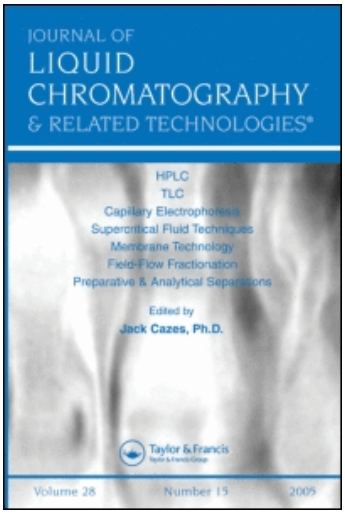

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P. Elödi[a]; T. Karsai[a]
[a] Department of Biochemistry, University Medical School, Debrecen, Hungary

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APPLICATION OF THIN-LAYER ION EXCHANGE CHROMATOGRAPHY AND VIDEO-DENSITOMETRY /CV-TECHNIQUE/ IN BIOCHEMISTRY AND RELATED FIELDS. A REVIEW ARTICLE

P. Elődi and T. Karsai
Department of Biochemistry
University Medical School
H-4012 Debrecen, POB 6, Hungary

INTRODUCTION

The various chromatographic procedures have made most significant contributions to the elucidation of fundamental facts and relationships in both theoretical and practical fields of biology and chemistry. The analysis of the extremely complex systems of biological origin or the preparation in pure form of substances that occur in small amounts in nature is practically unfeasible without the inclusion of one or more chromatographic steps.

Particular attention is paid to procedures which improve the efficiency of analytical work by simpli-

fying already existing principles and techniques without unfavourably affecting accuracy and sensitivity. In this context efficiency means one or more of the following:

1. A considerable reduction in the amount of specimen required for the analysis, in case of substances that are not readily available or are at our disposal only in small quantity;
2. The shortening of time period required for the analysis, particularly if large series comprising a great number of specimens are to be analyzed;
3. Last but not least, in serial analyses the question of expenses is also important.

In this survey a brief review will be given of the fields of application and results obtained of two recently introduced techniques. As can be judged from the experiences accumulated so far these two methods, which are variations on the theme of ion exchange chromatography and densitometry, satisfy the above three conditions. This renders the accomplishment of certain tasks possible which have so far been hampered by the limitations of available methods, such as the determination of enzyme content of tissue specimens on the milligram scale and others.

One of the novelties is the application of strong cation exchange resins in thin layer. Under such conditions the basic properties, the resolving power, of ion exchanger do not change but the amount of specimen and usually the time required for chromatography are reduced. The other novelty is the development of a densitometric technique that is new in principle and which is naturally suitable for the quantitation of any family of spots or bands that are geometrically regular, either in transmitted or in reflected light. It is also applicable to substances absorbing in the ultraviolet range or having fluorescent properties. In the following, the combination of chromatography with video-densitometry will be referred to as CV-technique.

Thin layer ion exchange chromatography

One of the most wide-spread field of application of ion exchange chromatography is amino acid analysis, particularly since the development of automatic amino acid analyzer by Hirs, Moore and Stein /1/. The amount of specimen required for analysis has since undergone considerable reduction, still the method is rather demanding in respect of fine chemicals. The instrument can handle only one specimen at a time and one analysis

takes at least 2-3 hours. These features become especially important if a great number of samples are to be analyzed. It was Dévényi /2/ who raised the possibility that the properties of ion exchangers can be favourably combined with the advantages offered by thin layer technique. In this way, while the ion exchanger preserves its good resolving power during chromatography /3/, both the quantity of specimen, along with the requirement for chemicals, and the time needed for the analyses can be markedly decreased. This is due to the fact that several specimens can be run on the thin layer chromatoplate simultaneously, moreover, many chromatoplates can be chromatographed at the same time. It is thus feasible to perform the chromatographic analysis of as many as several hundred samples at a time under identical conditions.

At present there is a Dowex 50 x 8 type, strongly acidic cation exchange thin layer chromatoplate commercially available under trade names Fixion 50 x 8 /Chinoin, Budapest, Hungary/ and Ionex SA-25 /Macherey and Nagel, Düren, F.R.G.//4/. These contain a resin layer consisting of spherical beads of 8-15 μm in diameter fixed by the aid of an indifferent adhesive onto 20 x 20 cm plastic foils. The plates /or sheets/ are available in Na^+

or Li^+ form, but the counterion can be exchanged at will by pre-chromatography with the appropriate solution /5/. In case of not particularly aggressive chromatographic solutions, in water or media also containing organic solvents, a run lasts for 1-2 hours. On a chromatoplate 6 to 8 /in case of need also 10/ samples 2 to 20 µl each can be loaded.

