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CYCLIC AMINO ACID DERIVATIVES IN GAS CHROMATOGRAPHY

PETR HUŠEK and VLADIMÍR FELT
Research Institute of Endocrinology, Národní třída 8, CS-116 94 Prague 1 (Czechoslovakia)
and
MIROSLAV MATUCHA
Institut for Research, Production and Application of Radioisotopes, Přístavní 24, CS-170 01 Prague 7 (Czechoslovakia)
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SUMMARY

The basic protein amino acids, together with more than twenty other α-amino acids, were divided according to chemical similarity into characteristic groups and the particular mixtures were first condensed with 1,3-dichlorotetrafluoroacetone and, if necessary, treated with reactive anhydrides in a subsequent reaction step. The rate of condensation of ^{14}C-labelled protein amino acids was followed by flat-bed chromatography. Approximately half of the tested compounds were converted into cyclic forms within a few minutes and analysed immediately. Amino acids with reactive side-chain groups were acylated in the condensation medium at room temperature instantaneously and subjected to analysis after a simple extraction procedure. The imidazolyl group of histidine was effectively blocked when, during extraction, a minute amount of chloroformate was added. Polyorganosiloxane phases coated on silanized supports of the usual particle size (80–100 mesh) enabled most of the derivatized amino acids to be determined. Analysis of diaminodicarboxylic acids, histidine and tryptophan required the use of larger support particles (45–60 mesh).

INTRODUCTION

Previous studies[1–5] have revealed that cyclic amino acid derivatives (substituted oxazolidinones) are very useful for the rapid determination of α-amino acids by gas chromatography. Condensation with 1,3-dichlorotetrafluoroacetone (DCTFA) proceeds readily at room or slightly elevated temperature[1,4,6] and even the heavy protein amino acids, which are difficult to determine by gas chromatography, can be analysed after several minutes of chemical treatment[5]. The weakly basic aprotic medium of the condensation reaction permits the further treatment of the sample with acylating reagents[1,2] and provides possibilities for distinguishing between dicarboxylic amino acids and their amides[3].

In view of the multifunctional nature of the protein amino acids, it is not easy to devise a uniform reaction scheme that would deal successfully with all of the side-chain reactive groups in a mixture of amino acids condensed to oxazolidinones. Numerous previous studies dealing with amino acid derivatization[7] have shown that the varying reactivities of hydroxyl and amino groups in particular must be taken in account. Considering the chemical differences, unification of the reaction conditions often necessitates a compromise that does not always allow the quantitative derivatization of all of the amino acids occurring in a mixture. Optimization of the procedure can be accomplished if the derivatization requirements of the particular reactive groups are known. For this purpose the protein amino acids and many other α-amino acids were divided into groups that would require exactly defined reaction conditions for the second acylation step. In order to succeed in the analysis, it was necessary to introduce an efficient extraction procedure and to find a convenient column filling for the elution of the perhalogenated, absorption susceptible compounds from the chromatographic column. The influence of the particle size of the support on the completeness of elution of some amino acid oxazolidinones is demonstrated graphically and results of the chromatographic analysis are presented.

EXPERIMENTAL

Materials and equipment

Reagents, glassware and the chromatograph were the same as reported earlier[2], and the reaction tubes were silanized as mentioned in a preceding study[5]. Silufol aluminium foils (5 × 15 cm) with a thin (100 μm) silica layer were obtained from Kavalier (Votice, Czechoslovakia) and heated at 110° for 15–30 min before use[6]. Methyl and isobutyl chloroformate were obtained from E. Merck (Darmstadt, G.F.R.).

Supelcoport (80–100 and 60–80 mesh) and Chromosorb W AW DMCS (45–60 mesh) chromatographic supports were purchased from Supelco (Bellefonte, Pa., U.S.A.) and coated with 3% (w/w) of SE-30 methylsilicone gum or OV-17 (or SP-2250) methylphenylsilicone fluid in the laboratory. Two columns were employed: (A) 2 m × 2 mm I.D., 3% OV-17 (or SP-2250) on 80–100-mesh Supelcoport; (B) 1 m × 2 mm I.D., 3% SE-30 on 45–60-mesh Chromosorb W AW DMCS.

The nitrogen flow-rate (30 ml/min), detector (FID) and injector temperatures (250° and 200°, respectively), and linearly programmed temperature increase (8°/min) were the same for both columns. The temperature ranges used in each of the analytical runs are given in the legends to the figures.

Amino acids of grade A quality were purchased from Calbiochem (Lucerne, Switzerland), E. Merck or Fluka (Buchs, Switzerland). α-Aminocaprylic acid was obtained from Sigma (St. Louis, Mo., U.S.A.). ^{14}C-Labelled amino acids were supplied by the Institute for Research, Application and Production of Radioisotopes (Prague, Czechoslovakia) or the Radiochemical Centre (Amersham, Great Britain), the specific activity being 1.5 GBq (40–50 mCi) per milligramatom of carbon.

Standard equimolar solutions (5 μmol/ml of each amino acid) of groups of selected non-labelled amino acids in 0.1 *M* hydrochloric acid were prepared with the following composition:

(1) Amino acids with carbon-linked chain:
  (A) branched: α-aminoisobutyric acid (AIBA), valine (VAL), leucine (LEU), isoleucine (ILE) and glycine (GLY);
  (B) straight: alanine (ALA), α-aminobutyric acid (ABA), α-aminovaleric acid (norvaline, NVAL), α-aminocaproic acid (norleucine, NLEU) and α-aminocaprylic acid (ACA).
(2) Amino acids with alkyl substituent on:
  (A) α-amino group ("imino" acids): N-methylglycine (sarcosine), proline (PRO) and pipecolinic acid (PIPA);
  (B) other chain-amino group: N^{ε}-methyllysine, 1-methylhistidine and 3-methylhistidine;
  (C) thiol group: S-methylcysteine (CYSM), methionine (MET) and ethionine (ETH).
(3) Diaminodicarboxylic acids:
  (A) with intermediate sulphur atom(s): lanthionine (LAN), cystine (CYS) and homocystine (HCYS);
  (B) with intermediate carbon-linked chain: diaminosuccinic (DAScA), diaminopimelic (DAPA) and diaminosuberic (DASbA) acids.
(4) Amino acids with indolyl or imidazolyl group: tryptophan (TRP) and histidine (HIS).
(5) Amino acids with O- or S-reactive groups in the chain:
  (A) serine (SER), threonine (THR), hydroxyproline (HYP) and tyrosine (TYR);
  (B) cysteine (CYSH) and penicillamine (β,β-dimethylcysteine).
(6) Amino acids with N-reactive group in the chain:
  (A) α,ω-diamino acids: diaminobutyric acid (DABA), ornithine (ORN) and lysine (LYS);
  (B) with amino-carbonyl group: asparagine (ASN), glutamine (GLN), citrulline (CIT) and homocitrulline (HCIT).
(7) Amino acids with two reactive groups in the chain:
  (A) arginine (ARG) and homoarginine (HARG);
  (B) dihydroxyphenylalanine (DOPA) and hydroxylysine (HLYS).

