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The Present State of the Gas-chromatographic Analysis of Aminoacids

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Problems of the general and enantiomeric gas-chromatographic analysis of aminoacid mixtures are considered. Data are presented concerning the accuracy, reproducibility, and linear range of the determination of aminoacids. The applicability of various types of derivatives is investigated from the standpoint of the suitability for quantitative analysis. Since analysis of aminoacids includes, apart from the chromatographic procedure and the final integration, their isolation from the natural material and the preparation of volatile derivatives, particular attention is devoted in the review to errors at each stage of the analysis and their contribution to the resulting reproducibility of the analysis.
The review is of interest to persons working in the field of the chemistry of natural products, biochemistry, medicine, as well as specialists developing analytical methods associated with the preparation of volatile derivatives.
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I. INTRODUCTION

Considerable advances have been achieved in recent years in the biochemistry and chemistry of proteins and aminoacids as a result of the application of modern analytical methods based on column and thin-layer chromatography. Nevertheless the search for more sensitive and accurate quantitative analytical methods is being continued. The principal trend in this search involves gas–liquid chromatography (GLC). Although conditions for gas-chromatographic analysis of general utility have not so far been found, it is already clear that the GLC of aminoacids is significantly more accurate than other methods, has an exceptionally low sensitivity theshold over a wide linear range, and is susceptible to full automation.

In contrast to an aminoacid analyser, the gas chromatograph is a universal instrument and can be used for the analysis of not only aminoacids but also of a multiplicity of other biochemically important substances of different types. A gas chromatograph can be very successfully combined with a mass-spectrometer, which gives rise to unique possibilities for the identification of minor components and anomalies in aminoacids. Finally GLC is the most accurate method for the analysis of mixtures of optical isomers (aminoacid enantiomers).

However, one must stipulate immediately that all the advantages of GLC in the analysis of aminoacids can be achieved only when instruments of sufficiently high quality are used. Thus relatively insensitive detectors, metallic columns with a high percentage content of the liquid phase, and coarse carriers combined with isothermal separation conditions were used in early attempts, these features being in general characteristic of the state of GLC at the end of the 1950's and the beginning of the 1960's. These studies showed that aminoacid derivatives are thermally unstable compounds with a wide range of vapour pressures and their analysis is therefore possible only on columns with a very low content of the [liquid] phase and very inert carriers and phases combined with programmed temperatures. This required the use of highly sensitive detection with a low noise level and compensation of background changes, which is best achieved with the aid of flame-ionisation detectors in compensation (two-column) schemes. Stabilised phases of the type of very pure polysiloxanes and polyesters as well as special carriers, ensuring minimal adsorption combined with a high homogeneity of the surface and the dimensions and shapes of the particles and lacking catalytic activity, have been widely employed. These are diatomaceous carriers such as Chromosorb W, Supelcoport, Anachrome, Gas Chrom, etc. The advantages of not only glass columns but also of a seamless separating channel became apparent.

Overall, it became clear that the gas chromatography of aminoacids is one of the most difficult ("delicate") chromatographic tasks and is comparable in its complexity and requirements with regard to equipment and materials perhaps only to the GLC of steroids. In view of the complexity and importance of such tasks, special chromatographs began to be manufactured in recent years for their solution (the so called chromatographs of the biomedical class), which constitute the highest achievements of chromatographic engineering in a certain sense as regards the rationality [of the design] and the quality of the separating channel.

This does not of course imply that satisfactory results cannot be achieved on ordinary industrial or home-made apparatus of suitable design. It is important that such apparatus should be provided with a seamless glass separating channel and a sensitive linear detector and should be based on a two-column scheme (for experiments with programmed temperatures). A unit for reproducible programming of the temperature, preferably with provision for the automatic return to the initial isothermal conditions, is essential. For quantitative analysis, the velocities of all the gases must be stabilised by accurate flow-rate regulators. Electronic integration combined with monitoring of the drift of the base line is necessary. In the latest studies carried out using modern research techniques, the most reliable results have of course been obtained.

Several reviews exist of the GLC of aminoacids[1-5] and the early studies are fairly fully described in Blau's

review[3]. The authors of the present review endeavoured to examine as fully as possible the latest and most important data concerning the development of the GLC method for aminoacids, concerning both the separation and preparation of derivatives and problems of quantitative analysis. Unfortunately not all the published studies have been performed at a sufficiently high quantitative level. This remark applies also to published reviews, since they discuss mainly problems concerning the preparation of derivatives and their chromatography rather than the characteristics related to the accuracy, reproducibility, sensitivity threshold, and linear range, i.e. all the factors which constitute the subject of the present review, which deals with standard protein aminoacids. Anomalous aminoacids, and their iodine- and selenium-containing analogues, have been considered in the review of Coulter and Hann[6].

II. VOLATILE AMINOACID DERIVATIVES AND THEIR CHROMATOGRAPHIC SEPARATION

a. Standard Protein Aminoacids

Since aminoacids are virtually involatile thermally unstable compounds, their direct gas-chromatographic analysis is impossible. It is therefore quite natural that the main bulk of published work on the analysis of aminoacids by GLC consists of studies on their transformation into volatile substances suitable for separation. The majority of these studies have been carried out at a qualitative or semiquantitative level, covering more than eighty types of aminoacid conversion products (see, for example, Blau[3]). Many of the results are of merely historical interest. However, on the whole they are useful both as a source of ideas for the solution of specific problems and the development of new methods for the identification and quantitative analysis of aminoacids.

There are several publications on the pyrolytic GLC of aminoacids in which the feasibility, in principle, of analysis based on the hydrocarbon or amine "profile" of the thermal decomposition products has been demonstrated. However, the complications arising from the fact that the same pyrolysis products are common to different aminoacids and the side reactions between the aminoacids and their decomposition products require special laborious investigations of the pyrolysis of aminoacid mixtures using a complex mathematical technique for the interpretation of the results, so that this procedure is on the whole rather unpromising as a standard method for the quantitative analysis of mixtures[3].

The development of the analysis of aminoacids by GLC followed a different pathway, involving chemical transformations of aminoacids into volatile compounds on the "one aminoacid–one derivative" principle, but attempts to employ oxidative, reductive, and other transformations of aminoacids into compounds of other classes (amines, aldehydes, aminoalcohols, etc.) did not yield satisfactory results, primarily because the methods are insufficiently universal and quantitative. The most promising procedures proved to be those based on the protection of the carboxy- and amino-groups as well as other functional groups.

However, here too serious difficulties arise owing to the fact that natural aminoacids are substances of an extremely wide variety of chemical types, the common feature of which is merely the amino-group in the α-position relative to the carboxyl. For this reason, as noted by Blau[3], it is difficult to find modifying reagents and conditions for quantitative reactions involving the synthesis of volatile derivatives which are common to all natural aminoacids. It is no less difficult to find conditions for the separation of such non-homologous mixtures.

Experiment has shown that only a very few of the proposed derivatives are really suitable for a simple and reliable quantitative analysis, i.e. satisfy the essential requirements listed below:

(1) simplicity, quantitative yield of derivatives, reproducibility, and, if possible, rapid conversion of all the natural aminoacids in the mixtures;

(2) optimal volatility of the derivative, suitable for chromatography without losses;

(3) stability of the derivative during the experiment and storage under the usual laboratory conditions;

(4) stability under the conditions of the gas-chromatographic experiment, i.e. on contact with the liquid phase and the solid carrier at temperatures up to 200–250°C;

(5) the possibility of obtaining individual peaks on the chromatogram on the "one aminoacid–one derivative–one peak" principle.

Requirements (4) and (5) determine the accuracy and reproducibility of the chromatographic analysis proper and taken together determine the accuracy and reproducibility of the entire method.

At the present time only two types of derivatives which satisfy sufficiently fully the above requirements are known. These are some of the alkyl esters of *N*-acylated (usually *N*-perfluoroacylated) aminoacids and to a lesser extent trimethylsilyl (TMS) derivatives. Subsequently we shall consider mainly studies using such derivatives as well as derivatives of other types which have been published after the publication of the reviews of Blau[3] and Coulter and Hann[6].

In essence, the gas-chromatography of aminoacids has begun to develop on the basis of Fischer's study[7] of the separation of methyl esters of aminoacids by a fractionation procedure. In 1957 Bayer et al.[8] separated a mixture of such esters. They also pointed out[9] the suitability for GLC of *N*-trifluoroacetyl (TFA) derivatives of methyl esters of aminoacids. Since then derivatives of this type have been studied very intensively. Formyl, acetyl, trifluoroacetyl, pentafluoropropionyl, heptafluorobutyryl and other groups have been used in various combinations for the protection of the amino-group (and also OH and SH groups) in methyl, ethyl, propyl, butyl, isopentyl, and other aminoacid esters. At the present time only butyl esters of *N*-trifluoroacyl derivatives can be separated on filled columns satisfying at the same time requirement (5), which is in fact the reason for their exceptional importance in quantitative analysis. As a result of studies mainly by Gehrke's school and also Darbre, Blau, and Islan[10], the n-butyl esters of *N*(*O*, *S*)-trifluoroacylated aminoacids (*N*-TFA n-butyl esters)† and to a lesser extent the corresponding methyl esters[12] have come to be used most frequently. The conditions for the preparation of these derivatives, giving the highest yields, the possible complications due to the presence of impurities and the oxidising action of atmospheric oxygen, stability during storage and gas-chromatographic analysis, and also optimal separation conditions have been studied in greatest detail.

