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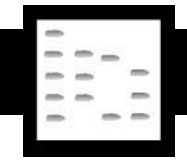
AMINO ACIDS



Gas Chromatography

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During the 1950s and 1960s, significant progress was made in the development of automated amino acid analysers based on separation by ion exchange. However, such instruments were dedicated to the task of amino acid analysis and were of limited application to the analysis of other types of compounds. Furthermore, they were expensive. During the same period, gas chromatography (GC) was being rapidly developed following the demonstration in 1952 by James and Martin that fatty acids could be assayed by GC. There followed a vast expansion in the application of GC to the analysis of other types of compounds. Amino acids were a logical target. In the intervening years, methods have been developed for assaying amino acids in protein hydrolysates and physiological fluids, and for determining the proportions of amino acid enantiomers in racemic mixtures. Some landmark developments are listed in **Table 1**.

Proteic and Physiological Amino Acids

Derivative Development

Amino acids are not sufficiently volatile or stable at the temperatures required for analysis by GC. Thus, they must be converted to derivatives having the desired characteristics. It was to be no simple task to derivatize or mask the several functional groups in even the 20 proteic amino acids. Carboxy, amino, hydroxy and sulfhydryl groups all need to be converted to eliminate internal zwitterionic charges and hydrogen bonding, and thus increase the volatility of the derivatives. It was thought in those early years that the molecular mass also required to be reduced but it was later realized that this was not an absolute requirement. As new reagents became available, it was found that volatility could be significantly increased while increasing the derivative mass. Apart from the multiplicity of functional groups, it is also necessary that each group should be quantitatively converted.

The first report of amino acid analysis by gas liquid chromatography was published in 1956. Hunter, Dimick and Corse oxidized isoleucine and leucine with ninhydrin to form volatile aldehydes. These were resolved using a 10 ft long silicone oil–celite column operated isothermally at 69°C. Peaks were detected at about 44 and 48 min (**Figure 1**). The aldehydes were generated using 2–5 mg of each amino acid. Either of the leucines could be assayed in the presence of 10-fold quantities of the other. However, only about eight simple amino acids yield volatile aldehydes.

From this simple but momentous beginning, there followed, in the next two decades, a proliferation of reaction schemes to prepare stable, volatile amino acid derivatives. Various oxidation, hydrocracking, pyrolysis and reduction reactions were explored but significant progress was to evolve from those procedures which focused on substituting the exchangeable protons of the reactive groups. In 1957, Bayer, Reuther and Born separated glutamic acid, leucine, methionine, norleucine, norvaline, phenylalanine, sarcosine and valine methyl esters on a silicone oil–sodium caproate packing. The use of an acyl ester constituted the first report of a key component in

Table 1 Advances in gas chromatography of amino acids

1956	First GC analysis (Hunter, Dimick and Corse)
1959	Acyl amino acid alkyl esters separated (Youngs)
1965	Resolution of alanine, leucine and valine enantiomers (Gil-Av, Charles and Fischer)
1962–79	Development of derivatization and separation procedures for the proteic amino acids (Gehrke)
1971	First single column separation of all proteic amino acids (Moss, Lambert and Diaz)
1971–76	Further improvements in resolution
1977	Development of Chirasil Val® (Frank, Nicholson and Bayer)
1989	Use of cyclodextrins for enantiomer resolution (König, Krebber and Mischnick)
1991	4 min analysis of proteic amino acids (Hušek)

a derivatization strategy which would eventually prove to be successful. One year later, Bayer reported that good resolution could be achieved using *N*-trifluoroacetyl (TFA) amino acid esters. This work represented the first use of *N*-TFA derivatives, representatives of a class of compounds which would feature strongly in later developments.

In the next decade, *N*-formyl and -acetyl derivatives were combined with a variety of alkyl esters such as methyl, ethyl, propyl, isopropyl, isobutyl, amyl and isoamyl. The work of Youngs in 1959 was the first in which *N*-acyl derivatives were combined with alkyl amino acid esters. *N*-acetyl ethyl and butyl esters of six simple amino acids were separated on hydrogenated vegetable oil. This approach was to provide the foundation for developments leading, over the next several years, to the quantitative resolution of all the amino acids in a protein hydrolysate. In 1964 Karmen and Saroff showed that excellent yields of *N*-TFA amino acid methyl esters were obtained when the esters were first prepared and then acylated. This general protocol remains in use.

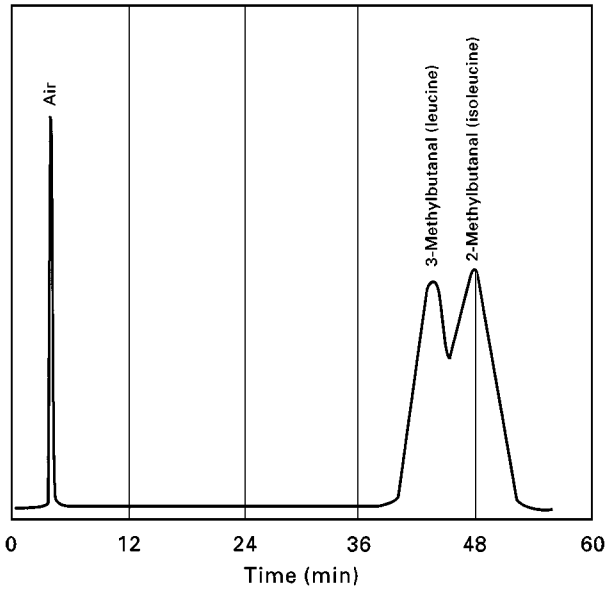

Figure 1 Separation of 3-methylbutanal and 2-methylbutanal using a 10ft column filled with a silicone–celite mixture. (Reproduced with permission from Hunter IR, Dimick KP and JW Corse (1956) Determination of amino acids by ninhydrin oxidation and gas chromatography. *Chemistry and Industry* 294–295.)