Phenylalanine (PHE) was added as an internal standard in an equimolar amount to each of the particular group of amino acids being analysed on column A or B; DAPA was added to groups the members of which were analysed on column B (these standards are given in parentheses on the chromatograms). *n*-Hexadecane was added in one instance (group 1A) in order to estimate the absolute molar responses in coulombs per mole and the relative molar response of PHE[10].

Procedure

Condensation. To the dry residue of amino acids (50 nmol of each amino acid in the mixture) were added 60 μl of solvent (acetonitrile–pyridine, 52:8), 20 μl of dichloromethane and 20 μl of DCTFA and the sample was held at room or slightly elevated temperature (40°) for 1–15 min (DAScA 30 min, PRO 45 min). A sample aliquot (2 μl) was subjected to analysis either after the condensation or after subsequent extraction or acylation and extraction. If the ^{14}C-labelled amino acids were

condensed, the sample aliquot was transferred on to a Whatman 3 MM paper sheet (3 × 12 cm) or Silufol foil (5 × 15 cm) and run in chloroform. The chromatograms were evaluated with the help of a Radioscanner II (Berthold, Wildbad, G.F.R.).

Acylation. Approximately 50 μmol of reactive anhydride (*i.e.*, 10–12 μl of HFBA or 5–7 μl of TFAA) were added either directly to the polar condensation medium (procedure A) or the condensation medium, prior to anhydride addition, was diluted with 500 μl of extraction medium (procedure B). As the acylation is instantaneous (except for CIT/HCIT, which required additional heating at 40° for 5 min) the sample can be extracted immediately.

Extraction. A 500-μl volume of extraction medium (25% of dichloromethane in light petroleum) is added to the sample (in procedure B the organic phase is already in the vial) and the contents are shaken with 400 μl of 1 *M* sodium carbonate solution for 10–15 sec. If HIS is present in the sample, 1 μl of IBCF or MCF is then added and shaking is continued for a further 20–30 sec (until the organic phase becomes clear). The aqueous carbonate solution is then discarded and the contents are shaken further with 400 μl of 1 *M* hydrochloric acid and 400 μl of water, always for 10–15 sec. After removing the aqueous layer, the organic extract is transferred into another vial and reduced in volume to a small drop (10–20 μl) at room temperature using a current of nitrogen. The last drop is then evaporated by rotating the vial along the longitudinal axis (the dispersed drop can be dried more easily when, during this rotation, 10–20 μl of extraction medium are added). This process is necessary in order to prevent losses of the simplest aliphatic amino acids.

Sample treatment before analysis. To the evaporated residue of the cyclic amino acid derivatives 100 μl of *n*-heptane and 5 μl of the appropriate anhydride are added and, after heating the sample at 70° for 2–3 min (ASN and DOPA for 10 min), an aliquot of 2 μl (1 nmol of each amino acid injected) is taken for gas chromatographic analysis.

RESULTS AND DISCUSSION

Analysis after condensation

Approximately half of the α-amino acids studied can be analysed directly after condensation by direct injection of the reaction medium into the column. However, pyridine tailing can have an adverse effect on the analysis of the simplest members, especially when their concentration in the medium is low. In such a case isolation of the derivatives via extraction is more likely to succeed.

Derivatization studies with dicarboxylic amino acids[2] have shown that the amount of pyridine in the medium should not be less than approximately 1 mol/l (*i.e.*, 8 μl of pyridine in 100 μl of medium) when 15–20 μl DCTFA are used for reaction. In the presence of polar acetonitrile the condensation proceeds most rapidly, whereas in the mixed solvent with benzene[2,3] some amino acids are difficult to dissolve. Partial replacement of acetonitrile with dichloromethane does not lower the dissolution rate too much and provides a good compromise with respect to subsequent acylation of the polyfunctional amino acids and the following extraction.

Condensation of ^{14}C-labelled amino acids with subsequent development of the sample aliquot in chloroform on a paper sheet (oxazolidinone moves with the

solvent front) or the silica layer of the Silufol foil (polar groups in the side-chain interact with the active surface and the mobility of oxazolidinones is diminished) enable the time dependence of the conversion rate to be evaluated.

Amino acids with carbon-linked chain. Results of the analysis of aliphatic amino acids with 2–8 carbon atoms in the molecule and a branched (A) or a straight (B) side-chain after 10 min of condensation are shown in Fig. 1. The studies revealed that the side-chain structure affects to a certain extent the course of the condensation reaction. A straight-chain amino acid with 8 carbon atoms (ACA) and surprisingly also GLY required a reaction time of 10 min to be fully (*i.e.*, more than 97%) cyclized. Straight-chain compounds with a smaller member of carbon atoms (ALA, ABA, NVAL and NLEU) and an amino acid branched in the γ-position (LEU) were converted into oxazolidinones within 3–5 min. Amino acids branched in the β-position (AIBA, VAL and ILE) were condensed most quickly, within 1–2 min at 20°.

