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ISOTACHOPHORESIS

THE SEPARATION OF AMINO ACIDS

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SUMMARY

There are several instrumental methods of analysis available for amino acids. The most important ones are ion-exchange chromatography (STEIN AND MOORE), gas chromatography and paper chromatography. These techniques all have various disadvantages; the time for analysis may be long or the sample treatment complicated and non-reproducible or the detector may be poor.

Electrophoretic methods using gel or paper are also available for separating amino acids or their derivatives, but as in chromatography none of them is entirely satisfactory.

Hence separation of amino acids by isotachophoresis is being investigated and various approaches are described in this paper.

INTRODUCTION

The amphoteric character of amino acids is the main problem, but also an advantage, in all liquid separation systems, including isotachophoresis. In isotachophoresis the amino acids may be analysed either as anions or cations. Anion analysis has been chosen because there is a greater differentiation of the pK values of the carboxyl groups.

To ensure that the amino acids run as anions, high pH values are required in aqueous solutions, (*i.e.* at least pH 10), and even then the net charge of several of the amino acids is small. The higher the pH, the larger the proportion of the current carried by hydroxyl ions will be and the slower acids will be separated, unsatisfactorily, by zone electrophoresis. The pH in an aqueous solution is expressed by

$$K_a = \frac{[B]\,[H^+]}{[BH^+]}$$

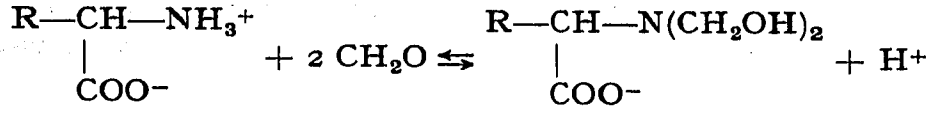
Here K_a is the acidic dissociation constant, and $[BH^+]$ and $[B]$ are the dissociated and undissociated forms of the buffer. Once the separation has reached a steady state

the pH is determined solely by the concentration in the leading solution of the ionised and unionised forms of the buffer, the mobilities of all the ions present in the leading solution and the mobilities and dissociation constants of the various zones.

EVERAERTS[1] has shown how the pH values may be calculated in detail. In general the concentration of each ion is lower than the preceeding one and the pH is higher. If the dissociation constants of succeeding zones are very different, however, there may be a rise rather than a fall of concentration and pH. The pH within the zones may differ by as much as 1 to 2 pH units from the pH of the leading electrolyte.

At the new pH (about 11), the hydroxyl ion concentration becomes 0.001 *M* which is actually comparable with that of the amino acid ions. The effective concentration of the hydroxyl ions is enlarged because their mobility is 4 to 5 times greater than that of an average amino acid ion. This thus causes an eluting effect. To avoid this problem the amino acids have to be transferred into compounds with a lower p*K* value, *i.e.* a less amphoteric character. Of the several methods of doing this, decarboxylation, esterification and deamination immediately spring to mind, replacing the amino group for a hydroxyl group is another possibility.

The method chosen is the equilibrium reaction with formaldehyde because of its non denaturating character, which can be of importance if preparative isotachophoresis is wanted; furthermore no sample treatment is necessary. The formaldehyde reacts with the amino group of the amino acids and decreases the p*K* value of this group. Thus a much lower pH is obtained with the same amount of ionised material. According to WHITE *et al.*[2] a possible explanation for the reaction is:



The shift in p*K* depends on the quantity of formaldehyde present.

The problem in using a formaldehyde solution is finding a buffer to work with at a pH of about 8. This buffer must have a great buffering capacity and not show any reaction with formaldehyde. Most buffers, suitable for this pH and buffering on cations, are based on an amine and therefore react with formaldehyde. So far good buffers have only been found among the tertiary amines.

A lower working pH has another advantage in that the eluting effects of the carbonate ion will disturb the analyses much less. The carbonate ion is present because carbon dioxide enters the system *via* the pressure valves for filling the capillary tube at high pH values or penetrates through the teflon capillary tube itself. Teflon is permeable to various gases, carbon dioxide being among them[3].

APPARATUS

The apparatus basically consists of a teflon capillary tube with a total length of 60 cm, an outside diameter of 0.75 mm and an inside diameter of 0.45 mm. This capillary tube is mounted between a cathode and an anode compartment, respectively. The effective length (*i.e.* from the injection point to the detector) is 50 cm.

A set of copper–constantan (0.001 in. diam.) thermocouples wound around the capillary tube and fixed with a suitable elastic glue are used as the detector. One thermocouple (giving the step-wise signal) is mounted with one junction on the cap-

illary tube. The temperature of the other junction is kept constant at 18°. The other thermocouple (giving the differential signal) is mounted with both junctions on the capillary tube[1].

The power supply consists of a 20 V d.c. power unit (Philips PE 1512), the voltage of which is lead to a 20 V d.c.–20 kV d.c. transformer (Spellman UHM 20 N 10 D). The output of the power unit as well as the input of the transformer are connected with a trigger unit because a current stabilised d.c. voltage is required.

The electrode compartments

The cathode consists of a platinum wire mounted in the compartment of the terminal electrolyte. This compartment is constructed[4] in such a way, that a sample can be injected into the apparatus with a syringe *via* a septum.

The anode compartment differs from the normal type used for isotachophoresis in that no membrane is present between the anode and the capillary tube. In this way pH effects, caused by the higher permeability of the membrane for protons, are avoided.

