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Journal of Chromatography A 819 (1998) 35–44

JOURNAL OF
CHROMATOGRAPHY A

On-line coupling of liquid chromatography with thin-layer chromatography

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Abstract

On-line coupling of high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC) represents a very promising two-dimensional technique because it allows the combination of two completely different separation principles. This is especially so for the combination of *reversed-phase* HPLC for the first separation and HPTLC with automated multiple development on a *normal-phase* thin-layer plate for the second separation. The system consists of a microbore HPLC syringe pump and a modified TLC autosampler (CAMAG ATS3-C) which is controlled by new software for a personal computer running under MS Windows. The new dedicated software enables the analyst to spray the effluent from the HPLC column in fractions onto the plate in a freely definable manner. In order to address different analytical problems, six transfer modes are made available. A complete profiling analysis can be carried out with the whole effluent from the liquid chromatographic column being transferred in fractions to the TLC plates. On the other hand, the program supports typical target compound analyses with multiple switching activated according to individual elution times. Typical applications are pesticide residue analysis in foodstuffs or environmental samples. The new dedicated software is expected to fill the gap considered to be the main obstacle for the widespread application of on-line coupling of HPLC with TLC.
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Keywords: High-performance liquid chromatography–thin-layer chromatography coupling; Interfaces, HPLC–TLC; Computer-assisted chromatography; Pesticides; Ipodione

1. Introduction

Determining individual analytes in complex biological or environmental samples requires some method of separation as a prerequisite to the analytical measurement. The necessary resolution depends on the selectivity of the final detection method. The highest resolution in separation is achieved by combining two high-performance chromatography systems. According to Giddings [1], the maximum peak capacity of a two-dimensional chromatography system is the product of the capacities of the individual chromatographic dimensions. The prerequisite in achieving that maximum is to combine two systems of greatest difference in selectivity.

Multidimensional separation systems may be used in either the profiling mode or the target mode. With most two-dimensional chromatographic systems, the critical operation is the selection and transfer of effluent fractions from the first to the second dimension. The key feature of a multidimensional system applicable for profiling is that it should be based upon the transfer of all relevant analytes composing the initially injected sample to the second separation mode.

On-line coupling of high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC) represents a very promising two-dimensional

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PII: S0021-9673(98)00446-4

technique. It allows the combination of two completely different separation principles as postulated to achieve highest possible peak capacity. There is no problem in fulfilling the requirements of a multidimensional system as stated by Giddings and cited above, namely to transfer all analytes composing the sample from one dimension to the other. This is because the second analyses of all fractions can run in parallel at any convenient time totally independent from the analysis in the first dimension.

The effective coupling of liquid chromatography to TLC was described by Hofstraat et al. [2]. They applied narrow-bore columns for normal phase and reversed phase separation and deposited the effluent from the column on the TLC plate without serious loss of chromatographic resolution. The interface was a fused silica capillary which connected the column outlet to the spray jet assembly of a Linomat applicator for TLC plates. They made the point that the stored chromatogram can serve as starting point for a new separation and that after the development of the TLC plates new detection principles can be used which are normally not compatible with HPLC. The new technique was applied to the analysis of polyaromatic hydrocarbons which were detected with fluorescence emission and individual compounds characterised by their fluorescence excitation emission spectra [3]. The construction of the interface between the narrow-bore HPLC and the TLC was later optimized based on the Linomat III applicator which, however, allows only the spraying of a line as the starting band. This lead to distortions of the deposited bands by the limited solvent evaporation from the column effluent [4].

The on-line coupling of reversed-phase HPLC and HPTLC with automated multiple development (AMD) was introduced by Burger as a new technique of multidimensional chromatography [5,6]. It combines two different separation principles in an easy manner. The first separation takes place on a reversed-phase column and the second on a normal-phase thin-layer plate. The method fulfils all requirements for profiling complex biological and environmental samples and also for target and multitarget compound analysis. The system consists of a microbore HPLC syringe pump and a modified TLC autosampler (CAMAG ATS3-C) which is used to apply the HPLC effluent on the TLC plates. The HPLC and the TLC autosampler are coupled via a capillary tubing. The earlier difficulties with distorted starting bands were overcome by the use of AMD-TLC in the second dimension which exhibits autofocusing into narrow bands during the separation procedure. The merits of the new technique were also demonstrated by Jaenchen and Issaq with the separation of a mixture of 56 pesticides into 17 fractions on a narrow-bore reversed-phase column followed by the separation on a silica gel TLC plate with AMD [7]. A review on multidimensionality in planar chromatography has recently been presented by Poole and Poole [8].