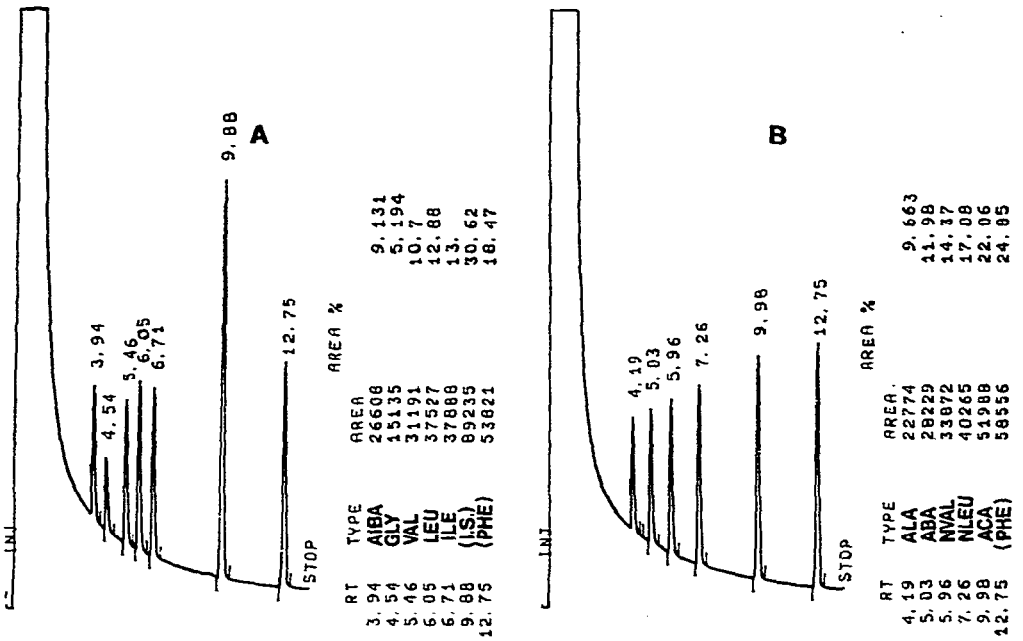
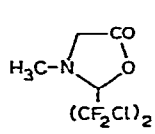


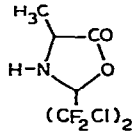
Fig. 1. GC analysis of aliphatic amino acids with (A) branched (plus glycine) and (B) straight carbon-linked chains on column A after 10 min of condensation. *n*-Hexadecane (internal standard, I.S.) added in order to evaluate the absolute molar responses[10]. Temperature range 80–200°.

The OV-17 silicone phase was found to be convenient for the separation of ALA–GLY and LEU–ILE. Retention of branched-chain amino acids in the chromatographic column was less than that of the straight-chain compounds. With AIBA the shift to lower retention times is so pronounced that it is eluted even before ALA. Concerning branching, it is interesting, however, that the γ-branched LEU is eluted before the β-branched ILE, contrary to expectation. On the other hand,

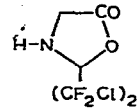
elution of GLY behind ALA agrees with the results of numerous previous studies on the analysis of amino acids in the form of acylated alkyl esters[7]. This anomaly is probably caused by an enhanced interaction of the secondary amino group with the column packing, whereas with ALA the presence of the neighbouring methyl group decreases such an interaction and, with sarcosine (N-methylglycine), the N-methylation completely eliminates this interaction. The retention times found (relative to sarcosine) confirm this conclusion:



N-methylglycine (sarcosine)
$r_t = 1.00$



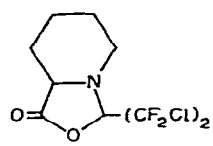
α-methylglycine (alanine)
$r_t = 1.08$



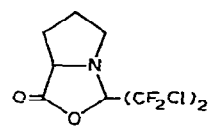
glycine
$r_t = 1.17$

Amino acids with alkyl substituent on polar group. Condensation of "imino" acids should theoretically be more difficult because of the low reactivity of the "imino" hydrogen atom. However, N-methylglycine is in fact condensed to the oxazolidinone 3 to 5 times faster than is GLY, its retention time on column A is identical with that of AIBA and its molar response is identical with that of ALA. Likewise, condensation of PIPA proceeds very easily (5 min) and the 6-membered piperidine ring does not hinder the formation of the oxazolidinone ring.

In contrast, the oxazolidinone formation from the 5-membered pyrrolidine of PRO is not a smooth process. The reaction course is influenced by medium polarity, reaction temperature and the concentrations of pyridine and DCTFA in the medium. In the chosen mixed solvent full cyclization was accomplished only after 45 min, and in acetonitrile alone after 30 min. As the reaction proceeds exponentially, two thirds of PRO is derivatized after 10 min at 40° (see Fig. 5). The oxazolidinones of PRO and PIPA have nearly identical retention times on column A and thus cannot be distinguished (Fig. 2A and B).



PIPA



PRO

The analysis of the oxazolidinones of N^{ε}-methyllysine, 1-methylhistidine and 3-methylhistidine was unsuccessful on both columns. The methyl group alone does not decrease the imidazole polarity to such an extent that the oxazolidinone of HIS will elute from the column (see below). However, with the methylated terminal amino group (N^{ε}-methyllysine) the column absorption is surprising.

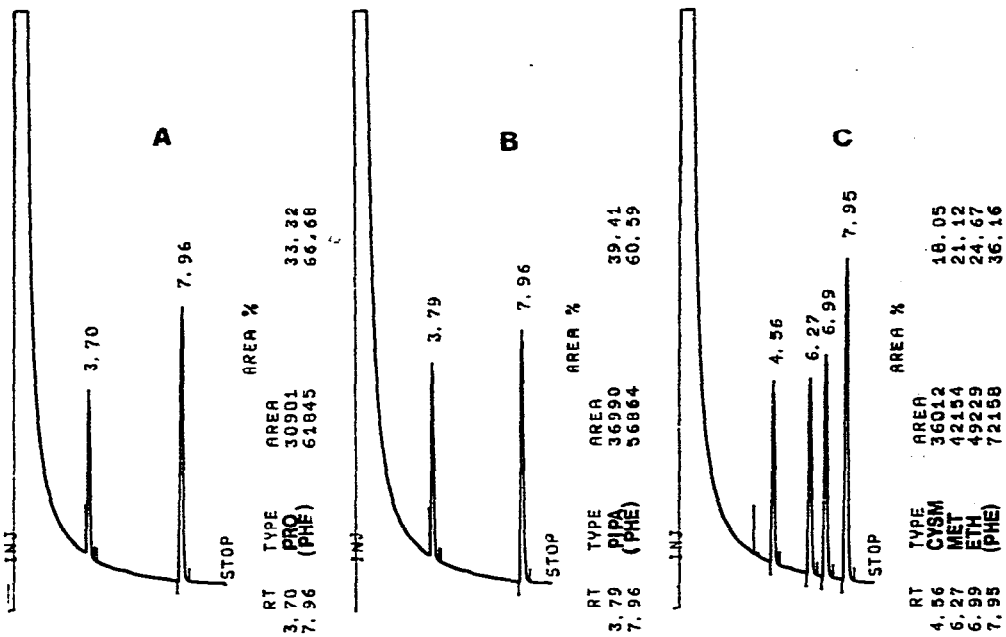
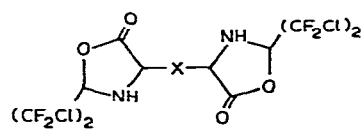


Fig. 2. GC analysis of (A) proline, (B) pipecolinic acid and (C) S-alkylated amino acids on column A after (B, C) 10 min and (A) 45 min of condensation. Temperature range 120–200°.