† The *N*-TFA n-butyl esters have been proposed and separated for the first time by Zomsely et al.[11]

The alkyl esters of *N*(*O*, *S*)-acyl derivatives of aminoacids are usually prepared in two stages [13]. In the first stage, the aminoacid is converted into an alkyl ester by treatment with the corresponding alcohol in the presence of HCl. Methyl esters of aminoacids are also transesterified with higher alcohols [14]. After the removal of the excess reagent *in vacuo*, the product is acylated with a solution of the corresponding anhydride in methylene chloride, chloroform, or another suitable solvent.

It is noteworthy that new agents for the protection of functional groups have been discovered recently. In particular, Birkofer and Donike [15] recently suggested that bistrifluoroacetamide and its *N*-methylated analogue be used for trifluoroacetylation. The authors considered the application of these reagents in relation to various compounds, including certain aminoacids. The mildness of the acylation conditions, the possibility of obtaining mixed derivatives (the hydroxy-groups protected by trimethylsilyl groups are not affected during acylation), and the absence of the side reactions observed when trifluoroacetic anhydride is used have been noted among their advantages [16,17]. Both reagents are fairly volatile and emerge from the columns soon after the solvent.

The aminoacids must be freed from proteins, nucleotides, sugars, lipids, and other possible impurities before the synthesis of derivatives in the analysis of mixtures of natural products. Metal ions must also be eliminated, since most of them prevent a quantitative yield of the derivatives [18]. Furthermore, after entering the evaporator and the column, these ions catalyse the decomposition of the samples injected. The purification process and the complications arising in the analysis of aminoacids by GLC have been examined in detail [19].

The polyesters of ethylene glycol and neopentyl glycol with succinic, adipic, and sebacic acids exhibit the highest selectivity in the separation of the alkyl esters of *N*-acylaminoacids [13,20,21]. Neopentyl glycol sebacate, the phase with the highest thermal stability, is suitable for the separation of the *N*-TFA n-butyl esters of twenty protein aminoacids [21]. Columns with the mixed EGSS-X phase—diethyleneglycol succinate—have been used for the same purpose [22]. Polysiloxane phases (OV-1, OV-3, OV-7, OV-17, OV-25, OV-101, OV-210, OV-225, SE-30, SE-52, etc.) as well as their mixtures have been employed mainly for the analysis of mixtures containing heavy aminoacids, the separation of the derivatives of which on polar polyesters is difficult.

The majority of modern studies on the separation of *N*-TFA n-butyl esters culminated in the investigation by Stefanovic and Walker [23], who showed that the selectivity of columns with poly(ethylene glycol adipate) (PEGA) depends on the amount of phase deposited on the solid carrier. In particular, columns containing 0.65% of PEGA relative to the weight of the solid carrier (Chromosorb W) proved to be the most suitable for the separation of the *N*-TFA n-butyl esters of protein aminoacids (except tryptophan, arginine, histidine, and cystine). The optimal PEGA concentration for the separation of *N*-TFA n-butyl esters depends on the solid carrier employed—it amounts to 0.325% for Chromosorb G. [13]

Many modern studies on the separation of alkyl esters of *N*-acyl derivatives of aminoacids on columns with polar polyester phases with a low content of the phase have been carried out using a carrier heated to a specified temperature. Vitt et al. [24] showed that even a relatively inactive carrier, such as Chromosorb W, exhibits a sufficiently high capacity for adsorption, which is important for selective separation. It follows from the data in Table 1 that the free energy and enthalpy of solution on columns with a low content of the [liquid] phase are higher and that the order in which the *N*-TFA n-butyl esters emerge (i.e. the free energies of solution) under conditions of isothermal separation depends on the amount of the phase.

Table 1. The energies ΔG (kcal mole^{-1}) (at 126°C) and heats of solution ΔH (kcal mole^{-1}) (at 105–170°C) of the butyl esters of certain trifluoroacetylated aminoacids on columns with different PEGA contents on Chromosorb W(A.W.) [21].

Aminoacid	−ΔH	−ΔG	−ΔH	−ΔG
	0.8% PEGA		3% PEGA	
Glycine	15.0	5.5	14.9	5.4
Leucine	17.9	6.2	14.2	5.2
Methionine	20.2	7.4	18.0	6.9
Hydroxyproline	19.0	7.6	18.0	6.5
Phenylalanine	20.0	8.6	18.7	7.1

These data undoubtedly show that carriers with a low percentage of the phase behave in essence as modified sorbents a controlled selectivity of which is achieved by altering the content of the [liquid] phase or by heat treatment of the carrier. The separation on such carriers is sensitive to the type of temperature programme and the length of the chromatographic column. In particular, for a 3 m column with activated Chromosorb W, the optimal concentration ensuring the most effective separation of *N*-TFA n-butyl esters proved to be 0.58% [24] (for 1–1.5 m columns, Stefanovic and Walker [23] and Gehrke et al. [13] showed that the optimal PEGA concentration is 0.65%). The highest selectivity for a column length of 1.5–1.8 m is achieved at a rate of temperature rise of 2–4 K min^{-1} [13], while for a column 3 m long the optimum rate is 2–2.5 K min^{-1}.

According to our data, a change in the type of carrier for the stationary phase alters the nature of the separation. Presumably the future use of modified carriers of this type will extend the scope of the analysis of various volatile derivatives of aminoacids.

Gehrke et al. [25] proposed a scheme for the separation of *N*-TFA n-butyl esters of aminoacids based on the use of a single four-column apparatus or of two instruments, each comprising two columns. The first pair of columns with 0.65% of stabilised PEGA on Chromosorb W (A.W.), heat-treated for 12 h at 140°C, is used for the separation of *N*-TFA n-butyl esters of 17 aminoacids. The second pair of columns with 2% of OV-17 (OV-101) and 1% of OV-210 on silylated Chromosorb G is used for the separation of the *N*-TFA n-butyl esters of arginine, histidine, and cystine. Both pairs of columns can operate under the conditions of a single temperature programme and can therefore be combined in a single four-column apparatus, which permits the complete analysis of the protein aminoacids in a single cycle. The temperature programme is as follows: 70°C for 10 min followed by heating at a rate of 2–4 K min^{-1} to 235°C. The aminoacids are injected into the heated zone of the evaporator (at temperatures up to 240°C). According to the results of Vitt et al. [24], in the

analysis of *N*-TFA n-butyl esters of aminoacids it is more convenient to employ a cold evaporator, having selected suitably the initial isothermal treatment.

Studies designed to choose conditions for the analysis of the *N*-TFA n-butyl esters of all twenty protein aminoacids on a single pair of columns are being continued. However, so far they have not been successful. Gerhrke and Roach[26] recently reported the analysis of the BTA derivatives of all twenty aminoacids on a single column with 10% of Apiezon M on Chromosorb W(HP), but, judging from the chromatograms presented, the separation of the *N*-TFA n-butyl esters was insufficient for accurate quantitative analysis; 13 peaks of the *N*-TFA n-butyl esters of protein aminoacids were incompletely separated.

The alkyl esters of pentafluoropropionyl and heptafluorobutyryl aminoacid derivatives, which became available subsequently, will probably prove more promising in this respect. Pollock and coworkers[27] were the first to suggest butyl esters of *N*(*O*, *S*)-pentafluoropropionyl derivatives of aminoacids and investigated their separation on silicone phases. Moss et al.[28] demonstrated the possibility of using n-propyl esters of heptafluorobutyryl derivatives of aminoacids. They carried out an analysis on a 3.6 m column with 3% of OV-1 on Chromosorb W(A. W.) treated with dimethylchlorosilane (DMCS), the temperature being programmed in the range between 100° and 250°C. In order to obtain a single histidine peak, the authors injected acetic anhydride together with the sample. We believe that, in order to convert monoacylhistidine into the diacyl derivative on the column, it would be necessary to inject together with the sample the anhydride used for the modification, as suggested for the first time by Gehrke and coworkers[29] for *N*-TFA n-butyl esters and as was done by Jonsson et al.[30] in the analysis of aminoacids in the form of the n-propyl esters of the *N*-heptafluorobutyryl derivatives.

Jonsson et al.[30] carried out the analysis on a short glass capillary column (6 m × 0.25 mm) to the walls of which a dimethylsiloxane polymeric phase has been grafted chemically. They achieved a satisfactory separation of derivatives of all the protein aminoacids except tryptophan; for the determination of cysteine, the latter was converted into the *S*-methyl derivative before modification.

Zanetta and Vincendon[14] demonstrated a satisfactory single-column separation of isopentyl esters of heptafluorobutyryl derivatives with the aid of 3% of SE-30 on Gas Chrom Q. Compared with *N*-TFA n-butyl esters, these derivatives are less volatile (which lowers the losses during evaporation), are less polar (which promotes a faster analysis), and give rise to symmetrical peaks.

Studies using alkyl esters of perfluoroacyl derivatives (particularly heptafluorobutyryl derivatives) lead at the same time to the possibility of a sharp reduction of the sensitivity threshold (down to 10^{-12}–10^{-13} g) when an electron capture detector is used.

Despite the clear achievements in the separation and, as will be shown below, in the quantitative analysis based on esters of perfluoroacyl derivatives of aminoacids, the development of a single-stage method for the synthesis of volatile derivatives constitutes an attractive proposition. The studies by Rogozhin and coworkers[31], who showed that alkyl esters of *N*-acyl derivatives of aminoacids can be obtained in a single stage by condensing orthocarbonate esters with aminoacids, are of interest in this respect.