Although the technique shows great potential, the research in coupled chromatographic systems with TLC is very small at present. It is likely that a lack of access to the necessary equipment together with a misunderstanding of the separation capacity of AMD-TLC is the reason for this disappointing situation. One other important point is certainly the lack of access to the software necessary to control the transfer of the effluent from the column separation in fractions to the TLC plate in a way that the transfer can be freely defined by the analyst.

In this paper, new dedicated software for a personal computer running under MS Windows is described which enables the analyst to spray the effluent from the HPLC column in fractions onto the plate in a freely definable manner applying commercially available equipment for interfacing.

2. Experimental

2.1. Reversed-phase high-performance liquid chromatography

RP-HPLC analyses were performed with a microbore pump (Dual Syringe Solvent Delivery System, 140 B, Applied Biosystems) equipped with two syringe pumps of 10 ml volume that can be programmed for gradient elution. The system allows reproducibly low flow rates of 10–50 μl/min. Separations were done by gradient elution starting with methanol–water (50:50) and ending with methanol at a flow-rate of 40 or 50 μl/min on a 250 mm×2.1 mm I.D. column packed with 5 μm RP-C_{18} material (Spherisorb-5, Applied Biosystems).

Injections were done either manually with an injection valve with a sample loop of 100 μl or using an HP 1050 autosampler (Hewlett-Packard) with 100 μl injection volume. A UV detector was used for on-line monitoring of the column effluent. The analog signal was converted into digital numbers in the personal computer applied for controlling the interface by means of an A/D converter card. These values were used for peak recognition and presentation of the chromatogram on the computer screen.

2.2. On-line coupling of HPLC and TLC

The setup is depicted schematically in Fig. 1. It consists of a modified ATS3-C autosampler applicator for TLC plates (CAMAG, Muttenz, Switzerland) containing a special sprayhead which can be moved on a sliding bridge. The system allows location of the sprayhead at any position over the three TLC plates (100 mm×200 mm) on the autosampler table. The sprayhead can be moved horizontally on one line within a defined bandwidth but also can be made to deposit the spray over a defined rectangular area, usually 8 mm×6 mm. The effluent can be directed either to a defined spot (starting band) or to a waste bottle. A spray jet is produced by nitrogen gas flow around the tip of the capillary. This assembly ensures accurate application of the effluent onto the TLC plate. The evaporation of the solvents of the effluent is promoted by the enlarged area of the starting band.

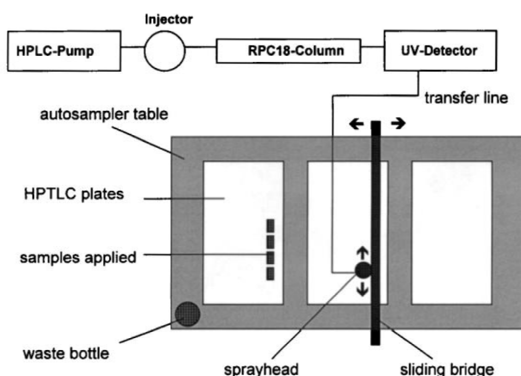

Fig. 1. Schematic presentation of on-line coupling.

2.3. Automated multiple development thin-layer chromatography

An AMD system is applied together with a TLC scanner II with an IBM-compatible computer and the evaluation software Cats 3.04 including the multiwavelength option all from CAMAG. HPTLC development is carried out with various multistep gradients, for instance those previously described for pesticide residue analysis [9,10].

HPTLC silica gel plates (10×20 cm with 0.2 mm sorbent thickness) from Merck (No. 5641) were developed with dichoromethane–methanol (1:1) before analytical use.