S-Alkylated amino acids can be converted into oxazolidinones very smoothly (2–3 min at 40°) and analysed on column A without problems with no anomalies in chromatographic behaviour (Fig. 2C).

Diaminodicarboxylic acids. Condensation results in the formation of the following bicyclic structures:



X	*Compound*
—	DAScA
$-(CH_2)_3-$	DAPA
$-(CH_2)_4-$	DASbA
$-CH_2-S-CH_2-$	LAN
$-CH_2-S-S-CH_2-$	CYS
$-(CH_2)_2-S-S-(CH_2)_2-$	HCYS

Because of the low solubility of these amino acids in the reaction medium the condensation rate is low (10–15 min at 40°), and with DAScA very low (30 min). The large number of halogen atoms in the molecule seems to be responsible for the enhanced absorptivity of the derivatives on column packings of the usual particle size (80–100 mesh). Absorption increases with increasing molecular weight and is higher for sulphur-containing diaminodicarboxylic acids. DAScA, the simplest diaminodicarboxylic acid, can be determined equally successfully on both columns (on column A eluted before PHE; on column B both of these amino acids are co-eluted). DAPA is partially absorbed (20%) on column A. LAN and especially CYS and HCYS show high absorption on this column, and the last two are absorbed partially

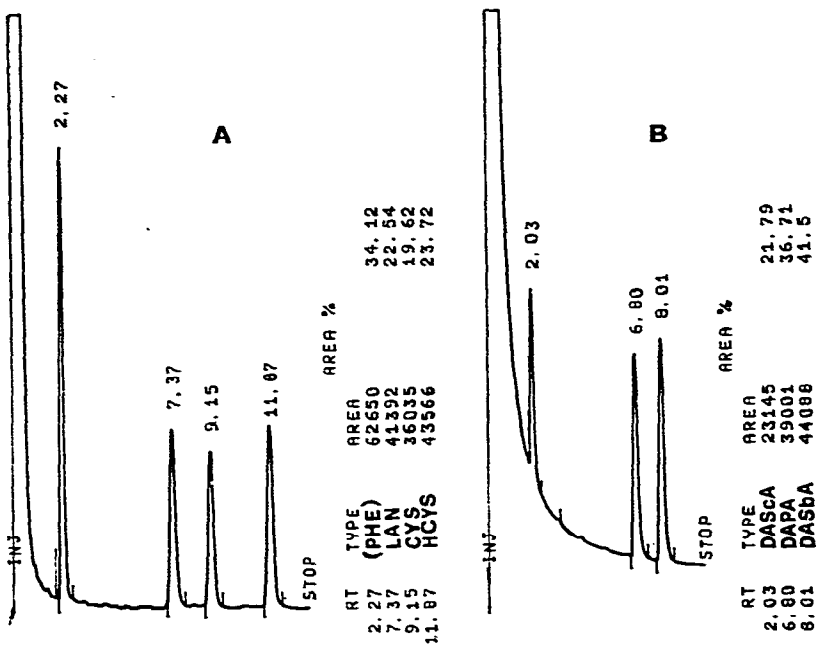
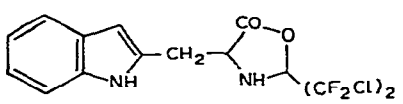


Fig. 3. GC analysis of diaminodicarboxylic acids with (A) intermediate sulphur atom(s) or (B) carbon-linked chain on column B after (A) 15 min and (B) 30 min of condensation. Temperature range 120–200°.

even on column B (losses of 10–20%). The derivatives were analysed after condensation (10 min at 40°) and subsequent extraction (Fig. 3A) or by direct injection of the condensation medium into column B (Fig. 3B) after 30 min of reaction.

Amino acids with indolyl or imidazolyl group. TRP and HIS, together with CYS, are the most difficult protein amino acids to determine by gas chromatography owing to their large molecules (TRP, CYS) or the polar and unstable derivative formed (HIS). The possibility of determining these amino acids in the form of oxazolidinones was considered in a previous paper[5]. TRP can be eluted from the column even with a free indolyl group, whereas free imidazole causes adsorption of HIS in the packing (Fig. 4A). The analysis of HIS on column B was successful after extraction if, during the extraction process, a small amount of methyl chloroformate (Fig. 4B) or isobutyl chloroformate (Fig. 4C) was added, as mentioned under Experimental. Alternatively, even acetic anhydride (5 μl) could be added to the organic phase after the carbonate wash and left to act for 3 min. Treatment of the organic layer with either of the three reagents was followed by [^{14}C]HIS. Without treatment a cation is formed on the imidazole group during shaking with hydrochloric acid and the oxazolidinone derivative of HIS passes into the aqueous phase. In contrast, the use of either of the