The appropriate separation of substances and the occurrence of geometrically regular spots needed for quantitation are, just like in the other varieties of ion exchange, dependent upon the experimental conditions, i.e. pH, ionic strength, presence of specific ions and/or organic solvents, temperature, etc. In certain instances two successive chromatographic runs in different media proved useful.

For the application of thin layer ion exchange chromatography experience has accumulated in the analysis of amino acids, nucleosides and their derivatives, antibiotics, various drugs, aminosugars and inorganic ions. As regards the applicability of the CV-technique the main body of works is related to the determination of amino acids.

Video-densitometry

This technique is unique among densitometric procedures both in principle and from the practical

point of view /6/ and can be characterized briefly as follows. In contrast to various commercially available densitometers in which usually the specimen is moved against a light beam narrowed by a slit and some kind of detector monitors the transmitted or reflected light, in the TELECHROM video-densitometer /Chinoin, Budapest, Hungary/ scanning is performed electronically so that the specimen placed into the illumination chamber of the instrument is scanned by an electronic system. The detector of the system is a Vidicon-tube governed by the electronics: on the one hand, it determines the area to be scanned and, on the other hand, it performs three-dimensional integration in which the x and y axes correspond to the geometry of the spot, whereas axis z corresponds to density. The size of specimen that can be placed into the instrument may vary between 200 x 200 mm and 24 x 36 mm. The Zoom-optics of the instrument renders it possible to magnify small spots to the required size.

On thin layers of size used in everyday practice usually several samples are resolved chromatographically or electrophoretically. The amount of the individual components of each sample can be measured by setting the electronic "window" /the so-called dark window/ of the instrument, which can be varied

at will both in size and shape, according to the size and shape of the sample to be analyzed. In this way we get the sum of densities of all coloured spots present in the sample /total density/. Then the so-called light windows of the instrument are positioned above each spot in turn, when we get the densities of the individual spots in percentage of the total density. The determination of the density of an individual spot takes in this way about 4 sec. Having set the windows, the instrument automatically repeates the programme. This feature is important above all in measuring large series /7/, since after the initial setting of the instrument any number of similar chromatoplates can be evaluated within a relatively short time period. As calculated from a large number of measurements, the error of video-densitometric determinations, as regards reproducibility, is less than 1 %. Density values may be directly read from the digital display of the instrument, but they may also be recorded by the aid of an on-line calculator and printer. Moreover, the programme given in the computer also enables one to get the percent values or appropriate concentration units directly. If a plotter is attached to the instrument the density profile can also be recorded.

The quantitative evaluation is only reliable, of course, if the colour, whether natural or developed by some reagent, of various substances present in the sample is identical and if the density values are proportional to the amount of material. These two conditions are met, with the exception of proline, for the amino acids, which rendered it possible, for example, to screan the amino acid composition of several thousand specimens of corn at the expense of reasonable time and labour /8, 9/. In fact, as compared with other techniques video-densitometry copes with such tasks not only much faster but also with greater accuracy /10/. The set of filters available with the instrument makes it possible to eliminate false densities due to spots of anomalous colour. Apart from the visible light sources, which can be operated either in transmission or reflectance mode /Fig. 1/, the instrument also has ultraviolet light sources. Thus one can determine substances that absorb in the not too far ultraviolet $/\lambda > 250$ nm/. Furthermore, the ultraviolet light sources are useful in the assay of fluorescing compounds. The electronic system can also be operated in the reverse mode when light areas appear dark and vice versa. Hence, in this mode fluorescing spots are evaluated as densities.

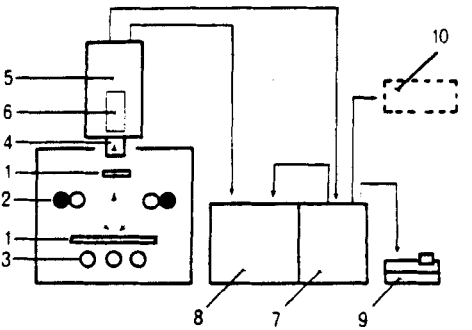

FIGURE 1 - Block diagram of the Telechrom OE 976 type video-densitometer. 1, sample holders for plates of 200 x 200 mm or 24 x 36 mm size; 2, visible and ultraviolet light sources for reflectance operation mode; 3, light source for transmission operation mode; 4, optical system; 5, television camera; 6, Vidicon tube; 7, electronic detector; 8, monitoring screen; 9, calculator and printer; 10, X-Y plotter.