The use of *N*-TFA derivatives in combination with amino acid alkyl esters was first reported by Ettre in 1962. Starting in the same year, Gehrke and his colleagues systematically studied the derivatization and chromatography of the *N*-TFA *n*-butyl amino acid esters. TFA derivatives were used in amino acid chemistry by Weygand as early as 1952 but were first applied in the context of GC analysis in 1960. In the first report, 22 naturally occurring amino acid derivatives were resolved in less than 45 min using a 2 m column packed with Gas Chrom A coated with 1% neopentyl succinate. Subsequently, the esterification reaction was simplified by using direct esterification instead of methylation followed by interesterification. Direct on-column injection and an all-glass system were demonstrated to avoid degradation of some derivatives. Rigorous exclusion of water is necessary both for complete derivatization and to prevent hydrolysis of derivatives once formed. These and other procedures developed by Gehrke formed a solid quantitative foundation for subsequent studies by others.

Continued refinement of both the reaction chemistry and the columns culminated in the complete separation of the 20 proteic amino acids in 1971. Seventeen amino acids were resolved using a 4 mm i.d. × 1.5 m glass column packed with 0.65% ethylene glycol adipate (EGA) on 80–100 mesh Chromosorb W–AW. The derivatives of arginine, histidine, tryptophan and cystine were separated from those of the other amino acids on a 4 mm i.d. × 1.5 m glass column packed with a mixed stationary phase of 2% OV-17 and 1% OV-210 coated on 100–200 mesh Supelcoport. In particular, histidine could be directly assayed. The two columns were operated simultaneously, resulting in an analysis time of 15–30 min. In 1979, the same derivatives were separated on a single EGA liquid phase but no significant improvement over other available procedures was obtained.

Gehrke also conducted a thorough assessment of possible sources of contamination. As detection sensitivity increased, contamination became a significant problem. At the nanogram level, contamination was shown to derive from laboratory reagents such as butanol, methylene chloride and water, and from human sources such as dandruff, fingerprints, hair, saliva and skin fragments.

The stationary phases used during the early years of development fell into three main classes: silicones, polyglycols and polyesters. Because it was difficult to separate even the proteic amino acids on a single phase, mixed phases were common. Eventually, however, the silicone phases, in nonpolar or slightly polar forms, became favoured and were essential for quantitative elution of arginine, cystine and histidine derivatives. Dual- and triple-column procedures were to give way in the search for a single column separation of the proteic amino acids. The first such resolution was achieved in 1971 by Moss, who prepared the *N*-heptafluorobutyryl (HFB) *n*-propyl esters. These were resolved on a $10 \times 1/4$ in glass column packed with 3% OV-1 coated on 80–100 mesh HP Chromosorb W (**Figure 2**). No quantitative data were provided. There followed other variations on the same theme. The *N*-HFB isoamyl (1973), isobutyl (1974) and isopropyl (1979) esters provided similar resolutions but with subtle separatory advantages depending on the relative proportions of specific amino acids present. Resolution was primarily a function of the ester, while the acyl group mainly moderated the volatility.

The search for a single-column resolution of the proteic amino acids was paralleled by a search for a single reaction which would derivatize all the functional groups present in amino acids. Trimethylsilylation was introduced as early as 1961 by Rühlman and Giesecke who reacted trimethylchlorosilane with amino acid salts. Six amino acids were separated in less than 30 min. A fuller account in 1963 reported that tyrosine and histidine derivatives tended to decompose in the presence of moisture or oxygen. The early reagents were generally silylated amines or monosubstituted amides and double derivative formation was a significant problem. However, newer reagents, for example *bis*-(trimethylsilyl)trifluoroacetamide, were considerably more potent and derivatization became quantitative. In more recent work (1993), all 22 proteic amino acids, including glutamine and asparagine, which would not be present in protein hydrolysates, have been quantitatively resolved as the *N*(*O*)-*tert*-butyldimethylsilyl derivatives in 41 min on a DB-1 column. The derivatives are formed in 30 min at 75°C.

Other approaches have also been used in the search for the simplest derivatization commensurate with reproducibility and stability, and with good chromatographic characteristics. Reaction with dichlorotetrafluoroacetone forms stable 2,2-*bis*(chlorodifluoromethyl)-4-subst-1,3-oxazolidine-5-one derivatives. All the proteic amino acids and more than 30 other α-amino acids have been studied. However, a second reaction with HFB anhydride is required and analysis of the diaminodicarboxylic acids histidine and tryptophan required a second column.

Alkoxycarbonyl alkyl esters, specifically the isobutoxycarbonyl methyl esters, were first prepared by Makita in 1976. Twenty proteic amino acid derivatives were separated using a dual-column system but the derivatization procedure involves multiple extraction. Arginine was first converted to ornithine. At that time, this procedure offered no significant advantage over the other protocols available. However, the method was subsequently improved so that, in 1996, all the proteic amino acid derivatives were resolved as single peaks in 9 min using a DB-17 capillary column. Serum amino acids could be assayed without any prior clean-up except for deproteinization. The isobutoxycarbonyl derivatives have also been effectively combined with *tert*-butyldimethylsilyl esters.