2.4. Personal computer controlling the ATS3-C autosampler for on-line transfer of HPLC effluent fractions onto the TLC plate

A IBM-compatible computer from 486DX upwards with 4 MB RAM, 1 MB free memory on hard disk, VGA color monitor, mouse, gameport, two serial interfaces (RS232), A/D converter card running under Windows 3.1 or higher was used for the development of the software to control the on-line coupling by means of the ATS3-C autosampler. Programming was carried out with the computer language Delphi.

2.5. Chemicals

Acetone, acetonitrile, ethyl acetate, dichloromethane, toluene, *n*-hexane and cyclohexane were purchased from Riedel-de Haen (Seelze, Germany) in Pestanal quality, acetic acid, ammonia(25%) in analytical-reagent quality from Merck (Darmstadt, Germany). Iprodione and the carbamate insecticides were from Dr. Ehrenstorfer (Augsburg, Germany), water and methanol as solvents for RP-HPLC were Baker analyzed HPLC reagents.

2.6. Clean-up

An aliquot of 20 g of a food sample was extracted with acetone and cleaned up according to the DFG multimethod S 19 [12]. Fractions 3 and 4 of the silica minicolumn fractionation were combined and evaporated to dryness and dissolved in 200 μl

methanol. 20 μl of this extract was injected onto the HPLC column.

2.7. Detection of carbamate insecticides by cholinesterase inhibition

Carbamate insecticides were detected by dipping the plate into a solution of choline esterase in Tris-HCl buffer pH 7.8, kept for 30 min at 37°C in a humid incubator and finally dipped into a mixture of 1-naphthyl acetate and Fast Blue B salt solutions according to Weins [13]. Carbamate insecticides appear as white spots on a pink background and can be easily seen and scanned with the TLC scanner at 533 nm for evaluation.

3. Software ATS3-C

The TLC autosampler ATS3-C is controlled by means of a personal computer using a new dedicated software program to be described. It enables the analyst to spray the effluent from the HPLC column in fractions onto the plate in a freely definable manner.

The method is suitable for characterizing complex samples as well as for the determination of target compounds like pesticide residues or environmental pollutants in complex matrices such as foodstuff or water samples.

There are usually three thin-layer plates on the table of the modified autosampler interface.

The analyst decides how many fractions from the column effluent are to be transferred and to which positions on the plates. A few bands on each plate are generally used for test mixtures.

The software runs under Windows. After starting, a typical Windows display appears with the name of the data file in the top line of the frame and the main menu in the second line containing the pull-down menus *File, Method, Transfer, Configuration* and *Help*. Immediately under this line is the toolbar with icons which can be activated by mouse click. Such a screen is shown in Fig. 2. The description on this screen is in German but it should not be an obstacle for understanding what the program is about. The icons have the following meaning from left to right: new method, opening of a method already created, save method, print method. The next two icons mean definition of instrument parameters and documentation while the following box contains push buttons for starting the analysis of the HPLC separation in the first dimension and the stop symbol stands for stopping the analysis. If the left peak icon is activated, the transfer of an effluent fraction is started and the icon appears activated as long as the transfer onto the plate takes place. The transfer is finished by clicking the same left peak symbol again. There are two options, namely to proceed with transferring the following effluent to the next position on the plate by pushing the right peak symbol or to stop effluent transfer just by ending the transfer with the left peak symbol as described. In this case the sprayhead moves to the waste bottle and must be activated again with the left peak symbol. The right hand box in this line reports the runtime of an analysis in progress.

The three plates laying on the autosampler table show the positions where the fractions from the column separation are transferred to, usually a few bands are reserved for running standards with the TLC separation. The progress of the analysis can be followed on the computer screen, the starting band where the effluent is deposited is flushing on the screen and at the end of the transfer the band appears with a different color.

Before the on-line coupling can be started, a method must be created with defining the instrument parameters. A new screen window can be opened either by activating the pull-down menu under *Method* or directly with mouse click to the icon. Instrument parameters include the number of plates and bands, the distance of the bands from the edge and between them as well as band width and band height. Additionally, the speed of the sprayhead and the delay of starting the transfer must be defined. Finally, starting time and the end of the analysis must be given, because usually only a part of the chromatogram is of interest.