According to our preliminary experiments the video-densitometer can be applied not only in chemical work, but may also be helpful in the quantitation of organelles in stained histological preparations, in the determination of the ratio of light and dark areas on X-ray pictures, and probably in the analysis of several other types of specimen amenable to densitometry.

Amino acid analysis by the CV-technique

Out of the twenty different kinds of amino acids that constitute proteins 16 are excellently separated in a single run on the cation-exchange chromatoplate

in the Na^+ form in sodium citrate buffer, pH 3.3 /11/. Under these conditions only serine and threonine are not resolved from each other /Fig. 2/. This phenomenon does not mean that the resolving power of cation exchanger is less in thin layer than in a column. Namely, the distance of run on the plates is only about 14 to 18 cm, whereas in the amino acid analyzers it is more than twice as long. Moreover, in the

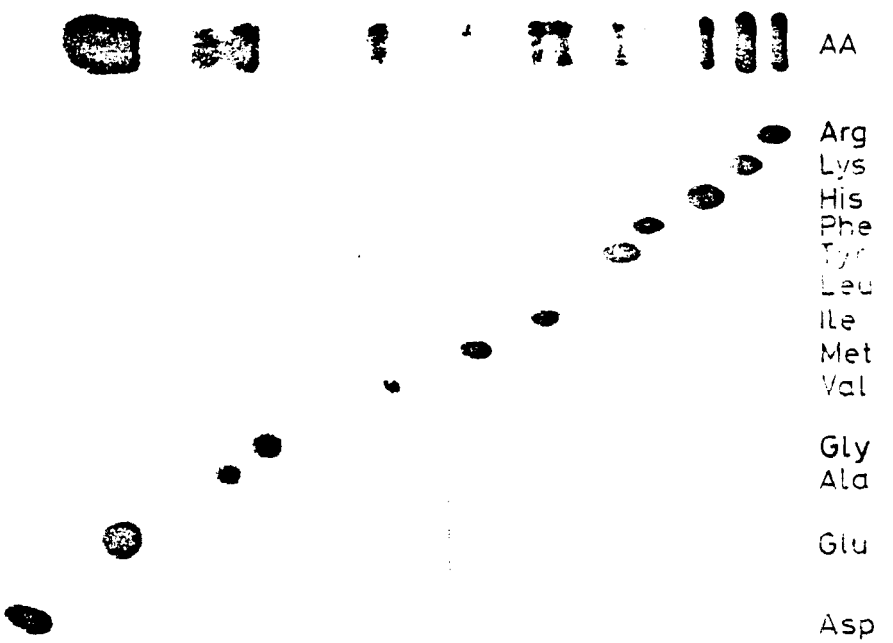

FIGURE 2 - One-dimensional separation of amino acids by thin layer ion exchange chromatography. Chromatography was carried out on ion exchange thin layer in the Na^+-form, in sodium citrate solution, pH 3.3, at 50°C. The plate was developed with cadmium-ninhydrin reagent. AA: mixture of amino acids.

analyzers there is one or more change of buffer, depending on the instrument, whereby the chromatographic properties of amino acids are compared in solutions of different pH and ionic strength values.

Based essentially on experiences obtained with automatic amino acid analyzers special running solutions can be developed which, by adopting the appropriate pH, ionic strength and quality permit the "aimed" resolution of certain groups of amino acids /12/. Thus, for example, acidic amino acids such as aspartate and glutamate, and their amides, are excellently resolved in a system of 200 mM lithium citrate-formate, pH 2.8, on a plate in the Li^+ form /5, 13/.

The thin layer ion exchange chromatoplate can be advantageously and economically used first of all in the analysis of large series, where the amount or a change in the amount of certain amino acid/s/ is to be determined. A typical example for this is provided by the chemical determination of the biological value of corn species. The biological value is defined in terms of the lysine, methionine and tryptophan content out of the essential amino acids. The programmes developed for tryptophan /14, 15/, methionine /15, 16/ and lysine /17/ made it possible to evaluate several thousands of corn specimens

within a very short time and with accuracy comparable to that of other methods. This is witnessed by a serial analysis of 37 000 specimens carried out in 21 laboratories and initiated by FAO in 1978-1979 /8, 9/, in the frame of which the results of eight different techniques have been compared. It emerged from this study that the CV-technique is capable of assaying 2 to 50 nmoles of amino acid with an accuracy of $\pm$ 6 % in specimens of various origin.