N- and *O*-Trimethylsilyl (TMS) derivatives can be obtained in a single stage. A fairly large number of studies have been made on the preparation and separation of TMS derivatives‡ of aminoacids[32-36]. In particular, new silylating agents have been proposed—bistrimethylsilylacetamide[36], bistrimethylsilyltrifluoroacetamide[34], etc.

These reagents give rise to a peak on the chromatogram which does not interfere with the peaks of the TMS derivatives of aminoacids. Furthermore, the use of bistrimethylsilyltrifluoroacetamide reduces the deposition of silica in the detector. The application of TMS derivatives of aminoacids in quantitative gas-chromatographic analysis is hindered by the fact that certain aminoacids form a mixture of silylation products, the proportions of which depend on the nature of the solvent employed[34].

In order to carry out the analysis of twenty protein aminoacids on a single column, Hardy and Kerring[37] used mixed derivatives—n-butyl esters of the *N*(*O*,*S*)-trimethylsilylated acids. The entire analysis on a ~2.5 m glass column with an internal diameter of 2 mm, filled with textured glass beads containing 0.2% of OV-7, was performed in less than 35 min.

The authors note that, in contrast to the free aminoacids, methylene chloride may be used as a solvent for the n-butyl esters in the silylation stage. On the one hand, the guanidino-group of arginine and the ω-amino-group of lysine are silylated under these conditions, which makes it possible to analyse the aminoacids, and, on the other hand, one can avoid the formation of the tristrimethylsilyl derivative of glycine, which emerges together with isoleucine derivatives during chromatography. However, as in the case of purely trimethylsilyl derivatives of aminoacids, the peaks of the serine and threonine derivatives are hardly separated at all, which naturally great complicates the quantitative analysis of these two aminoacids. Furthermore, the chromatograms show very small peaks corresponding to the trimethylsilyl derivatives of the non-esterified aminoacids, showing that the esterification reactions did not go to completion, which naturally constitutes an additional source of error in the analysis.

Thenot and Horning[38] used for GLC the alkyl esters of *N*-dimethylaminomethylene derivatives of aminoacids which are obtained in a single stage when *NN*-dimethylformamide reacts with aminoacid dialkyl acetals. They obtained derivatives of glycine, alanine, valine, leucine, isoleucine, aspartic and glutamic acids, phenylalanine, and lysine and separated them on filled and capillary columns with SE-30. We believe that, as long as the possibility of the rapid and quantitative formation of similar derivatives of "heavy" aminoacids (tryptophan, arginine, histidine, and cystine) has not been demonstrated, there is no point in trying to assess their practical value (with the exception, of course, of specific analytical problems). However, since the dialkyl acetals of *NN*-dimethylformamide do not react with hydroxy- and cercapto-groups under suitable conditions, the use of the above derivatives will always be limited.

The isopropylation of aminoacids has been recently proposed[39,40] as a single-stage reaction for the synthesis of derivatives to be used in GLC. However, these derivates have also scarcely found extensive applications, since satisfactory yields are obtained only for aminoacids without functional groups other than the carboxy- and α-amino groups. Furthermore, certain aminoacids give rise to several derivatives.

‡ The separation was as a rule carried out on silicone phases.

Mitchell's suggestion[41] that the Schiff bases of aminoacids, obtained by coupling benzaldehyde or penta-2.4-dione to methyl esters of aminoacids be used for GLC, has also apparently not found many applications. It is hardly possible to extend this method to all the protein aminoacids and to achieve sufficiently reproducible results.

Williams and Halpern[42] suggested that the gas-chromatographic analysis of aminoacids be carried out by pyrolysing in the evaporator of the chromatograph the trimethylanilinium salts of *N*-neopentylidene derivatives of aminoacids. These salts are obtained in a single stage by reaction with 2,2-dimethylpropanal and trimethylanilinium hydroxide in methanol for 15 min and 80°C in the presence of 3 Å molecular sieves. After being injected into the hot evaporator of the chromatograph, they decompose with formation of the *N*-neopentylidene derivative of the methyl ester of the corresponding aminoacid. The hydroxy-groups are also methylated. The nitrogen atoms in the proline molecule and in the indole component of the tryptophan molecule are likewise methylated. The derivatives obtained were successfully chromatographed on a ~2.5 m stainless steel column filled with 0.5% of OV-17 on Chromosorb W(DMCS). However, in this case too it is impossible to chromatograph all the protein aminoacids: histidine does not yield a volatile derivative. Furthermore, arginine and cystine give rise to several products. Incidentally, we believe that these difficulties may be obviated by replacing the column and particularly the evaporator by components made of glass and by a careful selection of the pyrolysis temperatures.

Finally, one should note that in recent attempts to discover a convenient single-stage method for the synthesis of volatile aminoacid derivatives various investigators have again shown interest in the preparation of cyclic aminoacid derivatives. Thus Grahl-Nielsen and Solheim[43] investigated the chromatographic behaviour of various 2-alkyloxazolin-5-one derivatives of leucine [44,45] and showed that, although these substances are extremely reactive, nevertheless their thermal stabilities make them quite suitable for chromatographic analysis. The authors recommend 2-trifluoromethyloxazolin-5-one, obtained by the reaction of leucines with trifluoroacetic anhydride, as the preferred derivative for the separation of leucine isomers. However, we believe that it is hardly possible to obtain similar derivatives of more complex aminoacids and to achieve quantitative yields. Furthermore, the high volatility of the derivatives, which facilitates chromatogrphy, may lead to significant losses at the unavoidable stage involving the distillation of the trifluoroacetic acid formed.

Husek[46] used the reaction of aminoacids with dichlorotetrafluoroacetone, which also yields in a single stage analogous derivatives[47,48], in order to determine tyrosine and its mono- and di-iodo derivatives. The use of this reaction is probably still more limited, since in this case additional reactions are needed to protect the hydroxy-, ω-amino-, etc. functional groups. (Husek protected the hydroxy-group of tyrosine by reaction with perfluorobutyric anhydride). However, one should recall that similar derivatives with a large number of halogen atoms in the molecule make it possible to reduce sharply the sensitivity threshold when an electron capture detector is used and may therefore prove to be extremely useful in the solution of specific problems.

The interest in the GLC of thiohydantoin derivatives of aminoacids is noteworthy. In general, these derivatives are not very suitable for gas-chromatographic analysis but are important in connection with the determination of the primary structure of peptides and proteins by the method of Edman and Begg. We shall not consider problems concerning the analysis of these derivatives, since a fairly complete review by Pisano et al.[49] was published recently. Furthermore, the application of GLC for the determination of *N*-terminal aminoacids of proteins and peptides has been examined by Rosmus and Deyl[50]. Among the latest studies, the analysis of phenylthiohydantoins on short capillary columns is noteworthy[51].

b. Aminoacid Enantiomers

Two methods for the separation of enantiomers by gas chromatography are known at present. The first is based on the quantitative conversion of the enantiomers into diastereoisomers under the influence of a dissymmetric agent. The diastereoisomers are then separated on a usual chromatographic phase[52-54]. The other method is based on the use of a dissymmetric stationary phase[55].

Weygand et al.[53] achieved for the first time the separation of diastereoisomeric aminoacid derivatives by the GLC of dipeptides; at the same time the possibility of determining the enantiomeric purity and the configurations by the GLC method was demonstrated by a number of investigators using as an example the much more suitable *N*-trifluoroacetyl (*N*-TFA) derivatives of the diastereoisomeric aminoacid esters[56-63].

In particular, the present authors[63], Pollock et al.[59], and Gil-Av and coworkers[56] described in 1965 the separation of *N*-TFA derivatives of aminoacid esters using optically active s-butyl alcohol and menthol[61]. After this, numerous investigations of this problem were published[63-78].

As regards the determination of the configuration by the GLC method, one should note the relation between the elution order of the diastereoisomeric derivatives and their configurations established by a number of workers. Thus, it has been shown that, in a series of aminoacid esters[59,64,65], the diastereoisomers with the *RR*- and *SS*-configurations have the lowest energies of solution in the chromatographic phase and hence the lowest retention volumes, while the highest retention volumes are characteristic of the *RS*- and *SR*-forms. The 2-butyl ester of phenylglycine[64], a non-natural aminoacid, is an exception. It has been established, for a series of amides obtained from aminoacid esters and amines, $RCH(NHCOCF_3)CONH-\underset{\displaystyle R''}{\underset{|}{CH}}-R'$ (R' = alkyl and R'' = alkyl or COOR), that the *SS*-diastereoisomer is eluted after the *SR*-form.[66] According to the authors, the explanation is that the *SS*-structure of amides has a more open form, as a result of which the amide hydrogen in this structure is more accessible for interaction with the carbonyl group of the polyester phase (it has been established that such interaction occurs[66]).

Although the *N*-TFA derivatives of s-butyl esters of aminoacids can be separated on both filled[68,69,71] and capillary columns[59,61,64,65], in practice only capillary chromatography yields satisfactory results, i.e. results suitable for quantitative analysis, which is due to the low value of $\Delta(\Delta G)$ for the majority of the phases investigated[69] (Table 2). Another disadvantage of the separation of aminoacids in the form of the *N*-TFA derivatives of their s-butyl esters is the low availability of the optically pure s-butyl alcohol. On the other hand, the separation of diastereoisomers of this type makes it possible to analyse the enantiomers of heavy aminoacids, which is difficult in the case of the more bulky L-menthyl derivatives.

N-TFA-L-menthyl derivatives of aminoacids have very high values of $\Delta(\Delta G)$ (Table 3)[69], which makes it possible to use the usual (filled) columns[63]. The preparation of the derivatives constitutes the difficulty in this instance. The value of $\Delta(\Delta G)$ is partly influenced by the conformational immobility of the groups.