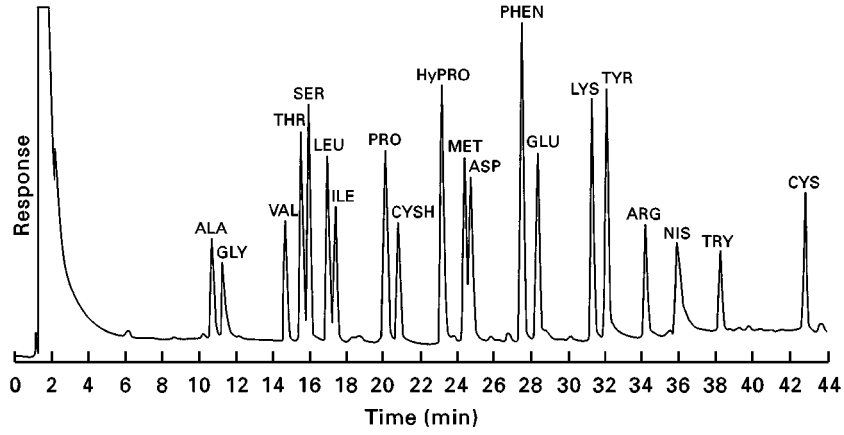

Figure 2 Separation of *N*(*O*, *S*)-heptafluorobutyryl *n*-propyl amino acids. (Reproduced with permission from Moss CW, Lambert MA and Diaz FJ (1971) Gas-liquid chromatography of twenty protein amino acids on a single column. *Journal of Chromatography* 60: 134–136.)

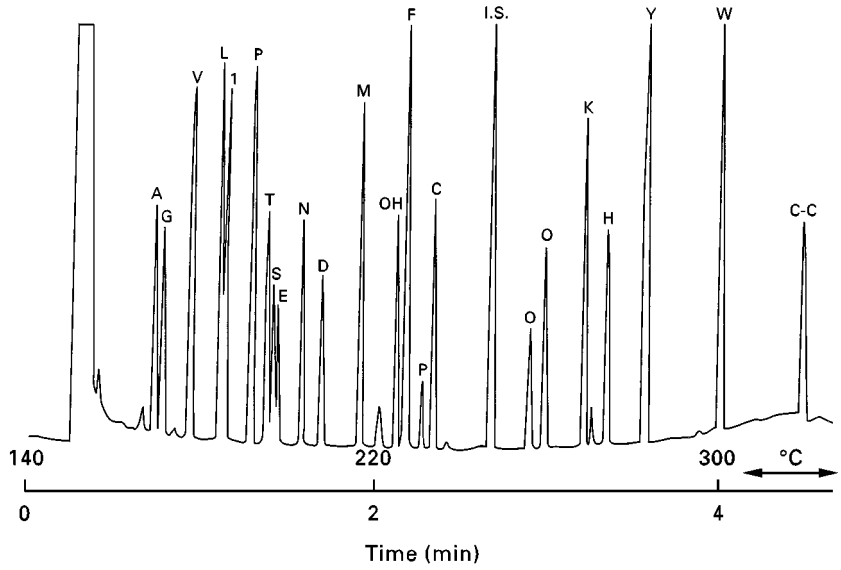

Figure 3 Analysis of *N*(*O*, *S*)-ethoxycarbonyl amino acid ethyl esters on a 10 m × 0.25 mm i.d. capillary column coated with OV1701. (Reproduced with permission from Hušek P and Sweeley CC (1991) Gas chromatographic separation of protein amino acids in four minutes. *Journal of High Resolution Chromatography* 14: 751–753.)

In 1991, Hušek prepared derivatives in the same general class but using ethyl chloroformate which reacts with all the functional groups found in amino acids. The *N*(*O*, *S*)-ethoxycarbonyl ethyl esters are formed in seconds in an aqueous medium. The derivatives were resolved in less than 5 min using a moderately polar OV1701 capillary column (**Figure 3**).

A variety of derivatization options are now available. The *N*-HFB isoamyl, isobutyl or isopropyl esters are equally effective for the relatively simple task of assaying the standard proteic amino acids. However, procedures requiring only a single derivatization step are more convenient and are preferred. Either the isobutoxycarbonyl methyl esters or the ethoxycarbonyl ethyl esters can be quickly prepared and resolved in less than 10 min using moderately polar capillary columns.

The several hundred amino acids which are present in physiological fluids cannot be resolved by any single method. Each method has advantages in a specific context. Frequently, however, the target is a single or a few structurally related amino acids. In such a context, any of the methods cited above may be appropriate, depending on the specific separations required. However, methods based on alkoxycarbonyl alkyl esters are more convenient to implement. Furthermore, some physiological samples, such as sera, can be assayed directly after deproteinization.

Very few amino acids are not amenable to being analysed by GC. Furthermore, the resolving power of capillary column chromatography cannot be matched by any other separatory medium. GC remains the method of choice for assaying amino acids in complex physiological samples.

Resolution of Optical Isomers

The determination of the configuration of amino acids and the relative proportions of the D and L forms is important in both natural and synthetic contexts. Proteins in living organisms commonly contain only the L-amino acids but D-amino acids occur in antibiotics (e.g. antiamoebin, gramicidin, valinomycin), bacterial cell wall peptidoglycans and in animals and insects. They have also been detected in human urine and blood. On death, the L-amino acids racemize, but so slowly that a racemic mixture is only produced over a geological time scale. The racemization rate is a function of temperature and the structure of each amino acid. Aspartic acid, which has a racemization half-life of about 15 000 years at 20°C, is most commonly used for archaelogocial dating, but there is considerable controversy over the results obtained.

