161].

3.2.3.3. N-Diethylphosphorylmethyl esters. Diethyl chlorophosphate was used as the phosphorylating agent in diethyl ether or acetonitrile solution in the presence of triethylamine as HCl acceptor[79]:

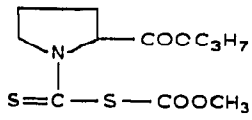


The reaction proceeded at room temperature for 30 min. The yields were not quantitative (approx. 45% for Gly). However, qualitative detection of 11 amino acids (the simple ones plus Asp, Glu and β-Ala) at the picogram level was possible using an AFID (with Rb_2SO_4 salt). The minimum detectable amount of Ala was $5 \cdot 10^{-12}$ g. The best separation of the derivatives was achieved on a 2-m column filled with 6% OV-17 on Chromosorb G HP, at 210–250°. The method was applied to the analysis of five amino acids in gramicidin S (250 pg of the peptide were subjected to hydrolysis and subsequent derivatization).

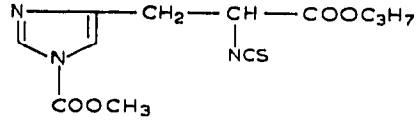
3.2.3.4. N-Thiocarbonylpropyl esters. The following scheme can be written for this derivatization technique[127]:



The reaction involves esterification of amino acids with propanol that is 8 *N* in HCl (I) followed by treatment of the dry residue with CS_2 (10 μl) and TEA* (8 μl) in 100 μl of CH_2Cl_2. After 1 h at 20°, the product II is treated with methyl chloroformate (2 μl), which results in the formation of the carbomethoxy dithiocarbamate (III) after an additional 1 h at 20°. The solution is then washed twice with citric acid (1 ml, 20%, w/v) and CH_2Cl_2 (5 ml), leading to the decomposition of III into the N-thiocarbonylpropyl ester (IV). After subsequent evaporation of the solvent, the derivatives were analyzed on a 1.83-m glass column filled with 5% QF-1 on Gas-Chrom P DMCS with temperature programming from 94 to 235° (rate 4°/min, final hold at 235° for 25 min). Only the Leu–Ile pair was not separated; Arg gave no volatile product. The other derivatives were fully characterized by MS; during treatment with the chloroformate ester, the hydroxyamino acids, CysH (and also Cys) react further to yield carbonate esters (–O–$COOCH_3$). The imidazole nitrogen of His is protected (the indole function of Trp is unchanged) and Pro remains as the stable carbomethoxy dithiocarbamate:



Pro



His

The reproducibility of the method was found to be 5%. A quantitative study using FID and ECD revealed that amounts of 10^{-10} and 10^{-13} moles, respectively, can be detected by this technique.

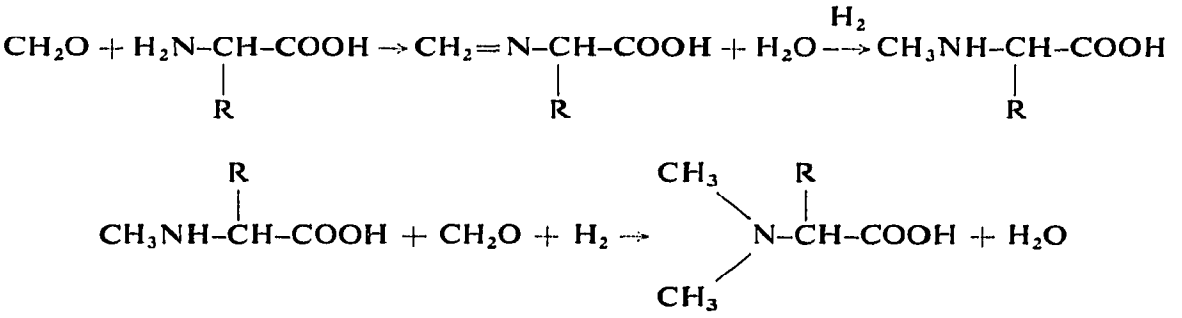
3.2.4. Condensation of amino acids or their alkyl esters with carbonyl compounds, resulting in formation of Schiff bases

Schiff-base formation, one of the well known basic reactions of organic chemistry, was also evaluated with respect to the determination of amino acids. Despite the distinct advantage of such a chemical process that the coupling between a carbonyl and an amino group usually proceeds under very mild conditions and almost quantitatively, this approach is less convenient owing to the multifunctional nature of protein amino acids. In the preceding section it has already been stated that all of the procedures that aim to modify the amino group alone are disadvantageous because two additional reaction steps are required. The only chemical treatment that could be used in a subsequent operation for both the carboxy and the other protonic groups would include, for example, use of a strong silylating agent such as BSTFA. It is to be regretted that the silylation of Schiff bases was not examined in any of the studies mentioned as this step could bring a considerable improvement to the methods. Thus, the condensation reactions are restricted to the determination of only a few amino acids, documented, for example, by analysis of Lys in biological material[316,409,410].

3.2.4.1. N,N-Dimethylmethyl esters (via N-methyleneamino acids). Aqueous formaldehyde condenses with an amino acid in the presence of palladised charcoal in

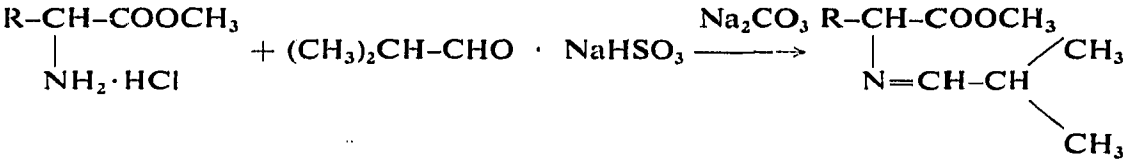
* TEA = Triethylamine.

a hydrogen atmosphere (the period of reduction varying from 3 to 12 h at 20°) to yield the corresponding N,N-dimethylamino acid (which can be further esterified, for example, with diazomethane):

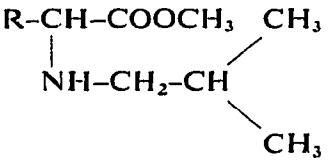


The dimethylation reaction was found to be limited for general purposes and the N,N-dimethylamino acid methyl esters were found to be very volatile[25]. The undesirable volatility of these compounds led to low recovery of derivatized Ala, Gly, Val and Leu when evaporating the ethereal CH_2N_2 solution at room temperature. No other work has been reported in this area.

3.2.4.2. N-isobutylidene (to N-isobutyl)-, N-neopentylidene- and N-benzylidene-methyl (or -ethyl) esters. The methyl ester hydrochlorides of amino acids react with isobutyraldehyde and sodium bisulphite in sodium carbonate solution according to the following equation[66]:



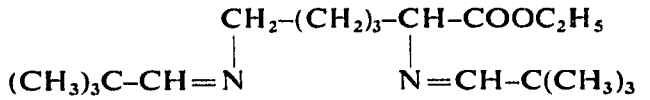
The alkylidene amino acid esters formed were chromatographed either as such (Leu) or after reduction with zinc powder in methanolic HCl to give the alkyl ester:



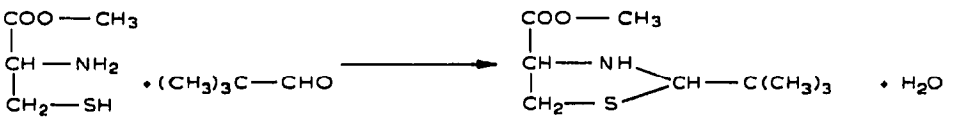
The N-isobutylmethyl esters of most of the amino acids except His, Arg, Trp, Tyr and Hypro were separated on capillary columns coated with Carbowax 1540 at 102–165°. The derivatives, prepared in bulk (in gram amounts), were obtained in yields between 50 and 98%.

In other studies a method for the quantitative determination of Lys in crude acid hydrolyzates of wheat and rice seeds has been developed[316,409,410]. The ethyl

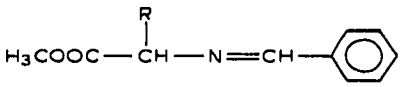
ester of Lys was dissolved in ethanol and then condensed with pivalic aldehyde at 50° for 90 min. The resulting bisneopentylideneethyl ester:



was chromatographed on a column coated with OV-17 on Chromosorb W HP, using phenanthrene as an internal standard. Jellum *et al.*[168a] treated methyl esters of sulphur-containing amino acids (CysH, CysM, Cys, homoCysH, homoCys, homoCys-thiol-actone, Met and a mixed disulphide formed from cysteamine and CysH ethyl ester) with pivaldehyde in the presence of Bio-Rad AG 1-X8 resin (HCO_3^-) and a molecular sieve (3 Å) at room temperature for 10 min. The Schiff bases were then chromatographed on a 1.5-m column filled with 5% SE-30 on Aeropack 30 and they could be well resolved at 80–250° (10°/min). Condensation of CysH methyl ester with the pivaldehyde led to formation of a cyclic derivative, 2-*tert.*-butyl-4-methyloxycarbonyl-1,3-thiazolidine:

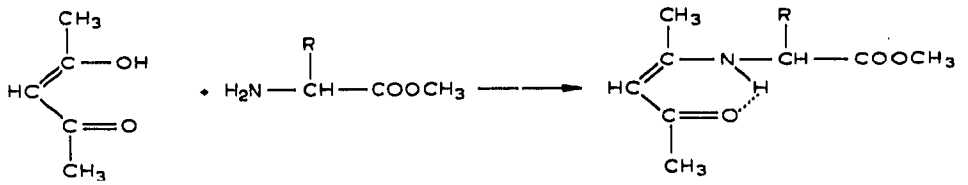


The coupling of amino acid methyl esters with benzaldehyde was stated to proceed under very mild conditions[240]. Mere mixing of an amino acid methyl ester with pyridine–benzaldehyde–methanol (1:1:10) was reported to be sufficient for derivative formation. The resulting derivatives, the N-benzylidenemethyl esters:



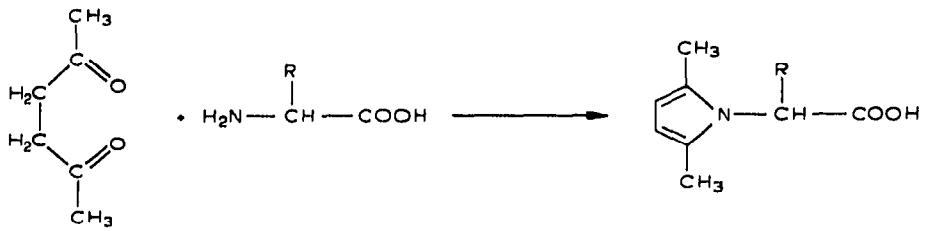
of protein amino acids were chromatographed between 100° and 280° on a column coated with SP-400. Pro and Hypro gave no condensation products (owing to the secondary amino group present in the molecule); Try, Arg and His gave only very poor peaks.

3.2.4.3. N-Isopentenonyl- and α-dimethylpyrrolylmethyl esters. Because the condensation of the secondary amino group of Pro and Hypro with benzaldehyde led to no volatile products, Mitchell examined the use of acetylacetone (2,4-pentanedione) for this purpose[240]. The pentanedione Schiff bases are present in the form of enamines stabilized by a hydrogen bond, so that Pro and Hypro could also be chromatographed in the enamine form:



The reaction between amino acid methyl esters and pyridine–acetylacetone–methanol (1:1:10) proceeded at 80°. After 10 min, the resulting N-2-isopentene-2-one-4-yl-methyl esters of all protein amino acids were subjected to GC analysis on SP-400. The analysis of Thr, Hypro, His, Tyr and Arg failed.

Walle[386] reported the condensation of amino acids with 2,5-hexanedione:



The condensation product, α-2,5-dimethylpyrrolylcarboxylic acids, was then esterified with methanol–HCl. Only a few amino acids were reported and no quantitative data were given.

3.3. Methods based on derivatization of the α-amino and carboxyl groups in one reaction step

There has been a persistent effort to simplify the derivatization technique by introducing new reagents that will allow the reaction to proceed faster and with a minimum of successive reaction steps. Investigations in this area are virtually unrestricted as they are in close relationship with progress in organic chemistry. Each new derivatization reagent developed in this branch of chemistry can be evaluated also for microscale analytical procedures. A classical example is the rapid development of the silylation technique (the technique is more correctly termed trimethylsilylation). Formerly, only two reagents were available, namely trimethylchlorosilane (TMCS) and hexamethyldisilazane (HMDS); strictly bistrimethylsilylamine. Later, two silylated dialkylamines were synthesized, trimethylsilyldiethylamine and -dimethylamine (TMSDEA and TMSDMA). Finally, as the most potent silylating agents, the silylated amides of acetic acid, N,O-bistrimethylsilylacetamide (BSA) and N-methyl-N-trimethylsilylacetamide (MSA) and their corresponding analogues derived from trifluoroacetamide (BSTFA and MSTFA), were brought onto the market, particularly by the Pierce Chemical Company (Rockford, Ill., U.S.A.)[282]. All of these silylating agents were applied to amino acid derivatization; the usefulness of MSTFA for the analysis of the protein amino acids is now under study (M. Donike, personal communication).

The "single-medium, single-reagent" one-step conversion of amino acids into their corresponding derivatives is still attractive, although there are limitations, *e.g.*, in the silylation technique. Apart from silylation, two other single-step methods were also adopted for the chemical modification of amino acids, *i.e.*, acylation and alkylation. This approach, however, seems to be more promising than it actually is. If all aspects of such procedures are taken into account, it can be stated that the easier the derivatization technique the more difficult it is to use it generally for the quantitation of all protein amino acids. Let us compare briefly the three methods.

Acylation is the shortest derivatization method. Unlike the silylation technique which requires a prolonged high-temperature treatment, acylation with TFAA is completed within 10 min at 150°. The simple amino acids gave good chromatographic peaks, indicating that the cyclic oxazolinon-5-es, although chemically very reactive, are thermally stable[117,118]. Unfortunately, no additional studies were conducted with regard to yields or with the other, multifunctional amino acids.

The silylation of amino acids is one of the oldest derivatization techniques used in the chromatography of amino acids. It has been developed into a sophisticated procedure that permits the quantitative analysis of the 20 protein amino acids[99,100]. However, satisfactory conversion into the derivatives requires a prolonged (2.5 h) high-temperature treatment and a long column (6 m) for their separation. The use of a polar silylating solvent (obligatory for Arg derivatization) leads to double-derivative formation with several amino acids[18,101] and in the case of Orn, for example, even to three derivatized forms[17]. Lastly, the persilylated derivatives are very sensitive to moisture, especially the silylated amino group. Even the best septa, when newly installed, cause the complete breakdown of silylated His, which is one of the most sensitive derivatives[346]. Some of the silanized supports were found to cause a partial breakdown of, for example, silylated Lys[343]. Thus, except for the advantage of a single-step operation, neither the derivatized compounds nor the reaction time encourages one to choose this procedure for general use.

The use of alkylated derivatives presents an opposite picture. The peralkylated compounds might be the best derivatives for GC analysis with regard to their stability and resistance to hydrolysis, provided that the alkylating agent used affords a sufficiently volatile compound. Their synthesis, based on the use of sodium hydride together with a halogenated alkane (and carried out in a solvent convenient for amino acids, such as DMSO), is known to proceed smoothly in general. However, the prognoses were unfortunately too optimistic. The peralkylated derivatives of amino acids exhibited excellent GC properties[280,281] but their preparation was far from quantitative and some multiple peaks were formed.

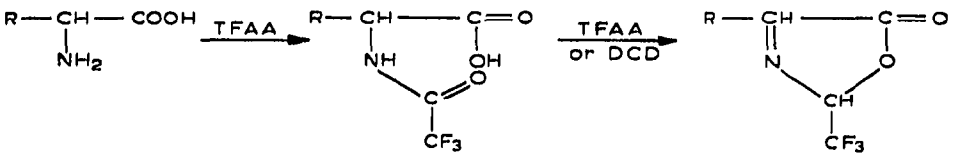
Having reviewed the single-step procedures doubts remain as to whether "universal" chemical treatments are the best choice. It seems, rather, that a compromise would be better. Two other reagents are now under examination, dimethylformamide dialkylacetals (DMF-DAA) and 1,3-dichlorotetrafluoroacetone (DCTFA), which are capable of derivatizing the α-amino and carboxyl groups simultaneously using an aprotic solvent, so that any subsequent derivatization of the remaining groups (*e.g.*, by acylation or silylation) can be accomplished in the same reaction medium. This derivatization technique can be assigned as a "single-medium, two-step" procedure.