TRP

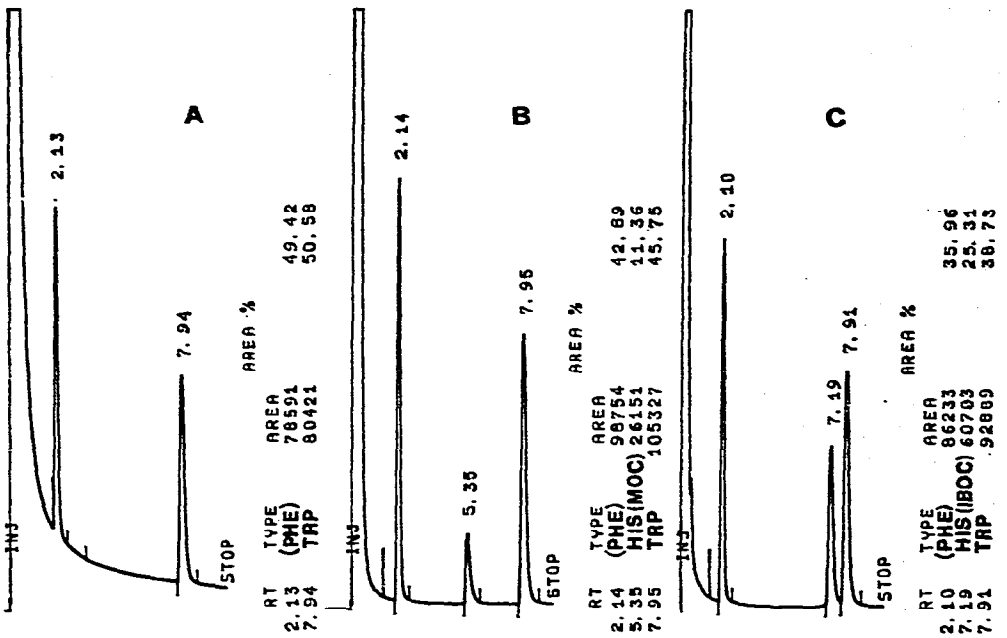
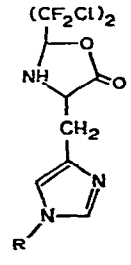


Fig. 4. GC analysis of tryptophan and histidine on column B after (A) condensation or condensation and extraction with added (B) methyl chloroformate or (C) isobutyl chloroformate. Temperature range 120–200°.

reagents was effective in blocking the imidazolyl hydrogen and the radioactively labelled compound remained in the organic phase. Reaction with chloroformates proceeds instantaneously: 1 μl of the corresponding reagent was found to be sufficient. The simultaneous analysis of TRP and HIS revealed that the indolyl group of TRP was not attacked by the reagents and remained unmodified.

Despite of the effective imidazole treatment, not all of the attached groups enable HIS to be analysed successfully. The bulkiness of the attached radical was decisive for decreasing the initial polarity of the oxazolidinone derivative of HIS. Only the N^{im}-IBOC (isobutyloxycarbonyl) derivative allowed HIS to be eluted from column B satisfactorily (90%). Substitution of the isobutyl group by methyl, *i.e.*, formation of the N^{im}-MOC (methyloxycarbonyl) derivative by treatment with MCF, results in less efficient masking of the imidazolyl nitrogen and HIS absorption increases. Acetylation of the imidazole group was ineffective in decreasing the polarity of the compound and, as in the analysis of 1-methyl- and 3-methyl-HIS oxazolidinones, the acetylated compound did not elute from the column. The following scheme summarizes the results clearly:



HIS

Radical (R)	*Elution yield (%)*
$-CH_3$ (methyl)	0
$-COCH_3$ (acetyl)	0
$-COO\cdot CH_3$ (MOC)	≈50
$-COO\cdot CH_2\cdot CH(CH_3)_2$ (IBOC)	≈90

In comparison with our previous study[5], where HIS was extracted after condensation without performing the acylation step, the anhydride addition leads to losses of about 10%. The reason for this phenomenon is not known. Derivatives of HIS can be analysed only on column B. TRP give a small response also on column A (see Fig. 8).

Analysis after acylation and extraction

With the exception of TRP and also TYR, that could be analysed successfully even without esterification of the hydroxyl group on both columns, the amino acids with side-chain reactive groups require further chemical treatment. The reactive perfluorinated anhydrides, HFBA and TFAA, proved to be most convenient for this purpose[2,3]. After having performed the acylation, which, with only one exception (CIT, HCIT), occurred simply on addition of the particular anhydride in the condensation medium, it was necessary to remove salts of pyridine and the perfluorinated acids by extraction. At the same time the derivatives had to be transferred quantitatively and without degradation losses into the organic phase, which should be volatile enough to prevent losses of the simplest amino acids by evaporation of the extraction medium. Considering these requirements, the combination of light petroleum with dichloromethane (3:1) proved to be the best for this purpose[5]; partial substitution of dichloromethane with benzene was also possible[2,3].

The choice of the first aqueous wash was decisive for the success of the extraction. If an acidic medium (dilute hydrochloric acid) is used first to remove pyridine, the extraction yields of some amino acid oxazolidinones are low and HIS passes into the aqueous phase owing to the cation formed on the imidazole end. On the other hand, neutralization of the excess of reagent by formation of salts from the reactive anhydrides and DCTFA [the ketone is converted into a bisodium salt, $(CF_2Cl)_2C(ONa)_2$] during the first alkaline wash proved useful because of the salting-out effect of nearly saturated sodium carbonate solution. The salting-out effect, being greater with 1 *M* sodium carbonate than 1 *M* potassium carbonate solution, plays an important role as the acetonitrile present partially hinders the extraction. The subsequent shaking of the organic phase with dilute hydrochloric acid removes pyridine without deterioration of the extracted forms and, following the water wash, the organic extract was prepared for evaporation. Drying of the extract with anhydrous sodium sulphate before the evaporation[2,3] was found to be unnecessary; however, transfer of the organic phase into another vial is recommended because of a thin aqueous layer sticking on the walls of the silanized reaction tube. Evaporation should be watched carefully in order to prevent blowing of gas into the evaporated residue. Stopping the process before the last drop has dried following manual evaporation as described is the best means of preventing losses of aliphatic amino acids. Direct evaporation of pyridine with the condensation medium always leads to complete loss of the oxazolidinones of most protein amino acids.

The oxazolidinones in the residue are readily soluble in hydrocarbon solvents and, because the extraction proceeds without degradation of the N,O-HFB- and even N,O-TFA-acylated forms, the derivatives can be subjected to analysis after dissolution in *n*-heptane or *n*-hexane only. Co-addition of about 5% of the particular anhydride to the solvent is, however, recommended (both agents are miscible after heating to 70°) in order to suppress the tendency of some acylated forms to undergo partial

column absorption. The analysis of three protein amino acids (ARG, GLN and ASN) is impossible without the additional acylation; *n*-heptane proved to be a suitable solvent for this purpose.