Animal bones and shells and certain sediments contain proteins, for example, collagen and conchiolin. Extraction of the residual protein and determination of the enantiomer ratio of aspartic acid following hydrolysis can, when combined with knowledge of the thermal history of the sample, be used to determine the age of the fossil. Recemization age dating is generally more sensitive and less expensive than the radiocarbon method. Typical examples of the use of this technique have been analysis of Apollo 12 lunar material and dating of the Dead Sea scrolls.

Amino acids also racemize under various conditions such as prolonged acid hydrolysis and during solid-phase peptide synthesis. Chemical procedures such as asymmetric synthesis require proof of enantiomeric purity, especially if the product is to be used for pharmaceutical purposes. Configurational analysis of peptide antibiotics and establishing retention of configuration during peptide synthesis are other contexts in which it is important to determine the enantiomeric composition of amino acid samples.

Enantiomeric amino acid mixtures are resolved using two approaches. The first is to derivatize (acylate or esterify) with optically active reagents to form diastereoisomers or diastereomers which are resolved on an optically inactive stationary phase. The reagents must be of high optical purity and conversion must be quantitative. The second approach is to derivatize with optically inactive reagents, for example the *N*-TFA isopropyl esters, and then conduct the separation on columns containing optically active stationary phases.

Most amino acid optical isomers result from asymmetry at the α-carbon atom and depend on the presence of an α-hydrogen atom. However, some contain two optically active centres. Thus, the *threo* and *erythro* forms of the hydroxy amino acids and isoleucine and *allo*-isoleucine can be resolved on conventional columns. Similarly, isovaline, which contains one asymmetric centre but no α-hydrogen, has also been resolved. The mechanism has been postulated to depend on the formation of transient diastereomeric hydrogen-bonded association complexes but other factors such as dipole–dipole interactions and dispersion forces may also play a role.

Resolution of Diastereomers

All four diastereomers cannot be resolved using optically inactive stationary phases: the DD + LL and DL + LD enantiomer pairs usually coelute. The elution order depends on the specific derivatives. A diastereomer can be formed by esterification or by acylation.

Optically Active Esterification Reagents

Initial studies focused on forming active esters of *N*(*O*)-acyl amino acids and these were subsequently to be the most widely used derivatives. In 1965, Gil-Av reported the first resolution of amino acid diastereomers by GC (**Figure 4**). The 2-butyl and 2-octyl amino acid esters of alanine, glutamic acid, leucine, phenylalanine, proline and valine were resolved on capillary columns coated with either poly(trifluoropropylmethylsiloxane) or poly(propylene glycol) operated isothermally at 140 or 180°C. In the same year, Pollock reported the resolution of the *N*-TFA 2-butyl esters of 13 amino acids but those of aspartic acid, serine and threonine were only partially resolved. A study by Westley (1968) concluded that the resolution was directly proportional to the size of the groups attached to the alcoholic asymmetric carbon and to the proximity of the branching to the asymmetric centre. Thus 3,3-dimethyl-2-butanol gave superior resolution. In 1968, Pollock extended his study to the resolution of all the proteic amino acids except arginine, histidine and cystine. Three years later, 37 amino acid diastereomers were resolved as the *N*-TFA 2-butyl esters.

In 1977, König separated the *N*-pentafluoropropionyl (PFP) (+)-3-methyl-2-butyl esters of all the

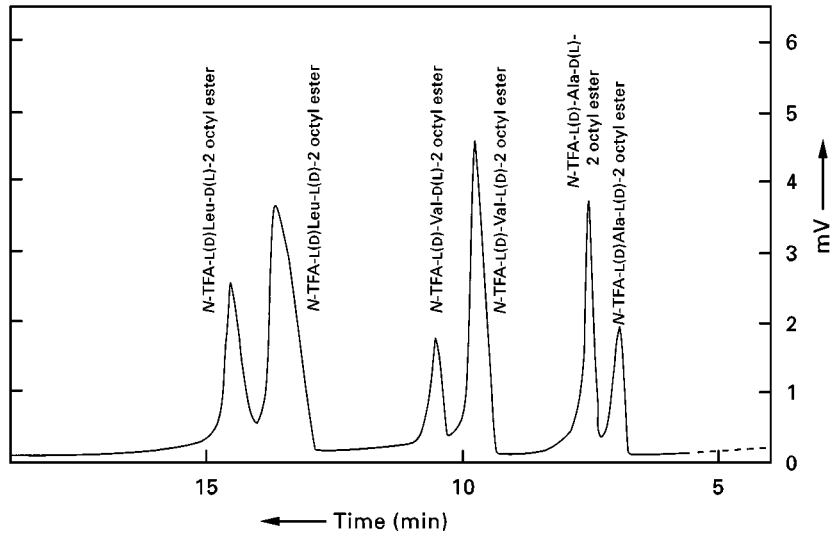

Figure 4 Resolution of diastereomers of *N*-trifluoroacetyl amino acid (*N*-TFA) 2-octyl esters. (Reproduced with permission from Gil-Av E, Charles R and Fischer G (1965) Resolution of amino acids by gas chromatography. *Journal of Chromatography* 17: 408–410.)

common proteic amino acids, including arginine, histidine and tryptophan. Excellent resolution was obtained using a 25 m column coated with SE30 and temperature programming from 85 to 220°C at 2°C min^{-1}.