The DMF-DAA reagents have been developed recently and they seem to be very promising for use in the treatment of various reactive groups. The reagent is capable of alkylating the carboxyl group(s) and condensing with the amino group(s) simultaneously[369]. The hydroxy groups are not affected in the derivatization and they require a further treatment, *e.g.*, with silylating agents. A lower volatility of the reagent causes the Ala and Gly to be obscured in the reagent peak on some types of column packing. Another disadvantage is the necessity to prolong the derivatization time to 1 h because of difficulties with Asp solubilization. Further details on this technique would be highly desirable.

The unique features of halogenoacetones (among them also DCTFA) have already been described[342]; however, the use of these reagents for the derivatization of amino acids was not successful owing to the lack of a convenient solvent[78]. This problem has now been solved[152,153] and the cyclic derivatives of all 20 protein amino acids have been prepared quantitatively by a mild temperature treatment within several minutes. Similar excellent results were achieved when the iodoamino acids were subjected to derivatization (see 4.3). The subsequent treatment of the other functional groups with TFAA or HFBA was considered to be a very promising additional method. Among the advantages of the DCTFA treatment are particularly the desirable features of the derivatives in GC analysis (stability, volatility, high sensitivity to ECD) and the simplicity and rapidity of their preparation.

3.3.1. Acylation of amino acids, leading to 2-trifluoromethyloxazolin-5-ones

The 2-trifluoromethyl-4-substituted-oxazolin-5-ones were synthesized directly by heating amino acids with TFAA[117,118] (for 10 min at 150°) or by the action of dicyclohexylcarbodiimide (DCD) on an N-acylated amino acid (Val[399] or Leu[118]):

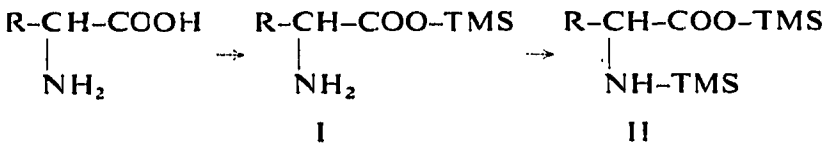


Eleven oxazolinones of simple amino acids were chromatographed on 0.325% EGA on 80–100 mesh H.T. Chromosorb G between 40° and 140° [117]. The 2-H-, 2-methyl- and 2-phenyloxazolinones of Leu were analyzed together with its 2-trifluoromethyloxazolinone on OV-17 silicone. The latter compound exhibited the shortest retention time.

The 2-trifluoromethyloxazolinones of Cysh and Ser were found as artifacts during analysis of N-TFA-amino acid methyl esters on a capillary column coated with Carbowax[125]. The derivatives were found to be very volatile.

3.3.2. Silylation of amino acids, leading to N-trimethylsilyltrimethylsilyl esters

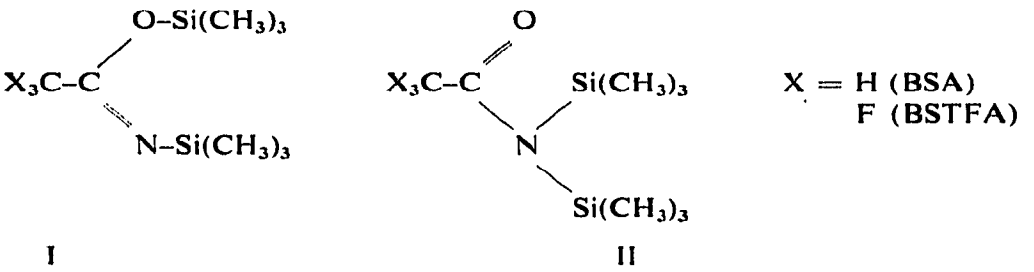
The TMS group was introduced into GC, in connection with derivatization of amino acids, in 1961[317]. Rühlmann and co-workers[317,322,323] pioneered the silylation technique using TMCS, HMDS or TMSDEA as silylating agents. The usefulness of these reagents, either alone or in combination, was examined further by Smith and co-workers[219,344] using both amino acids (Leu, Ser, Asp) and model compounds (*n*-octylamine, *n*-octanol and hexanoic acid). Valuable conclusions can be drawn from all of these earlier studies. Firstly, the silylation of an amino acid can be considered to proceed in two stages:



I II

Thus, on silylation, an amino acid can yield two products, as the carboxyl group is silylated more easily than the amino group. For silylation of the carboxyl group, a mild silylating agent, *e.g.*, HMDS or TMCS, is usually sufficient (see also 3.1.7). Silylation of other functional groups, such as –OH and –SH, requires stronger conditions; the amino groups as well as guanidine are the most difficult to silylate. Secondly, TMSDEA was found to be the preferred reagent of all those investigated. HMDS, either alone or together with TMCS and pyridine as solvent, did not give satisfactory results. Thirdly, the lability of one silylated group in comparison with another in the molecules of completely silylated amino acids varies markedly. The Si–N bond was found to be the most labile and therefore the N-TMS groups were hydrolyzed very easily. On the other hand, the O-TMS group was relatively very stable and resistant to hydrolysis. The silylated carboxyl group exhibited intermediate stability. In all instances, methylsilicone liquid phases were used for GC analysis.

Whereas in the procedures used earlier the silylated dialkylamines occupied a significant position, since 1966 they have been replaced by silylated amides. After being treated with MSA by shaking at 20° (in some instances heating to 60–100° was necessary), single peaks were obtained with all of the protein amino acids except Arg, Trp and Cys[23]. A revolution among the silylation techniques brought the introduction of BSA[181] and later also BSTFA[353] on to the market. These reagents were found to be 50 times more potent as silyl donors than any of the monosubstituted amides. Nuclear magnetic resonance and infrared spectra of BSA favours structure I rather than its isomer II, with rapid intramolecular exchange of the TMS groups between the oxygen and nitrogen atoms; on the other hand, with BSTFA formula II was considered to be more probable:



I II

Using BSA, and CH_3CN as solvent, 22 amino acid derivatives were prepared by heating the medium near to the boiling point; single chromatographic peaks were produced on SE-30[181]. Arg underwent decomposition on the column and, more serious, Ala and Gly derivatives were obscured by the peak of one of the reaction products, N-TMS-acetamide. When BSTFA was used for silylation of 18 amino acids by conducting the reaction in CH_3CN at 125°, the corresponding reaction product was eluted before Ala and Gly[353]. Using both bissilylated amides, Shahrokhi[333] and Shahrokhi and Gehrke[335] prepared quantitatively the TMS derivatives of 12 sulphur-containing amino acids by heating them at 150° for 5 min in a closed tube. The yields ranged from 95.5 to 99.2% with BSA as silylating reagent and CH_3CN as solvent (reagent: solvent ratio 1:3). BSTFA was recommended as the silylating agent for all of the sulphur amino acids (see Fig. 14) except for Met, Met sulphoxide and CysM.

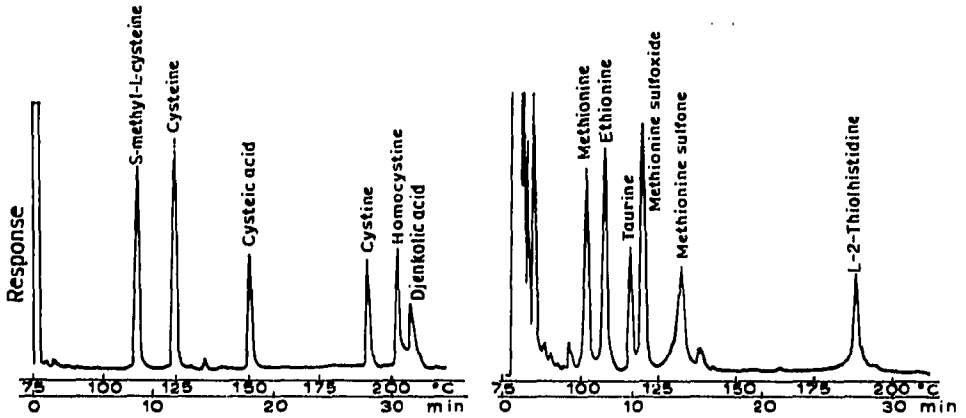
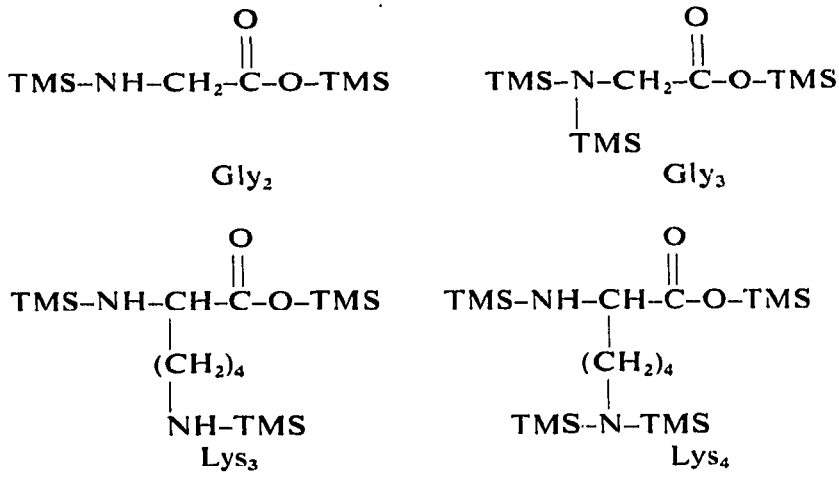


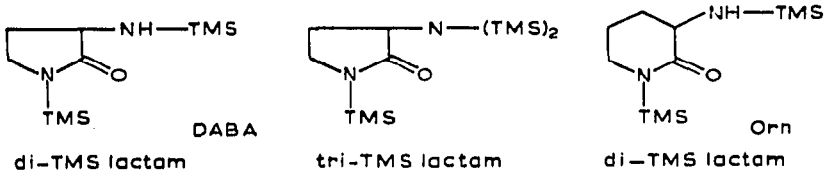
Fig. 14. Chromatography of N-TMS-TMS esters of sulphur amino acids. Column: 1 m × 3.5 mm I.D. with 0.5% SE-30 on 80–100 mesh Chromosorb G AW DMCS. Initial temperature, 75°; programme rate, 4.6°/min. Carrier gas (N_2) flow-rate: 40 ml/min. Reproduced from *J. Chromatogr.*, 36 (1968) 31, by kind permission of the authors.

Smith and Shewbart[345] reported in 1969 a quantitative comparison of TMS reagents for protein amino acids using Phe, Tyr and Lys as representative amino acids. This work compared TMSDMA, TMSDEA, BSA, BSTFA, MSA and TMSI (-imidazole) under various reflux conditions. It was concluded that the TMS amines were to be preferred to the TMS amides owing to the stability of the resulting derivative solution and the greater volatility of the silylated amine reagents. However, acetonitrile as a polar solvent was not used in connection with TMS amides and the optimal silylating conditions for these compounds were not presented. Albro and Fishbein[3] studied 11 different silylation systems for the silylation of Tyr and its 12 metabolites and Try and its 16 metabolites. HMDS, TMCS, TMSI, TMSDEA and BSTFA were employed in various combinations with or without pyridine as a solvent. The best yields were obtained with TMSDEA–TMCS (8:1) and BSTFA–TMSDEA–TMCS–pyridine (99:30:1:100) after 3 h at 20° or 10 min at 100°. This is the only report in which simultaneous treatment with a silylamide and a silylamine has been described. Bhatti and Clamp[19] studied the effects of solvent, temperature and length of heating on the preparation of N-TMS-TMS esters of 11 amino acids. The use of BSA alone or in combination with acetonitrile (1:3) was said to be equally effective (for Phe and Glu, however, the results with BSA alone were better) when reacted at 75° for 15 min.

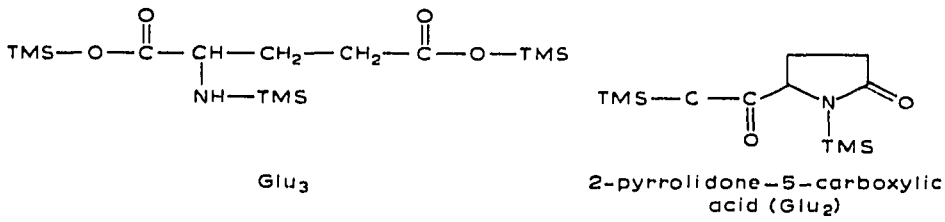
Bergström *et al.*[18] reported on the trimethylsilylation of 18 protein amino acids using BSTFA with and without acetonitrile as solvent at 125° for 15 min. In accordance with a subsequent study[17] concerning the GC and structural properties of silylated α-, ω- and α,ω-diamino straight-chain carboxylic acids with 2–6 carbon atoms, the double-step silylation of the ω-amino group was confirmed. This gave rise to double-derivatives of Gly (Gly_2 and Gly_3) and Lys (Lys_3 and Lys_4):



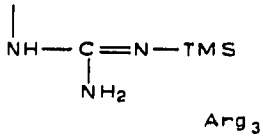
The silylation of these two protein amino acids proceeds to an equilibrium in which a few percent of the less silylated (more volatile) compound still remains[18]. More difficulties arise with the silylation of α,ω-diamino acids with 4 and 5 carbon atoms, where inner cyclizatio.. to lactams may occur. Therefore, 2,4-diaminobutyric acid, for example, forms, in addition to the persilylated compound (tetra-TMS-DABA), two more volatile cyclic compounds identified as di- and trisilylated lactams. Orn (2,5-diaminovaleric acid) yields, in addition to the Orn_3 and Orn_4 silylated forms (as also in the case of Lys), a third more volatile derivative, the di-TMS lactam[17]:



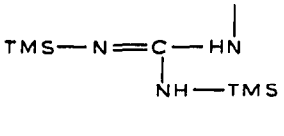
Similarly, Glu can form two products on silylation, the persilylated compound (Glu_3) and the cyclic derivative, 2-pyrrolidone-5-carboxylic acid[101]:



His and Trp may (His_3, Trp_3) or may not (His_2, Trp_2) be silylated at the imidazolyl or indolyl ring. Likewise, the double-silylated guanidino group gives the Arg_4 derivative; the less silylated guanidine gives Arg_3:



Arg_3



Arg_4

All the peaks can appear on the chromatogram. Gehrke and co-workers carried out the most complete study of the silylation technique and established the exact conditions for quantitative conversion of the 20 protein amino acids into N-TMS-TMS esters, including their separation on a single silicone-coated column[99–101,201,255,311,411].

In the first comprehensive report[101] on silylation with BSTFA-CH_3CN (1:1), 14 amino acids were derivatized at 135° for 15 min. However, Glu, Arg, Lys, Trp, His and Cys required a 4-h treatment at 135° in order to appear on the chromatogram as measurable peaks. Despite this, Gly and Glu formed two peaks even after such a prolonged treatment. Gly_3 was poorly separated from Ile and Pro so that its quantitative analysis in samples containing it in large amounts (*e.g.*, urine) was very difficult[255,411]. Some aspects of His quantitation, in connection with the analysis of ribonuclease, were treated in other reports[255,311]. The same GC conditions and silylation techniques were employed.

In their most recent work, Gehrke and Leimer[99,100,201] developed a superior silylation technique for the quantitative determination of the 20 protein amino acids. They showed the effect of polar and non-polar solvents on the derivatization of amino acids using BSTFA[99,201]. The number of chromatographic peaks corresponding to the TMS derivatives of Gly and Arg is determined by the polarity of the solvent. With hexane, methylene chloride, chloroform and 1,2-dichloroethane, one peak is obtained for Gly (Gly_2) and two peaks (Gly_2 and Gly_3) with six other more polar solvents. Arg gives no peak in the four less polar solvents studied (unlike the silylation of its butyl ester; see 3.2.2) and one peak in the other six more-polar solvents. The best and most reproducible silylation of 17 amino acids was achieved within 15 min at 150° using BSTFA–CH_3CN (1:1). However, for Gly, Arg and Glu, 2.5 h at 150° are necessary for reproducible derivatization. It is recommended that silylation for 2.5 h at 150° should be used for all 20 protein amino acids[100,201]. The N-TMS-TMS-amino acid esters were found to be stable for 8 days when stored at room temperature in a tightly capped vial. Gly, CysH, Tyr and Lys were the most susceptible to variations in $RMR_{a.a./I.S.}$ (see 2.5).

