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# AMINO ACID DETERMINATION BY CAPILLARY GAS CHROMATOGRAPHY ON CHIRASIL-VAL

## ENANTIOMER LABELLING AND NITROGEN-SELECTIVE DETECTION

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SUMMARY

Amino acid analysis by enantiomer labelling and capillary gas chromatography on Chirasil-Val is superior to conventional gas chromatography and ion-exchange chromatography with respect to sensitivity, accuracy and speed. Employment of an alkali flame-ionization detector allows the selective detection of amino acids and suppression of background peaks; in addition, the detectability of amino acids is enhanced. Most nitrogen-selective detectors require meticulous adjustment of the operating conditions, but in combination with enantiomer labelling this is less critical. Maximum sensitivity and selectivity of the alkali-bead flame-ionization detector is achieved with a minimal flow of hydrogen. When using it as the carrier gas in capillary gas chromatography, flow control instead of the common pressure regulation is recommended to avoid a continuous fall of the baseline during temperature programming. Accurate flow control is achieved with a micro-aperture. The benefit of nitrogen-selective detection is especially apparent for histidine and arginine.

## INTRODUCTION

The determination of amino acids in biological fluids or protein hydrolysates is of great interest in many areas, such as peptide synthesis, protein chemistry, clinical analysis and food chemistry. Amino acid analysis is usually performed by ion-exchange chromatography, a procedure introduced by Spackman *et al.* 25 years ago[1]. Although gas chromatography was proposed relatively early[2,3] as a method potentially superior with respect to sensitivity and speed, until recently it suffered from several drawbacks associated with the need for derivatization of amino acids. While sample preparation for ion-exchange chromatography is relatively simple, the pretreatment for gas chromatography includes additional physical and chemical steps such as sorption of the amino acids on a cation exchanger, desorption with ammonia,

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evaporation of water, esterification of the carboxyl group, acylation of amino and other functional groups in trifunctional amino acids, evaporation of reagents, dissolution to an appropriate concentration, withdrawal of an aliquot and injection into the gas chromatograph[4,5]. All these steps pose risks of incomplete recoveries or yields and only by adherence to meticulously standardized conditions is the reproducibility of analysis comparable to that of ion-exchange procedures. Additional sources of errors arise during sample introduction into a capillary, usually termed discrimination. Hence the advantages of gas chromatographic analysis are outweighed by the need for a time-consuming pre-chromatographic treatment and the difficulties associated with different injection techniques for quantitative capillary gas chromatography. Especially for users not experienced in gas chromatography, this represents a barrier to employing this potentially faster and more sensitive method.

In principle, all of these drawbacks can be overcome with appropriately selected internal standards. Of course, internal standardization has often been employed in quantitative gas chromatography, but for amino acids with their widely differing chemical properties one or two analogues as internal standards serve virtually only for compensation of physical losses, without reflecting the different chemical behaviour of the amino acids at the various stages of analysis.

Some years ago we synthesized a chiral stationary phase of high thermal stability that permits the separation of all individual amino acids and exhibits sufficient enantioselectivity to resolve all their enantiomers[6,7]. This opened up the possibility of determining in two consecutive gas chromatographic runs the enantiomeric composition of the common amino acids and, by enantiomer labelling with the optical antipodes as internal standards, their exact amounts. Of course, determination of the enantiomer ratios is not required when the presence of only the natural L-enantiomers can be assumed, as is the case for biological fluids[8].

The accuracy and precision of amino acid determinations in protein hydrolysates, serum, urine and cerebrospinal fluid by enantiomer labelling proved to be equal and for most amino acids superior to those by the ion-exchange method. At the same time, the stringently defined conditions of sample preparation required for reproducible yields could be relaxed. Nevertheless, some difficulties arose from extraneous peaks in the chromatograms. When a more extensive sample purification was employed, the accuracy and precision could be further increased; lipids were extracted and the acid for precipitation of proteins, usually picric or sulphosalicylic acid, was adsorbed on the anion exchanger, both prior to cation exchange. This indicates that small amounts of coeluting substances may interfere with quantitative determination[8,9]. In ion-exchange chromatography these do not present any difficulties as the post-column reaction with ninhydrin leads to selective detection of the amino acids without interference by other contaminants.