In order to address the different analytical problems, six transfer modes are made available.

3.1. Automatic transfer

In this mode the whole effluent from the liquid chromatographic column is transferred in equal

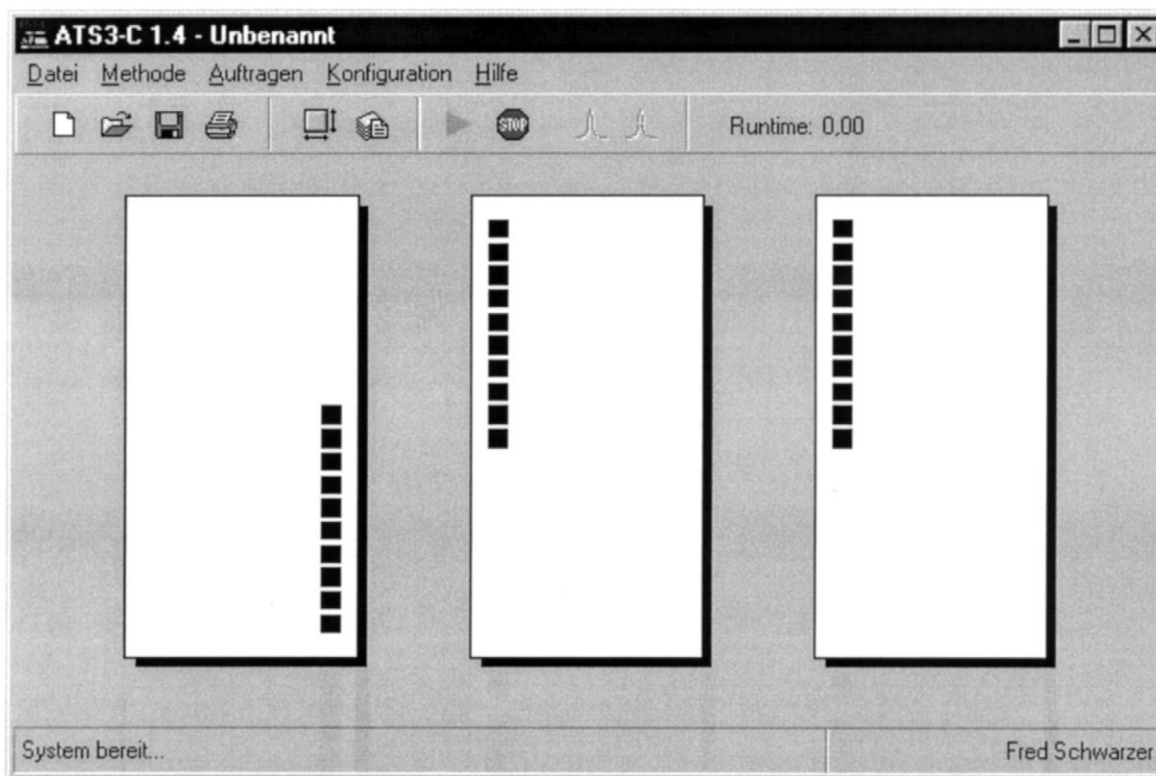

Fig. 2. Display on the personal computer monitor after starting the application. Starting bands of fractions from the on-line coupling are on three TLC plates indicated, they change colour when the sample transfer has taken place. Free lanes are reserved for reference compounds.

fractions to the TLC plates. The analyst tells the software to how many plates and starting bands the effluent is to be deposited and the program calculates the time switching table and then runs the analysis automatically. This is a typical profiling analysis such as from a complex waste water sample which may be cut into 100 or even 150 fractions on 10 plates. In this particular case, TLC plates already used for effluent transfer have to be substituted with new ones.

3.2. Manual transfer

The analyst follows the chromatogram of the column separation on the monitor and activates the transfer of a peak (fraction) to the plate in real time by means of the peak symbols in the tool bar as already described. The other effluent goes to waste.