The persilylated amino acid derivatives, being very sensitive to moisture, were found to decompose even on the column with some types of column packing. Only the best available supports, for example Gas-Chrom Q or Chromosorb W HP, should be used if satisfactory quantitation of the compounds is to be achieved; even the carefully silylated supports were said to cause partial breakdown of the derivatives (Lys_3 was used as the model compound)[343]. Subsequently, silicone phases alone, with higher loadings and larger columns, were used in the determination of the N-TMS-TMS esters of the protein amino acids. They were separated with relatively good success on a 3.7-m column filled with 3% DC-550 on Chromosorb W AW DMCS[18] or on a 1.75-m column with 4.5% of mixed silicones OV-7 and OV-22 (2:1) on Chromosorb G HP[255,411]. A superior column packing consisting of 10% OV-11 on Supelcoport, 100–120 mesh, in a 6 m × 2 mm column was described by Gehrke and co-

workers[99,100,201]. Using this column, the 20 protein amino acids could be quantitatively determined (Fig. 15).

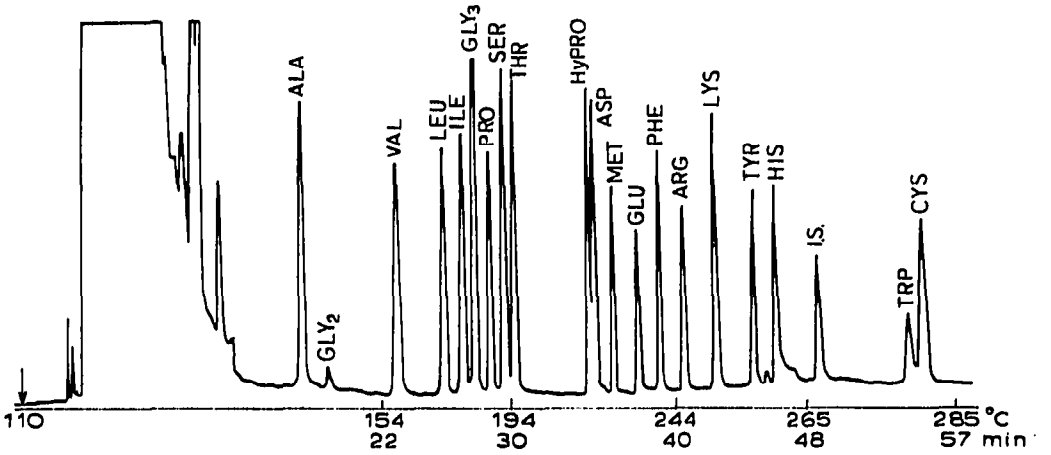


Fig. 15. GC analysis of N-TMS-TMS esters of 20 protein amino acids. Column: 6 m × 2 mm I.D. packed with 10% OV-11 on 100–120 mesh Supelcoport. Conditions: initial temperature 110°, programme rate 2°/min for 22 min and then 5°/min to 285°. Injection port temperature: 275°. FID temperature: 300°. Carrier gas (N_2) flow-rate: 20 ml/min. I.S. = phenanthrene. Reproduced from *J. Chromatogr.*, 57 (1971) 219, by courtesy of C. W. Gehrke.

Other silicones, *i.e.*, OV-1 (7%), OV-17 (10%) or OV-210 (10%), on Gas-Chrom Q or Supelcoport packed in a 3.1-m column were used for determining the persilylated urinary metabolites of Trp and Tyr[3]. A 2-m column with 3.8% SE-30 on Diatoport S was used to separate 11 amino acids released from insulin and oxytocin by hydrolysis[19].

Butts[39] recently reported GC retention data, presented as methylene unit (MU) values, for the TMS derivatives of 250 biochemically important compounds. The protein amino acids were subjected to silylation with BSTFA (1% in TMCS) using pyridine as a solvent for 16 h at 60° (overnight) and analyzed on OV-1 and OV-17 silicones. The differences in methylene unit values (ΔMU) were calculated from MU values on the moderately polar OV-17 and the non-polar OV-1 columns. Some relationships between ΔMU values and structure were discussed. In general, highly polar compounds showed large ΔMU values whereas the less polar persilylated amino acids exhibited low ΔMU values. A similar study on the retention properties of α-, ω- and α,ω-diamino acids, which were silylated and studied with combined GC–MS, was described elsewhere[325].

The silylation technique was also used in the GC analysis of some sulpho- and selenoamino acids[40] (the selenium analogues of sulphamino acids are important in animal health and nutrition) and in a GC–MS study of ^{13}C-enriched and deuterium-containing amino acids[376–378]. CysH and Met were resolved from their selenium analogues; the FID response to the sulphur compounds was about a third greater than to the selenium compounds[40]. The study with the deuterated amino acids revealed, surprisingly, that in many instances the retention times of the deuterated compounds were measurably shorter than those of their hydrogen analogues. Under the experi-

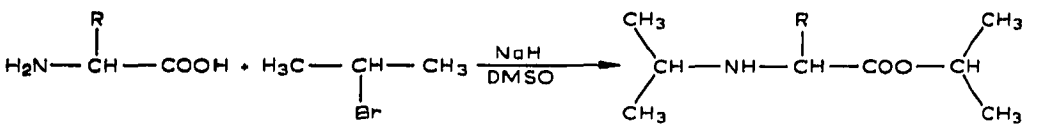
mental conditions used (separation studies were performed using a 3.66-m column coated with 2% F-60 and 1.5% SE-30 on Gas-Chrom P), the separation of mixtures was observed with only two pairs of compounds, Leu and Leu-D_{10} and Ile and Ile-D_{10}[376–378]. The technique was applied to analyses of amino acids in biological material[1,3,198].

3.3.3. Alkylation of amino acids, leading to N-alkylalkyl esters

Alkylation with an alkyl halide, a technique that introduces the same alkyl moiety to all reactive centres of a molecule, has already been mentioned in the introductory section (3.3). The permethylation technique, usually employed for chemical modification of peptides, was found to be inconvenient owing to the possible formation of quaternary amine salts (it would be difficult to stop the reaction after the addition of one or two methyl groups)[280,281]. Using a larger isoalkyl halide, it has been possible to stop the N-alkylation at the secondary or tertiary stage by steric hindrance. The isopropyl group was found to satisfy the steric requirements very well.

The two other methods treated in this section afford derivatization of the α-amino and carboxyl groups in one reaction step; however, different alkyl moieties are introduced on to each group. Not all other reactive groups that occur in the amino acid side-chain, however, are affected in the derivatization process. From this point of view, these two techniques cannot be considered as universal methods. One of them (3.3.3.2) has been developed rather for a special amino acid profile only, whereas the other (3.3.3.3) could well be adopted for general use with the protein amino acids. However, this method still needs to be further developed and, in view of its possible usefulness, more needs to be published.

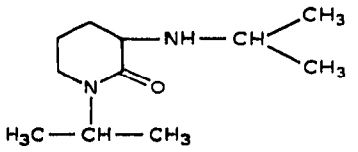
3.3.3.1. N-Isopropylisopropyl esters. Pettitt and Stouffer[280,281] reported on this new approach in the amino acid field in 1970. The amino acids (1–10 mg) were suspended in DMSO and subjected to treatment with sodium hydride (57% oil dispersion washed four times with hexane) and 2-bromopropane:



With the exception of Arg, all amino acids investigated yielded the expected derivative (including the iodinated amino acids, MIT and DIT, which were detected in nanogram amounts using an ECD). However, the hydroxy group in Hypro appeared not to be derivatized. The derivatives were found to be stable for reasonably long periods. For GC analysis, the silicone OV-17 (3% on Chromosorb W HP) was used; however, no data were reported on the analysis and separation of all protein amino acids.

Using a GC–MS system, other investigators subsequently critically assessed this procedure[29]. By extending the original work, Blessington and Fiagbe[29] were able to derivatize and separate in a single programmed GC analysis a mixture of 23 common amino acids using 5% Carbowax 20M on silanized Chromosorb G. Several amino acids (Glu, Gln, Asp and Asn) did not give any detectable derivatives, while

the others showed multiple peaks. Moreover, significant amounts of side-products that could confuse the analysis were produced. Arg yielded a simple small peak whose MS spectrum was almost identical with that of Orn; this derivative corresponded with lactam formation:

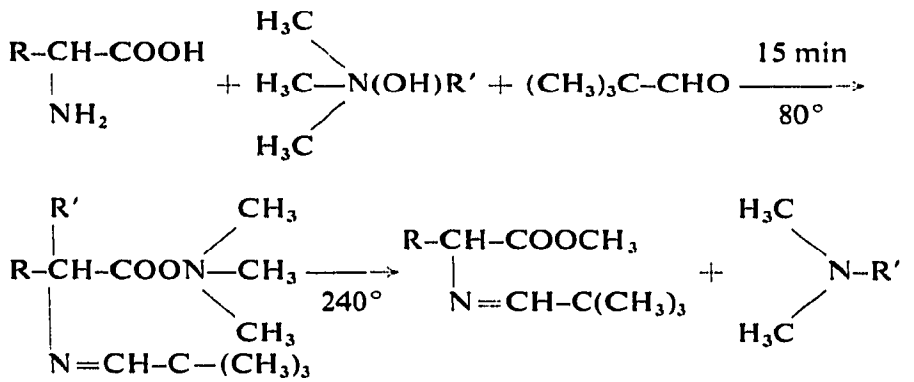


Orn

diisopropyl lactam

Despite these less encouraging findings, the same workers used the technique for the examination of the urinary amino acid profile by GC–MS[30].

3.3.3.2. N-Neopentylidenemethyl esters. This rapid and quantitative reaction process was developed originally for the determination of Phe in serum[128] but later it was adapted to the analysis of most of the protein amino acids[400]. The amino acids were dissolved in trimethylanilinium[400] (or tetramethylammonium[128]) hydroxide (0.2 *M* in methanol) and, after addition of a molecular sieve, they were treated with pivaldehyde at 80° for 15 min (for Phe, treatment for 5 min at 20° was sufficient)[128]. The resulting neopentylidene quaternary ammonium salt was subjected to pyrolysis in the flash heater of a GC, yielding the N-neopentylidenemethyl ester derivative ("pyrolysis esterification"):

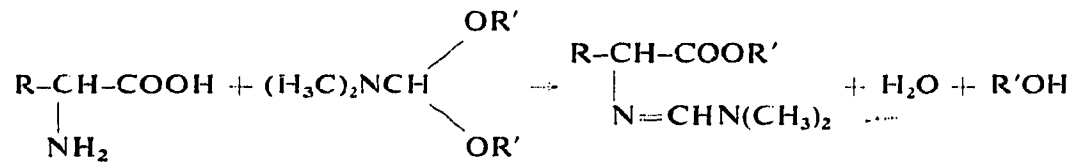


where R′ = methyl or phenyl.

Neutral, dibasic and acidic amino acid derivatives were chromatographed on OV-17 silicone (5% on silanized Chromosorb W). The hydroxy groups and the ring nitrogen atoms of Trp and Pro were also methylated. His, Arg, and Cys yielded no derivatives.

This method was suggested for the screening of amino acids in biological fluids. By this technique, Phe was analyzed in serum[128,355] and most protein amino acids in urine and serum[401].

3.3.3.3. N-Dimethylaminomethylenemethyl(-butyl) esters. Dimethylformamide-dialkyl acetals (DMF-DAA) were shown to react with amino acids to produce N-dimethylaminomethylene(DMAM)alkyl esters[369].



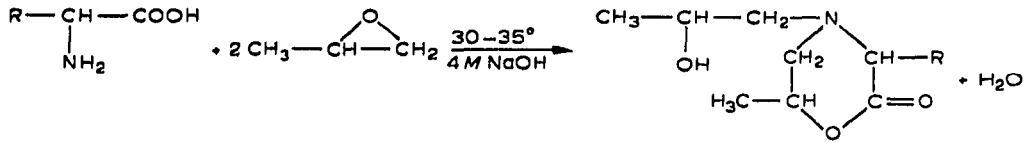
Complete and rapid reaction was observed at 100° (1:1 ratio of reagent to CH_3CN). Most compounds were dissolved within 20 min at 100°, Asp required more time for dissolution (almost 1 h). Under these conditions, hydroxy groups did not react; however, the hydroxy-substituted amino acids were said to yield appropriate derivatives without the reaction of hydroxy groups (nevertheless, methods are under study for the silylation of compounds of this type[369]). Using 1% SE-30 silicone, data were given for the analysis of N-DMAM-methyl esters of Gly, Ala, Val, Leu, Ileu, Phe, Asp, Glu (both the latter being N-DMAM-dimethyl) and Lys (di-N-DMAM-methyl). Moreover, Val, Leu, Phe and Glu were analyzed as N-DMAM-butyl esters on a capillary column coated with SE-30.

3.3.4. Condensation of amino acids with (thio)carbonyl compounds (or oxides), resulting in heterocycle formation

The three-stage Edman procedure[75] leading to the formation of PTH amino acids can hardly be imagined as being selected for the derivatization of the 20 protein amino acids. In keeping with this, the separation of PTH amino acids on a single column is not of prime importance. Simply, an analysis would be satisfactory if a single peak for each amino acid were obtained. The procedure has been developed thus far at present; however, the PTHs of the multifunctional amino acids need to be treated with a powerful silylating agent prior to GC analysis in order to make them volatile. The silylation of PTHs was adopted successfully for their GC analysis performed with fully automated apparatus, the sequence analyzers. Acylation was also used, but not to the same extent as the silylation technique. Unfortunately, the PTH ring is also included in the process but its chemical modification is not always complete, which is the reason for some difficulties and multipeak formation. The analysis of MTHs, *i.e.*, the more volatile analogues, was found to be preferable with regard to the GC analysis of the protein amino acids.

As the PTHs were the only cyclic derivatives of amino acids to be analyzed for many years and as they did not exhibit desirable GC features, less attention was paid to cyclic derivatization techniques. However, preliminary results with the oxazolidinones indicate that the formation of cyclic derivatives deserves more attention. Unlike the hydantoin ring, the oxazolidinone ring undergoes no changes even after subsequent acylation or silylation with strong reagents. The secondary amino group, involved in this cycle, becomes less polar and is sterically hindered by the presence of strongly negative, bulky halogen atoms at the adjacent carbon atom. The formation of the heterocycle is, moreover, very easy without any side-reaction and the derivatives show outstanding features with regard to GC analysis.

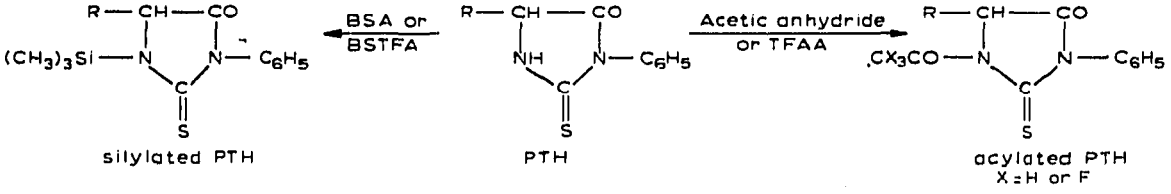
3.3.4.1. Morpholones. Some 2-morpholones have been prepared by Coussement and Renard[55] by the reaction of two molecules of propylene oxide with the amino acid:



Unfortunately, single peaks were obtained only from the simple amino acids. The multifunctional molecules gave multiple peaks. No further report has appeared.

3.3.4.2. Phenylthiohydantoins. Procedures for the GC analysis of PTHs have been under development since 1962[288]. Only three reports appeared up to 1968[84,288,328] in which PTHs were subjected to chromatography in the native form. Asp and Glu were introduced on to the column as methyl esters prepared with the aid of diazomethane[288]. The failure to obtain satisfactory results with Asn, Gln, Ser, Thr and the basic amino acids was the limitation of the procedure. GC–MS analysis of five PTH amino acids was reported[237].