In microbiological materials or in studying the metabolism of higher organisms, but also in other cases, it is often necessary to detect the so-called minor amino acids, in addition to the twenty amino acids occurring in proteins. For the separation of non-protein basic amino acids a convenient method has been developed by Tyihák et al. /18/.

In the sequence determination of proteins the amount of material available for analysis may be a limiting factor. By virtue of its small specimen requirement the thin layer ion exchanger proved helpful in the analysis of both the C-terminal /19/ and the N-terminal /20/, as well as in the solution of other tasks /21/.

Application of CV-technique in clinical laboratories

The experience accumulated so far suggests that the CV-technique can be successfully applied in clinical laboratories for broadening the scope of diagnosis. In respect of possible applications, two groups have to be considered. In part of the cases the material to be analyzed /blood, serum, urine, spinal fluid, etc./ is abundantly available, but a great number of samples are to be analyzed, for example, in the examination of a given population, family, etc. Among these the most common example is the screening for phenylketonuria disease of newborns /22, 23/. We have also undertaken the screening of two Homes of Disabled Children accomodating 256 and 280 patients, respectively /unpublished result/. In each of the two cases, the work including the withdrawal of specimens, analysis of serum and urine samples, and the performance of retardation tests required three days. In contrast to the conventional, microbiological phenylketonuria test we could reveal, in addition to the phenylketonuria disease, other amino acid disorders, too: namely, two prolinaemia, one tyrosinaemia and one non-ketotic glycinaemia cases. By the family analysis of the propositi, by loading with amino acid the members of the often quite large families, we were

able to establish within four hours after blood withdrawal the heterozygote carriers of the metabolic disorder /24/. It is worth mentioning that the heterozygote carriers, who are actually not ill, excreted a load /100 or 200 mg per kg body weight/ of amino acid much faster than did the patients, but considerably slower than did the control normal individuals.

Another possible clinical application may attract perhaps even greater attention. There are cases in which the amount of sample to be analyzed is very small, for example, the amount of tissues obtained by biopsy. For chemical or enzymological assays in such instances only part of the specimen /about 10 mg/ is available, because the larger part is taken for histology. The case is analogous, however, if the tissue or organ to be analyzed is very small. The minute quantities required by video-densitometry allowed us to examine the amino acid content of amnionic fluid samples from early pregnancy /25/. The study of amino acid content of amnionic fluid provides valuable data already in early pregnancy about the feto-maternal transport processes and for the prenatal diagnosis of amino acidopathies /26/ including the indication for interruption.

Recently we have elaborated procedures by the aid of which the activity of enzymes catalyzing the synthesis or degradation of amino acids can be measured with reasonable accuracy. Homogenates or extracts were made from the available tissue specimens /a few mg/ and the enzymes were assayed in reaction mixtures of 50 to 100 µl final volume, by taking aliquots /5 to 20 µl/ at the appropriate time intervals and putting them on ion-exchange chromatoplates. After the chromatographic run the change in the amount of amino acid tested was determined video-densitometrically. In the above way changes as low as 1-2 nmoles in the amount of amino acids can already be reliably detected. For these enzymes none of the other methods currently in use is able to quantitate enzyme activities from such minute biological specimens and with comparable sensitivity. The technique has so far been applied to the study of arginase /27/ and other enzymes of the urea-cycle /28/, the enzymes of polyamine and pyrimidine synthesis /29/, as well as amino acid dehydrogenases, transferases and ammonia lyase /30/.

The ion exchange thin layer can be used, in addition to the study of enzymes acting on amino acids, for the investigation of other enzymes, too. For example, Tomasz and Farkas /31/ employed it

successfully for assaying adenylate cyclase. In this procedure it has been exploited that the reaction products can be rapidly and effectively separated from all other substances in the system by ion exchange, without the need to perform the complicated and time-consuming manipulations inherent in other methods. Likewise, the activity of the enzyme catalyzing ATP-pyrophosphate exchange can readily be monitored by the technique /32/.

Analysis of nucleosides and their derivatives

The methodology of thin layer ion exchange separation of the building blocks of nucleic acids has been developed by Tomasz. In contrast to the analysis of amino acids, here the chromatoplate in H^+-form /or possibly NH_4^+-form/ is more convenient. Such plates can be readily prepared by equilibrating the commercially available plates /in Na^+-form/ in 1 N hydrochloric acid, then the excess of hydrochloric acid is washed out with distilled water and the plate is dried /33/. The chromatographic runs can be made simply in deionized water or in a hydrochloric acid solution of suitable concentration.