Table 2. The values of $-\Delta(\Delta G)$ (cal mole^{-1}) for the s-butyl esters of *N*-TFA derivatives of aminoacids (at 125 °C) on different chromatographic phases.

Aminoacid	PEGA	PEG	NPGA
Alanine	44.0	57.0	42.0
Valine	52.0	44.0	44.0
Leucine	45.0	57.0	50.0
Isoleucine	70.0	86.0	55.0
Norvaline	35.0	52.0	32.0
Norleucine	47.0	44.0	32.0
α-Aminooctanoic acid	67.0	39.0	—

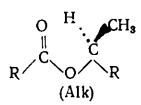
Note: PEGA = poly(ethylene glycol adipate), PEG = poly-(ethylene glycol), and NPGA = neopentylglycol adipate.

In the chromatography of *N*-TFA-L-prolyl-2-methylindone, $\Delta(\Delta G^\circ) = 298$ cal mole^{-1}, which is the highest value noted in all instances of the separation of diastereoisomers, was obtained[72]. In this compound, the asymmetric centres are located in the ring, so that the conformational immobility of the groups linked to them is greater.

Table 3. The values of $-\Delta(\Delta G)$ (cal mole^{-1}) for the L-menthyl esters of *N*-TFA derivatives of aminoacids (at 160°C) on different chromatographic phases.

Aminoacid	PEGA	PEG	NPGA	APIEZON-L
Alanine	109.0	117.0	59.0	99.0
Valine	188.8	127.0	88.0	109.0
Leucine	145.0	—	124.0	108.0
Isoleucine	141.0	—	124.0	138.0
Norvaline	145.0	—	118.0	104.0
Norleucine	109.0	—	112.0	118.0
α-Aminobutyric acid	149.0	—	138.0	89.0

If the dissymmetric agent is acyclic, the groups linked to the asymmetric centre must be as large as possible. The point is that free rotation about the O–(Alk) bond in the molecule of the diastereoisomeric ester is hindered owing to the presence of the neighbouring carbonyl group.



When the substituent R′ is varied from CH_3 to t-C_4H_9, the separation is improved as a result of the decrease of the conformational mobility caused by the increase of the energy barrier to free rotation about the Alk–O bond[66,72].

As already pointed out, the other method for the gas-chromatographic analysis of enantiomers involves their separation on a dissymetric phase. The first reliable result was obtained by Gil-Av et al.[78] in 1967 in the separation of the ethyl, n-propyl, isopropyl, n-butyl, and 2-butyl esters of the *N*-TFA derivatives of alanine, valine, and leucine and the lauryl ester of *N*-TFA-L-isoleucine. Derivatives with a single asymmetric centre gave two peaks and those with two centres gave four peaks. The separation was specific: the methyl esters were not separated at all and only the cyclopentyl esters were fully separated.

Four types of dissymmetric stationary phases are now used for the gas-chromatographic analysis of enantiometric derivatives of aminoacids (Table 4):

I. Esters of *N*-TFA derivatives of aminoacids and C_6–C_{12} alcohols[78,79].

II. The ureide of the isopropyl ester of valine[55,80,81] (for the separation of amines and aminoacids).

III. Esters of *N*-TFA derivatives of dipeptides and C_1–C_6 alcohols[58,80-90].

IV. Amides[91,92].

The isopropyl esters of *N*-TFA-derivatives of aminoacids are most frequently separated. On peptide and ester L-phases, the D-isomer is eluted before the L-isomer, which makes it possible to determine the configurations of the aminoacids. On the ureide phase, the elution sequence of the enantiomers is related to the size of the alkyl substituents at the asymmetric centre and in the ester group of the enantiomer[80].

According to Gil-Av and coworkers[82,84,85], the mechanism of the separation process consists in the formation of diastereoisomeric complexes of the aminoacid enantiomer with the dissymmetric phase. It is suggested that the linkage between the molecules in these "complexes" is formed via hydrogen bond bridges. In other studies, hypotheses have been put forward concerning the structures of the "complexes" formed[79,84,85] and studies have been made in order to elucidate the role of the amide and ester components of the dipeptide[85,86,91] in the formation of the "complexes" with the enantiomeric aminoacid derivatives. Regardless of whether we attribute the dissolution of the aminoacid derivatives in the peptide phase to the formation of a "complex" or whether we explain the observed difference between the dissolution energies by specific solvation and a statistical distribution of different types of association, it is clear that the amide component of the dipeptide is in fact responsible for the stereoselective effect[86,91], although important secondary steric contributions are made by the ester end[85,86]. This is why tripeptide and even more so polypeptide phases proved to be less suitable for the separation of enantiomeric aminoacid derivatives than dipeptide phases[84,86].

Best results in the separation of aminoacid enantiomers (in terms of the highest values of $\alpha_{L/D}$§) have been obtained using dipeptide stationary phases of type (III), in which the hydrocarbon substituents at the asymmetric centres of the dipeptide and the ester groups are fairly bulky and branched (for example the cyclohexyl or t-butyl esters of the *N*-TFA derivatives of L-valyl-L-valine), and also on stationary phases of type (IV).

§ $\alpha_{L/D}$ is the ratio of the retention volumes of the L- and D-enantiomers of aminoacids.

Table 4. The separation of derivatives of enantiomeric aminoacids on dissymmetric phases.

Aminoacids and their analytical forms	$\alpha_{L/D}$	Remarks
90°C; 50–100 m Pyrex column; d = 0.25 mm; chromatographic phase—lauryl ester of *N*-TFA-L-leucine [79, 82]		
2-Butyl esters of N-TFA derivatives of:		complete separation
D- and L-alanine	–	
D- and L-valine	–	
D- and L-leucine	–	
Methyl and ethyl esters of *N*-TFA derivatives of:		almost no separation
D- and L-alanine		
Isopropyl esters of *N*-TFA derivatives of:		
D- and L-alanine	1.001	poor separation
n-Butyl esters of *N*-TFA derivatives of:		
D- and L-alanine	1.012	*inflection*
Cyclopentyl esters of *N*-TFA derivatives of:		
D- and L-alanine	1.069	complete separation
100°C; 2 m x 1 mm column; 5% of chromatographic phase on Chromosorb W; chromatographic phase—cyclohexyl ester of *N*-TFA-L-valyl-L-valine [80]		
t-Butyl esters of *N*-TFA derivatives of D- and L-alanine	–	in complete separation
110°C; 150 m x 0.5 mm steel capillary; He pressure 1.5 atm; chromatographic phase–cyclohexyl ester of *N*-TFA-L-valyl-L-valine [82]		
Methyl esters of *N*-TFA derivatives of:		7 components out of 31 are not determined (the peaks are superimposed)
D- and L-alanine	1.067	
D- and L-aminobutyric acid	1.056	
D- and L-valine	1.031	
Glycine	–	
D- and L-threonine	1.053	
D- and L-n-valine	1.054	
D- and L-alloisoleucine	1.054	
D- and L-isoleucine	1.059	
D- and L-leucine	1.057	
D- and L-serine	1.045	
D- and L-norleucine	1.055	
D- and L-proline	1.044	
D- and L-aspartic acid	1.039	
D- and L-methionine	1.072	
D- and L-phenylalanine	1.082	
D- and L-glutamic acid	1.081	
110°C; 150 m x 0.5 mm steel capillary; chromatographic phase—cyclohexyl ester of *N*-TFA-L-valyl-L-valine [82]		
Isopropyl esters of *N*-TFA-derivatives of:		
D- and L-Alanine	1.096	
D- and L-aminobutyric acid	1.089	
D- and L-valine	1.080	10 components out of 34 are not determined
Glycine	–	
D- and L-threonine	1.104	
D- and L-n-valine	1.095	
D- and L-alloisoleucine	1.097	
D- and L-isoleucine	1.077	
β-Alanine	–	
D- and L-leucine	1.114	
D- and L-serine	1.080	
D- and L-allothreonine	1.080	
D- and L-n-leucine	–	
D- and L-proline	1.041	
110°C; 30 m x 0.5 mm steel capillary; chromatographic phase—cyclohexyl ester of *N*-TFA-L-valyl-L-valine [82]		
Isopropyl esters of *N*-TFA derivatives of:		
D- and L- aspartic acid	1.062	
D- and L-methionine	1.116	
D- and L-phenylalanine	1.138	
D- and L-glutamic acid	1.123	
110°C; 120 mm x 0.5 mm steel capillary; chromatographic phase—cyclohexyl ester of *N*-TFA-L-valyl-L-valine [82, 93]		
n-Butyl esters of *N*-TFA derivatives of:		
D- and L-alanine	1.087	4 components out of 23 are not determined
D- and L-valine	1.056	
Glycine	–	
D- and L-threonine	1.095	
D- and L-alloisoleucine	1.083	
D- and L-isoleucine	1.059	
D- and L-leucine	1.099	
D- and L-serine	1.079	
D- and L-proline	1.045	
110°C; 60 m x 0.5 mm steel capillary; chromatographic phase—cyclohexyl ester of *N*-TFA-L-valyl-L-valine [82]		
n-Butyl esters of *N*-TFA-derivatives of:		
D- and L-methionine	1.110	
D- and L-phenylalanine	1.120	
D- and L-aspartic acid	1.060	

Table 4 cont'd.