3.3. Target compound transfer

The analyst knows the elution characteristics of the target compounds and cuts the fractions to the plate by creating a form for activating multiple switching in time. This program supports typical target compound analyses such as those for pesticide residues in foodstuff or environmental samples.

3.4. Autosampler mode with target compound transfer

Many samples are run on HPLC with autosampling and selected fractions from each sample are cut to the plates according to individual elution times. This program supports typical target compound analyses with routine samples.

3.5. Peak transfer after automatic peak recognition

Peaks which are detected in the column effluent with any detector are automatically cut to the plate. A simple peak recognition algorithm is implemented that processes the chromatographic signals from the detector after analog to digital conversion. On appearance of a peak, the transfer is started and ended after its disappearance. By using the typical delay of 20–30 s which an analyte experiences between leaving the HPLC column and arriving at the spray jet, the program has enough time to find out if a slope increase represents the beginning of a peak in the chromatogram above a set threshold or not. This program can be used for checking the purity of peaks in any HPLC separation.

3.6. Peak transfer after recognition of added chromatographic markers

When analysing samples which tend to overload the column or samples containing coextractives impairing the chromatographic separation, analysts may want to check for shifts in retention of analytes. Therefore, fractions which elute between added chromatographic marker compounds can be transferred to the plate in relation to the retention time of such internal standards. Selected dyes or a series of homologues that do not interfere with the detection of the target analytes may be suitable as markers.

A special feature of the software is the presentation of the chromatogram from any in-line HPLC detector. The detector must produce an analog signal for the A/D converter to create the chromatogram on the computer monitor. This comes together with a graphic indication of the fractions to be transferred to the TLC plates as shown in Fig. 3.

4. Results and discussion

The software has been developed to serve our own needs with a special focus on pesticide residue analysis in foodstuff and water samples. Multiresidue methods for thermostable volatile active ingredients are well established in this field and rely on gas chromatography with sensitive selective detectors. Positive results can be easily confirmed by coupling gas chromatography with mass spectrometry, a combination with unrivalled selectivity. The method shows its limitation when analytes are very polar and not stable to thermal burden. In these cases HPLC and TLC are the techniques of choice. The combination of mass spectrometry with liquid or thin-layer chromatography is, however, more difficult to bring into operation although considerable progress has been made in the last couple of years in coupling HPLC with mass spectrometry. The latter combination, however, needs very expensive equipment.

Another approach is to compensate for a lack in detection selectivity by increasing chromatographic resolution by multidimensionality.

As stated above, the merits of the new technique of combining RP-HPLC with AMD-TLC were demonstrated impressively by Jaenchen and Issaq with the separation and identification of a mixture of 56 pesticides [7]. The lack of access to software necessary to control the transfer of the effluent from the column separation in fractions to the TLC plate was obviously an important obstacle for broad application of this technique. The new dedicated software for a personal computer which allows the analyst to freely define the transfer procedure may bring a breakthrough.

It is clear that on-line coupling of HPLC with AMD-TLC is an analytical technique of general applicability. Here, two examples are given from the field of pesticide residue analysis.

Iprodione can be reliably identified in food samples using the German DFG multimethod S19, if the extracts are analyzed by capillary gas chromatography with electron capture and nitrogen-specific detectors in parallel and then easily confirmed by coupling to mass spectrometry. Quantitative determination, however, can be difficult because iprodione often disintegrates in the gas chromatographic system. Quantification of iprodione is possible with UV absorption spectroscopy following liquid chromatography or TLC, but often suffers from interference by matrix components. These can be totally separated by on-line coupling of RP-HPLC and AMD-TLC as demonstrated in Fig. 4. The upper trace on the TLC plate shows a lettuce extract after cleanup containing 70 ppb iprodione overlapped by matrix compounds while the middle trace shows the iprodione well