Optically Active Acylation Reagents

A variety of carbonyl chlorides have been used to generate optically active dipeptides. *N*-TFA-L(−)-prolyl chloride was first used in 1965 by Halpern and Westley who separated the isomers of alanine, leucine, methionine, phenylalanine, proline and valine. The reagent was chosen because it was thought that the cyclic nature of the derivative would preclude recemization via an oxazolinone mechanism. This reasoning was later shown by Bonner to be incorrect but the problem was overcome by modifying the derivatization procedure. The reagent subsequently came into fairly common use. It was extensively studied by Iwase and Murai who combined TFA, PFP and HFB forms with methyl, *n*-propyl, *n*-butyl, *tert*-butyl and cyclopentyl esters. By assessing the resolution of alanine, valine, leucine and isoleucine, they concluded that the esters of *n*-alkyl alcohols gave better resolution than branched or cyclic chain alcohols.

König introduced a second asymmetric centre into amino acid methyl esters using the chiral reagent L-α-chloroisovaleryl chloride. Formation of the 3-methyl-2-butyl esters enabled resolution of all the proteic amino acid diastereomers, including arginine, on an SE-30 capillary column in less than 1 h. A separate analysis was required for the basic amino acids. Nevertheless, the diastereomer approach was to be overtaken by the more direct and absolute method of enantiomer resolution on chiral phases.

Resolution of Enantiomers on Optically Active Columns

In 1966, Gil-Av demonstrated the first resolution of amino acid enantiomers on an optically active stationary phase. The *N*-TFA-2-butyl esters of alanine, valine and leucine were resolved on an *N*-TFA-L-isoleucine lauryl ester phase coated on a capillary column. However, phases of this type quickly gave way to dipeptide phases such as *N*-acyl-L,L-dipeptide alkyl esters which were first introduced by Feibush and Gil-Av in 1967 and which produced better resolution.

In 1970 Nakaparskin and colleagues separated 17 amino acid enantiomers on an *N*-TFA-L-val-L-val-cyclohexyl ester phase (val-val). In earlier studies, stainless-steel columns up to 500 ft long were used. Consequently, analysis times were prolonged and the cystine, serine and threonine derivatives were degraded. In addition, dipeptide stationary phases such as val-val were functional over a limited temperature range or a limited maximum operating temperature. Columns were usually operated in isothermal mode.

König addressed the problem of temperature stability by introducing the *N*-TFA-L-phenylalanyl-L-leucine cyclohexyl ester which could be operated at 140°C. A later modification, the *N*-TFA-L-phenylalanyl-L-aspartic acid *bis*-(cyclohexyl) ester, was stable over the range 96–165°C and allowed the use of temperature programming. In addition, the introduction of glass capillary columns reduced degradation of the amino acid derivatives. The high boiling *N*-PFP isopropyl esters of aspartic acid, methionine, phenylalanine, glutamic acid, tyrosine, ornithine and lysine were eluted using a 20 m column. However, the diamide phases still left room for improvement in thermal stability and in peak resolution.

Another generation of phases was introduced by Frank, Nicholson and Bayer who linked the diamide moiety, L-valine *tert*-butylamide, to a polysiloxane backbone. Later termed Chirasil Val®, phases of this general type became predominant and are still in use. Early versions of this phase resulted in the overlap of D- and L-proline, D-isoleucine and L-*allo*-isoleucine, and L-threonine and D-*allo*-isoleucine. Nevertheless, the enantiomers of all the other proteic amino acids were resolved as the *N*-PFP *n*- or isopropyl esters in about 30 min by temperature programming from 90 to 190°C (**Figure 5**). Acid treatment of the glass capillary followed by methanol washing was necessary rigorously to exclude basic sites and thus to obtain satisfactory elution of cysteine, serine, threonine and tyrosine and to obtain a sharp peak for arginine. The relative retention times of the amino acids can be manipulated by including polar modifiers such as cyanopropyl and phenyl groups but the effect varies with specific amino acids. The L-valine *tert*-butyl moiety was subsequently grafted to chloropropionylmethyl phenylmethyl silicone, a modified OV–225, and to Silar 10C, but no overall improvement was achieved.

Chirasil-Val® was further improved by the incorporation of 15% phenyl groups substituted for methyl groups in the dimethylsiloxane units and the introduction of fused silica capillary columns. Thermal stability, ease of handling and separation efficiency were improved. The product is commercially marketed as Heliflex™ Chirasil-Val®.