Improved results could be obtained only by the use of two powerful silylating agents, BSA and BSTFA. Pisano[287] and Harman *et al.*[143] first used these reagents to silylate PTH amino acids and obtained peaks of a number of them, including Ser and Thr. It was observed that, in addition to the protonic functional groups, the hydantoin ring was also silylated. In a similar manner, the PTH ring was acylated by treatment with TFAA[34,312] or acetic anhydride[34,158]:



The TMS-PTHs have been further investigated by Guerin and Shults[122], who reported on the GC behaviour of 26 derivatized amino acids using a sulphur-specific photometric detector sensitive to nanogram amounts of the thiohydantoins (there is a limitation of a maximum operating temperature of 160°). The most detailed studies on PTHs were described by Pisano and Bronzert[283–286]. The technique of silylation has been examined in greater detail and extended to all of the amino acids[283,284]. BSA and BSTFA were tested with solvents of varying polarity; in a mixture (1:1) with pyridine or acetonitrile, a 10–15-min treatment at 50° was found to be sufficient. The compounds were separated into three groups according to their volatility and the number of functional groups to be silylated. For Asp, Glu and $CysO_3H$, silylation prior to GC analysis was essential, while for Ser, Thr, His and CysCM it was helpful. For the separation of the derivatives, a blend of the silicone phases SP-400, OV-210 and OV-225 proved to be very convenient[285,286], the operating temperatures being high (usually between 190 and 270°). The SP-400 silicone phase was found to be very useful for the separation of the PTHs of simple amino acids including Tyr and Trp in the GC 55/65 system of the Beckman 890c sequencer. Other workers have conducted studies aimed at the resolution of silylated PTHs of the protein amino acids[83,300]. Using a packed column (3.25 m), no stationary phase was found to separate them[300];

the best results were obtained with OV-17 (5%) or Dexsil 300 (1%) liquid phases. When a capillary column (4.5 m × 0.13 mm) with a mixture of OV-101 and OV-225 was employed, 19 amino acids of the 20 silylated were separated in one operation; His could be identified only by starting the analysis at a higher temperature, CysH was analysed as CysCM and Arg after prior conversion into Orn[83].

In all instances, only the silicone phases were useful for the analysis of PTHs. Analysis of the silylated PTH of Arg failed in all studies. It is therefore surprising that in the Jeol Chromatography Bulletin[172] PTH-Arg was reported to give a signal (two peaks) on a 10% SE-30 on Gas-Chrom Q column after silylation with equal amounts of BSTFA and CH_3CN for 6 min at 80°.

The analysis of acetylated PTHs revealed also a peak for Arg. Inglis and Nicholls[158] studied a variety of reaction conditions that were necessary for the conversion of PTHs into the acetylated products. Using PTH-Val as a model compound, complete acetylation with acetic anhydride–pyridine (4:1) was observed after 6–14 h at 20°, 1 h at 50° or 5 min at 100°. For His and Arg, requiring otherwise 3 h to 6 days for satisfactory derivatization, the on-column derivatization technique (the acetylating reagent was co-injected) was the only recommendable one that yielded a high percentage of the acetylated PTH. However, it was impossible to acetylate the PTH ring completely by this technique (*e.g.*, 30% of PTH-Val did not undergo acetylation). The derivatives had approximately 6% lower retention times compared with those of their non-acetylated parent compounds.

Lequin and Niall[201a] reported on the GC analysis of more volatile analogues, the pentafluoro-PTHs, which were prepared by a modification of the Edman degradation procedure using PFPITC as the coupling reagent. Except for Arg and His, the PFPTHs of all other protein amino acids could be chromatographed and separated satisfactorily on a single column (1.22 m × 2 mm) packed with either 10% DC-560 or 2% OV-25 on Chromosorb W (100–120 mesh) between 160 and 240°. Silylation with BSA was employed to allow the determination of Asp and Glu (and also Asn and Gln) and also for the resolution of the Leu–Ile pair. Although the derivatives possess more desirable features with regard to GC analysis, the perhalogenated reagent did not come into general use.

3.3.4.3. Methylthiohydantoins. MTHs have two advantages over the PTHs. Firstly, their preparation by the three-step Edman procedure is easier and more rapid and, secondly, they have more desirable features for GC analysis owing to their higher volatility. In four papers[83,285,286,300] the MTHs and PTHs were treated together and analyzed under the same conditions, as mentioned in the preceding section. Waterfield *et al.*[389] at first studied the use of MITC for the sequential degradation and analyzed the products after subsequent treatment with BSA or TFAA (derivatization of MTHs with TFAA proceeded in the inlet heater after co-injection of the reagent with the sample) on a 5% QF-1 column packing[388]. Attrill *et al.*[7] showed that silylation was unnecessary for identifying 16 MTHs of protein amino acids when using two columns coated with SE-30 and OV-17 silicones. The five remaining MTH-amino acids, *i.e.*, Asp, Ser, Arg, CysCM and $CysO_3H$, gave unique peaks after silylation. Vance and Feingold[374,375] studied in detail the reaction of peptides and proteins with MITC and identified the MTHs after silylation with BSA–CH_3CN reagent (5 min at 20°) on OV-17 and OV-1 phases. The yield of His was increased two-fold by co-injection of BSA.

The most detailed studies of the GC analysis of silylated MTHs of all amino acids so far found in proteins appeared recently[191,192]. Silylation was performed with BSA–CH_3CN (1:3) and heating to 100° for 10 min. GC separation was accomplished in a single run on a 1.65-m column filled with 2% OV-17 on Gas-Chrom Q at 145–230°. The only unresolved pair (Asn and Phe) could be separated by an additional procedure requiring only 4.5 min. Arg, as in all previous instances, did not yield a volatile derivative. Using the flame photometric sulphur detector, some extraneous peaks observed occasionally with the FID were absent[191] (Fig. 16).

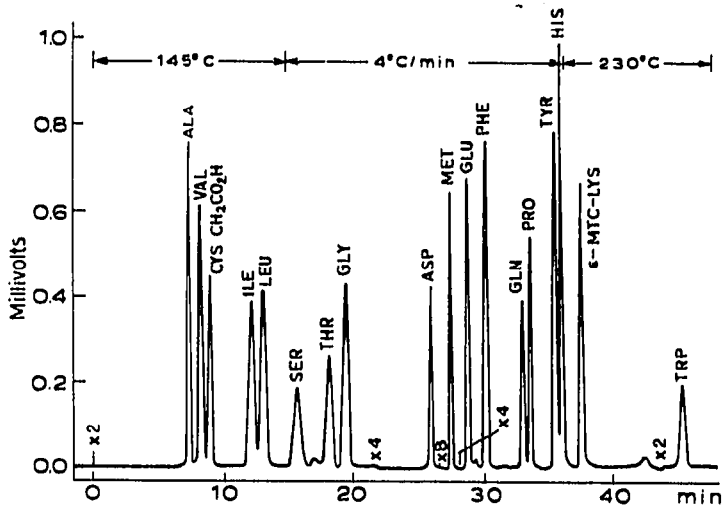
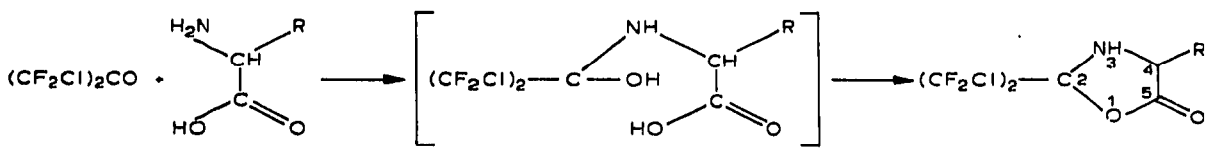


Fig. 16. GC separation of trimethylsilylated MTHs with flame photometric detection. Each peak represents 2.5 nmoles of the derivatized amino acid, the MTH of His represents 5 nmoles. Column: 165 cm × 4 mm I.D. with 2% OV-17 on 80–100 mesh Gas-Chrom Q. Carrier gas (N_2) flow-rate: 50 ml/min. Temperature programmed from 145° to 230° at 4°/min. Temperature of FPSD: 220°. Temperature of injection port: 240°. Reproduced from *Anal. Biochem.*, 58 (1974) 549, by courtesy of W. M. Lamkin.

3.3.4.4. 2,2-Bis(chlorodifluoromethyl)oxazolidin-5-ones. The reactions of the halogenoacetones were studied extensively by Simmons and Wiley[342]. They described the preparation of the oxazolidinone from Ala. A condensation reaction between 1,3-dichlorotetrafluoroacetone (DCTFA) and an amino acid proceeds by the formation of a five-membered ring, the 2,2-bis(*cdf*-methyl)-4-substituted-1,3-oxazolidin-5-one:



Another halogenated acetone, hexafluoroacetone (HFA), reacts in the same way, and the products of its treatment were analyzed on a capillary column[394,395].

In addition to the simple amino acids Ser (with an unreacted –OH group) also appeared on the chromatogram. The use of HFA, however, is not very practical as the reagent is a gas, relatively expensive and the derivatives of the simplest amino acids are too volatile. DCTFA, with a boiling point of 45°, proved to be an excellent cyclization reagent[152,153].

Engelhardt[78] successfully prepared the oxazolidinones from some simple protein amino acids and chromatographed them. However, there were difficulties in dissolving the acids in the reaction medium, as the acids themselves are not soluble in DCTFA. In a solvent such as acetonitrile, the conversion of amino acids into the derivatives occurred at an elevated temperature after several hours. Moreover, with amino acid hydrochloride salts, no conversion was observed under the same reaction conditions.

The optimal conditions for the condensation reaction with respect to a solvent, catalyst, time and temperature, etc., were established recently using Tyr and its mono- and diiodinated analogues as model compounds[153]. GC analysis of the oxazolidinones of these hydroxylated amino acids was successful after subsequent treatment with acylating or silylating agents in the same reaction medium. Both ECD and FID molar responses were evaluated. The application of this unique and selective chemical reaction to the quantitative determination of the protein amino acids is now under study[152]. The reaction medium, consisting of 1–4 vol.-% of pyridine in CH_3CN and DCTFA (reagent to solvent ratio 1:5 to 1:15), was effective in converting all 20 protein amino acids into their oxazolidinones at 50° within several minutes. The yields were high (>95%, followed by radioactivity measurement) and the same results were obtained with amino acids and their hydrochlorides. For the subsequent treatment, acylation with TFAA or HFBA (completed within 5 min) proved to be the most useful method. Silicone phases are assumed to allow the complete separation of protein amino acids in a single run. Some difficulties were observed with elution of His from the GC column. The extent to which the secondary carboxyl group of Asp and Glu is involved in the derivatization is at present being investigated using a GC–MS system. It is believed that a procedure for the quantitative determination of the protein amino acids by this promising derivatization technique will be published soon.

4. ANALYSIS OF IODOAMINO ACIDS

Six amino acids together were subjected to derivatization studies and GC analysis: mono- and diiodotyrosine (MIT and DIT), diiodo-, 3,5,3′- and 3,3′,5′-triiodothyronine and thyroxine (T_2, T_3, T'_3 and T_4). However, only two of them, T_3 and T_4, are of clinical importance, Their accurate determination in biological material, especially in blood or in pharmaceuticals, for example, is of great importance. T_3 exhibits about a 5-times higher physiological activity than T_4 and its concentration in human serum is very low. A mean serum concentration for euthyroid subjects was found to be approximately 1.4 ng/ml; thus, its concentration is only about 2% of that of T_4 (average 70 ng/ml). As a routine clinical method, convenient also for the assay of both hormones in unextracted serum, radioimmunoassay has been generally adopted. Photometry based on the ceric–arsenite colour reaction is often still employed. The use of GC analysis, however, has diminished. The main papers dealing with the GC determination of thyroid hormones appeared between 1966 and 1973 and since

then no further papers have appeared. The main reason for this is very probably the recent progress with radioimmunoassay procedures and the necessity to isolate the material from biological sources if subjected to GC. However, the derivatization technique and the less desirable features of the derivatized compounds also contributed to an abandonment of GC. By treatment with pivalic anhydride (trimethylacetic anhydride, TMAA), in the only method adopted for blood assay, the very large molecules are converted into the least volatile derivatives in comparison with the other acyl moieties (TFA-, HFB-). Moreover, in spite of careful precautions, approximately 1–2% of the T_4 present in the serum sample is converted (de-iodinated) into T_3 during this analytical procedure, causing an increase of about 50–100% in the T_3 value[262]. The other derivatization techniques were not very successful because of the instability of the resulting compounds. However, perhaps the procedure only recently investigated using DCTFA and subsequent treatment with HFBA (see 3.3.4.4) under very mild reaction conditions could still maintain an interest in using GC in the determination of thyroid hormones in blood. The GC analysis of both hormones can be characterized as "high temperature" with respect to the operation mode (usually above 250°), choice of liquid phases (OV-1, SE-30, OV-17, Dexsil 300) and ^{63}Ni ECD required (nowadays also the Sc ^{3}H ECD could be employed). The newly developed linear ECD[210] with a range of linearity of 1:10,000 would be, however, the best choice for the quantitation of these compounds at the picogram level. The finding of an internal standard, for example a halogenated compound preferably emerging between T_3 and T_4, would also be highly desirable. The use of narrow (1–2 mm I.D.), short (up to 1 m long) columns[360], together with higher flow-rates of the carrier gas (50–150 ml/min)[93], is common.

4.1. Acylation of iodoamino acid methyl esters with anhydrides

The first studies were performed with artificial mixtures of iodoamino acids. The carboxyl group was in all instances methylated by heating with a 25% solution of HCl in methanol at 70° for 30 min (or 10 min[262] or 60 min[70,364]. The dry residue was then treated either with TFAA[70,307] or TMAA[70,148,167,241,262,360–362,364,382]. Although the trifluoroacetylation proceeds very easily under mild reaction conditions (20°) and satisfactory results were achieved with micromole amounts using an FID[307], the analysis of T_2, T_3 and T_4 at the picomole level using an ECD revealed considerable breakdown of the compounds during chromatography[70].

The N,O-TMA(pivalyl)methyl esters proved to be more suitable than the TFA species[70]. They were introduced by Stouffer *et al.*[361] in 1966 and analyzed at first with an FID with temperature programming. In three subsequent studies, an ECD and isothermal operating conditions were applied and the procedure was adapted to the analysis of serum samples using at first 3–5 ml of serum[167,362] and later only 1 ml of serum[360]. Solvent extraction and Sephadex LH-20 chromatography were suggested for the isolation of the material from serum. Acylation with TMAA (0.2 ml) with 10 μl of both methanol and triethylamine was complete within 30 min at 110°. A 90 cm × 2 mm column with 2% OV-17 on Gas-Chrom Q operating at 272° or 285° was used for the analysis. T_3 could be detected at levels as low as 20 pg by using the ECD-pulse method. The derivatives were found to be stable to moisture, heat and light and could be stored for a period of several months in dilute methanolic solution with-

out any detectable changes. Other workers[382] confirmed that the derivatives were sufficiently stable under atmospheric conditions and prepared them in macro amounts.

Hollander and co-workers[148,241,262] studied the procedure in detail and developed a sophisticated method using Bio-Rad AG 50W-X2 (100–200 mesh) ion-exchange resin for the isolation of the hormones from blood[262]. Acylation with TMAA–methanol–triethylamine (20:1:1) was performed at 70° for 10 min. The derivatives were purified using Amberlite IR-45 resin and benzene as eluent. The dry residue was dissolved in 100 μl of benzene and 5 μl of solution were injected directly into the column (60 cm × 4 mm, 5% OV-1 on Chromosorb W HP); after maintaining the temperature at 220° for 12 min, it was programmed at 3°/min up to 300°, which final temperature was maintained for 5 min. A calibration standard was injected immediately following the sample (Fig. 17). The injection port and detector temperatures were 280° and 350°. Comparison of the GC analysis with radioimmunoassay showed almost identical values for serum T_3[241]. In the latest report on this technique[364], the same procedure was adapted for serum assay, and the N-TMA-methyl esters of T_3 and T_4 were analyzed on an 81-cm column filled with 3% Dexsil 300 on 80–100 mesh Chromosorb W HP at 305°.

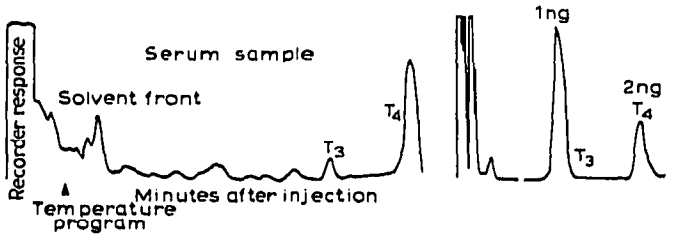


Fig. 17. GC analysis of T_3 and T_4 in human serum in the form of N,O-pivalylmethyl esters. Column: 60 cm × 4 mm I.D. with 5% OV-1 on Chromosorb W HP. Programme: 12 min at 220° then 3°/min to 300° with a final hold for 5 min. A calibration standard was injected immediately following the sample. Reproduced from *Anal. Biochem.*, 43 (1971) 435, by courtesy of C. S. Hollander.