The electrode is mounted in a glass T piece. Another T piece is mounted in such a way that the rinsing water will not pass the electrode, so that none of the reaction products, formed at the anode, are able to enter into the capillary tube. These products could also enter into the capillary tube during the analysis, giving cause to disturbances.

Various reactions can possibly occur at the anode and are discussed below. Gas is not developed, at least not visibly.

Copper will go into solution at the anode as follows:

$$Cu - 3e \rightarrow Cu^{2+} + Cu^{+}$$

It can be seen that both ions are formed, because a red and a blue colour is formed near the electrode. Cu^{2+} can give $Cu(OH)_2$ which could be responsible for the dark slurry that is seen near the electrode.

The copper ions will undoubtedly form complexes with the buffer and the formaldehyde. For example, with pyridine both copper ions together can form some 9 positive complexes. Some of these will be stable enough to move in the opposite direction to that of the sample. It is evident that the same kind of complex formation can be expected with other buffers than pyridine.

The copper–buffer and copper–formaldehyde complexes, migrating to the cathode, could be changed into copper–amino acid complexes on meeting them in the capillary tube. Then again different charges would be possible.

Formaldehyde itself can easily be transformed into formic acid, in the presence of oxygen. This could also explain why no visible evolution of gas occurs. Formic acid can also be formed in the reaction of formaldehyde with the amino acids. This can be avoided by mixing a small amount of methanol in the solution.

By having a large volume between the exit of the capillary tube and the anode, the potential gradient over this compartment will be small. Thus the positive complexes mentioned above are not able to enter into the capillary tube easily.

Solutions

The capillary tube and the anode compartment are both filled with the same

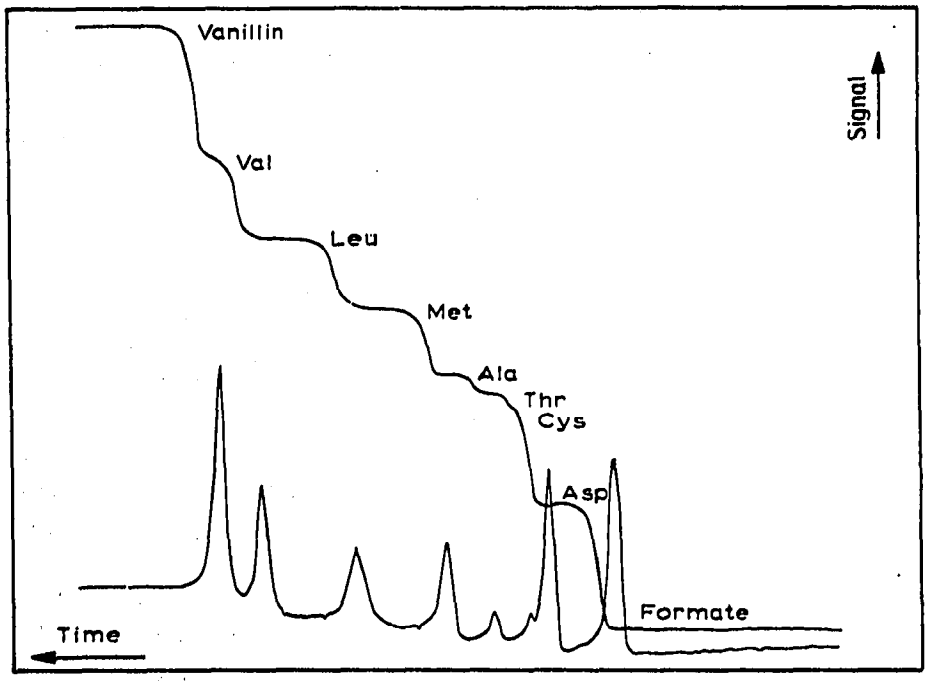


Fig. 1. Isotachophoretic analysis of some amino acids. For explanation see text.

solution, the leading electrolyte. This solution always contains 17.5% of formaldehyde. The buffer used is present in a concentration varying from 0.02 *M* to 0.06 *M*. The mixture of formaldehyde and buffer is first treated with a strongly basic ion exchanger, to remove foreign anions. Then the leading electrolyte and the anode solution are made by mixing the solution with an ion with a high net mobility like formate. The terminal electrolyte is made by mixing the buffer–formaldehyde solution with an ion with a low net mobility such as vanillin. Formate is used as leading ion because it can, as mentioned above, be formed during the analysis. Fig. 1 shows an example of an analysis of amino acids as it can be made at this stage.

It should be mentioned here that the electropherogram must be interpreted as follows. The stepwise curve (integral) gives the information about the zone temperature and this temperature is related to the ion species. The differential curve gives, from the distance between the peaks, the information about the concentration.

The conditions of the analysis were as follows: The leading electrolyte consisted of: *sym.* collidine 0.02 *M*; formic acid 0.01 *M*, and formaldehyde 17.5%; the pH of this solution is 7.1. The sample injected was 1 μl of a solution containing 0.4 mole of each of the following amino acids: aspartic acid, cysteine, threonine, alanine, methionine, leucine and valine dissolved in the mixture of collidine and formaldehyde.

CONCLUSIONS

Good buffers were found to be pyridine, 3-methylpyridine, *sym.* collidine and nicotine. The first two however have a p*K* value which is too low. Collidine is satisfactory however (about 7.2) and its reaction with formaldehyde is negligible. Nicotine has not been tested so far.

The solutions described above are satisfactory for qualitative analyses of amino acids. The method has yet to be developed for quantitative purposes.

ACKNOWLEDGEMENT

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