Later improvements included the enhancement of enantioselectivity and thermal stability by immobilization of the Chirasil-Val® by radical or thermal

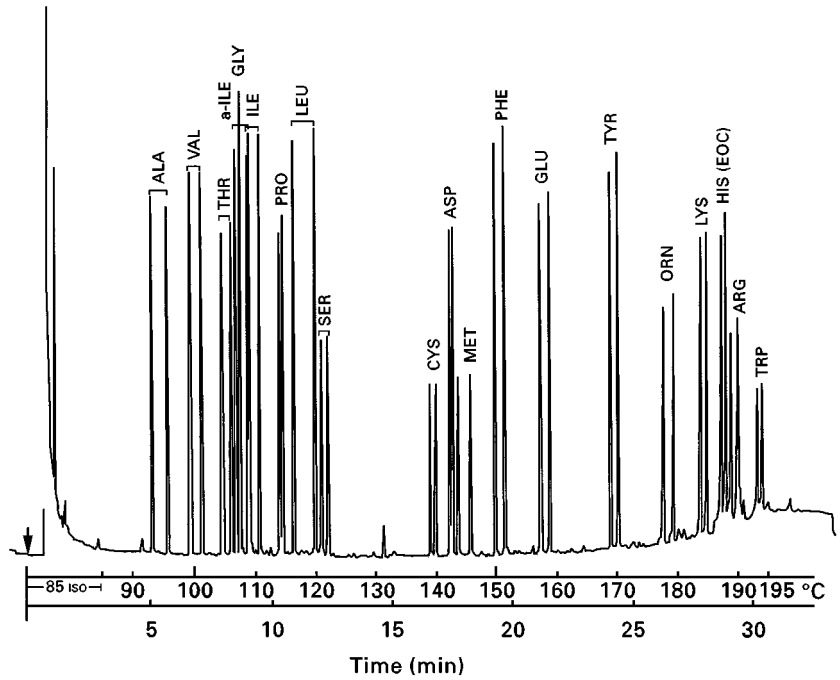

Figure 5 Resolution of a racemic mixture of proteic amino acids as the *N*-(*O*, *S*)-pentafluoropropionyl *n*-propyl esters. (Reproduced with permission from Bayer E, Nicholson G and Frank H (1987) Separation of amino acid enantiomers using chiral polysiloxanes: quantitative analysis by enantiomer labeling. In Gehrke CC, Kuo KCT and Zumwalt RW (eds) *Amino Acid Analysis by Gas Chromatography*, Volume II, pp. 35–53. Boca Raton, FL: CRC Press.)

reactions. Chiral polysiloxanes with regular repeat units, e.g. trifluoroethyl ester-functionalized polysiloxanes supporting L-val-*tert*-butylamide or L-α-naphthylethylamine liquid phases, have shown improved enantioselectivity. Backbone modification achieved by replacing one methyl group per dialkylsiloxy unit with a pentyl or hexyl group improved resolution of arginine and tryptophan *N*-TFA *n*-propyl esters but the overall separation of the other amino acids was not significantly affected. However, satisfactory results have been obtained by varying the proportion of L-val-*tert*-butylamide on the polysiloxane backbone. A ratio of about 6–7 dimethylsiloxane units per chirally modified dialkyl siloxane unit is effective for the complete resolution of all components present in a chiral mixture of the 20 proteic amino acids in about 35 min on a 20 m × 0.3 mm glass capillary column.

Most studies on amino acid enantiomer resolution on Chirasil-Val® type columns have used *N*-perfluoroacyl alkyl ester derivatives. However, other derivatives may present advantages in specific contexts. For example, the *N*-alkyloxycarbonyl alkylamide derivatives of proline are completely resolved on a Chirasil-Val® column. Similarly, König demonstrated the utility of isocyanate derivatives for resolving the enantiomers of *N*-methyl and β-hydroxy amino acids.

A radically different approach to enantiomer resolution has become possible with the development of cyclodextrins as stationary phases. Although suitable for liquid chromatography, the high melting point of cyclodextrins rendered them unsuitable for GC without further modification. König reduced the melting point and increased stability by introducing hydrophobic moieties by both partial and complete alkylation and acylation of the hydroxy groups. γ-Cyclodextrin substituted with 3-*O*-butyl and 2,6-di-*O*-pentyl residues was found to resolve most of the common amino acid enantiomers as the *N*-TFA methyl esters. Histidine enantiomers were only partially separated and arginine did not elute from the column. However, proline, 3,4-dihydroxyproline and pipecolic acid enantiomers were resolved, strongly suggesting that hydrogen bonding is not involved in the separatory mechanism. Atypical amino acids such as *N*-methyl and β-amino acids were also resolved.

More recently (1994), Abe explored capillary columns coated with four types of cyclodextrin derivatives of 6-*O*-*tert*-butyldimethylsilyl-2,3-di-*O*-acetyl or *n*-butyl-β- and γ-cyclodextrin. Depending on the phase, all proteic amino acid enantiomers except for those of tryptophan were resolved as the *N*-TFA isopropyl esters. Variants such as 2,6-di-*O*-pentyl-3-*O*-propionyl-γ-cyclodextrin have also been used to

separate a number of amino acid enantiomers. Molecular modelling has positively correlated the GC elution order of proline derivatives on 2,6-di-*O*-methyl-3-*O*-trifluoroacetyl-β-cyclodextrin with the energies of the host–guest complexes.

Several satisfactory methods now exist for the resolution of amino acid enantiomers. Typically, 0.1% of a minor enantiomer can be precisely determined and, depending on the context and the specific method used, it is possible to assay as little as 0.01% or less.

Detectors

By far the majority of GC amino acid analyses have been conducted using flame ionization detectors (FID). These have the advantage of being sensitive and economical, but are nonspecific and provide no structural information.

Selective detectors confer distinct analytical advantages but are most often used to address special, nonroutine analytical problems. For example, the ability to detect a specific atom or molecular property can simplify sample preparation. Thus, by using a nitrogen/phosphorus-selective detector, contaminating compounds not containing nitrogen or phosphorus are simply not detected in most samples. Furthermore, it can reasonably be assumed that those peaks which have been detected contain nitrogen. In addition, problems caused by overlapping peaks are reduced. Selective detectors can also provide additional sensitivity. The ultimate detector is a mass spectrometer which can, depending on the context, provide all of these advantages and also provide detailed structural information.