The non-selective detection by flame ionization (FID), commonly employed in gas chromatography, presents a drawback in amino acid analysis. The use of nitrogen-selective detectors has been proposed[10,11], but problems associated with the strong dependence of their response on gas flows, detector temperature, column bleed, design of the alkali source and operational parameters prevented their general acceptance. The rubidium silicate bead detector (alkali flame-ionization detector)[12] in the N-mode is extremely sensitive towards fluctuations of the hydrogen flow-rate. When hydrogen is used as the carrier gas, which has definite advantages[13], the de-

crease in flow-rate on temperature programming causes an unacceptably large negative drift of the baseline, even when the detector is supplied with additional hydrogen. Operation of the capillary with a flow-controlled carrier gas rather than pressure control alleviated this problem and enabled us to utilize fully the high selectivity and sensitivity of this type of thermionic detector.

## EXPERIMENTAL

### *Isolation of amino acids from serum*

A 100-$\mu$l volume of serum is mixed with 10 $\mu$l of a solution containing D-amino acids in accurately known concentrations, about ten times that expected for serum. A 0.5-ml volume of aqueous picric acid (1.2%) is added with vigorous swirling and precipitated protein is sedimented by centrifugation. The supernatant is poured on to a 10 × 5 mm column of Dowex 50W-X8 ($H^+$). After 3 min the resin bed is washed four times with 2 ml of water and the amino acids are eluted with 1 ml of aqueous ammonia. The eluate is collected in conical screw-capped vials and dried overnight in a vacuum centrifuge (Speed Vac Concentrator).

### *Derivatization*

*n*-Propanol (200 $\mu$l), containing 4 mol/l hydrogen chloride and prepared by mixing the appropriate volumes of alcohol and acetyl chloride[8,14], and 10 $\mu$l of ethanethiol are added to the dry amino acids. The closed screw-capped vial is heated to 110°C for 30 min. After cooling to about 50°C the vial is opened and the reagent is evaporated with a gentle stream of nitrogen at moderate temperature. The residue is dissolved in 50 $\mu$l of dichloromethane and 50 $\mu$l of pentafluoropropionic anhydride and the vial is tightly closed and heated for 10 min to 150°C. After cooling to room temperature, the solvent and reagent are evaporated under a gentle stream of nitrogen. For derivatization of histidine the residue is dissolved in 50 $\mu$l of dichloromethane, 10 $\mu$l of ethyl chloroformate are added and the mixture is kept at 100°C for 10 min. After cooling, the solvent and reagent are evaporated under a stream of nitrogen; excessive drying should be avoided as otherwise the more volatile amino acids alanine, valine and threonine may be partially lost. The residue is dissolved in an appropriate volume of toluene.

### *Gas chromatography*

Gas chromatography is performed on a Carlo Erba Model 4160 instrument, equipped with an alkali flame-ionization detector (P–N detector, Model 793). The carrier gas control unit is modified as shown in Fig. 1 to allow both pressure and flow regulation. Accurate flow control is achieved with a Siemens micro-aperture, class 5. At an inlet pressure of 350 kPa this micro-aperture delivers a flow of 1.5 ml/min of hydrogen as the carrier gas. Samples are introduced either by split injection or splitless with an automatic sampler for solid samples (Carlo Erba, Model 952). During split injection (splitting ratio 1:50) the carrier gas is pressure controlled; 3 min after injection, the split-bypass needle valve and the septum-flush needle valve are closed. Immediately afterwards the carrier gas supply is diverted to flow control by opening and closing of the corresponding needle valves; automatic synchronized actuation of the valves is possible for routine analysis. During splitless injection with the automatic sampler this change-over is not required.