Aminoacids and their analytical forms	$\alpha_{L/D}$	Remarks
110°C; 120 m x 0.5 mm steel capillary; chromatographic phase—cyclohexyl ester of *N*-TFA-L-valyl-L-valine [82]		
3-Pentyl esters of *N*-TFA derivatives of:		6 components out of 27 are not determined
D- and L-alanine	1.130	
D- and L-α-aminobutyric acid	1.119	
D- and L-valine	1.090	
Glycine	–	
D- and L-threonine	1.100	
D- and L-alloisoleucine	1.116	
D- and L-isoleucine	1.098	
D- and L-serine	1.123	
D- and L-n-leucine	1.130	
D- and L-proline	1.039	
110°C; 60 m x 0.5 mm steel capillary; chromatographic phase—cyclohexyl ester of *N*-TFA-L-valyl-L-valine [93]		
3-Pentyl esters of *N*-TFA derivatives of:		
D- and L-serine	1.123	
D- and L-aspartic acid	1.083	
D- and L-phenylalanine	1.099	
100°C; 60 m x 0.25 mm glass capillary; chromatographic phase—cyclohexyl ester of *N*-TFA-L-valyl-L-valine [93]		
Methyl esters of *N*-TFA derivatives of D- and L-alanine	1.080	
Ethyl esters of *N*-TFA derivatives of D- and L-alanine	1.106	
Isopropyl esters of *N*-TFA derivatives of D- and L-alanine	1.136	
t-Butyl esters of *N*-TFA derivatives of D- and L-alanine	1.207	
n-Propyl esters of *N*-TFA derivatives of D- and L-alanine	1.106	
n-Butyl esters of *N*-TFA derivatives of D- and L-alanine	1.113	
Cyclopentyl esters of *N*-TFA derivatives of D- and L-alanine	1.139	
107–109°C; 70 m x 0.25 mm glass capillary; chromatographic phase—isopropyl ester of *N*-TFA-L-valyl-L-valine [84]		
Methyl esters of *N*-TFA derivatives of D- and L-alanine	1.076	
Ethyl esters of *N*-TFA derivatives of D- and L-alanine	1.103	
Isopropyl esters of *N*-TFA derivatives of D- and L-alanine	1.126	
n-Propyl esters of *N*-TFA derivatives of D- and L-alanine	1.103	
t-Butyl esters of *N*-TFA derivatives of D- and L-alanine	1.214	
Cyclopentyl esters of *N*-TFA derivatives of D- and L-alanine	1.133	
139°C; 70 m x 0.25 mm glass capillary; chromatographic phase—isopropyl ester of *N*-acetyl-L-valyl-L-valine [84]		
Methyl esters of *N*-TFA derivatives of D- and L-alanine	1.072	
n-Propyl esters of *N*-TFA derivatives of D- and L-alanine	1.086	
t-Butyl esters of *N*-TFA derivatives of D- and L-alanine	1.130	
n-Butyl esters of *N*-TFA derivatives of D- and L-alanine	1.090	
Cyclopentyl esters of *N*-TFA derivatives of D- and L-alanine	1.095	
Isopropyl esters of *N*-acetyl derivatives of D- and L-alanine	1.103	
140°C; 120 m x 0.5 mm steel capillary; chromatographic phase—cyclohexyl ester of *N*-TFA-phenylalanyl-L-leucine [83, 90]		
Isopropyl esters of *N*-TFA derivatives of:		
D- and L-alanine	1.073	
D- and L-isoleucine	1.070	
D- and L-threonine	–	
D- and L-valine	1.045	
Glycine	–	
D- and L-leucine	1.083	
D- and L-serine	–	
D- and L-proline	1.00	
140°C; 30 x 0.5 mm steel capillary; chromatographic phase—cyclohexyl ester of *N*-TFA-phenylalanyl-L-leucine [83]		
Isopropyl esters of *N*-TFA derivatives of:		
D- and L-aspartic acid	–	
D- and L-methionine	–	
D- and L-phenylalanine	–	
D- and L-glutamic acid	–	
130°C; 10 m x 0.5 mm steel capillary; chromatographic phase—cyclohexyl ester of *N*-TFA-phenylalanyl-L-leucine [83]		
Isopropyl esters of *N*-TFA derivatives of:		
D- and L-phenylalanine	–	
D- and L-glutamic acid	–	
D- and L-tyrosine	–	
110°C; 120 m x 0.5 mm steel capillary; chromatographic phase—cyclohexyl ester of *N*-TFA-phenylalanyl-L-leucine		
Methyl esters of *N*-TFA derivatives of D- and L-leucine	1.082	
Isopropyl esters of *N*-TFA derivatives of D- and L-leucine	1.127	
t-Butyl esters of *N*-TFA derivatives of D- and L-leucine	1.176	
Methyl esters of *N*-pentafluoropropionyl (*N*-PFP) derivatives of D- and L-leucine	1.090	
Isopropyl esters of *N*-PFP derivatives of D- and L-leucine	1.123	
t-Butyl esters of *N*-PFP derivatives of D- and L-leucine	1.167	

Table 4 cont'd.

Aminoacids and their analytical forms	$\alpha_{L/D}$	Remarks
Methyl esters of *N*-heptafluorobutyryl (*N*-HFB) derivatives of D- and L-leucine	1.091	
Isopropyl esters of *N*-HFB derivatives of D- and L-leucine	1.129	
t-Butyl esters of *N*-HFB derivatives of D- and L-leucine	1.142	
Methyl esters of *N*-pentadecafluorooctanoyl (*N*-PDFO) derivatives of D- and L-leucine	1.100	
Isopropyl esters of *N*-PDFO derivatives of D- and L-leucine	1.137	
t-Butyl esters of *N*-PDFO derivatives of D- and L-leucine	1.177	
100°, 110°, 120°, and 130°C (the results are indicated in the corresponding sequence); 120 m × 5 mm steel capillary; chromatographic phase—cyclohexyl ester of L-norvalyl-L-norvaline [88]		
Isopropyl esters of *N*-TFA derivatives of:		
D- and L-alanine	1.110	
	1.087	
	1.075	
	1.063	
D- and L-valine	1.083	
	1.069	
	1.050	
	1.044	
D- and L-isoleucine	1.108	
	1.093	
	1.079	
	1.068	
D- and L-leucine	1.100	
	1.096	
	1.082	
	1.070	
D- and L-α-aminobutyric acid	1.097	
	1.086	
	1.071	
	1.060	
D- and L-norvaline	1.092	
	1.081	
	1.070	
	1.060	
D- and L-norleucine	1.108	
	1.096	
	1.083	
	1.070	
D- and L-t-leucine	1.044	
	1.040	
	1.034	
	1.028	
100°C; remaining conditions—as above [88]		
Isopropyl esters of *N*-TFA derivatives of:		2 components out of 15 are not determined
D- and L-alanine	–	
D- and L-valine	–	
Glycine	–	
D- and L-threonine	–	
D- and L-isoleucine	–	
D- and L-leucine	–	
D- and L-serine	–	
D- and L-proline	–	
120°C; 122 × 0.508 mm steel capillary; chromatographic phase—cyclohexyl ester of the *N*-TFA derivative of the L-α-aminobutyric acid dipeptide [89]		
Isopropyl esters of *N*-TFA derivatives of:		
D- and L-alanine	–	
D- and L-valine	–	
D- and L-threonine	–	
D- and L-isoleucine	–	
D- and L-leucine	–	
D- and L-serine	–	
D- and L-proline	–	
D- and L-aspartic acid	–	
Glycine	–	
D- and L-threonine	–	
145°C; 120 m × 0.5 mm steel capillary; chromatographic phase—caproylvaline *N*-n-hexylamide [90]		
Isopropyl esters of *N*-TFA derivatives of:		
D- and L-alanine	1.069	
D- and L-valine	1.047	
D- and L-n-valine	1.070	
D- and L-isoleucine	1.070	
D- and L-leucine	1.087	
D- and L-n-leucine	1.072	
130°C; 45 m × 0.5 mm steel capillary; chromatographic phase—*N*-lauroyl-L-valine-*N*-t-butylamide [91]		
Methyl esters of *N*-TFA derivatives of:		2 components out of 26 are not determined
D- and L-alanine	1.188	
D- and L-valine	1.170	
D- and L-threonine	1.177	
D- and L-t-leucine	1.084	
D- and L-alloisoleucine	1.186	
D- and L-isoleucine	1.159	
D- and L-leucine	1.280	
D- and L-proline	1.057	

Table 4 cont'd.