Amino acids with O- or S- reactive groups in the chain. In order to esterify the hydroxyl groups successfully, it was necessary to dilute the condensation medium with 500 μl of extraction medium prior to anhydride addition (procedure B). Without the dilution the acylation yields of especially THR and SER are low and THR affords two peaks, the second being co-eluted with HYP. The acylation yields bear a close relationship to the dilution of the condensation medium up to a volume of 500 μl; further addition of extraction medium does not enhance the yields any more. Likewise, methanol promotes successful acylation; its adding to the condensation medium together with light petroleum (*e.g.*, 4 and 60 μl, respectively) enables THR and SER oxazolidinones to be esterified nearly quantitatively at the hydroxyl group. This is an important finding in the search for a uniform reaction procedure that would cope successfully with all reactive groups present in mixtures of protein amino acids. Acylation of TYR was not influenced by the polarity of the medium to the same extent; when acylated in the condensation medium (procedure A) the molar response was about 70% of that obtained by procedure B. Further heating of the reaction medium with added anhydride did not alter the yields; the esterification proceeds instantaneously and only the polarity of the medium at the moment of anhydride addition determines the conversion rate.

THR and SER should be acylated just after dissolution of the compounds in the condensation medium (condensation is accomplished in 1 min at 20°) in order to obtain the maximal response. If these two amino acids remain in the medium any longer a progressive linear decrease in their molar responses occurs (see Fig. 5), the decrease with THR being more rapid than that with SER so that both responses have the same value after about 20 min. The reason for this effect is not known; the decrease accelerates the enhancement of pyridine, DCTFA and acetonitrile concentrations in the medium, but chromatographable side-products are not formed. Such a phenomenon was not observed with HYP and TYR, and it is thus characteristic of amino acids that contain a hydroxyl group adjacent to an α-amino group. Condensation of HYP is approximately five times more rapid as that of PRO because of an electron shift on the pyrrolidine ring due to hydroxyl group, acylation of which according to procedure B proceeds smoothly. Analysis of O-HFB- and O-TFA-esterified oxazolidinones after 10 min of condensation, acylation according to procedure B, extraction and additional acylation (1–2 min) is illustrated in Figs. 6A and 7A. When the derivatives are subjected to analysis in *n*-heptane only, the molar responses are lower by about 5–10% (O-HFB forms) or 15–20% (O-TFA forms).

Degradation of THR and SER oxazolidinones with time appears evident when they are present in large amounts in the sample, when the condensation medium turns progressively yellow. If the sample contains amino acids with thiol groups (CYSH, penicillamine = β,β-dimethylcysteine) the medium immediately turns yellow after addition of DCTFA. The subsequent acylation does not alter the fact that the chromatographic analysis affords no peaks. Thus, in order to succeed, the thiol group has to be blocked in advance. Eyem *et al.*[8] published a procedure for methylating the thiol group with dimethyl sulphate in order to convert CYSH into CYSM, which can easily be analysed as the oxazolidinone (see Fig. 2C). Alternatively, by

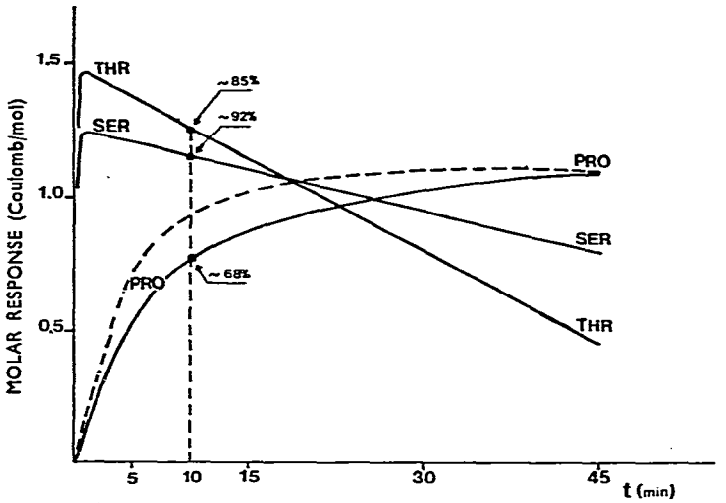


Fig. 5. Effect of polarity of the medium and the total condensation time on reaction yields of threonine, serine and proline cyclization. Condensation occurred in the given mixed solvent (full line) or in acetonitrile only (72 μl of acetonitrile, 8 μl of PYR, 20 μl of DCTFA; broken line).