A number of selective detectors have been used to assay amino acids. Their use will be illustrated using some examples.

A nitrogen/phosphorus-specific detector, operated in the nitrogen mode, has been used to assay free amino acids in conifer tissues. All the proteic amino acids and several biologically important nonproteic amino acids were assayed at the low picomole level as the *N*-HFB isobutyl esters. Comparison of the FID chromatogram with the NPD chromatogram enabled identification of those compounds which did not contain nitrogen (**Figure 6**). Similarly, 1-aminocyclopropane-1-carboxylic acid, a precursor of the plant hormone ethylene, has been assayed as the *N*-benzoyl *n*-propyl derivative in the leaves and xylem sap of tomato plants. More recently (1997), 21 proteic and 33 nonproteic amino acids have been resolved in less than 30 min as the *N*-isobutoxycarbonyl methyl esters at a detection limit of 6–150 pg per injection. Small urine samples were analysed without prior clean-up and with no detectable influence from any non-nitrogen-containing compounds present.

Flame photometric detection (FPD) is useful for analysing sulphur-containing amino acids but has rarely been used in that context. Amino acid phosphorylation is an important biochemical regulatory mechanism and is also important for correlating protein structure and function. The *O*-phosphoamino acids, specifically *O*-phospho serine, threonine and tyrosine, have been assayed as the *N*-isobutoxycarbonyl methyl esters using FPD. The detection limits

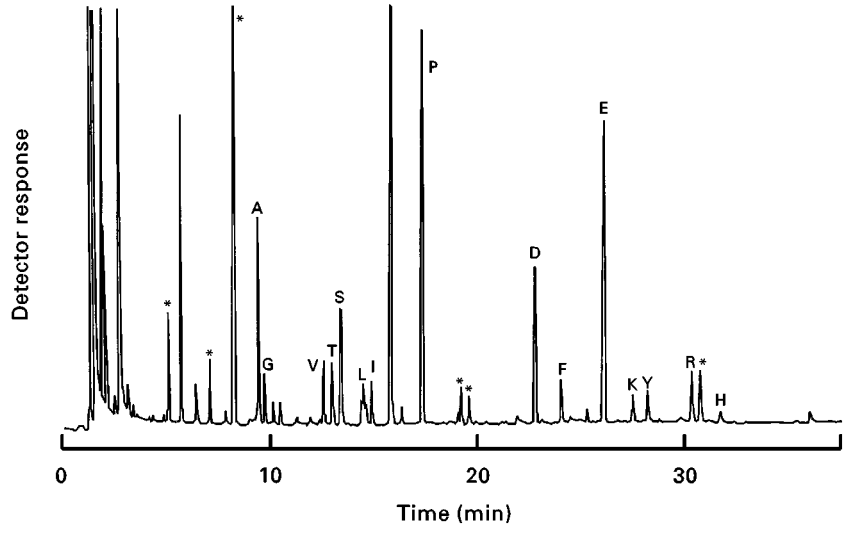

Figure 6 Resolution of White Spruce leaf-free amino acids as the *N*-(*O*, *S*)-heptafluorobutyryl isobutyl esters using a flame ionization detector. Peaks marked by asterisks were shown not to contain nitrogen by comparison with an analysis of the same sample using a nitrogen-selective detector. (Reproduced with permission from MacKenzie SL (1986) Amino acid analysis by gas-liquid chromatography using a nitrogen-selective detector. *Journal of Chromatography* 358: 219–230.)

ranged from 0.18 to 0.3 pmol, reflecting a sensitivity about 200 times greater than FID detection. The method has been applied to the determination of *O*-phosphoamino acids phosphorylated by protein kinase both *in vitro* and *in vivo* without radiolabelling. Other amino acids did not interfere. The secondary amino acids, proline, pipecolic, thioproline, hydroxyproline and hydroxypipecolic acids, have also been assayed using FPD. Detection limits for the *N*-dimethylthiophosphoryl methyl esters were 0.1–0.7 pmol per injection.

Electron capture detectors are particularly useful for detection of the strongly electronegative perfluoroacyl derivatives of amino acids, but few studies have been conducted. Typically, as little as 1.4 pmol of tyrosine has been detected in a standard amino acid mixture. γ-Aminobutyric acid and five other aliphatic acids have been assayed in small volumes of supernatants from brain homogenates following sequential reaction with isobutyl chloroformate and pentafluorophenol.

Mass spectrometric detection provides structural as well as quantitative information. It is most frequently used either to confirm the structure of derivatives during the development of new protocols or to identify unknown compounds. Detection limits are frequently in the femtomole range. Electron impact (EI) ionization is most commonly used but both positive and negative ion chemical ionization have also been applied. Selected ion monitoring (SIM) of diagnostic ions is often used to increase sensitivity.

Typical examples of the structural information role of a mass spectrometric detector are the identification of *O*-phosphoamino acids in urine hydrolysates, the identification of amino acid ethyl esters in wines, the determination of amino acid composition in small peptides, and the assaying of γ-aminobutyric acid in mouse brain synaptosomes following therapy with the antiepileptic drug valproic acid. The versality of GC-mass spectrometry (GC-MS) is further illustrated by the identification of 3-OH-4-methyldecanoic acid, a fungal cyclodepsipeptide, and by the simultaneous analysis of branched-chain carboxylic, α-oxo, α-hydroxy and α-amino acids in the urine of patients suffering from maple syrup urine disease. GC-MS has also been used to characterize binding media from medieval polychrome sculptures. Animal glues, casein, egg and drying oils were identified as components of the binders of paint and ground layers.