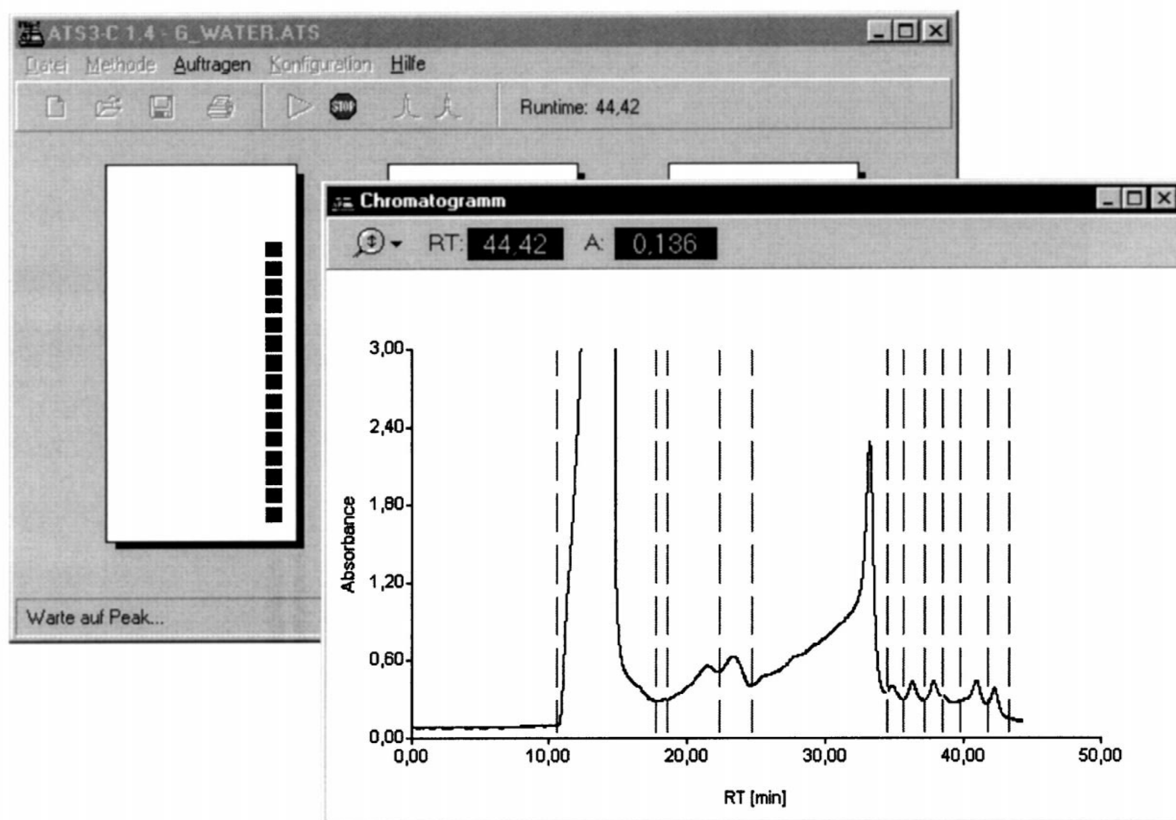

Fig. 3. Graphic representation of bands deposited in relation to the chromatogram from the coupled HPLC system.

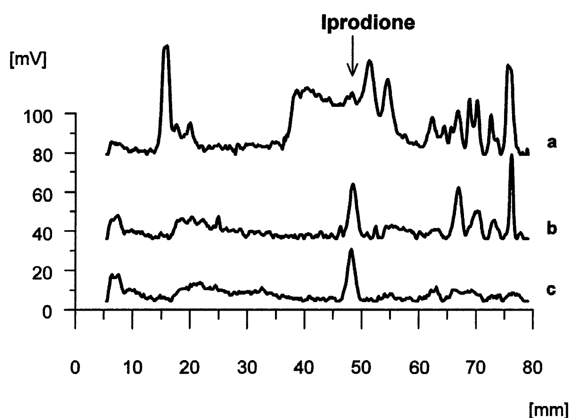

Fig. 4. Determination of iprodione residues in lettuce with on-line coupling RP-HPLC and AMD-TLC. AMD-TLC chromatograms: UV scan at 200 nm: a=extract from lettuce with 70 ppb iprodione; b=extract from lettuce with 70 ppb iprodione, after on-line coupling HPLC effluent from 40 to 45 min; c=standard solution of iprodione (70 ng). From [10], with permission.

separated from matrix compounds coeluted from the RP-HPLC C_{18} column in a 5 min fraction. Most of the coeluted matrix compounds exhibit longer migration distances in AMD-TLC development [10].