For the identification of purine and pyrimidine bases after chromatography, as it is well known,

there is no need for staining since they can be detected under an ultraviolet lamp /of short wavelength, with maximum at 254 nm/ on the basis of light absorption. By the aid of ion exchange chromatography the composition of fairly complex mixtures can be determined after chromatography for one to one and a half hour. Thus, for example, 5'-ribonucleotides can be excellently resolved by chromatography in deionized water /34/, whereas the bases, ribonucleosides and their monophosphates are satisfactorily separated in 1.0 N hydrochloric acid. The order of decreasing migration rate of bases and their derivatives is as follows: adenine, cytosine, guanine and uracil. The analysis of a mixture of nucleosides can be performed in 0.1 M ammonium carbonate or 0.4 M ammonium acetate solution /35/, while that of diribonucleotide monophosphates is conveniently made in running solutions containing ammonium ions in various concentrations /36/. The separation of modified /methylated, hydroxylated/ DNA bases can be achieved in relatively concentrated /2.8 N/ hydrochloric acid solution /37/, whereas by two-dimensional chromatography /1.0 N hydrochloric acid in one dimension and ammonium phosphate in the other/ 19 base-derivatives may be well resolved /38/. For the separation of

methylated ribonucleoside derivatives 0.4 M sodium acetate, pH 6.5, proved suitable /39/. Cyclic nucleotides can be separated in 50 mM oxalic acid /40/, which renders it possible to assay the enzyme cyclase catalyzing their formation /31/. The scope of application and limitations of the method have recently been reviewed by Tomasz /40/.

Analysis of miscellaneous substances on thin layer ion exchange chromatoplates

It has been reported from several laboratories recently that the thin layer ion exchange technique can be successfully applied in drug analysis. Thus Kádár-Pauncz /41/ analyzed twenty different antibiotic derivatives containing deoxystreptamine. To achieve good resolution relatively high ionic strength /1.5 M sodium acetate and 1.0 M sodium chloride/ and 10 % tert-butanol had to be used and chromatography was run at 40°C. Similarly, high ionic strength /0.5 M Na_2HPO_4, pH 6.5, and 1.5 M NaCl/, 5 % tert-butanol and 50°C running temperature were needed for the resolution of nebramycin components /42/./A detailed survey by Kádár-Pauncz and Bárdy of the chromatographic analysis of antibiotics can be found in this volume, p.

Pongor et al. /43/ developed a procedure for the determination of D-penicillamine required in

pediatrics, according to which the amount of drug can be reliably measured even from a drop of blood dried on filter paper. Dávid and Takácsy /44/ detected the contaminating substances in cephalexin by the aid of thin layer ion exchanger. Kádár-Pauncz /45/ elaborated the chromatographic analysis of 20 sulfonamide derivatives. For checking the purity of preparations, for the identification of the individual derivatives the running solution contained 20 % ethanol in 0.5 M sodium phosphate and 1.5 M sodium chloride, and by varying the pH and ethanol content the migration of components could be favourably influenced.

Aminosugars, similarly to amino acids, can be readily separated from one another and from amino acids occasionally present. Hrabák /46/ recommends the use of 0.25 M sodium citrate, pH 5.3, for their chromatographic analysis. Under such conditions both glucosamine and galactosamine are well resolved from basic and neutral amino acids.

On ion exchange chromatoplates equilibrated with 0.1 % acetic acid the organic dicarboxylic acids /malate, citrate and fumarate/ can be separated from one another and from amino-dicarboxylic acids /aspartate, glutamate, asparagine/. Himoe and Rinne /13/ chromatographed the sample first in 1 % acetic

acid for 75 min, then, after drying the chromatoplate, in 4.4 % formic acid for 28 min. By this time the front of second solvent ran about half way on the chromatoplate and separated those components that had not been resolved in the first run. According to Völkl and Berlet /47/ guanidino derivatives are well separated from one another in 0.12 M sodium citrate, pH 5.2.

In some laboratories attempts have been made to apply the thin layer ion exchange technique to the analysis of inorganic substances. Arbusti and Lederer /48/ studied the properties of rhodium/III/ compounds under a variety of conditions. Investigations of the ion exchange behaviour of other metal ions have been reported by Cardaci et al. /49/, whereas phosphates were studied by Kroschwitz et al. /50/.

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