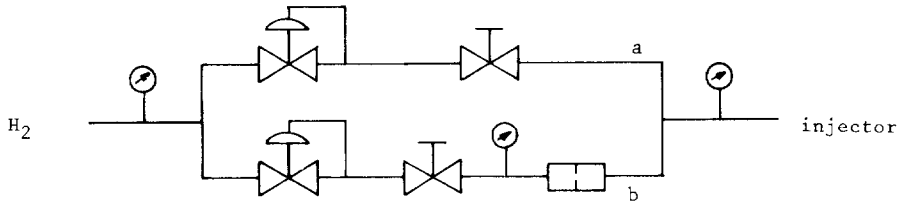


Fig. 1. Carrier gas control unit for alternative regulation of pressure (a) or flow with micro-aperture (b).

A Duran-glass capillary (25 m × 0.25 mm I.D.) is prepared by dynamic coating with colloidal silicic acid in acetone[15] and static coating with a 0.3% (w/v) solution of Chirasil-Val in pentane[16]. Temperature programme is as follows; 80°C isothermal for 5 min, followed by a temperature rise of 4°C/min to 210°C, isothermal for 10 min. At a hydrogen flow-rate of 1.5 ml/min, delivered through the micro-aperture, the inlet pressure with this glass capilary is 45 kPa at 80°C and 63 kPa at 210°C. The amounts of amino acids injected for gas chromatography are in the range 1–10 ng for splitless injection and correspondingly higher for split-injections. The operating parameters for the alkali flame-ionization detection (AFID) are make-up gas 15 ml/min nitrogen, air 130 ml/min, source power adjusted to give a detector current of 5 pA and detector temperature 275°C.

## RESULTS AND DISCUSSION

With the development of Chirasil-Val for the resolution of the optical antipodes of amino acids, hydroxy acids, alcohols, amines, glycols, sulphoxides and carbohydrates, and the introduction of the method of enantiomeric labelling, involving optical antipodes as internal standards, most of the problems associated with the quantitative analysis of chiral compounds by capillary gas chromatography have been eliminated. Optically active compounds of biological origin usually occur predominantly as one enantiomer; hence enantiomer labelling offers great potential for the analysis of chiral compounds in biological samples, especially as the necessarily extensive clean-up incurs the risk of incomplete and non-reproducible recoveries and yields.

Amino acid analysis in biological fluids and protein hydrolysates is a prime area of potential application of this method. However, ion-exchange chromatography is still the preferred method; the equipment is an expensive dedicated liquid chromatograph, but when operated correctly such an instrument yields accurate results, usually requiring little attention to sample preparation. Also, as this technique has profited from the rapid progress in liquid chromatography in recent years, the analysis times could be shortened considerably. In routine analysis the time and the reliability of results are the most decisive aspects in the selection of a particular method, only sometimes outweighed by the need for high sensitivity or low cost. Gas chromatography, the alternative to ion-exchange chromatography, is superior in the last two aspects (sensitivity and cost). The time required for chromatography alone is short, but as sample preparation is laborious, especially when high accuracy is sought, overall gas chromatographic analysis is more time-consuming. Also, the accuracy and reliability of gas chromatographic analysis are comparable only with meticulous ad-

herence to standardized procedures on all stages, which demands a widely experienced analyst. In fact, many potential users, after obtaining frustrating results with gas chromatography, have reverted to ion-exchange chromatography.

Capillary gas chromatography on Chirasil-Val in conjuction with enantiomer labelling changed this situation radically, permitting considerable simplification of sample preparation and at the same time providing enhanced sensitivity and accuracy. A primary prerequisite for the successful application of this method is the ability to resolve all enantiomers without overlapping of different amino acids. This depends largely on the characteristics of the stationary phase, such as its polarity, hydrogen-bonding properties and enantioselectivity; they must be adjusted judiciously to obtain a suitable silicone. Only total synthesis gives enough freedom to do this; by inclusion of cyanoalkyl or phenyls as modifiers in the silicone chain[17] the selectivity of the phase can be influenced within a sufficiently wide range.