Aminoacids and their analytical forms	$\alpha_{L/D}$	Remarks
D- and L-serine	1.101	
D- and L-aspartic acid	1.078	
D- and L-glutamic acid	1.170	
D- and L-methionine	1.215	
D- and L-phenylalanine	1.198	
120°C; 80 m × 0.25 mm glass capillary; chromatographic phase—carbonylbis-(*N*-L-valine isopropyl ester) ureide [80]		
Esters $RCH(NHCOCF_3)CO_2R$ [R = CH_3, C_2H_5, n-C_3H_7, n-C_4H_9, n-C_5H_7, iso-C_3H_7, or $CH(C_2H_5)_2$; the results are given in the same sequence] of:		
D- and L-alanine	1.017	
	1.031	
	1.034	
	1.039	
	1.042	
	1.046	
	1.060	
D- and L-α-aminobutyric acid	1.000	
	1.014	
	1.017	
	1.022	
	1.023	
	1.027	
	1.050	
D- and L-α-aminovaleric acid	0.976	
	0.994	
	0.997	
	1.002	
	1.004	
	1.005	
	1.030	
D- and L-α-aminohexanoic acid	0.968	
	0.985	
	0.989	
	0.994	
	0.997	
	1.002	
	1.027	
D- and L-α-aminooctanoic acid	0.956	
	0.973	
	0.977	
	0.979	
	–	
	0.990	
	1.016	
D- and L-valine	1.012	
	1.030	
	1.033	
	1.034	
	1.036	
	1.043	
	1.058	
D- and L-leucine	0.957	
	0.977	
	0.981	
	0.983	
	0.987	
	1.000	
	1.025	
D- and L-t-leucine	1.000	
	1.015	
	–	
	–	
	–	
	1.019	
140°C (the results are quoted in the same sequence); 17 m × 0.25 mm glass capillary; chromatographic phase—isopropyl ester of *N*-TFA-(L-valyl)$_2$L-valine (I) in mixture with isopropyl ester of *N*-TFA-L-valyl-L-valine (II) [84]		
t-Butyl esters of *N*-TFA derivatives of D- and L-alanine	1.288	$X^* = 0.42$
	1.269	$X = 0.42$
	1.242	$X = 0.28$
	1.239	$X = 0.42$
	1.216	$X = 0.55$
	1.214	$X = 0$
	1.205	$X = 0.28$
	1.206	$X = 0.42$
	1.203	$X = 0.55$
	1.187	$X = 0$
	1.180	$X = 0.28$
	1.179	$X = 0.42$
	1.176	$X = 0.55$
	1.165	$X = 0$
	1.156	$X = 0.28$
	1.159	$X = 0.42$
	1.154	$X = 0.55$
	1.142	$X = 0$
	1.139	$X = 0.28$

* $\frac{n_1}{n_1 + n_2}$; n_1 and n_2 are the numbers of moles of (I) and (II).

It has been shown that the structure of the volatile aminoacid derivative is important for the separation[84,85,88,91]. Thus the following features were noted in the separation of isopropyl esters of *N*-TFA-aminoacids on the cyclohexyl ester of *N*-TFA-L-norvalyl-L-norvaline[88]:

(a) The successive increase in the length of the hydrocarbon group and the replacement of the isopropyl ester of *N*-TFA-alanine by the corresponding α-aminobutyric acid and norvaline derivative leads to a decrease of $\alpha_{L/D}$.

(b) The replacement of the protons in the methyl group of alanine (the same derivatives) by methyl groups gives rise to the following series in terms of $\alpha_{L/D}$ [83]:

alanine > α-aminobutyric acid > valine ≫ t-leucine

(c) For leucines, we have the following series in terms of the values of $\alpha_{L/D}$:

leucine, isoleucine > n-leucine ≫ t-leucine.

Parr and Howard[88] explained these characteristics by the steric hindrance in the formation of a complex with the chromatographic phase.

Proline, the *N*-TFA derivative of which does not contain amide hydrogen, yields the lowest separation factor among all the aminoacids on the available chromatographic phases[91,93]. The separation of aspartic acid enantiomers is also comparatively poor[93] (which cannot be said about glutamic acid).

All the volatile aminoacid derivatives which are capable of satisfactory resolution into enantiomers contain the group $-CONHCHR-CO-$. The quality of the separation is closely related to the presence of this structure[84], as shown by the finding that, on the isopropyl ester of *N*-TFA-L-valyl-L-valine, *N*-TFA-2-aminoheptane gives rise to only a slight inflection, while the isopropyl esters of *N*-(2,2,2,-trifluoroethyl)alanine [$CF_3CH_2NH-CH-COOCH(CH_3)_2$] and *N*-TFA-β-aminobutyric acid are not resolved at all[82]. According to the authors, these derivatives do not form "complexes" with the chromatographic phase and are involved in three hydrogen bonds. The statistical weight of the selective association of dipeptides (which have a high capacity for the formation of hydrogen bonds) via two or three bridges, for example in the case of *N*-TFA-2-aminoheptane, is very low[84].

Whereas the presence of the group $-CO-NH-HCR-CO-$ (the acceptor and donor sections are located on either side of the asymmetric centre) is required for separation on a peptide phase, the presence of the group $-CO-NH-\overset{|}{C}{}^{*}$ is sufficient for the urea derivative obtained from L-valine,

$$(CH_3)_2CH-\underset{\diagdown\ \diagup}{CH}-NHCONH-\underset{|}{CH}-CH(CH_3)_2$$
$$\qquad\quad COOCH(CH_3)_2 \qquad\quad COOCH(CH_3)_2$$

on which both amines and aminoacids can be separated[80]. Here it is significant that the elution sequence of the D- and L-isomers of the aminoacid derivatives $RCH(NHCOCF_3)CO_2R'$ is influenced only by the size of the R and CO_2R' groups and to a certain extent by the degree of branching of the hydrocarbon substituents.

Tripeptide phases have been used to separate enantiomers[84,86]. Here the working temperature is higher by about 10 K than the working temperature of the dipeptide phase and the separation factor is slightly lower. Furthermore, tripeptide esters can be used to make up binary phase compositions with m.p. $< 80°C$.

Polypeptide phases have also been investigated; the separation on such phases proved to be poor, so that they can be scarcely used for the practical separation of enantiomers[86].

N-Pentafluoropropionyl and *N*-heptafluorobutyryl derivatives of dipeptide esters can be used to separate aminoacid enantiomers[86], while the corresponding *N*-acyl derivatives of aminoacid esters are more stable[85], the separation factors on these phases are higher, and the retention times are shorter than for the corresponding *N*-TFA derivatives[92].

The heats of reaction of the enantiomers with the dipeptide phases have been published in the literature[84,88,90,94]. For example, $\Delta(\Delta H)$ for the enantiomers of the *N*-TFA derivatives of the isopropyl esters of a number of aminoacids have the following values on the cyclohexyl ester of *N*-TFA-L-norvalyl-L-norvaline [88]:

Aminoacid	Aniline	α-Aminobutyric acid	Valine	Norvaline	Leucine	Isoleucine	t-Leucine
$-\Delta(\Delta H)\pm$ ± 5, cal mole^{-1}	342	339	302	293	366	348	156

Table 4 lists the aminoacid derivatives and the phases used in the enantiomeric analysis of aminoacids on dissymmetric phases.

Summarising the results published hitherto concerning the use of dissymmetric phases for the separation of aminoacids, the following factors must be noted particularly.

1. The differences between the excess energies of the interaction of enantiomeric aminoacid derivatives with the phases described in the literature range from several cal mole^{-1} to 150 cal mole^{-1} depending on the enantiomer–chromatographic phase pair selected and the experimental temperature (values calculated from the retention parameters)[93].

2. Effective separation of aminoacid enantiomers during a reasonable period is possible despite the fact that high values of $\alpha_{L/D}$ are obtained on capillary columns only (Table 4).

3. The attainment of effective separation of enantiomeric pairs has in many instances been accompanied by the superposition of the peaks of several aminoacids belonging to different enantiomeric pairs despite the large number of phases tested (Table 4).

4. Unfortunately, many phases have a narrow working temperature range (the lower limit of the range is determined by the melting point of the phase and the upper limit by its volatility or instability).

Gas-chromatographic determination of the enantiomeric composition of complex mixtures makes it possible to solve also the specific problem involving the determination of optical purity. In both instances gas chromatography has an undoubted advantage over polarimetry and NMR. This highly effective method of separation does not, in principle, entail a limit to the number of components of the mixture of both optically active and inactive components being analysed.

III. QUANTITATIVE ANALYSIS

Quantitative analysis is characterised by parameters such as accuracy, reproducibility, linear range of concentrations being determined, and the sensitivity limit. For a multistage analytical procedure (and the GLC of aminoacids is in fact such a procedure), these parameters are determined by the partial parameters of each stage: the isolation of aminoacids from a natural or synthetic mixture, the formation of derivatives, the chromatographic separation proper, and the determination of the areas of the chromatographic peaks. Comparatively few studies

have been made on the determination of these parameters[24,95-102] and those which have been carried out were mainly concerned with the separation of the butyl esters of the trifluoroacetylated derivatives by the Stefanovic–Gehrke method[12,13] and trimethylsilane derivatives and the preparation of such derivatives.

a. Reproducibility of the Analysis

The literature data on this problem are the most numerous and contradictory (coefficients of variation $\overline{w}$ ranging from 0.02 to 35% are quoted). This is not unexpected—there is also a large scatter in data concerning the reproducibility of the ion-exchange analysis of aminoacids. The results obtained in the separation of aminoacid enantiomers in the form of the isopropyl esters of their *N*-TFA derivatives on the cyclohexyl ester of *N*-TFA-L-valyl-L-valine [95] under the conditions of isothermal separation on capillary columns, i.e. under conditions ensuring the optimum signal/noise and signal/drift ratios with adequate separation of the peaks and equal chemical stabilities and volatilities of the components separated, have been the most reproducible (to within 0.02%, Varian 480 integrator).

The reproducibility of the separation of aminoacids in the form of the butyl esters of their trifluoroacetylated derivatives is somewhat less satisfactory. According to the results of Vitt et al.[24], the separation of such esters of twelve aminoacids on a 3 m glass column with stabilised PEGA (0.58%) has $\overline{w}$ = 0.17 rel.% (Infotronics CRS-100 integrator), the error (as well as the error in the separation of the enantiomers) depending on the amount of the sample injected into the chromatograph (see subsection c of the present Section). According to Gehrke's data[96], the average chromatographic reproducibility for 19 aminoacids, obtained from three replicate determinations, is to within 1.3%. The chromatographic reproducibility of the separation of trimethylsilane derivatives is to within 1.1 rel.% (for 20 aminoacids) according to Gehrke et al.[33]

Talbot et al.[97] investigated the reproducibility of the calibration coefficients in the analysis of the butyl esters of trifluoroacetylated aminoacids and showed that the coefficients vary on passing from one column to another (even with the same chromatographic phase). Thus the calibration coefficients of the threonine differed from one another by a factor greater than 2.5. The reason for this may be that the author failed to reach the complete separation of the butyl esters.

