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DETERMINATION OF AMINO ACIDS

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Amino acids attract the continuous interest of chemists, biologically and chemically. These compounds make up proteinaceous substances of all living matter, take part in metabolic functions of all tissues, and are, therefore, indispensable components of the diet of all animals and man.

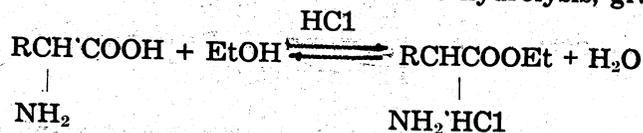
As chemicals, amino acids are very reactive, soluble and amphoteric and, therefore, enter into a wide diversity of chemical reactions. Because of these biological and chemical characteristics amino acids find a wide use as food additives to increase the nutritional value of food (1-11). Lately amino acids and derivatives of amino acids have been linked with cosmetic preparations (12), and a wide range of different chemotherapeutics (13, 14): the L-enantiomer of β -3, 4-dihydroxyphenylalanine, "L-DOPA", has been widely adapted for treatment of Parkinson's disease (15, 16). Preparations containing L-lysine have been used to treat fatigue, myocardial anomalies (17), and psoriasis (18). Preparations which stem from N-acetylcysteine are valuable hepatoprotective agents (19). Arginine dithiobenzoates have been introduced as antiinflammatory and antirheumatic agents (20). L-Sarcosin and L-phenylalanine mustards, known as "MELPHALANS" have been recognized as effective agents for treatment of tumors (21-26). Finally, amino acids are widely used for parenteral infusions and for many other applications. The complete list would exceed the aim of this paper. The examples cited should be sufficient to justify constant revitalization of research in this field.

Recent reviews of amino acids (27, 28) indicate that at present there are about 90 known amino acids, of which about 50 have been characterized. The original 20 or so amino acids known before 1950 (29) are often called the "classical amino acids". They are abundant in nature, and constitute the major part of all proteins, regardless of origin. A number of these classical amino acids are essential for nutrition (30). The optically active L-forms of the amino acids have nutritional and pharmacological value. Thus, it is more advantageous to prepare the L-amino acids by hydrolyses of proteins than by syntheses which yield racemic mixtures (31, 32).

This review will be limited to the classical amino acids and will focus on procedures that might be used to isolate these compounds from different industrial waste materials via esterification and subsequent acetylation. The N-acetyl amino acid ethyl esters are sufficiently stable for fractionation (33, 34).

Classical methods of esterification require anhydrous ethyl alcohol

and anhydrous hydrochloric acid gas (35, 36) to catalyse the reaction, and, therefore, are not suited for large scale preparation of amino acid esters, especially from aqueous hydrolysates of industrial waste materials. In addition these methods, because of reverse hydrolysis, give low yields.



Removal of water from the reaction mixture should improve the yields. Thus, Dymicky et al., (37) developed a method to esterify amino acids by continuous distillation of the azeotropic mixture, alcohol-benzene-water. This procedure, which uses non-anhydrous alcohols and ordinary hydrochloric acid is efficient, convenient, and economical.

N-Monoacetylation of Amino Acids

Methods of acylation described in the literature require free acid or free esters (33, 38-43). These methods also have some serious shortcomings, such as the effects of racemization, decomposition, and polyacylations (44, 46). Weygand and Röpsh (47) recently developed a procedure for acylation that used trifluoroacetic acid-phenylester as the acylating agent and phenol as a solvent. Usually an amino acid is dissolved or suspended in phenol, the acylating agent added, and the reaction mixture is heated for a few minutes at 120-150°C. Under these conditions acylation takes place smoothly, giving high yields of N-trifluoroacetyl (N-TFA) derivatives without any effects of racemization except with N-TFA histidine. In the presence of organic bases, e.g. triethylamine, the racemization is completely inhibited, due to the formation of double anions.

Although trifluoroacetic acid-phenylester fails to acylate asparagine, cystine, and glutamic acid, it is suitable to acylate peptides without the effect of peptide cleavage.

Weygand and Geiger later (48) introduced trifluoroacetic acid methyl ester as the acylating agent, which proved to be very useful for acylation of amino acid methyl esters in the presence of triethylamine with anhydrous methanol as solvent. The products, N-TFA-amino acid methyl esters, were sufficiently volatile for the isolation of 16 amino acids from casein and 13 from gelatin.

The N-TFA-amino acid methyl esters are suitable derivatives for gas-liquid chromatographic (GLC) determinations of amino acids; GLC later will be discussed in some detail. The trifluoro-reagents are expensive and, therefore, not suited for commercial isolation of amino acids. In that respect, innovations are still needed.

Determination of Amino Acids

Historically, methods for determination of amino acids can be divided into two categories; those developed and used before and after 1950. The

early classical methods were based on tedious chemical reaction and have been described in textbooks (29, 30) and reviews (49-51). The related microbiological methods were reviewed by Dunn (52) in 1949. Most of those methods developed before 1950 can now be regarded as obsolete except the procedure of Van Slyke (53).