It is surprising that HFBA has not been evaluated as a possible acylation reagent. The acylation of methyl esters of some iodoamino acids with HFBA, performed by us[153], proved to be very convenient with respect to both the reaction time and the GC behaviour of the derivatives. It could be accomplished within 5 min at 50°; the N,O-HFB-methyl esters were found to be stable enough and the most volatile. They give the highest response in the ECD, implying that it is not only the iodine atoms in the molecule of an iodoamino acid that are responsible for the magnitude of the signal. Amounts as low as 10^{-13} moles of T_3 and T_4 could be readily detected[152].

4.2. Silylation of iodoamino acids with bis(trimethylsilyl)acetamide or condensation with perhalogenated acetone

The silyl derivatives were prepared by treatment with BSA[4,93,334] or BSA–TMCS (traces)[9,141,145] alone[9,93] or in conjunction with a solvent (acetonitrile[4,334], pyridine[145] or tetrahydrofuran[141]). The silylation was completed within 1 min at 70° (ref. 4), or usually within 5–10 min at 50° (refs. 93, 145 and 334) when a solvent was

used together with the silyl reagent. Without a solvent, a 35-min treatment was necessary for T_4[93]. However, when picomole amounts are to be derivatized, the use of BSA alone is preferable[93]. The reaction products were found to decompose in dilute solutions, even if the dilution was carried out with pure BSA (breakdown of 40% in 1 h was observed at concentrations of 1 ng/μl; in the case of dilution with CH_3CN–BSA (4:1), 100% breakdown occurred within 20 min). Other silylating agents, *e.g.*, TMCS and HMDS in conjunction with pyridine, were also investigated[334] but conversion into the derivatives was not complete. In all instances, an FID and programmed-temperature operations were employed; moreover, the electron capture detection limit was also investigated[9,93,141]. A typical analysis of N,O-TMS-TMS esters of five iodoamino acids and Tyr using an FID is shown in Fig. 18.

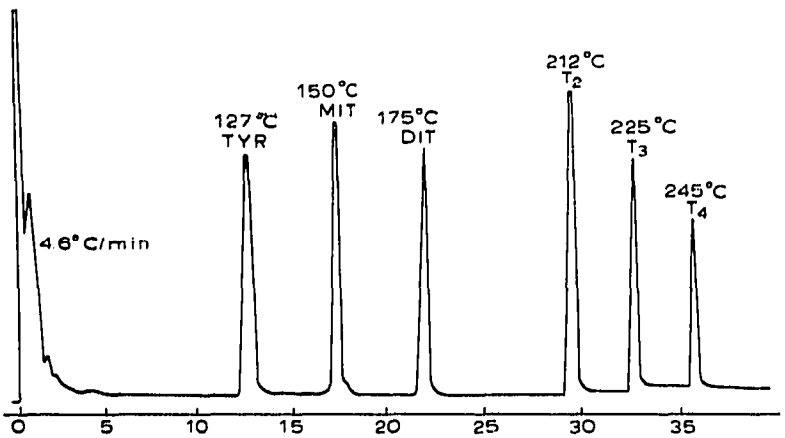
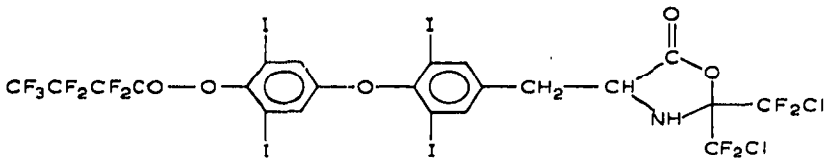


Fig. 18. Chromatography of five iodinated amino acids and Tyr in the form of N-TMS-TMS esters. Column: 1.0 m × 3.5 mm I.D. with 0.5% SE-30 on 60–80 mesh Chromosorb G AW DMCS. Programme as indicated on the figure. Reproduced from *Anal. Biochem.*, 24 (1968) 281, by the kind permission of the authors.

The FID showed a minimum detection limit of about 20 ng for T_4 and 5–20 ng for T_3[145], whereas with an ECD an amount of two orders of magnitude less could be detected[93]. The silylation procedure has been adopted as a routine method for the trace analysis of thyroid hormone preparations and drugs that contain thyroid hormones; a quantitative evaluation (FID) was achieved by the use of T_2 as an internal standard[145]. In none of the studies was the method applied to the analysis of the hormones in serum. Silylation seemed to be inconvenient for this purpose as partial destruction of the TMS derivatives on the column appeared when sub-nanogram amounts were injected[93].

With respect to simplicity of preparation, treatment of the iodoamino acids with dichlorotetrafluoroacetone seems to be the preferred method[152,153]. The iodoamino acids are converted quantitatively into the derivatives within 15 min at 50° including the subsequent treatment with HFBA. The heptafluorobutyrated oxazolidones are the highest possible halogenated compounds:
possessing some of the highest responses in ECD[153]. The analysis of this derivative



O — HFB — bis(chlorodifluoromethyl) — 1,3 — oxazolidinon — 5 — one of T_4

at the picomole level of T_3 and the occurrence of T_4 are at present under study[152]. Preliminary studies showed, however, that picomole amounts of such derivatized thyroid hormones are not easily eluted from the usual column fillings (unlike, for example, the N,O-HFB-methyl esters). It is believed that the use of textured glass beads with a 0.1 % coating of a silicone phase may solve this problem.

5. RESOLUTION OF AMINO ACID ENANTIOMERS

This analysis may be important for several reasons. Amino acids are known to racemize under various conditions, *e.g.*, by treatment with dilute alkali[293], under conditions of prolonged acid hydrolysis especially at higher temperatures[258] or, to a lesser extent, during solid-phase synthesis of peptides[15]. For the complete amino acid analysis of synthetic or natural polypeptides, the determination of the content of any D-amino acid is essential as the effects of isomeric contamination are cumulative; 1 % of D-amino acid in each amino acid built into a peptide of 10 units will result in 10 % of inactive material. The importance of D-amino acids in some antibiotics such as gramicidin is well known. More recently, a wide distribution of D-amino acids in nature (some bacteria, cell wall materials, insects and animals)[52] and even in human urine and blood (β-aminoisobutyric acid)[349] has been found. Another area of considerable interest relates to the analysis of extraterrestial material[184,200,290,292].

Optical isomers (enantiomers) of amino acids cannot be separated on the usual GC liquid phases when using any of the derivatization reagents described in section 3. The resolution of enantiomeric mixtures of amino acids has been accomplished by either of two alternative techniques that have been shown to be almost equally successful: (1) derivatization (esterification or acylation) of enantiomeric amino acids with optically active reagents to form suitably volatile diastereoisomers (diastereomers) following their GC separation on conventional, *i.e.*, optically inactive, stationary phases (see 5.1); (2) derivatization of the enantiomers with common optically inactive reagents, followed by GC separation of the derivatives on columns loaded with special optically active stationary phases (see 5.2). The disadvantages of the former method are that optically active reagents of high optical purity are not always readily available and that the conversion into derivatives must be strictly quantitative in order to avoid the risk of fractionation during the preparation of the diastereomeric derivatives. Optical impurities lead to more peaks and corrections are not always possible, especially when only small amounts of isomers are to be detected. The latter method suffers from the disadvantages of requiring relatively low operating temperatures owing to the low temperature limit of optically active stationary phases, and consequently long retention times. It uses optically inactive reagents to create suitable deriv-

atives, *e.g.*, N-TFA-isopropyl esters; thus, the only correction to be made is that for an eventual racemization during hydrolysis of peptides and proteins. Esterification with isopropanol that is 3 *N* in HCl, carried out mostly at 100° for several hours, has been reported to cause no apparent racemization; however, the completeness of this process has shown to be the critical factor[327].

All of these resolutions have been concerned with the optical activity associated with the α-carbon atom. However, it has been found[180] that hydroxyamino acids could be resolved into the *threo-* and *erythro-* forms without the need to use an optically active derivative as there are already two optically active centres in the molecule, involving the α-carbon and the carbon carrying the –OH group. Surprisingly, there was a reverse order of eluted compounds depending on whether or not the –OH group had been trifluoroacetylated. Also, Ile and *allo*-Ile have been noticed to be partially resolved on several stationary phases[62].

5.1. Addition of an asymmetric centre to the amino acid molecule

Treatment with the simplest member of the optically active alcohols, 2-butanol, was first applied to the separation of enantiomeric amino acids. Esterification with higher secondary alcohols was later found to be more effective in resolving the diastereomeric esters[112,135]; however, the choice of 2-butanol represented the best compromise with regard to the dissolution properties of the lower alcohols in comparison with the higher ones (*e.g.*, 2-octanol) and the higher volatility of the resulting derivatives. The formation of N-TFA-2-alkyl diastereomers involved esterification with a highly acidified (4–8 *M* HCl) optically active 2-alkanol at 100° for at least 1 h, followed by removal of the solvent and subsequent acylation with TFAA. The possibility that esterification with hot acidified alcohol might introduce an unknown degree of racemization led other investigators to use optically active acylating agents such as N-TFA-L-propyl chloride. The preparative procedures appeared to be simple and mild, and the resulting diastereomeric dipeptides could be readily separated on conventially packed GC columns. Thus, this procedure seems to be preferable also for those investigators who are not so familiar with the capillary technique, the use of which is usual in resolving N-TFA-2-alkyl esters. However, a loss of volatility due to dipeptide formation is disadvantageous for the analysis of the less volatile amino acids.

It has been observed in a number of systems that when LL, LD, DL and DD diastereomers are resolved on an inactive stationary phase, the LL and DD compounds are eluted together either before (for secondary alkanol esters) or after (for diastereomeric dipeptides) the LD and DL isomers, which are also eluted together.

5.1.1. Optically active esterification reagents

Secondary alkanols, particularly 2-butanol and 2-octanol, were found to be useful for the GC analysis of amino acids by Gil-Av and co-workers[45,111,112]. The N-TFA-2-butyl and -2-octyl esters of Ala and Val[45], of some simple amino acids and Glu[111] and of the simple amino acids including Hypro, Asp, Glu and Lys[112] were separated on a capillary column (50 m × 0.25 mm) coated with polypropylene glycol or FS-1265 fluorosilicone. The latter phase was found to be more convenient, giving retention times and higher values for the separation factor α (the ratio of the retention

time of the later eluted LD diastereomer to that of the previously eluted LL isomer). The $\alpha_{LD/LL}$ values were higher for the 2-octyl esters (1.03–1.10) in all instances. The procedure was applied to the determination of the configuration of the amino acids in antibiotics of the vernamycin group[46]. Pollock and co-workers[294,296,297] developed very similar methods in which the N-TFA-2-butyl esters were used to separate enantiomers of all of the protein amino acids except His, Arg and Cys. Three different pre-treatments of the trifluoroacetylated diastereomers were examined with the following results: (1) treatment with water led to destruction of the O-TFA bond so that the hydroxylated amino acids failed to be eluted or they were eluted with larger retention times than the monoacyl (N-TFA) compounds[296]; (2) with strict exclusion of moisture (CH_2Cl_2–TFAA treatment) the N,O-di-TFA derivatives remained unchanged and the resolution of 21 racemic amino acids was reported[297]; (3) after treatment with methanol for 20 h at 20°, complete cleavage of the O- and S-TFA bonds occurred and subsequent treatment with acetic anhydride resulted in the formation of O(S)-acetyl-N-TFA-2-butyl esters (decomposition of the N-TFA bond during acetylation did not occur)[294]; separation of the diastereomers of Asp was accomplished by formation of the N-TFA-3,3-dimethyl-2-butyl ester and the N-pentafluorobenzoyl-2-butyl ester. Capillary columns 50-m long coated with Ucon LB-550-X[297] or preferably with Carbowax[294,296] were employed for the separation. Raulin and Khare[304] used packed columns with EGA polyester as the stationary phase for the possible resolution of protein amino acid enantiomers in the form of N-TFA-2-butyl esters. They succeeded in partially resolving 14 amino acids; however, Asp, Orn and Lys were not separated (Fig. 19).

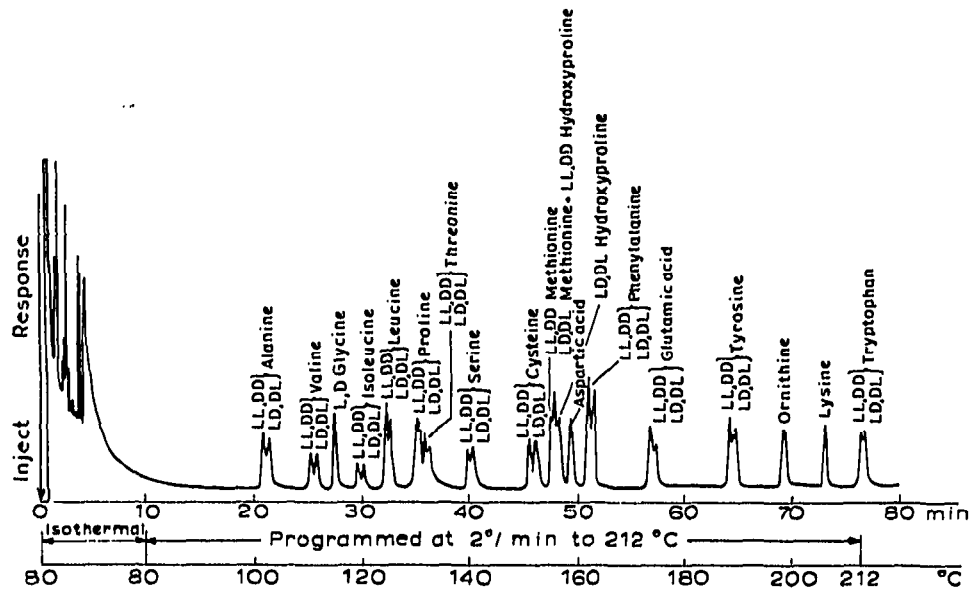
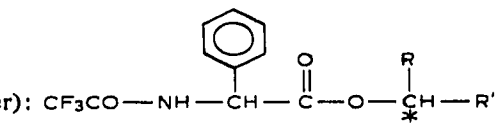


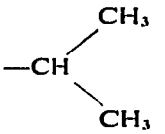
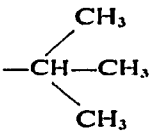
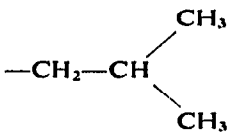
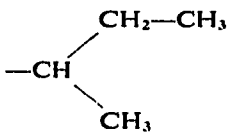
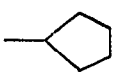
Fig. 19. GC analysis of N-TFA-2-butyl esters of 17 enantiomeric amino acids. Column: 3.36 m × 2 mm I.D. glass, coated with 0.325% stabilized EGA on 80–100 mesh H. T. Chromosorb G AW. Isothermal run for 10 min at 80°, then 2°/min up to 215°. Each peak corresponds to about 25 μmoles of D,L-amino acid. Reproduced from *J. Chromatogr.*, 75 (1973) 13, by courtesy of F. Raulin.

For the resolution of enantiomers of some simple amino acids, the higher alkanols proved to be more useful. On a conventional column packing, the N-TFA-2-pentanol ester of Val gave two well resolved peaks[64]. Esterification with C_5–C_8 2-alkanols with subsequent acylation with TFAA, HFBA, PFPA or chlorodifluoroacetic anhydride (CDFAA) was examined in another study[290]. N-PFP-2-pentyl and -2-octyl esters proved to be the most useful derivatives for the effective separation of enantiomers. Westley *et al.*[393] studied the influence of the structure of the alcohols on the separation of diastereomeric esters. Phe was used as a model compound and, after esterification with various 2-alkanols and acylation with TFAA, the products were analyzed on a polyester or fluorosilicone-coated column. The structural effects were followed according to two criteria: the separation factor (α) and the difference in standard molar free energies of GC partition behaviour for the diastereomeric pairs. The data are shown in Table I.