There is no doubt that the dependence of the calibration coefficients on the properties of the column may be reduced under the conditions of unified procedures for the preparation of the columns and by improving the efficiency of the separation. According to the present authors' results (with α-aminooctanoic acid as the internal standard), the calibration coefficients for the majority of aminoacids vary on passing from one column with PEGA to another by not more than 3%, the variation increasing to 10% only for threonine and serine. The stability of the calibration coefficients was achieved by a reproducible preparation of the columns and their prolonged conditioning (for at least 60 h at 190–200°C in a stream of helium).

The reproducibility of the conversion and the degree of racemisation in the preparation of derivatives for enantiomeric analysis may be investigated on the basis of data for the final procedure—the gas-chromatographic separation. However, the best procedure for the synthesis of the butyl esters of trifluoroacetylated aminoacids (direct esterification–acylation) is as follows. A dry mixture of aminoacids (between 2 μg and 50 mg of all the aminoacids) is covered by 3 *N* HCl solution in thoroughly dry n-butyl alcohol and heated at 100°C for 15–35 min. The excess reagent and water are distilled off on a rotary evaporator or in a stream of pure nitrogen, the mixture being heated twice with anhydrous methylene chloride in order to remove the residual water. The acylation is carried out with a 10% solution of trifluoroacetic anhydride in methylene chloride at temperatures up to 150°C for 5–60 min. There is a wide range of conditions associated with the different reactivities and stabilities of various aminoacids. Thus, when the mixture does not contain isoleucine, 15 min is sufficient for esterification (the maximum yield of the n-butyl ester of tryptophan is also obtained under these conditions); the quantitative esterification of isoleucine requires an increase of the reaction time to 35 min. On the other hand, the n-butyl esters of certain aminoacids (particularly histidine) are trifluoroacetylated quantitatively at a sufficient rate only at 100–150°C.[13]

The literature data for the preparation of the butyl ester of trifluoroacetylated histidine are contradictory (see, for example, the review of Coulter and Hann[6]). Even the structure of the volatile derivative is unknown. In view of the difficulty of the direct determination of histidine in the form of the butyl ester of trifluoroacetylated derivative, its preliminary conversion into aspartic acid by ozonolysis was suggested[6]. For the same purpose, arginine may be hydrolysed to ornithine in the presence of arginase[6].

Other complications in the quantitative analysis of the butyl esters of trifluoroacetylated derivatives are associated with the fact that the prolonged esterification necessary for the complete conversion of all 20 protein aminoacids into their esters is accompanied by partial charring of tryptophan. Therefore the esterification conditions must be rigorously standardised as regards the concentrations and amounts of reactants, the duration of reaction, and temperature[98].

According to our results, the calibration coefficients for the majority of butyl esters of trifluoroacetylated aminoacids do not change on storage of the derivatives (at room temperature) in the presence of a 2% solution of trifluoroacetic acid in methylene chloride for two weeks; however, the butyl ester of trifluoroacetylated methionine decomposes rapidly under these conditions. Dilute aqueous solutions of methionine are also unstable. Therefore methionine-containing mixtures must be analysed rapidly.

Together with the rigorous requirements which must be met by the gas chromatograph (seamless glass separation channel, minimal contact with the metal in the path traversed by the gases between the end of the column and the flame, an absolutely dry and filtered carrier gas), it is suggested that special attention should be devoted to the purity of the vessels used, the quality of the rotary evaporator which must not contain metallic components, and the purity of the derivatives of the reactants and gases used in the synthesis.

Vitt et al.[24] reported that samples without histidine and arginine can be acylated quantitatively with a satisfactory reproducibility at room temperature by two treatments with trifluoroacetic anhydride (initially in the form of a 10% and then a 2% solution in CH_2Cl_2) with intervening distillation of the CF_3COOH formed and the solvent on a rotary evaporator.

The esterification of aminoacids by isopropyl alcohol is much slower than the esterification with n-butyl alcohol. Fig. 1 presents kinetic curves for the esterification of a number of aminoacids[95], from which it follows that there

is virtually no complete conversion in the synthesis of these derivatives. The authors recommend that the esterification time be limited to 4 h, since after more prolonged esterification the change in the enantiomeric composition of the mixture in consequence of racemisation becomes comparable to the random chromatographic error.

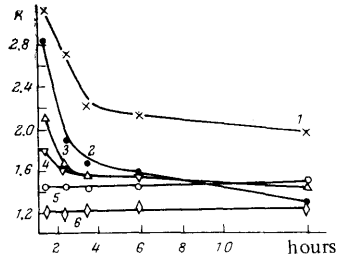


Figure 1. Dependence of the calibration coefficients of the isopropyl esters of *N*-TFA derivatives of aminoacids on esterification time; $K = S_{st}(m_i/m_{st})s_i$ (m_i is the aminoacid sample, m_{st} the sample of the standard, s_i the area of the aminoacid peak, and s_{st} the area of the peak due to the standard): 1) isoleucine; 2) valine; 3) threonine; 4) leucine; 5) glycine; 6) alanine.

In general aminoacid analysis, the reproducibility in the preparation of derivatives characterises the scope of the analysis of mixtures which do not require preliminary purification. The optimal overall reproducibility for two stages quoted in the literature[24] is $\bar{w}_{n=4,\, m=12} = 0.7\%$. According to the results of Gehrke and coworkers[96], the corresponding quantity for 19 aminoacids amounted to $\bar{w}_{n=6,\, m=19} = 1.5\%$. If the preparation of derivatives must be preceded by ion-exchange purification, the reproducibility of the analysis is impaired even more. Incidentally, this is no longer the "fault" of the gas chromatography—purification is a standard procedure also in analysis by ion-exchange chromatography.

Gehrke and coworkers[96,98] showed that the ion-exchange purification required for the isolation of the pure aminoacid fraction to be used in subsequent aminoacid analysis entails a partial loss of a number of aminoacids. The same workers established that arginine is completely lost in certain types of purification. The reproducibility of the gas-chromatographic analysis of synthetic and natural mixtures (urine, blood), obtained from three replicate determinations, amounted under these conditions to about 1.5% for synthetic mixtures and to 1.5–2.2% for natural mixtures. The reproducibility of the ion-exchange chromatography of the same specimens incorporating 17 aminoacids was to within 5–12%. Vitt et al.[24] obtained similar reproducibilities: 1.1% for the former case and 1.3% for the latter (for 12 aminoacids). Evidently the reproducibility of the ion-exchange purification is impaired for highly contaminated (natural) mixtures.

b. Accuracy of the Analysis

Strictly speaking the solution of the problem of accuracy is associated with the availability of standard procedures. Studies involving a standardisation procedure require standards whose purity approaches the reproducibility of the method and for this reason ion-exchange chromatography cannot serve as a standard for the determination of the accuracy of the gas-chromatographic analysis of aminoacids.

In all probability one must assume that gas chromatography itself constitutes the required standard procedure. The possibility of standardisation based on the equality of the concentrations of the compounds analysed was considered in the survey of Vitt et al.[95] Such instances include, for example, the equality of the concentrations of enantiomers in racemic mixtures. It has been shown that, in the chromatography of volatile D-aminoacid derivatives under isothermal conditions, a systematic error is observed, depending on the ratio of the peak variances and heights, i.e. on the concentration. This error is associated with the integration threshold—a sharp peak is integrated more fully than a broad peak. In the cases investigated the error was 0.02–0.3 rel.%.

When the internal standard method is employed (i.e. in general aminoacid analysis), the systematic error is determined by the purity of the standards and the operating point employed within the linear range of the method.

Under the conditions of large systematic errors which are known to exceed the uncertainty in the composition of the standards (this case is in fact characteristic of the stages involving the preparation of derivatives and particularly the purification on ion-exchange columns), the systematic errors can be readily determined from a small number of experimental results. This error (the average for 17 aminoacids) has been determined as 3.24 rel.% in the analysis of a standard mixture in the presence of urine aminoacids with removal of protein by ion-exchange[96]. The error can probably be reduced by extending the calibration over the entire analysis, including the purification stage.

In conclusion we shall consider the problem of the analysis of aminoacids in the case of overlapping peaks. The usual gas-chromatographic separation of the butyl esters of trifluoroacetylated aminoacids gives a satisfactorily resolved chromatogram for 18–20 natural aminoacids and is therefore suitable for an accurate quantitative analysis. Other methods, particularly the separation of enantiomers, where the number of peaks increases by a factor of two, do not exhibit this feature. The overall resolution depends in this instance on the separation of the components corresponding to various aminoacids and not on the selectivity of the separation within the enantiomeric pairs, i.e. is associated only with the efficiency of the column used. The reason is that the aminoacid mixtures are not homologous mixtures and therefore the retention volume of any one enantiomer of a single aminoacid may be close to the retention volume of other aminoacids. The likelihood of this situation increases with increase of the number of aminoacids present in the mixture. For a quantitative analysis of such partly resolved mixtures, simultaneous gas-chromatographic analysis on a column with a usual (not dissymmetric) stationary phase and the use of the available data for the aminoacid (but not enantiomeric) composition of the mixtures analysed has been suggested[95].