From the classical methods emerged the valuable chromatographic procedure of Stein and Moore (54-58), which was modified in 1954, and then finally developed into a universal commercial tool called "automatic amino acid analyzer" an automated version of ion-exchange chromatography. With the analyzer as many as 50 amino acids can be determined in a single sample within 2 days. With ordinary classical methods, time required for these analyses would be measured in years. This analyzer, which requires only a fraction of a gram of amino acid mixture, is extremely important for studies of composition of proteins. While the "automatic amino acid analyzer" may be regarded as the best tool for determination of amino acids, other less expensive methods have been developed; infra-red spectroscopy (59) paper chromatography, and paper electrophoresis (60, 61) summarized by Niederwieser and Pataki (62).

With paper chromatography good results have been reported from the two dimensional procedures as developed by Thompson and Morris (63)

From paper chromatography developed thin-layer chromatography (TLC). Stahl (64) has reviewed the literature on that subject up to about 1968. TLC is faster than paper chromatography, requires less space and plates can be developed with a wider selection of reagents than paper.

Other methods for analyses of amino acids, which have been introduced recently, include mass spectrometry, molecular-sieve chromatography, thinlayer gel filtration, nuclear magnetic resonance (NMR) and gas-liquid chromatography (GLC). The NMR method has been recently reviewed by Roberts and Jardetzky (65). This procedure has been very useful for conformational analyses of amino acids (66, 67).

Gas-Liquid Chromatography (GLC)

This procedure was introduced to analytical chemistry in 1952 by James and Martin (68) and now is regarded as an invaluable technique in every phase of chemistry. This procedure is applicable only to volatile compounds, and its basic principles have been described by Keulemans et al. (69, 70) and in a number of other textbooks (71-74). By 1960 GLC had been completely commercialized and its advantage over other tools established. First it can be used for qualitative and quantitative determinations of any class of compounds that are in a volatile form. GLC analysis requires only a fraction of an hour and the apparatus is much less expensive than an amino acid analyzer. Finally, the columns can be used for several determinations and a sample as small as a fraction of a milligram can be analyzed.

For analysis by GLC, amino acids must be converted to a volatile

form. In 1956 Hunter et al. (75) introduced a procedure in which amino acids were reacted with ninhydrin and converted to the corresponding aldehydes with one less carbon atom (76). The method which was used later by others (77, 78) has been abandoned because it could not be used for amino acids such as arginine, aspartic acid, cystine, glutamic acid, and tryptophan. In addition, formaldehyde, which stems from glycine, was polymerized by the reaction. In a further search for suitable derivatives attempts have been made to decarboxylate amino acids with N-bromosuccinamide (79), but this reaction yielded aldehydes and nitriles that introduced serious complications. There followed an attempt to esterify the carboxylic group and to substitute chlorine (80) or hydroxyl (81) for the amino group but this was time-consuming and reduced the yield. Similarly, the 2,4-dinitrophenyl methyl ester and the phenylthiohydantoin derivatives (82) were not entirely satisfactory. These two methods encountered some difficulties with determination of serine, threonine, glutamine, asparagine, and with the basic amino acids.

Emil Fischer (83) and Cherbulies (83, 84) suggested that amino acid methyl esters were sufficiently volatile, thus suitable for GLC analyses. Bayer et al. (85) reported that use of the methyl esters was limited, probably because they hydrolyzed readily. Weygand and Geiger (48) prepared a number of N-trifluoroacetyl amino acid methyl esters and showed that these derivatives were suitable for fractionation. From these derivatives they isolated, by fractional distillation, respectable yields of 13 amino acids from gelatin and about 16 from casein.

N-trifluoroacetyl amino acid methyl esters have been adopted by a number of workers (86-93) as the most suitable derivatives for GLC analyses of amino acids. Since some workers expressed concern that the methyl esters are too volatile, Young (94) utilized ethyl and n-butyl esters. A similar concern was expressed by Gehrke and Stalling (95), who recommended the n-butyl esters as the most suitable derivatives. In extensive work Gehrke et al. (96-99) developed techniques for acylation, development of the columns, and methodology, and their procedure has been widely accepted. Using that procedure, Cassagrande (100) separated 35 amino acids and two amino sugars. The N-trifluoroacetyl amino acid esters also are suitable for sequence analyses of polypeptides (101).

Some other derivatives of amino acids, the N-trimethyl-silyl n-butyl esters (102, 103), also gave good reproducible results; with these derivatives, however, some of the peaks overlapped (104) and arginine failed to separate (105).

Johnson et al. (106) undertook extensive studies to evaluate the suitability of N-acetyl esters for GLC analyses. They prepared and chromatographed the esters of n-butyl, isobutyl, n-amyl and isoamyl, and concluded that the n-amyl esters were the most suitable. With this ester good separation was achieved and 33 amino acids were identified. These authors reported that preparation of the derivatives and GLC took about 2 hours,

and that 10^{-10} moles of amino acid could be detected. The most advanced "amino acid analyzers" (58) can detect levels as low as 10^{-10} moles in 2.5 hrs.