The second example is suitable for the demonstration of a unique feature of TLC. Since the many chromatographic separations in the second dimension are frozen, they can be investigated by various non-destructive spectroscopic methods and additionally with subsequent use of chemical or biological assays.

Carbamate insecticides are not amenable to gas chromatography due to their instability under thermal burden. Therefore, they are usually analyzed by means of HPLC followed by a chemical reaction in order to increase the detection sensitivity. The method needs expensive dedicated equipment which cannot be applied for other types of analysis [11].

AMD-TLC opens another approach by applying cholinesterase inhibition as detection principle. Interferences from matrix compounds are not usually observed. The separation of 23 carbamates on one TLC plate, however, can not be achieved without critical pairs resulting, as demonstrated in Fig. 5. The carbamates which form such critical pairs were therefore distributed between four different test mixtures in order to check their migration distances in parallel to real samples on the TLC plate. In the following example, onions were spiked with the six carbamates carbofuran (k), furathiocarb (p), fenoxycarb (m), bendiocarb (o), fenobucarb (s) and prosulfocarb (w), four of which cannot be resolved with the TLC method applied. The on-line coupling of RP-HPLC with AMD-TLC results in satisfactory resolution. The respective carbamates appear well separated in different fractions from the RP-HPLC column as can be drawn from Fig. 6. With the TLC scan at 533 nm in all lanes the solvent front produces ugly peaks which can, however, be easily distinguished from insecticide spots by visible inspection.

Summarizing, critical pairs appearing in the RP-HPLC separation are resolved with AMD-TLC and vice versa.

5. Conclusion

On-line coupling of HPLC and TLC represents a very promising two-dimensional technique because it allows the combination of two completely different separation principles. This is especially so for the combination of *reversed-phase* HPLC for the first separation and HPTLC with AMD on a *normal-phase* thin-layer plate for the second separation. This combination of two completely different separation principles guarantees highest possible peak capacity. Critical pairs appearing in the RP-HPLC separation are resolved with AMD-TLC and vice versa. There is no problem to transfer all analytes composing the sample from one dimension to the other. This is because the second analyses of all fractions on the TLC plate can run in parallel at any convenient time totally independent from the analysis on the HPLC column. The method fulfils all requirements for

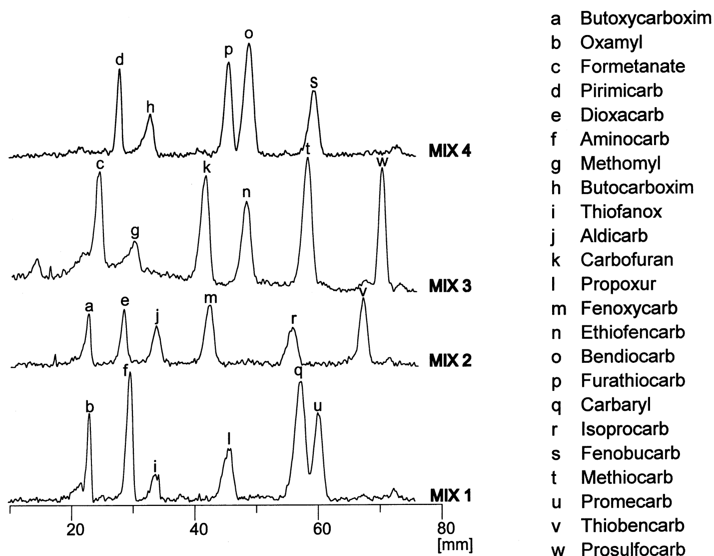

Fig. 5. AMD-TLC chromatograms of 23 carbamate insecticides in four test mixtures. Gradient of acetonitrile, dichloromethane, *n*-hexane, ammonia as described elsewhere [9]. Detection with cholinesterase inhibition [13]. Insecticides appear as white spots on pink background. TLC scan at 533 nm.