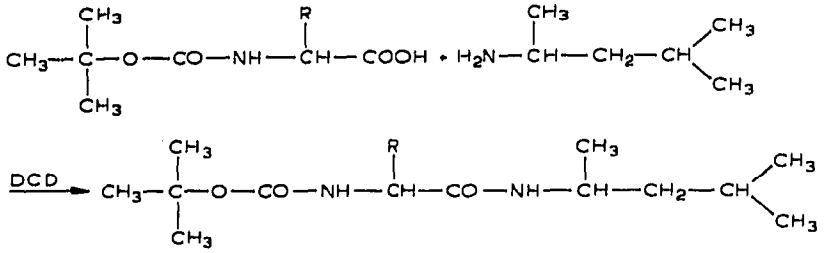
TABLE I

EFFECT OF THE STRUCTURE OF ALCOHOLS ON THE SEPARATION OF DIASTEREOMERIC ESTERS[393]

Model compound (N-TFA-S-Phe ± alkyl ester): 

2-Alkanol	*R*	*R'*	$\alpha = \frac{r_{t/s(+)}}{r_{t/s(-)}}$	$\Delta(\Delta G^0)$
2-Butanol	—CH_3	—CH_2—CH_3	1.04	−35
3-Methyl-2-butanol	—CH_3		1.10	−83
3,3-Dimethyl-2-butanol	—CH_3		1.17	−134
4-Methyl-2-pentanol	—CH_3		1.025	−22
3-Methyl-2-pentanol	—CH_3		1.07	−60
1-Cyclopentyl-1-ethanol	—CH_3		1.11	−90

It could be concluded that: (1) Increases in the size differential of groups attached to the alcoholic asymmetric carbon atom of esters (*i.e.*, R *vs.* R') cause increases in the degree of separation of diastereomers; and (2) the closer the branching is to the asymmetric centre, the larger will be the change in size from the straight-chain group, which consequently aids the separation. Thus, superior resolution is evidently obtained by using 3,3-dimethyl-2-butanol. Almost the same findings were reported later by Ayers *et al.*[8], who recognized that replacement of 2-octanol with 3- and 4-octanol led to a decrease in resolution (the difference between R and R' is diminished), and that the use of 3,3-dimethyl-2-butanol gave the best possible separation. Fourteen amino acid diastereomers, each in the form of N-TFA-3,3-dimethyl-2-butyl esters, could be resolved to the extent of 93% or better; Asp and Pro derivatives could be resolved to the extents of 70 and 82%, respectively. Arg, His and Cys derivatives were not studied. GC analysis was carried out using a 3-m column with 10% OV-17 silicone on Chromosorb W AW; this arrangement appeared to be the best choice. When testing various stationary phases, no column filling was found to be capable of resolving all of the diastereomers. The analysis of amino acid N-TFA-menthyl esters was reported in two papers[134,381]. On columns packed with a polyester[381] or a polyethylene glycol phase[134], only the most volatile amino acids (Ala, Val, Leu and Nleu) were resolved with a high degree of separation. Studies on racemization during peptide bond formation were responsible for the development of the *tert.*-butyloxycarbonyl (BOC) amino acid (+)-4-methyl-2-pentylamides as volatile derivatives for GC[126]. These derivatives were formed by condensation of *tert.*-BOC-L-amino acid with sterically pure (—)-2-amino-4-methylpentane:



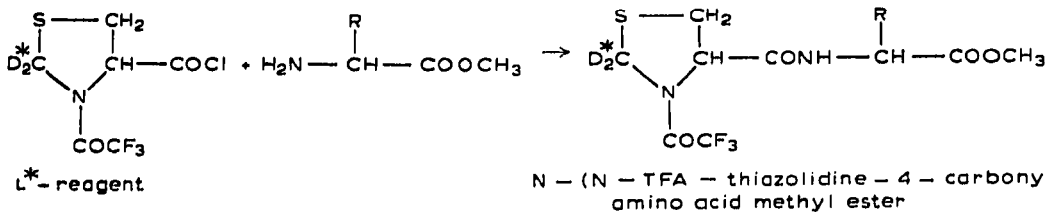
and thereafter analyzed on a packed column with EGA phase.

The formation of diastereomers by treatment of amino acid enantiomers with optically active esterification reagents has been widely used in application studies[126,129,131,135,184–188,200,290–293].

5.1.2. Optically active acylation reagents

The following carbonyl chlorides, prepared from the chosen antipode of a carboxylic or amino acid by treatment with thionyl chloride, have been used for coupling with the α-amino group of an amino acid methyl ester to create optically active dipeptides: α-chloropropionyl-[130,138,193,392], α-bromopropionyl-[194], α-chloroacyl-(others)[133,138,392], N-TFA-thiazolidine-4-carbonyl-[139] and N-TFA-propyl chloride[31,32,59,132,136,137,165,166,349,392].

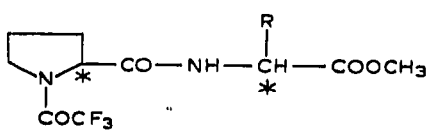
Halpern *et al.*[139] were the first to use this technique to resolve five simple enantiomeric amino acids by GC and MS. The procedure consisted in the preparation of methyl esters followed by acylation with an artificial mixture of deuterium-labelled N-TFA-L*- and unlabelled N-TFA-D-thiazolidine-4-carboxylic acid chloride:



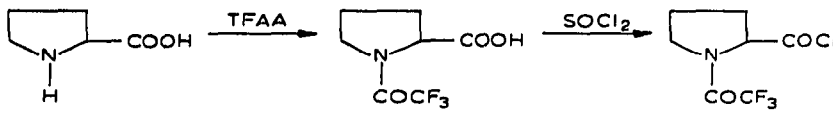
The derivatives were separated by GC on a 1.5 m × 3 mm column with 5% SE-30 at 180° and the peak effluent was collected and passed into a mass spectrometer. For each symmetrical molecule (*e.g.*, Gly), the D and L* reagents were unresolved and the label ratio remained constant through the peak. If an asymmetric molecule was encountered, which gave rise to resolvable diastereomers, the deuterated reagent was concentrated in one peak, distorting the ratio. Thus, if the target molecule was racemic (D, L), two peaks were formed (one containing L* D plus D, L, the other L*L plus D, D), but the label ratio in each peak remained constant.

The same group of workers also investigated various α-chlorocarboxylic acid chlorides as resolving agents[133,138,392]. Experiments on the influence of the steric effects about the amide bond of α-chloroalkanoyl-Val-methyl esters showed that differences between the retention times of diastereoisomers were enhanced by increased crowding about this bond. Using 1.5-m columns packed with either non-polar SE-30 or polar free fatty acid phase, the separation coefficient, $\alpha_{LL/LD}$, exerted higher values on the polar phase, ranging from 1.13 for the 2-chloropropionylamide group to 1.30 for the 2-chloro-3-dimethylbutyryl moiety[138,392]. Some simple amino acid enantiomers were separated on the same columns after coupling with L-α-chloroisovaleryl chloride (prepared from L-Val by treatment with thionyl chloride)[133]. Lande and Landowne[193] used the L-α-chloropropionyl derivatives of the amino acid methyl esters. Acylation of the amino acids, released from the newly synthesized peptide by hydrolysis, was achieved using a mixed anhydride of L-chloropropionic acid and pivalic acid, and after esterification with diazomethane these derivatives were separated on a 46 m × 0.25 mm capillary column coated with XE-60 silicone or polypropylene glycol. The N-α-bromopropionyl-Phe-methyl ester could be separated very well under the same conditions[194]; however, the difficulties in obtaining quantitatively the optically pure α-bromopropionic acid limited the practicability of this method.

The last, widely used and today generally adopted procedure for the preparation of optically active dipeptides is, however, treatment of amino acid methyl esters with N-TFA-L (or S)-prolyl chloride (L-TPC) yielding the N-TFA-L-prolyl amino acid methyl esters (TPAM):



The reagent can be easily prepared by the action of TFAA on L-Pro followed by treatment with thionyl chloride:



Derivatization with L-TPC to TPAM had already been introduced by Halpern and Westley in 1965[132]. The main reason for their choice was an assumption that the cyclic nature of the derivative precluded racemization. The preparation procedure appeared to be simple and mild: (1) the amino acid enantiomers are converted by reaction with methanol and thionyl chloride into their methyl ester hydrochlorides[33], and (2) the latter are converted into TPAM by reaction at room temperature with L-TPC in dichloromethane in the presence of triethylamine. However, in neither the first report[132] nor subsequent papers[136,137,392] did the authors test the question of racemization experimentally, nor in fact did they present exact experimental details for the preparation of the derivatives. This critical statement appeared in a recent paper by Bonner[31], where it was shown that both the treatment of N-TFA-L-Pro with thionyl chloride and the subsequent coupling of L-TPC with an amino acid methyl ester in the presence of triethylamine may be accompanied by extensive racemization. The temperature maintained throughout the procedure and the triethylamine concentration in the second reaction step were found to be the critical factors, so that racemization of either the proline moiety, the amino acid moiety, or both, accompanied the preparation of the dipeptide. A modified procedure was then suggested in which racemization of L-TPC in the triethylamine step was subsequently eliminated by the gradual addition of approximately the theoretical amount of the base in very dilute dichloromethane solution at dry-ice temperature. Dabrowiak and Cooke[59] had already mentioned earlier some problems encountered during the synthesis of the L-TPC reagent and they described a similar low-temperature synthesis leading to a reagent of high optical purity (99%). This method was adopted in the recent work of Iwase and Murai[166], who investigated the TPAM diastereomeric derivatives of most of the protein amino acids (except Arg, His, Trp and Cys).

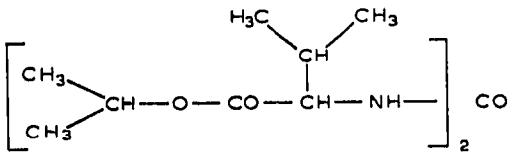
For the separation of the diastereomeric dipeptides, packed GC columns of usual length (1.5–2 m) were found to be sufficient. They were packed either with nonpolar silicones (SE-30, OV-1)[132,166,392] or with polyesters (DEGS, EGA)[59,136,392]. In one instance, the use of a 15-m SCOT column coated with NPGA was reported[31]. Polyglutamates were recently found to be useful stationary phases for the separation of TPAM also[165]. The polyfunctional amino acids, after conversion into TPAM diastereomers, were treated with silylating agents[59,136,166]. The use of BSTFA in conjunction with acetonitrile (on addition a few drops of TMCS) was found to be most effective[166].

The N-α-chloropropionyl esters were utilized in some enzymatic studies[130]; the N-TFA-L-prolyl amino acid methyl esters have been found to be useful in various applications [11a,32,137,349].

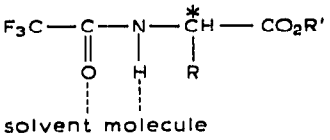
5.2. Addition of an asymmetric centre to the stationary phase

The problem of separating enantiomeric amino acid derivatives, where the original α-carbon atom represents the single optically active centre in the molecule, has been solved by introducing a second asymmetric centre into the column in the form of an optically active stationary phase. The phases can be divided into three groups: (1) simple esters, *i.e.*, N-acyl amino acid alkyl esters or -alkylamides; (2) ureides *i.e.*, carbonylbis(L-amino acid alkyl esters); and (3) dipeptides, *i.e.* N-acyl-L,L-dipeptide alkyl esters. The stationary phases of the latter group are the preferred ones with the best separation characteristics. Most papers have dealt with them; however, in the pioneering studies the first group of phases were utilized.

N-TFA-D- or L-isoleucine lauryl ester and N-TFA-L-phenylalanine cyclohexyl ester appeared in 1966. Gil-Av *et al.*[114,115] coated them on glass capillary columns 50–100-m long and subjected the N-TFA-methyl to -cyclopentyl esters of five simple amino acids to analysis on these phases at 90°. The *tert.*-butyl esters have shown the highest separation factor; the N-TFA-L-Phe-cyclohexyl ester phase gave a higher resolution of the enantiomers. The L enantiomers always emerged last on the L phase and first on the D phase. The mechanism of separation has been explained in terms of hydrogen bridging between the molecules of the solute and the solvent. The replacement of $-COCF_3$ by $-CH_2CF_3$ in the case of Ala-isopropyl ester reduced the resolution to zero, implying that hydrogen bonding was necessary for the separation[114]. By condensation of L-valine isopropyl ester with phosgene, another optically active stationary phase called "ureide" was produced and its interaction with N-TFA(±)-alkyl (linear or branched chain) esters of amino acids was studied by using the same capillary column as described above[86,88]:



ureide (solvent)



N–TFA–alkyl ester (solute); interaction with the stationary phase (solvent)

The experimental data fully confirmed the prediction, that for derivatives with the same configuration the compounds in which CO_2R' is larger than R would emerge in the opposite order to those in which CO_2R is smaller than R[88].

In accordance with the finding that most of the separation occurs at the amide end of the dipeptide[50], the synthesized N-caproyl-L-Val *n*-hexylamide phase had high separation efficiencies that were comparable with those of the dipeptides and were better than those achieved with the simple ester phases[121]. Moreover, the phase exhibited a superior thermal stability (145°) and, despite the use of a long (122 m) capillary column, the enantiomers of six simple amino acids could be eluted with reasonable elution times and well resolved peaks.

Feibush and Gil-Av[85,87,113] at first introduced a dipeptide phase, N-TFA-L-valyl-L-Val isopropyl ester[113], which was, however, found to be thermally very un-

stable. It was therefore replaced with N-TFA-L-valyl-L-Val cyclohexyl ester (abbreviation: Val-Val), a phase that become very popular and more frequently used than any other[15,22,50,51,85,87,113,256–258,269,271,327]. When coated in an amount of 5% on Chromosorb W in a packed 2-m column, it was possible to achieve almost complete resolution of N-TFA-Ala-*tert.*-butyl ester[85,113]. It was stated later[87] that the phases derived from the di- and tripeptides of L-Val permitted the best separation reported thus far for the antipodes of N-TFA amino acid esters; the use of the N-acetyl analogue of Val-Val, however, gave worse results, and elongation of the peptide chain by synthesizing N-TFA-(L-Val)$_3$-O-isopropyl ester gave, in addition to better thermal stability, also a slight decrease in the resolution[87]. The Val-Val phase showed its best separation properties when operated at its temperature limit, *i.e.*, at 110° (refs. 22, 182, 256 and 257). Because of the low permissible operating temperature, the mixtures were analyzed best in two steps. Capillary columns of 120–150 m served for the resolution of the volatile amino acids up to Pro and CysH (Fig. 20), whereas for Met, Phe, Asp and Glu, columns of 30–60 m were recommended[257]. A short capillary column (10 m) was recommended for the analysis of Phe, Glu, Tyr and Arg[182]. The influence of the alcohol used for esterification has been also examined[87,257,271]. It was found that the resolution increases in the order primary, secondary, tertiary alcoholic group. Suitable derivatives were N-TFA-amino acid methyl, isopropyl and *tert.*-butyl esters. However, the latter are difficult to prepare[271], and therefore the isopropyl esters represent the best compromise as they combine reasonable retention times with good resolution properties[257].

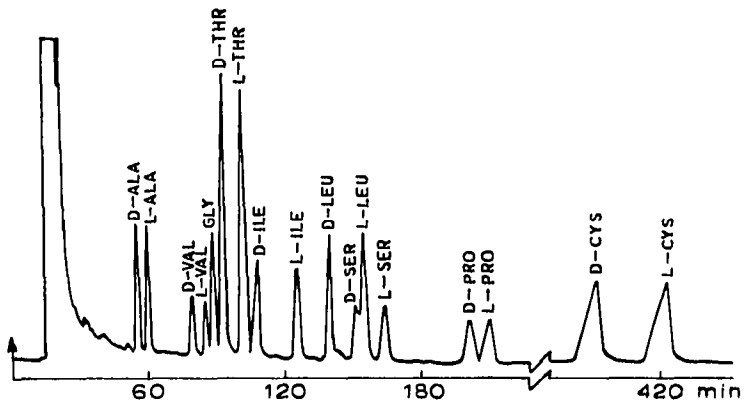


Fig. 20. Resolution of enantiomeric amino acids as N-TFA-isopropyl esters in a 122 m × 0.5 mm I.D. capillary column coated with N-TFA-L-valyl-L-valine cyclohexyl ester at 110°. Reproduced from *J. Chromatogr. Sci.*, 9 (1971) 141, by courtesy of W. Parr.