The first step in the preparation of a serum sample for analysis is precipitation of proteins. Quantitative recovery of the amino acids at this stage is difficult to achieve; in fact, in a recent round-robin experiment it turned out that ion-exchange analyses yielded values 5–10% lower than those obtained by enantiomer labelling, which is clearly due to occlusion of amino acids during precipitation of serum proteins. In enantiomer labelling these losses are compensated for by the use of optical antipodes as internal standards. The subsequent adsorption of the amino acids on a cation exchanger can now be performed in a batch procedure[9] instead of a chromatographic mode, significantly shortening the time required. Further, quantitative yields of both derivatization reactions are no longer necessary. Finally, effects such as discrimination or decomposition of derivatives in the injection port of the capillary gas chromatograph have a much less severe impact on the analytical results. Some difficulties may still arise from unidentified contaminants in the prepared samples, which cause noisy baselines on the chromatograms and may overlap with the peaks of the amino acids. Also, sometimes an excessive drift of the baseline at high temperatures is observed, resulting from accumulation of non-volatile compounds from relatively dirty samples, which gradually decompose in the hot injector.

These problems could be alleviated by employment of an AFID with high, selective sensitivity for nitrogen-containing compounds. Except in a few instances[10,11,18], nitrogen-selective detectors have found little application to amino acids, for several reasons. The long-term stability of the rubidium silicate bead detector is relatively poor, mainly associated with ageing of the bead. Also, adjustment and operating parameters have a strong influence on the response, especially depending on gas flow-rates and, correspondingly, bead temperature. As this detector exhibits its optimum sensitivity and selectivity at a very low hydrogen flow-rate of below 2 ml/min, a constant supply of this gas is of utmost importance. When using hydrogen as the carrier gas at a constant pressure, as is usual in capillary gas chromatography, a specific problem is the decrease in flow during temperature programming; the result is a steadily falling baseline and, more important for quantitative amino acid analysis, a concomitant change of the sensitivity of the detector. In a previous investigation[10] helium was tried as the carrier gas and 100–200-fold enhancements of sensitivity, depending on the amino acids, relative to FID were found. Another recent paper[11] described capillary gas chromatography with hydrogen as the carrier gas, but 35 ml/min of hydrogen were added as make-up gas. Under this condition our alkali

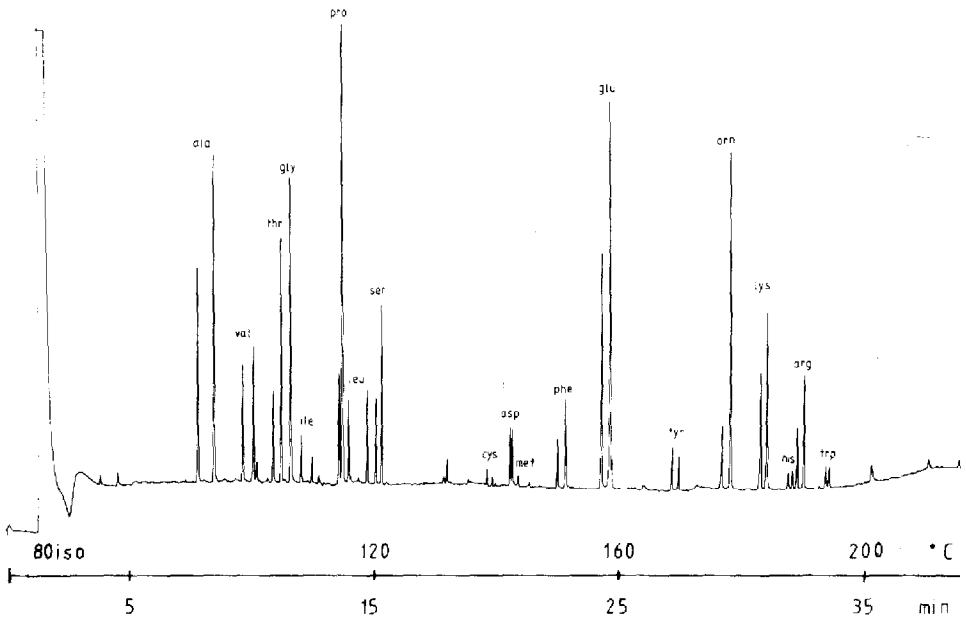


Fig. 2. Capillary gas chromatogram of serum amino acids as N(O,S)-pentafluoropropionyl amino acid *n*-propyl esters, histidine as $N^{im}$-ethoxycarbonyl derivative. Injected amounts about 125–500 ng; splitting ratio 1:50. Nitrogen-selective detection with an alkali flame-ionization detector.

flame-ionization detector exhibited characteristics of flame ionization, with only a 2–3-fold enhancement of sensitivity over a normal flame-ionization detector of the same geometry.