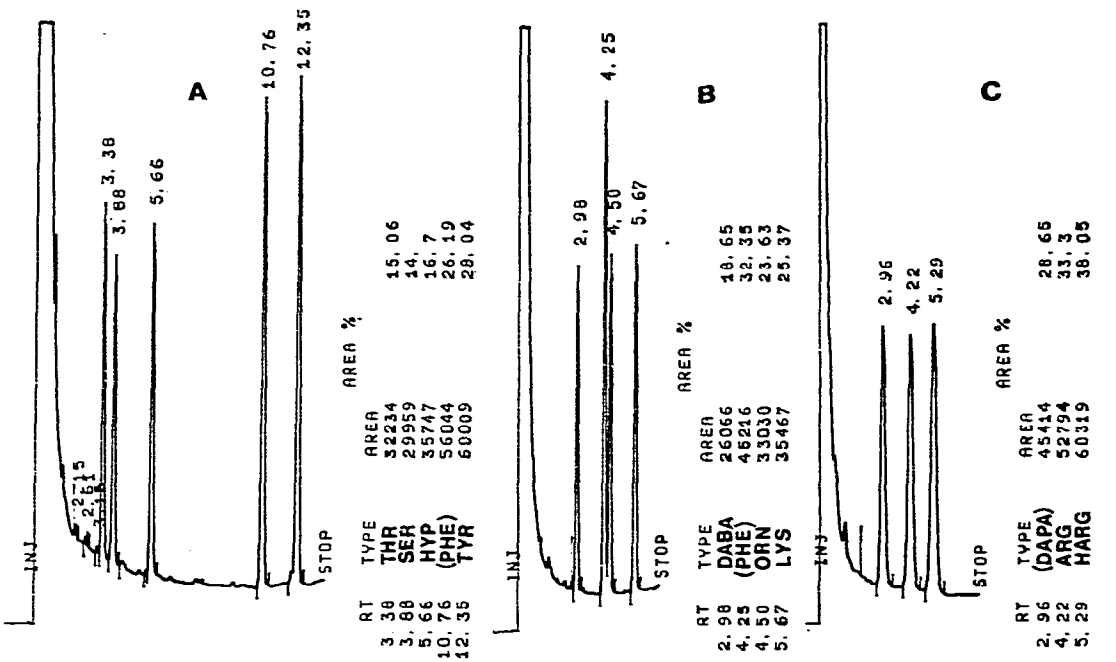


Fig. 6. GC analysis of (A) hydroxyamino acids, (B) α,ω-diamino acids and (C) arginine and homoarginine condensed and treated with HFBA according to procedure A (B, C) or procedure B (sample A). After extraction and additional acylation at 70° for 3 min the samples were analysed on column A or B (sample C) in the temperature ranges 100–200° (sample A) or 160–220° (samples B and C).

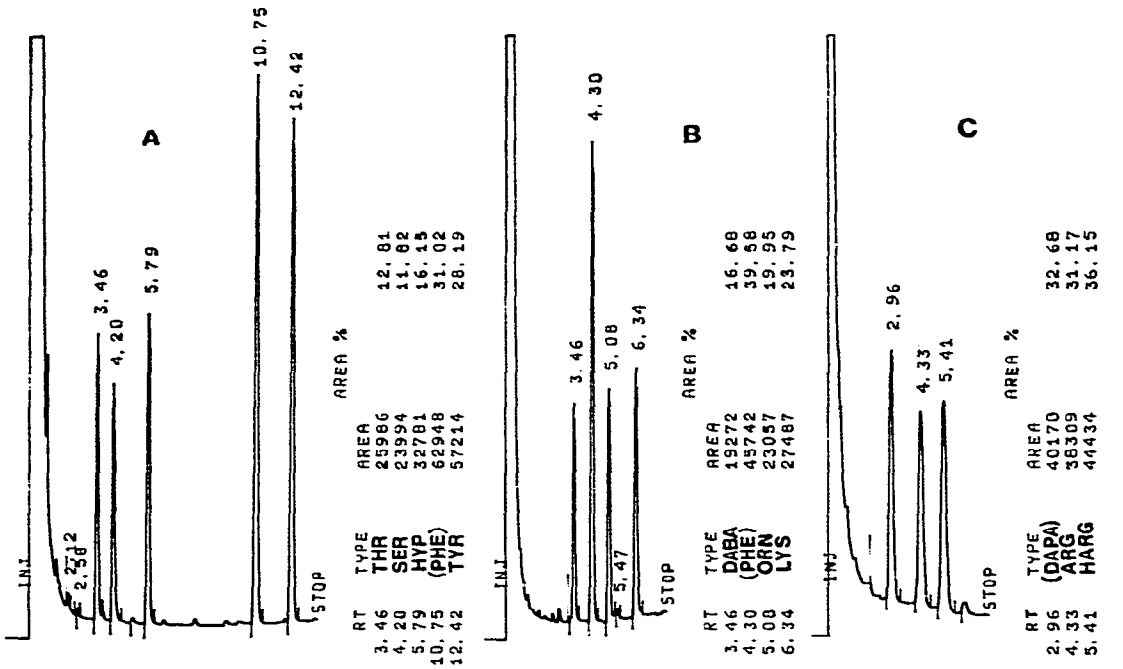
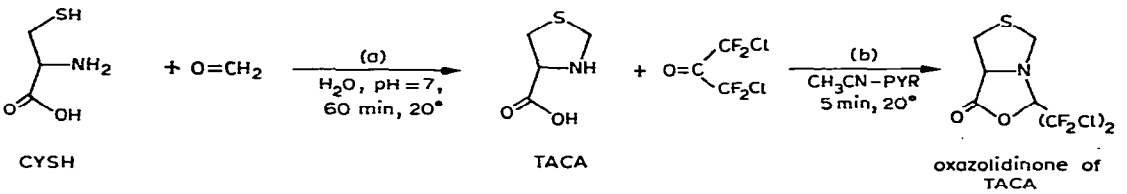


Fig. 7. GC analysis of the same amino acids as in Fig. 6 except that they were treated with TFAA. Procedure and analytical conditions as in Fig. 6.

treatment with dilute formaldehyde solution[9], CYSH is converted into thiazolidine-4-carboxylic acid (TACA), the condensation of which is, like PIPA and unlike PRO, very smooth (3–5 min), the retention time on column A being similar to that of CYSM. The reaction sequence is as follows:



CYSH TACA oxazolidinone of TACA

Amino acids with N-reactive group in the chain. Oxazolidinones of amino acids with an additional amino or amido group are acylated effectively in a polar condensation medium (procedure A). Acylation of the α,ω-diamino acids (DABA, ORN, LYS), ASN and GLN is instantaneous, whereas modification of the ureidic group ($R–NH \cdot CO \cdot NH_2$) in the molecule of CIT and HCIT requires longer anhydride action (5 min at 40°). CIT and HCIT are the only amino acids that require an anhydride treatment with heating, during which the ureidic group is converted into an amino group so that ORN and LYS are formed. However, the presence of methanol in the medium prior to addition of anhydride hinders the acylation of CIT and HCIT

and thus their successful determination. In contrast, in accordance with a previous study[3], acylation in the absence of methanol results in double peak formation with GLN. Both the amides ASN and GLN require the additional acylation step for a chromatographic response to be obtained.

Analysis of α,ω-amino acids after 10 min of condensation, treatment with HFBA or TFAA, extraction and application of the sample in column A in *n*-heptane in the presence of anhydride is shown in Figs. 6B and 7B, respectively. If injected in *n*-heptane only, a small absorption, especially of the N^{ω}-TFA forms, occurred and the results were less reproducible.