The expense of mass spectrometers mitigates against their use as routine analytical detectors and many real sample analyses by GC-MS (as distinct from the analysis of standard mixtures) have been directed to addressing analytical problems which cannot be resolved using other types of detectors.

The ratios of the stable isotopes of C and N are used in the assessment of *in vivo* protein turnover studies, and in identifying the sources and history of organic matter. Both natural abundances and the ratios obtained after enrichment with singly or multiply labelled amino acids or other compounds such as ^{13}C-glucose, pyruvate or acetate have been determined. The ratios may be determined after online combustion following GC and introduction of the resultant gases into a conventional isotope ratio mass spectrometer. This approach has been used to study ^{15}N: ^{14}N isotopic ratios in plasma-free amino acids and, by eliminating many preparative steps, requires only about 500 μL-of plasma, whereas preparatory methods may require as much as 60 mL.

Alternatively, the intact labelled compounds can be introduced directly into the mass spectrometer. For example, by combining stable isotope dilution with the use of EI and SIM to monitor the $[M\text{-}57]^+$ peak, homocysteine sulfinic acid, homocysteic acid, cystine sulfinic acid and cysteic acid have been shown to be agonistic to *N*-methyl-D-aspartate receptors in brain tissue. This approach also enabled the identification and quantitation of these compounds in normal human serum. Similarly, endogenous and newly synthesized concentrations of the neurotransmitter amino acids γ-aminobutyric acid, glutamate and aspartate have been assayed in brain slices following incubation with ^{13}C-labelled precursors.

Future Developments

The techniques for derivatizing and separating the standard amino acids in protein hydrolysates are mature and there is no significant room for improvement. Given the existence of quantitative derivatization protocols which proceed very rapidly, it is doubtful whether the development of on-column derivatization would constitute a significant advantage. Similarly, proteic amino acids can now be assayed in less than 10 min, so, given the availability of reliable automatic injectors, a reduction in analysis time is not of significant value.

Physiological samples may contain several hundred amino acids and these cannot, at present, all be resolved on any one single column. Frequently, however, only a subset is of interest. Thus, although the simultaneous derivatization and separation of all the proteic amino acids and as many as 50 of the more common nonproteic amino acids are now possible, it is likely that procedures targeted at specific subsets will remain important in specific contexts. The sensitivity of the FID detector is adequate for most analytical purposes but mass spectrometric detectors

will remain important for specialized applications requiring femtomole sensitivity.

See also: **III/Amino Acids:** Liquid Chromatography; Thin-Layer (Planar) Chromatography. **Amino Acids and Derivatives: Chiral Separations. Amino Acids and Peptides: Capillary Electrophoresis.**

Further Reading

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Liquid Chromatography

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The first approach to the automatic liquid chromatography (LC) of amino acids (AAs) – known today as ion exchange chromatography (IEC) – was published by Spackman *et al*. in 1958. In over 40 years later, it now takes less than 5 min (**Figure 1**) to separate and quantitate the essential protein AAs instead of 2 days. Early separations were carried out by post-column derivatization.

Over the last 20 years LC has offered unlimited possibilities in both the preparative and analytical scale. The wide choice and sophisticated columns, detectors, derivatization procedures, development of modern instrumentation and data-handling systems reduce time and costs, and give versatility and automation in Good Laboratory Practice (GLP)- controlled conditions for selectivity, sensitivity and reproducibility. It is the responsibility of the researcher to choose the most appropriate method for the given task. The most popular LC method for analysis of both free AAs (present in many natural matrices, biological fluids and tissues, feed and foodstuffs) and of those constituents of protein hydrolysates is now reversed-phase (RP) chromatography after pre-column derivatization of the AAs.

Numerous methods for derivatization are available in the literature. This article will discuss the advantages and drawbacks of the commonly used derivatives.

Current trends in AA analysis identify the best conditions for enantiomer separation and the development of LC-mass spectrometry (LC-MS).

LC of Underivatized AAs

To attain one of the main advantages of LC – separating the 'classical 20' as underivatized AAs – has appealed to chromatographers. In spite of a number of efforts, the simultaneous LC of underivatized AAs has remained of secondary importance. Determination of a few selected AAs, such as tryptophan or sulfur-containing AAs, has proved to be fruitful for special tasks.

The aim of various investigations was to render unnecessary the time-consuming derivatization techniques. However, the characteristics of the free AAs are considerably different from each other and their various structural properties did not permit their easy resolution. Thus, in attempting to achieve better separation of free AAs, further means of discrimination were needed. For this purpose special techniques have been introduced, such as the use of various phase systems, ion pair and ligand exchange chromatography, column-switching techniques or anion exchange chromatography with electrochemical detection.

The solvent-generated ion exchange phase system ensured the gradient elution of 19 AAs (**Figure 2**A): some, but not all, are baseline-separated. A simple isocratic method using aqueous, copper acetate/alkylsulfonate additives containing acetate buffer (pH 5.6) as mobile phase, a conventional RP column and UV detection (230–240 nm) at different temperatures and varying the concentrations of additives was unable to separate the classical 20 protein amino acids. Significant improvement in the separation can be obtained by column switching (Figure 2B), as well as by using an anion exchange column, a quaternary