Then it follows from the experimentally demonstrated equality of the relative sensitivities of flame-ionisation detectors that

$$s_L + s_D = \omega s^*,$$

where $\omega = s_{st}/s^*_{st}$ is a proportionality coefficient, which remains unchanged for all the components in the given chromatographic experiment, s^* represents the peak areas for a column with a dissymmetric phase, s_{st} the peak area of the standard, and s the peak areas for a column with an optically inactive phase. Hence

$$S^*_L = \omega s - s^*_D.$$

In this case the systematic error depends on the accuracy of the determination of the peak areas under the conditions of chromatography on a usual phase.

c. The Linear Range and the Limits of Sensitivity

Depending on the concentrations being determined, the gas-chromatographic analysis of aminoacids in the form of the butyl esters of their trifluoroacetylated derivatives [13] is carried out using samples in the range 2 μg–50 mg. The size of the sample injected into the chromatograph then varies from 0.125 to 500 μg.

Chromatograms showing that individual peaks for 6–8 ng of aminoacids, injected into the gas chromatograph in the form of the butyl esters of their trifluoroacetylated derivatives, can be obtained using a flame-ionisation detector have been described in the literature [97]. In order to improve the signal/noise ratio, the bulk of the solvent vapour was discarded via a valve located between the analytical column and the fore-column [97].

According to the results of Vitt [110], the minimum amount of aminoacid which can be determined (in the form of the butyl ester of its trifluoroacetylated derivative) using an electron capture detector is 1–50 pg (evidently for a very narrow linear range).

It has been established [24] that the systematic and random errors in the gas-chromatographic analysis of the butyl esters of trifluoroacetylated aminoacids pass through a minimum (Fig. 2), i.e. there is an optimum concentration of the samples to be analysed as regards accuracy and reproducibility. The errors in the range of concentrations below the otpimum value are probably due to interference (noise and drift of columns), while the right-hand branch of the curve is due to the impairment of the resolution of the peaks with increase in concentration.

It follows from Fig. 2 that the linear range of the method is determined by the permissible concentrations and, conversely, the accuracy and reproducibility are determined by the linear range adopted and can be improved by operating within a narrower range of concentrations in the region of the minimum. The wide scatter of the literature values as regards accuracy and reproducibility is probably due to the fact that the investigators have not always worked in the concentration range corresponding to the optimum and also a satisfactory resolution and an adequately high signal/noise ratio were not always achieved. When the experimental parameters are incorrectly selected, the curve relating the errors to concentration is located at a higher level and has sharper minimum.

These limitations apply not only to the accuracy and reproducibility but also to threshold sensitivity: we are still unable to isolate aminoacids quantitatively and to obtain their derivatives at the level of 10^{-10} g.

Evidently relations between accuracy and reproducibility on the one hand and concentration on the other should also exist for processes involving the synthesis of aminoacid derivatives and the isolation of aminoacids from natural mixtures; in the region of low concentrations, there is a possibility of a sharp increase of errors owing to oxidation and residual sorption on glass and the materials of the columns [101]. One must recognise that, in a multistage procedure including gas chromatography, the stages preceding the gas chromatography proper are rate-limiting and have been least thoroughly developed.

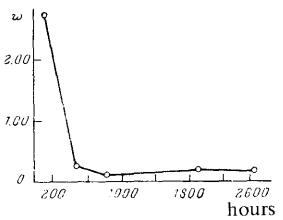


Figure 2. Variation of the reproducibility of the calibration coefficients of aminoacids with the concentration of the sample injected.

It follows from the above results that the accuracy and reproducibility of gas chromatography as a quantitative method are at least an order of magnitude higher than those of polarimetry and NMR in cases most favourable for these methods (in mixtures of two enantiomers, in determinations of optical purity). This can also be said of the sensitivity thresholds of the methods considered.

IV. EXAMPLES OF APPLICATIONS OF THE GAS-CHROMATOGRAPHIC ANALYSIS OF AMINOACIDS

In the first studies on the gas chromatography of aminoacids (see, for example, Gehrke et al. [13]), the analyses of natural mixtures (usually acid protein hydrolysates) were mainly carried out in order to demonstrate the applicability and reliability of the method, comparison with the results of ion-exchange chromatography being used as a criterion. As a result of numerous investigations, it became clear that the analysis of aminoacids by GLC is very accurate and reproducible and the number of studies in which GLC is used as an independent analytical method (not requiring a check by comparison with other methods) is at present increasing. Furthermore, certain investigations became possible (or at least became greatly facilitated) only after the application of the universal and highly sensitive gas-chromatographic method for the analysis of aminoacids.

We shall endeavour to demonstrate on a number of characteristic examples the present level of studies using GLC for the analysis of aminoacids and the scope of this method.

In 1971 Pellizzari and coworkers [13] used GLC to analyse blood plasma aminoacids in the form of the n-butyl esters of their *N*-trifluoroacetyl derivatives and carried out a statistical assessment of the results. They showed that only 0.2 ml of plasma is needed for the analysis and that

the reproducibility of the results is quite satisfactory for clinical applications, for example for the diagnosis of metabolic disturbances (particularly in diseases associated with a disturbance of the aminoacid composition of proteins).

Subsequent studies confirmed the usefulness of GLC for the analysis of aminoacids in blood, urine, and other biological liquids in clinical tests.

For example the dynamics of aminoacid contents of the blood of patients operated on for cancer of the stomach and fed parenterally has been studied[69]. The aminoacids were determined in the blood plasma before the parenteral administration of the Aminosol preparation (1 g per kg of body weight in the form of a 10% solution) and in the course of its administration. It was found that the sum of the free analysable aminoacids in the blood plasma of the majority of the patients investigated decreased following the introduction of the aminoacid preparation into the blood supply, i.e. the injection of the mixture of aminoacids into blood accelerates sharply their assimilation. The blood plasma of the patients contained exclusively aminoacids having the L-configuration.

The increasing application of the GLC of aminoacids in biological studies is associated with the determination of the aminoacid composition and the primary structure of peptides and proteins and this is by no means the least important application. We have already noted[46] the success of the GLC of the thiohydantoin derivatives of aminoacids—the products of the stepwise degradation of proteins by the Edman method. The greater sensitivity of GLC compared with thin-layer chromatography makes it possible to use smaller amounts of the test substance and to determine the structures of proteins having higher molecular weights.

The advantages of gas chromatography may be even greater in the analysis of the composition of molecules of complex substances such as lipoproteins, protein–nucleic acid complexes, glycoproteins, etc. For example, Zanetta and Vincendon[14] showed that, in the case of glycoproteins, it is possible to analyse initially the hydrocarbon component and then the protein component of the same specimen. The universality of the GLC method is extremely convenient also in the analysis of products of aminoacid metabolism. Thus Albro and Fishbein[103] determined simultaneously with the aid of gas chromatography about 30 tyrosine and tryptophan metabolites—substances of very different chemical types.

In order to illustrate the scope of GLC in complex biological studies involving the determination of the aminoacid compositions, we may mention the study of Butler et al.[104], who used this method to analyse the aminoacids attached to tRNA.

Analysis of aminoacids by GLC has been used successfully in geochemical and paleontological investigations. Thus Pocklington[105] used this method for the quantitative analysis of the aminoacids in the water taken from the North Atlantic and Gardner and Lee[106] determined aminoacids in lake water. In both cases the aminoacid concentration was about 10^{-8} M (with respect to each aminoacid) and GLC proved to be a convenient and accurate analytical method. Gehrke's group[19] analysed the aminoacids in aqueous extracts of a specimen taken from the ancient Onverwacht chart (whose age is 3.4×10^9 years). GLC has demonstrated with a high degree of reliability the presence of aminoacids at the level of 10^{-8}–10^{-9} g. Bearing in mind that the minimum detectable amount of aminoacids has been reduced to 1–50 pg with the aid of the electron capture detector, the advantage of GLC in the analysis of aminoacids at an ultramicrolevel over other methods becomes clear. The new mass-spectrometric degradation method, usually called the "mass fragmentation" method, has a similar sensitivity limit. This technique has already been applied to the gas–liquid analysis of aminoacids in biological compounds[107] and in soil.

The unique possibility of determining by GLC the enantiomeric composition of aminoacid mixtures has been used in the analysis of collagen fossils[108], deposits on the sea bottom[109], and also the enantiomeric composition of the aminoacids in blood plasma and the hydrolysates and autolysates of baker's yeast[110].

The GLC of aminoacids will undoubtedly find extensive applications in studies associated with the origin of life on Earth and the possibility of life on other celestial bodies. Thus Raulin et al.[111] used this method to analyse the aminoacids formed in the photolysis of a gaseous mixture simulating Earth's primitive atmosphere. They showed that, in a single chromatographic experiment, it is possible to analyse together with the twenty main protein aminoacids also at least 18 non-protein aminoacids, which is particularly important in the study of chemical evolution and gives to gas chromatography an important advantage over aminoacid analysers.

The gas-chromatographic method has been used to seek aminoacids and other organic substances in the lunar dust supplied by the American Apollo spaceships[19,100] and we believe it will undoubtedly prove suitable for the analysis of soil specimens taken from the planets.

In conclusion we may note that the literature concerning the uses of the analysis of aminoacids by GLC is extremely extensive. This also shows that the reliability of the method and its suitability for the usual tasks in aminoacid analysis and for the solution of special problems requiring a universal, highly sensitive, accurate, and reproducible analytical procedure have been adequately demonstrated.

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