In order to retain the advantages of hydrogen as the carrier gas[13] and at the same time to operate the alkali flame-ionization detector under optimum conditions,

TABLE I

COMPARISON OF THE DETECTABILITY OF SEVERAL AMINO ACIDS WITH A RUBIDIUM SILICATE BEAD DETECTOR (AFID) AND A FLAME-IONIZATION DETECTOR (FID)

Amounts injected: 100 pg each.

| *Amino acid* | *Signal-to-noise ratio* | | *Enhancement* |
|---|---|---|---|
| | *FID* | *AFID* | |
| Ala | 12.4 | 1320 | 106-fold |
| Thr | 11.3 | 1320 | 116 |
| Pro | 10.0 | 2000 | 200 |
| Leu | 14.1 | 1370 | 97 |
| Ser | 7.6 | 1430 | 188 |
| Cys | 4.5 | 610 | 135 |
| Met | 7.6 | 690 | 90 |
| Glu | 10.0 | 910 | 91 |
| Lys | 7.3 | 1360 | 186 |
| Arg | 3.7 | 780 | 210 |

we incorporated a micro-aperture for accurate flow control of the carrier gas[19]. This ensured a constant flow-rate of 1.5 ml/min of hydrogen and resulted in a straight baseline. A typical chromatogram of serum amino acids obtained under these conditions is shown in Fig. 2. The chromatogram is considerably cleaner than a similar one obtained with FID. Peak tailing, usually more pronounced with AFID, is barely perceptible.

In the past we have sometimes encountered difficulties with histidine and arginine, mainly owing to an unstable baseline at elevated temperatures and low derivatization yields. In the present instance these two are detected with a sufficiently high signal-to-noise ratio. The high nitrogen content of these two amino acids further increases their detectability with an alkali flame-ionization detector.

The sensitivity of the alkali flame-ionization detector under the adopted operating conditions for several selected amino acids was compared with the response of a flame-ionization detector of the same geometry under identical chromatographic conditions. The results are shown in Table I; similar enhancements have been reported previously[10].

Flow regulation of the carrier gas did not prevent the possibility of using split injection; in this case only a change-over from pressure control to flow control was necessary, usually 3 min after injection. With an automatic sampler for solids these manipulations are not required. Obviously, another possibility for maintaining a constant flow of hydrogen to the alkali flame-ionization detector is to compensate the increasing flow resistance of the capillary by pressure programming.

The approach described is equally well suited to the quantitative determination of other nitrogen-containing compounds, which can be resolved into their enantiomers, such as amines, amino alcohols, amino sugars, hydroxycarboxylic acids as amides[20] or alcohols as carbamates[17].

## CONCLUSIONS

A combination of enantiomer labelling, flow-regulated capillary gas chromatography on Chirasil-Val and nitrogen-selective detection is suitable for quantitative amino acid analysis. The main disadvantages of the alkali flame-ionization detector, the poor long-term stability and the strong dependance of its response on the operating conditions, are unimportant as the determination of relative response factors is not required, in contrast to conventional quantitative gas chromatography. The high selectivity of the alkali flame-ionization detector greatly increases the reliability of quantitative determination, especially of the more "troublesome" basic amino acids. In most routine analyses the amounts of the amino acids available are high. In some special instances, such as in structure elucidation of proteins or with amino acids in unusual matrices, the enhanced sensitivity of the alkali flame-ionization detector adds another favourable facet to gas chromatographic amino acid analysis.

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