Amino acids with two reactive groups in the chain. In order to succeed in the determination of ARG and HARG, both the guanidine [–NH–C(=NH)–NH_2] terminal groups, *i.e.*, the amino and the imino groups, must be acylated. Effective blocking of the guanidino group takes place after addition of anhydride to the condensation medium, so that the oxazolidinones of ARG and HARG do not pass into the acidic medium during shaking with dilute hydrochloric acid. The acyl moiety on the imido group is nevertheless cleaved during this process, as analysis in *n*-heptane only produces no response from the extracted forms. By means of brief heating (2–3 min at 70°) with the medium of the additional acylation, the imino group is re-acylated and the N,N-diHFB- (Fig. 6C) or N,N,-diTFA- (Fig. 7C) oxazolidinones of ARG and HARG can be analysed on column B successfully. When analysed on column A, partial absorption (30% of ARG and 50% of HARG, see Fig. 8) occurs.

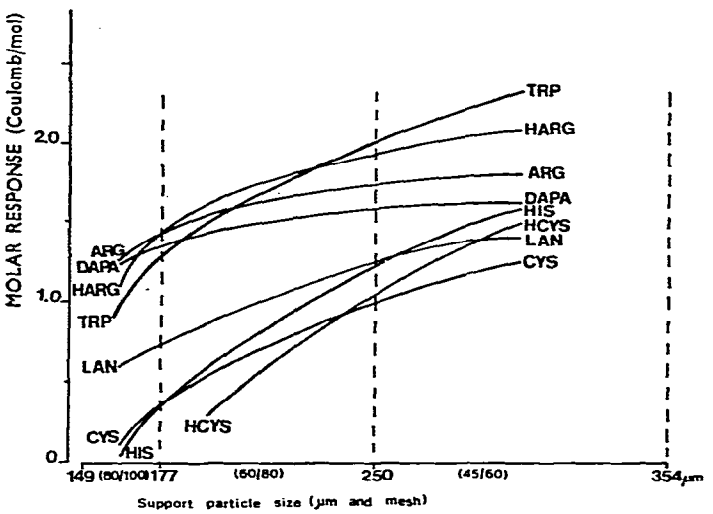


Fig. 8. Effect of particle size (80–100, 60–80 and 45–60 mesh fractions) of a silanized chromatographic support on the elution of amino acids from a 1 m × 2 mm I.D. glass column packed with 3% SE-30. Nitrogen flow-rate, 30 ml/min; temperature range, 120–240°.

Acylation of the two hydroxyl groups in the molecule of DOPA and the one hydroxyl and one amino group in the molecule of HLYS gave mostly unsatisfactory results with either of the two acylation procedures. Analysis of HLYS on column B gave only some minor peaks after the additional acylation; analysis of DOPA

afforded the same response on both columns, but only about 50% of the expected response. The diHFB acylated oxazolidinone of DOPA is eluted just before TYR on column A and in front of DAPA on column B.

CONCLUSIONS

In view of this study and some previous results[2,3,5], it can be concluded that the analysis of α-amino acids as the cyclic oxazolidinone derivatives is a useful technique. Evidence has been presented that all protein amino acids, including many other α-amino acids, can be converted into convenient analytical forms successfully and very rapidly. With regard to the amino acid composition of the protein set, the chemical modifications carried out need to include the following four steps: condensation, acylation, extraction and additional acylation. Condensation with DCTFA in acetonitrile as a solvent and pyridine as a catalyst proceeds at room or slightly elevated temperature very smoothly and rapidly. Acylation with reactive anhydrides is accomplished simply by addition of the reagent in the polar aprotic condensation medium, in which the basic amino acids are acylated well. For hydroxyamino acids, however, it is necessary to decrease the polarity of the medium by addition of the extraction medium prior to anhydride treatment. In order to esterify dicarboxylic amino acids the presence of a small amount of methanol is also needed[2]. Elaboration of a uniform derivatization procedure will thus require optimization of the acylation step.

Extraction of the derivatives into light petroleum–dichloromethane enables excess of reagent and the pyridine to be removed by shaking with aqueous carbonate solution and dilute hydrochloric acid, respectively. Addition of a minute amount of chloroformate contributes to the formation of a stable derivative of histidine. The organophilic oxazolidinones are readily soluble in *n*-heptane, and the addition of a small amount (5%, v/v) of anhydride and subsequent brief heating at 70° also allows arginine, glutamine and asparagine to be determined.

Although amino acid oxazolidinones exhibit good chromatographic features, their analysis requires more exactly defined conditions than is usual[7]. Glass as the column material and silanization of the chromatographic support are basic requirements, without which the analysis fails. The best silanized supports with the usual particle size (80–100 mesh) allow most of the oxazolidinones to be eluted quantitatively; employment of finer particles (100–120 mesh) causes partial absorption of some basic amino acids in the column. Amino acid derivatives with a greater abundance of halogen atoms (two oxazolidinone rings, N,N-diacylated forms) especially exert an enhanced interaction with the support surface. Only by decreasing the surface area by increasing the particle size of the support (60–80 to 45–60 mesh) and shortening the column length to 1 m could the largest and most polar amino acids be analysed[5] (Fig. 8). Homocystine and histidine are the most influenced by the particle size, and their elution is not complete even from the coarse batch of the support. The same is also true, although to a lesser extent, for cystine and tryptophan.

The simultaneous analysis of *n*-hexadecane with amino acid oxazolidinones (see Fig. 1A) enabled the phenylalanine molar response to be evaluated, and this compound was then added as the internal standard to the particular amino acids. From the values of the corresponding molar responses of amino acids in a homol-

ogous series it was possible to calculate the effective carbon number and to judge the completeness of the derivatization and elution. The results will be presented in a subsequent paper[10].

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REFERENCES

1 P. Hušek, *J. Chromatogr.*, 91 (1974) 475.
2 P. Hušek and V. Felt, *J. Chromatogr.*, 152 (1978) 363.
3 P. Hušek and V. Felt, *J. Chromatogr.*, 152 (1978) 546.
4 M. Matucha and E. Smolková, *J. Chromatogr.*, 168 (1979) 255.
5 P. Hušek, *J. Chromatogr.*, 172 (1979) 468.
6 P. Hušek and V. Felt, *Clin. Chim. Acta*, 72 (1976) 195.
7 P. Hušek and K. Macek, *J. Chromatogr.*, 113 (1975) 139.
8 J. Eyem, J. Sjödahl and J. Sjöquist, *Anal. Biochem.*, 74 (1976) 359.
9 S. Ratner and H. T. Clarke, *J. Amer. Chem. Soc.*, 59 (1937) 200.
10 V. Felt and P. Hušek, *J. Chromatogr.*, submitted for publication.