In commenting on the procedure of Youngs (94) and on the suitability of N-acetylamino acid n-butyl esters, Johnson et al. (106) reported that when these derivatives were analyzed the peaks for alanine and valine tended to overlap. When the corresponding n-amyl esters were used however, separation was excellent. These authors used a 6-foot column packed with 5% carbowax 1540,* coated on chromosorb W, 60-80 mesh.

Because the higher esters, e.g. hexyl, heptyl, etc. have such high boiling points, the N-acetyl derivatives of the lower alcohols have been studied. Thus, Shlyapnikov et al. (107, 108) undertook extensive studies of the N-acetyl esters of methyl, ethyl, propyl, and isopropyl alcohols and also of the corresponding N-formylamino acid esters. They used the 12 most common amino acids and five columns packed with materials of different polarities. The esters of the N-formylamino acids gave poorer separations than the corresponding derivatives of N-acetylamino acids. The authors concluded that all the N-acetylamino acid esters studied were suitable derivatives for GLC analyses of amino acids if the stationary phases contained a polar material.

The works of Johnson et al. (106) and of Shlyapnikov et al. (107, 108) have been of particular importance to us. The suitability of N-acetylamino acid ethyl esters for GLC analyses of amino acids supplemented our work with esterification (37) and provided a convenient way to isolate, and simultaneously quantitate, these products.

In general, GLC is now regarded as the most important and the most convenient procedure for analyses of any classes of compounds including amino acids. Chemical & Engineering News (109) stated that the procedure offers many advantages over all other known methods.

By GLC, determination of amino acids, or any class of compounds, is reduced to two basic problems: selection of a column and conversion of the samples into a derivative of suitable volatility. Generally it is a matter of choice; Gee (92) and Darbre & Islam (93) preferred to use N-trifluoroacetyl amino acid methyl esters, whereas Zumwalt et al. (99), who considered the methyl esters too volatile, preferred the n-butyl esters. Gee (92) pointed out that the methyl esters are suitable for GLC examinations because they are more volatile than the higher esters, and are easier to prepare. Darbre (93) pointed out that an important advantage in the use of methyl esters is that chromatography may be effected at lower temperature. If these derivatives are considered from the economic point of view, trifluoroacetic anhydride is much more expensive than acetyl chloride and other alcohols are much more expensive than commercial ethanol. For

*Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

isolation of amino acids on a large scale, several factors must be considered.

For esterification and acetylation of pure amino acids, it would be advantageous to perform first acylation then the esterification (110).

GLC Separation of Enantiomers of Amino Acids

The latest development in analysis of amino acids is GLC separation of their enantiomers. This procedure demonstrates best the superiority of gasliquid chromatography over other known methods in analytical chemistry.

Gil-Av et al. (111-113) and Nakaparksin et al. (114) have shown that enantiomeric amino acids can be separated on capillary columns, coated with an optically active stationary phase, e.g. N-trifluoroacetyl-L-valyl-L-valine cyclohexyl ester. Since that ester has limited stability at about 110°C, Noenig et al. (115) developed a new, more temperature-stable dipeptide, N-TFA-L-phenyl-alanyl-L-leucine cyclohexyl ester. Using a 375-foot, 0.02-in. i.d. stainless steel capillary column, these authors have established that TFA-amino acid isopropyl esters are appropriate derivatives for the separation of D- and L-enantiomers.

Corbin and Rogers (116) studied the stationary phases and concluded that both esters mentioned above could be used to separate the enantiomers of amino acid derivatives. The ureide phase (117) may be considered as the next best material. These findings have been confirmed by Parr et al. (118). These authors stated that amino acid enantiomers could be resolved by GLC, using N-TFA-L-valyl-L-valine or N-TFA-L-phenylalanyl-L-leucine cyclohexyl ester, therefore, the most suitable derivatives for the resolution are N-TFA-amino acid isopropyl esters.

Investigation of the influence of the alcohol used for esterification (119) showed that resolution increased in the order, primary, secondary, tertiary alcohols; however the t-butyl esters of amino acids were rather difficult to prepare. Practically, TFA-amino acid isopropyl esters represented the best compromise, although there has been concern about their low volatility.

There are two basic approaches to resolution of the enantiomers, either an optically active derivative of amino acids with an optically inactive stationary phase is used or vice versa. Usually an optically active stationary phase is used with inactive TFA-amino acid isopropyl esters. Detailed information on this procedure has been published by Parr et al. (118) and Raulin & Khare (119). Also most recently, Parr and Howard (120) described a rapid method for determination of the optical purity of amino acids, utilizing a new optically active stationary phase, a dipeptide, cyclohexyl N-TFA-L- α -aminobutyrate. This phase is suitable for determination of small amounts of D-amino acids in the presence of larger amounts of L-isomers, or small amounts of L-amino acids in the presence of the D-isomers. Thus, these authors resolved eight racemic mixtures,

at one time, namely: DL-alanine, DL-valine, DL-threonine, DL-isoleucine, DL-leucine, D,L-serine, DL-proline and DL-aspartic acid.

Mass-spectrometric procedure, which recently has been introduced for determination of amino acids, has proven useful in the determination of the sequence of these acids in peptides and proteins (121).

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