A new, more temperature-stable dipeptide phase possessing a higher molecular weight and lower vapour pressure was synthesized[182] and evaluated for the separation of protein amino acids[268–272]. The phase N-TFA-L-phenylalanyl-L-Leu cyclohexyl ester (Phe-Leu) could be operated at temperatures up to 140°, which resulted in a better separation of less volatile amino acids. Moreover, the newly synthesized N-PFP (pentafluoropropionyl)isopropyl esters exhibited retention times up to 47% shorter

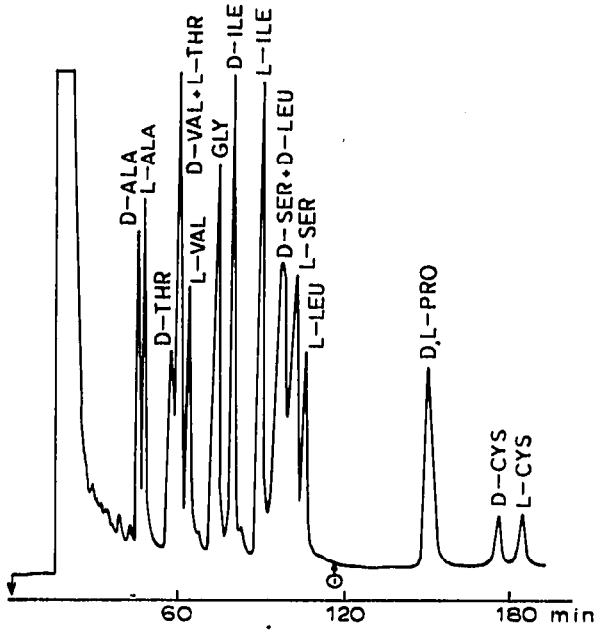


Fig. 21. Resolution of enantiomeric amino acids as N-PFP-isopropyl esters on the same column as in Fig. 20 coated with N-TFA-L-phenylalanyl-L-leucine cyclohexyl ester at 110°. Reproduced as for Fig. 20.

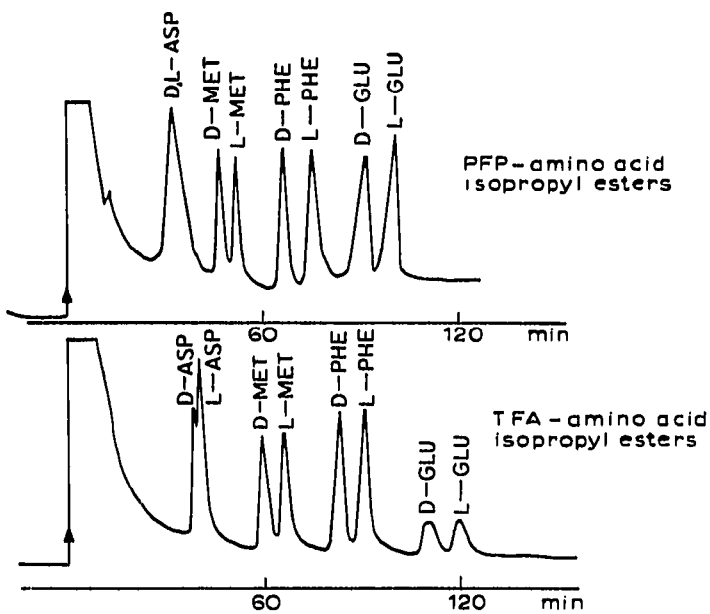


Fig. 22. GC analysis of N-TFA- and N-PFP amino acid isopropyl esters in a 30-m capillary column on the same phase as in Fig. 21. Reproduced as for Fig. 20.

compared with the N-TFA species, without a significant loss in the resolution factors[269,271,272]. By using two capillaries of lengths 30 and 120 m coated with Phe-Leu phase, 17 enantiomers of protein amino acids could be analyzed as N-PFP-isopropyl esters (Figs. 21–23)[269].

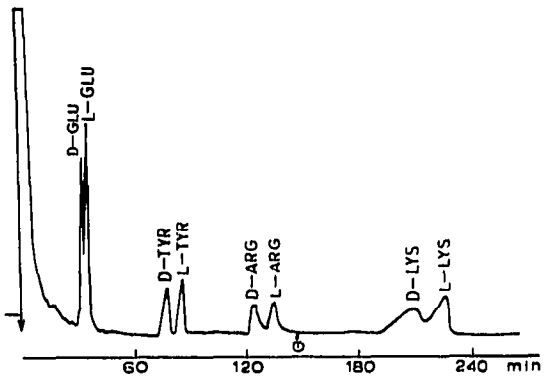
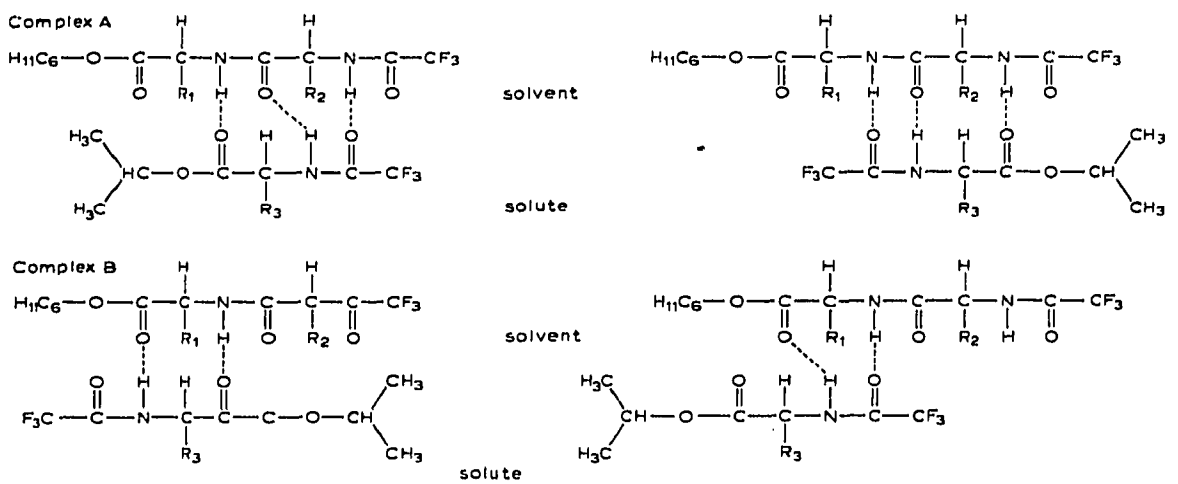


Fig. 23. GC analysis of enantiomers of Glu, Tyr, Arg and Lys in the form of N-PFP-isopropyl esters at 125°. The column and the phase were the same as in Fig. 22. Reproduced as for Fig. 20.

Fig. 22 shows that D,L-Pro could not be resolved on Phe-Leu, whereas on both Val-Val (Fig. 21) and the newly introduced Val-Leu[263] it has been satisfactorily separated. This could be explained only by giving the answer on the nature of molecular interactions in the unique solvent (stationary phase)–solute (amino acid enantiomer) system. The formation of a diastereomeric association complex has been confirmed[50,121]; the presence of the amide portion of the solvent molecule was found to be the segment actually participating in the formation of the complex. The separation of the D,L-isomers proceeds via formation of diastereomeric complexes possessing three sites in which hydrogen bonding may occur[263,266,267]:



Parr and Howard[266] showed that the structure of the solute derivatives had to contain two monocyclic carbonyl groups (configurational requirement) separated by two atoms (spatial requirement) in order to achieve the formation of the association complex. In accordance with this, other enantiomeric amino acid derivatives such as N-isopropylisopropyl esters or N-TMS-TMS esters (see 3.3), or even N-TFA-β-amino acid isopropyl esters, were not separated[266]. With Pro, only two of the theoretically possible hydrogen bonds can be formed (no hydrogen atom is available on the N-α-TFA secondary amide), which results in a decrease in the separation factor in comparison with the other α-amino acids. There is, moreover, a third, steric requirement. When R_1 and R_2 are small, as in Val-Val, there is still sufficient space for the formation of hydrogen bonds. When R_1 and R_2 are both lengthened (Phe-Leu), neither of the possible hydrogen bonds can be readily formed and no resolution is seen[263].

Finally, in a series of papers, Parr and Howard[150,264–267], and also Corbin *et al.*[50], evaluated the effect of the structure of synthesized selected dipeptides on the separation of amino acid enantiomers. The impetus for synthesizing four systematically substituted N-TFA-L,L-dipeptide cyclohexyl esters of Ala, ABA, Nval and Nleu was especially the failure to separate the enantiomers of *tert.*-Leu. Assuming that the bulky nature of its side-chain and even of the groups attached to the α-carbon atom of the dipeptide solvent were responsible for the failure to resolve it, phases with less bulky groups were supposed to achieve the desired separation. The phases together with the solutes examined are summarized in Table 2.

Capillary columns 122 (or 30) m in length and 0.5 mm in diameter coated with one of the phases were used throughout these studies, which were carried out isothermally at 110°. It was found that an increase in the size of the alkyl substituents (R_1, R_2) on the asymmetric centres of the dipeptide solvent produced greater solvent–solute interactions and thus a better separation. On the other hand, this modification, when applied to the side-chain on the α-carbon atom of the solute (R_3), caused a decrease in interaction. Similar findings have also been reported by other workers[50], who showed simultaneously that tripeptide phases such as $(Leu)_3$ did not contribute to large values of the separation factor. Two phases, Nval-Nval and ABA-ABA, permitted the complete separation of *tert.*-Leu. The separation factors, together with differential enthalpies of solution, have been found to decrease in the order Leu $\geqslant$ Nleu $>$ Ile $\gg$ *tert.*-Leu. Each phase has been investigated with respect to its ability to give a complete separation of a mixture of naturally occurring enantiomeric amino acid derivatives. The ABA-ABA phase has been found to be superior to the others, effecting a complete separation of seven enantiomeric amino acids and Gly in a reasonable time (Fig. 24). Using a 30-m capillary column, six additional amino acid enantiomers (Asp, Met, Phe, Glu, Tyr and Lys) could be readily separated on this phase. For the separation of the N-TFA-isopropyl esters of D,L-Arg and D,L-Trp, the Phe-Leu dipeptide remains the phase of choice (Fig. 23).

The low temperature limits of the optically active dipeptides are a limitation of this technique. However, investigations of more stable phases, *e.g.*, Phe-Phe (170° maximum), have shown that, in accordance with the previous studies on steric hindrance, the resolution was very poor[269]. On the other hand, the GC behaviour of Val-Val, ureide and N-TFA-L-Phe-cyclohexyl ester was investigated at column temperatures below their melting points[51]. Adsorption chromatography just below the melt-

TABLE 2

HOMOLOGOUS DIPEPTIDE STATIONARY PHASES (SOLVENTS) AND DERIVATIZED AMINO ACID ENANTIOMERS (SOLUTES) SUBJECTED TO GC BEHAVIOUR STUDIES[267]

Solvent: $CF_3CO-NH-\underset{R_1}{CH}-CO-NH-\underset{R_2}{CH}-COOC_6H_{11}$, *Solute:* $CF_3CO-NH-\underset{R_3}{CH}-COOC_3H_7$,

*N-TFA-*L,L*-dipeptide-cyclohexyl ester of*	R_1, R_2	*N-TFA-*D,L*-amino acid isopropyl ester of*	R_3
Ala-Ala	$-CH_3$	Ala	$-CH_3$
		ABA	$-CH_2-CH_3$
ABA-ABA	$-CH_2-CH_3$	Val	$-CH(CH_3)_2$
		Nval	$-CH_2-CH_2-CH_3$
Nval-Nval	$-CH_2-CH_2-CH_3$	Leu	$-CH_2-CH(CH_3)_2$
		Nleu	$-CH_2-CH_2-CH_2-CH_3$
Nleu-Nleu	$-CH_2-CH_2-CH_2-CH_3$	Ile	$-CH(CH_3)-CH_2-CH_3$
		tert.-Leu	$-C(CH_3)_3$

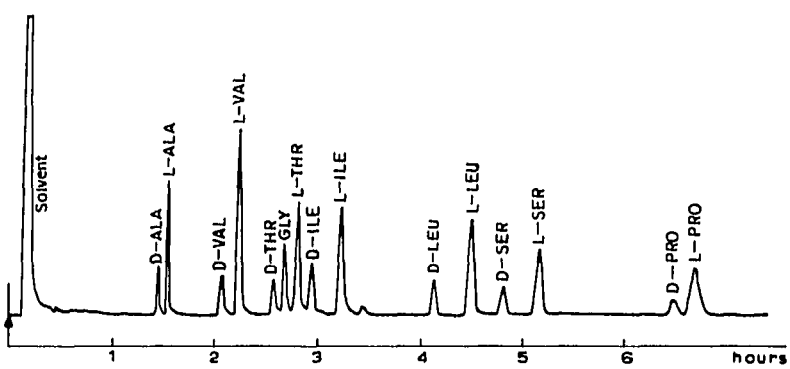


Fig. 24. N-TFA-D,L-amino acid isopropyl esters separated on N-TFA-L-α-amino-*n*-butyryl-L-α-amino-butyric acid cyclohexyl ester at 110° on a 122 m × 0.5 mm I.D. capillary column. Reproduced from *Anal. Chem.*, 45 (1973) 711, by courtesy of W. Parr.

ing point was found to be more convenient than partition chromatography because, in the case of ureide, it led to a six-fold decrease in elution times of enantiomers without significant alteration to the resolution.

Two optically active dipeptides, Val-Val and Phe-Leu, were useful in some studies on the racemization of amino acids under various conditions[15,72,258].

6. APPLICATIONS

Together with the first efforts to analyze amino acids by GC, there were frequent attempts to apply this technique to the analysis of protein and peptide hydrolyzates, the most commonly encountered analytical problem in this area. Such analyses were undertaken as soon as a method, often incomplete, was available and the only additional step was the employment of a proper hydrolysis technique. As the hydrolysis procedures have been treated in section 2.2.1 and applications to peptides and proteins have been mentioned in the text, we do not need to summarize the numerous papers separately here. They are well tabulated, for example, in the review article by Blau[24]. In this section are included those procedures which require a preceding isolation step in order to determine the amino acid content (*e.g.*, in biological fluids) without interfering substances. The section is completed with several studies on the occurrence of amino acids in different types of material. The papers dealing with this topic are listed in Table 3. For the derivatization forms used, see the remarks at the end of each of the preceding sections.

Biological material is varied in origin and may contain a wide range of potentially volatile compounds so that an isolation step, very often involving a clean-up procedure on ion-exchangers, has to be carried out prior to derivatization and subsequent GC determination. This is an apparent disadvantage as none of these steps is usually necessary in IEC (using amino acid analyzers), where at most a simple deproteinization may be required.

Physiological fluids for the determination of free amino acids were prepared by two alternative techniques: with or without preliminary precipitation of the protein followed in both instances by cation-exchange clean-up. The first technique was recommended for plasma amino acid GC determinations by Gehrke and coworkers[102,107,177,414]; the second has been developed by Pellizzari *et al.*[278] and found to be more convenient than the precipitation techniques.

The deproteinization of blood plasma samples can be performed simply by adding alcohol or, more effectively, by mixing a sample of plasma with saturated picric acid solution. In the former instance, ethanol was usually added to 3–4 times the sample volume (0.2–0.5 ml)[128,156,363]; the precipitated protein, removed by centrifugation, was washed repeatedly with aqueous alcohol and the combined supernatants were evaporated to dryness. The residue was subjected to derivatization either directly (in the case of Phe only)[128] or after dissolution in water[363] or 0.5 *N* HCl[156] (either the cation exchange or merely chloroform extraction in the latter instance was used for the clean-up). Similarly, as in picric acid deproteinization, the cation exchangers Dowex (AG) 50W-X12 (50–100 mesh)[277,363,414] or Amberlite CG-120 (100–200 mesh)[177,414], both in the H^+ form, were recommended for the clean-up. The following procedure has been developed as the most convenient for smaller plasma samples[177]. A 0.1–0.4-ml volume of plasma is denatured by adding 4 volumes of saturated picric acid

TABLE 3

GC ANALYSIS OF AMINO ACIDS AND THEIR ENANTIOMERS: APPLICATION STUDIES

Type of material and studies	*Topic*	
	Amino acids	*Their enantiomers*
A. Biological material		
(a) Biological fluids:		
Serum	8, 36, 102, 107, 128, 156, 157, 169–171, 177, 222, 233, 277, 278, 306, 355, 363, 371, 401, 412, 414; 148, 167, 241, 262, 360, 362, 364 (thyroid hormones)	129, 349
Urine	2, 3, 30, 36, 58, 107, 120, 157, 169–171, 222, 230, 233, 363, 371, 373, 401, 412, 414	349
Cerebrospinal fluid	371	
(b) Food and plants	2, 42, 89, 94, 102, 107, 177, 198, 316, 409, 410, 412	
(c) Tissue samples	1, 3, 38, 177, 215, 225, 228	
B. Geological material	279, 301	72, 186, 187
C. Extraterrestrial material	95, 107, 301, 413	184, 200, 290, 292
D. Pharmaceuticals	145 (thyroid hormones)	
E. Contaminants	301	
F. Studies concerning:		
(a) Synthesis (or degradation) of amino acids or peptides	199	15, 32
(b) Racemization of amino acids		11a, 126, 258, 293
(c) Enzyme–substrate interaction		130, 131, 137, 291

solution; the solution is centrifuged for 15 min at 4400 rpm, the supernatant decanted on to a 1–2-ml resin bed of Amberlite CG-120 (H^+), 100–200 mesh, and allowed to pass through the resin at 1 ml/min. The samples are subsequently washed with 10–20 ml of water and the amino acids are eluted with 1–2 ml of 7 *N* ammonia solution.

Other workers[36,278] found the picrate protein precipitation to be unsatisfactory as it gave poor recoveries, especially of the basic amino acids (Arg, Lys), and therefore a new purification method, involving dilution of the sample with acetic acid and thus eliminating the protein precipitation, was suggested as a replacement for the picrate method. Each aliquot (100 μl) from a plasma sample was diluted with 10 volumes of 1 *N* acetic acid (pH <2.4). The sample was then passed through a 1.5-ml bed volume of Dowex 50W-X12 (H^+) resin (200–400 mesh), washed with 8 ml of water and the amino acids were eluted with 4 ml of 1 *N* ammonia solution and rinsed with 3 ml of water. In spite of the fact that recoveries for some amino acids were better (followed with ^{14}C-labelled amino acids) than with picrate denaturation, the yields of plasma protein amino acids did not give such a distinctive picture in favour of the acetic acid method (some amino acids showed higher yields with the picrate method), so that the choice of one of the two methods remains open.

The procedure for isolating thyroid hormones from human serum (1 ml) in-

volves retention of the iodoamino acids on Bio-Rad AG 50W-X2 (H^+), 100–200 mesh, resin followed by washing with 15 ml of 0.15 *M* ammonium acetate solution (pH 8.5) in order to remove most of the lipids and proteins, and subsequent elution of the hormones with either 8.4 *N* ammonia solution (6 ml)[262] or, better, methanolic ammonia[152,364].

For the purification of urinary amino acid fractions, a procedure similar to that for the plasma amino acids was developed[414]. If the deproteinization with picric acid solution was employed, then a double ion-exchange clean-up was usual, first with the cation exchanger Amberlite CG-120 (H^+) and then with the anion exchanger Dowex 1-X2 (CH_3COO^-)[414]. However, a single-step ion-exchange clean-up with either Dowex 50W-X4 (H^+)[120] or Dowex 50W-X8 (Na^+) was also found to be satisfactory. A simple procedure for the removal of urinary pigments from urine hydrolyzates (equal amounts of urine and concentrated HCl were sealed under nitrogen and heated at 100° for 22 h or at 145° for 4 h)[233] was reported recently[230]. A mixture of Dowex 1-X8 (200–400 mesh) resin with Norite A charcoal and salicylic acid (4:2:1), stirred previously for 2 h and washed with 6 *N* HCl, ethanol and diethyl ether, was added (100 mg) to 8 ml of diluted urine hydrolyzate and then centrifuged at 3000 rpm for 15 min. An aliquot of the supernatant was evaporated to dryness and subjected to derivatization.

Analysis of biological substances and geochemical samples of both terrestrial and extraterrestrial origin for amino acids at the 1–10 ng/g level, and the concentration problems connected with such analyses, was the subject of a comprehensive study by Rash *et al.*[301]. With increasing sensitivity, contamination becomes a most important problem. The sources of contamination investigated were fingerprints (Fig. 25), laboratory dust (Fig. 26), cigarette smoke, skin fragments, hair, dandruff, saliva, muslin towel fibres, latex gloves, water, and an eluate from an ion-exchange column used to de-salt geological samples. The GC method developed by Gehrke and co-

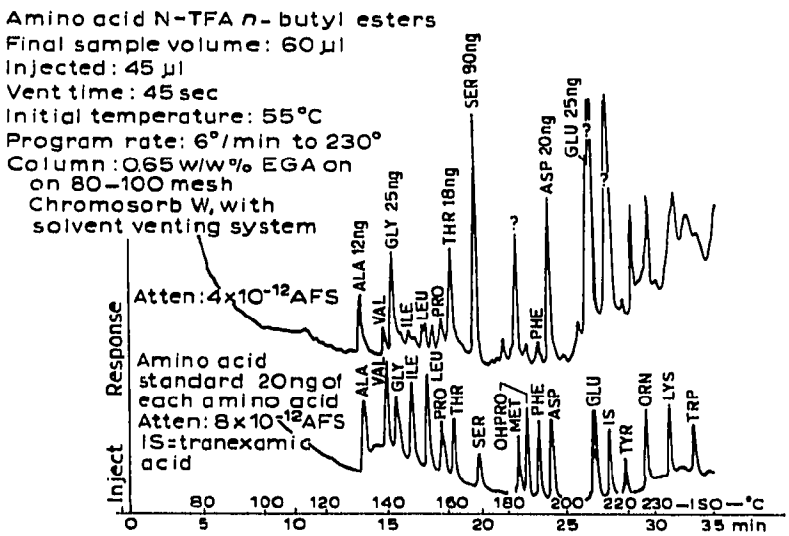


Fig. 25. GC analysis of a fingerprint. Reproduced from *J. Chromatogr. Sci.*, 10 (1972) 444, by the kind permission of the authors.

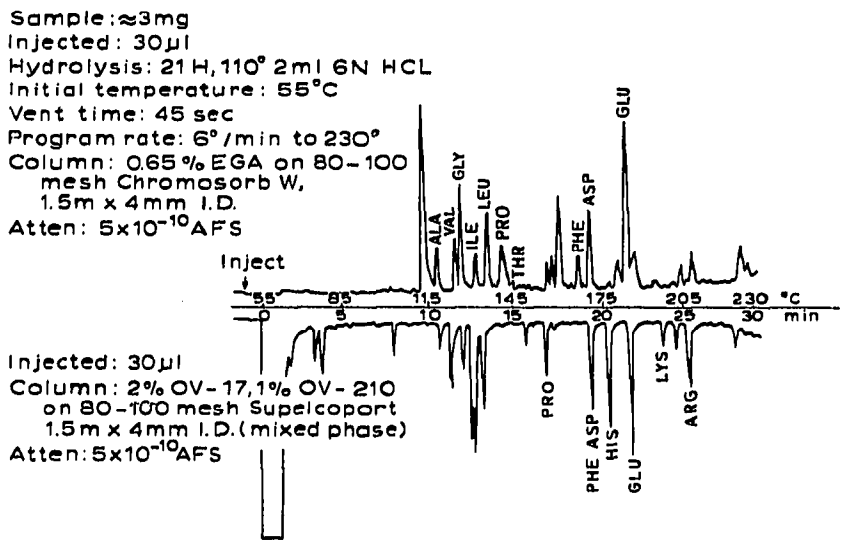


Fig. 26. GC analysis of hydrolyzed dust. Reproduced as for Fig. 25.

workers has been used for the analysis of amino acids in the lunar samples brought back by Apollo 11, 12 and 14[95,107,301,413] (Fig. 27), and also in water extracts of 3.4 billion-year-old Onverwacht chert (fossil)[301].

Very interesting studies have also been performed on the determination of the optical configuration of amino acids occurring in cherts[187] and meteorites[184,200,290,292] in order to gain some information concerning the origin and evolution of early life on Earth. In the precambrian fig tree chert[187], the amino acid concentration was found to be less than about 2 nmoles/g and the largely predominant form corresponded to the L(+) diastereoisomers; thus, the biological processes as we know them were active 3 billion years ago. On the other hand, in the hydrolyzates of meteorite residues, nearly equal amounts of the D and L isomers of some simple protein amino acids were present[184,200] (in microgram amounts per gram of the material) and additionally 11 non-protein amino acids were assessed, indicating an abiogenic character of the material.

In another application, GC and MS have been used to study the absolute configuration of β-AIBA in human serum and urine[349]. In the urine, almost exclusively the *R* isomer of β-AIBA was found (probably originating from thymine degradation), whereas the serum contained a mixture of the *R* and *S* enantiomorphs in a ratio of 1:4.

7. CONCLUSIONS

Twenty years have passed since the first report on the GC analysis of amino acids. During this time, the GC determination of these compounds has proved to be equally valuable, if not more convenient in some instances, than the classical IEC. GC analysis, in comparison with amino acid determination by IEC, is usually said to be a rapid and alternative method. However, this is only partly valid today and then only when we compare the conventional performance of both techniques. With suitable derivative, GC analysis, including the derivatization procedure, can be

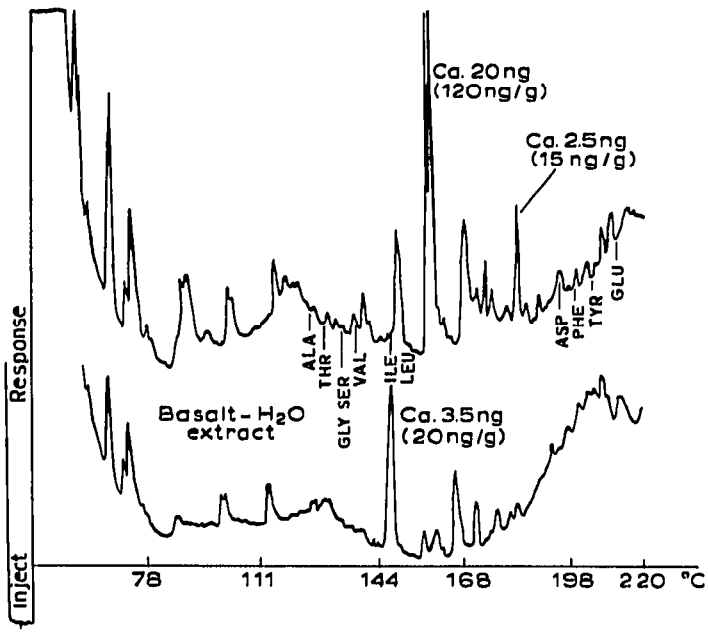


Fig. 27. Gas chromatogram obtained on analysis of the aqueous extract of Apollo 12 lunar material. Column: 1 % OV-17 on Chromosorb G HP, 2.5 m × 2 mm I.D. A 1.2-g sample of lunar fines was refluxed with triply distilled water for 13 h. Derivatization: 100 μl of butanol, 3 *N* in HCl, at 100° and treatment of the evaporated residue with 100 μl of TFAA–CH_2Cl_2 (1:200). Conditions: final volume, 30 μl; injected sample, 5 μl; injection port, 280°; initial temperature, 70° for 6 min then 4°/min to 220°; attenuation, $4 \cdot 10^{-12}$ a.f.s. Reproduced from *J. Chromatogr.*, 57 (1971) 193, by kind permission of the authors.

completed in less than 1 h with sensitivities reaching approximately 10^{-10} mole when the universal FID is used. Alternatively, the employment of higher pressures along with IEC nowadays gives rise to a high-pressure single-column ion-exchange analyzer (*e.g.*, Model D500, Durrum, Palo Alto, Calif., U.S.A.), which is capable of performing a complete amino acid analysis with the same rapidity (in less than 1 h) and almost equal sensitivity (1 nmole and less) as GC–FID. The very high cost of such apparatus is in direct contrast to the relatively low cost of a gas chromatograph, the versatility of which is its additional great advantage. Provided that the ultrasensitive ECD and high-resolution capillary technology are employed, the detection limit can be extended to 10^{-13} mole and, in the latter instance, a separation efficiency is obtained that is incomparable with any other technique. However, more work still remains to be done in order to combine high-resolution ECD-capillary chromatography with temperature-programmed systems.

The major advantage of the use of the GC technique for amino acid analysis should be, however, in developing the possibility of coupling a gas chromatograph with a mass spectrometer or with a sequence analyzer for other kinds of study. The GC–MS combination is unique in both the structure identification of unknown compounds emerging from the GC column and in the quantitation of femtomole amounts of chromatographed substances (mass fragmentography with multiple ion

detection), provided that the mass spectrometer is used as a detector of the GC system. GC is also the preferable identification method for the N-terminal amino acids in sequence studies which are aimed at elucidating the primary structures of peptides and proteins. After each completed Edman degradation step in an automatic sequencer, the PTH amino acid is subjected to analysis in the GC column. Lastly, GC is virtually the only technique available for the determination of the optical antipodes of amino acids.

Among the numerous procedures reviewed for the GC determination of the 20 protein amino acids, the techniques making use of acylation of the amino acid alkyl esters have been by far the most used until now. The most reliable and generally used method has been the use of N-TFA-butyl esters. Excellent precision and accuracy have been demonstrated when using this method for amino acid analysis in biological material. The N-HFB-propyl or -isoamyl esters have also proved to be very convenient for the quantitation of the 20 protein amino acids in a single-column programmed operation. The same is true for the N-acetylpropyl esters, but with some restrictions due to incomplete derivatization of Arg and no obtainable peak for Cys on the recommended column packing. The use of N-TFA-methyl esters is not usual because of possible losses of the too volatile derivatives and a rather complicated temperature programme along with the specified column.

The persilylated derivatives, however attractive the procedure may seem at first, seem to be much less convenient for the quantitation of all 20 amino acids. The N-TMS-TMS esters are extremely sensitive compounds, their preparation is a lengthy procedure and the packed separation column is unusually long. The trimethylsilylated butyl esters have been shown to be more stable; however, this technique is not used because of the superior possibility of using the acylation procedure. Concerning the cyclic amino acid derivatives, the PTH-amino acids occupied a high position among useful GC procedures, but rather for qualitative analysis only in conjunction with automatic sequenators. The MTHs have recently been shown to be suitable derivatives for the determination of 18 amino acids (except Arg) in a single column run. The formation of oxazolidinones (the procedure is not yet completed) seems to be very hopeful for the determination of both protein amino acids and thyroid hormones in blood. The evaluation of this technique will follow as soon as possible.

A little more remains to be done on the determination of all amino acid optical antipodes. The resolution of the enantiomers of His and eventually Cys has not yet been successful. As well as the possibility of finding a new, more temperature-stable optically active stationary phase, work on the other technique, the acylation of amino acid methyl esters with N-TFA (or PFP)prolyl chloride and the separation of the resulting L,D-dipeptide on temperature-stable silicone phases, is also now in progress. These two approaches are generally believed to be able to solve the problem successfully.

The only constant feature of science is that of change. What, then, of the position of GC for amino acid analysis in the future? Without doubt, GC will share its position with the two other techniques that are now rapidly entering the field, namely high-performance liquid chromatography and isotachophoresis. Which of them will be the preferred method will perhaps be more a matter of subjective attitude than of the actual progress of a particular method.

8. SUMMARY

This review summarizes all papers that have appeared on the gas chromatography of amino acids (including the iodoamino acids) and their enantiomers in the period 1956–mid-1974. It has been found that the methods used for analysis of amino acids can be divided into three classes: (1) degradative procedures and techniques for converting the amino acid into another chemical compound; (2) procedures based on esterification of the carboxyl group and derivatization of the α-amino and other reactive groups in at least two steps; and (3) procedures based on a simultaneous derivatization of the carboxyl and α-amino groups in one reaction medium. For the treatment of the amino acid or its alkyl ester, three approaches can be distinguished for the two latter cases, *i.e.*, acylation, alkylation (including silylation) and condensation. Of the procedures used for the resolution of optical antipodes, two methods are discussed, namely analysis of diastereoisomers on optically inactive stationary phases and separation of enantiomers on optically active stationary phases.

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