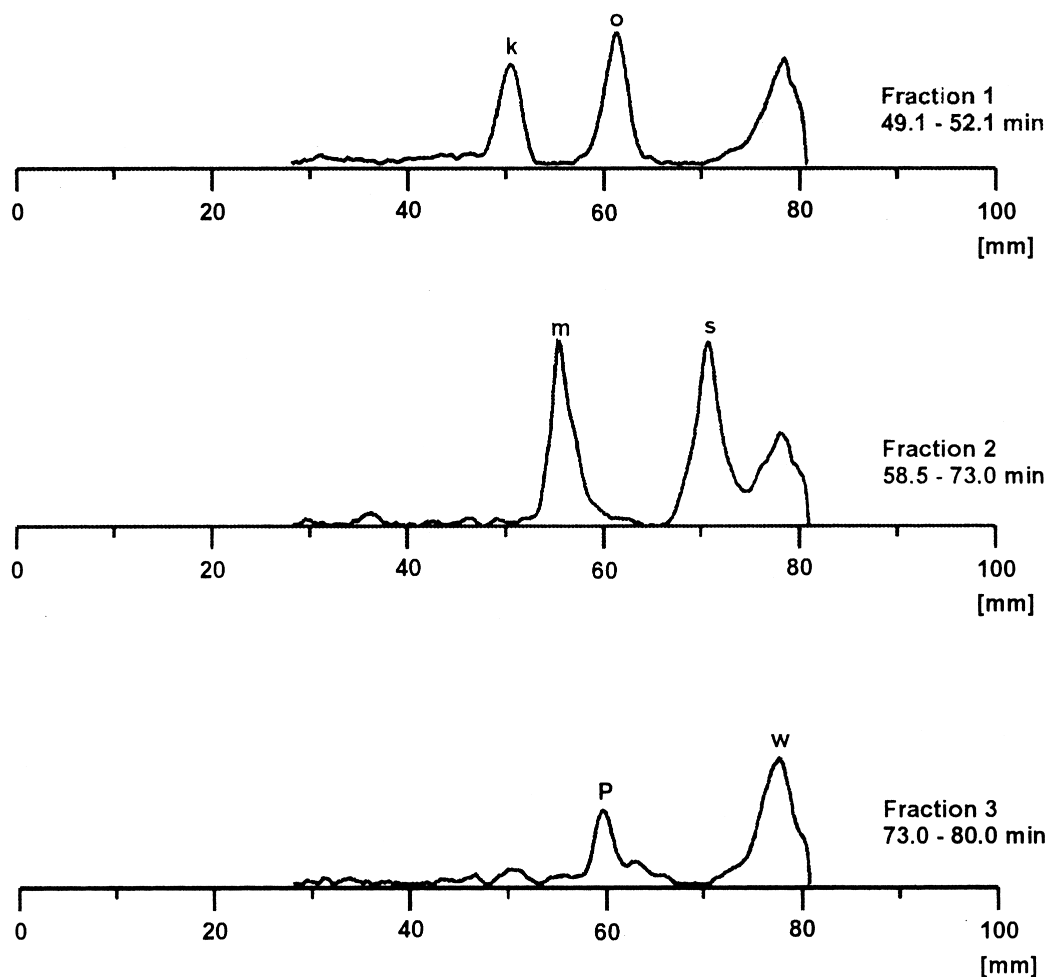

Fig. 6. Determination of six carbamate insecticides residues in onion with on-line coupling RP-HPLC and AMD-TLC. Detection of carbamate insecticides by cholinesterase inhibition. TLC scan at 533 nm; k=0.6 ppb carbofuran; o=1.5 ppb bendiocarb; m=15 ppb fenoxycarb; s=3 ppb fenobucarb; p=15 ppb furathiocarb; w=10 ppb prosulfocarb. Peaks representing the solvent front in all lanes are easy to distinguish visibly from insecticide spots.

profiling complex biological and environmental samples and also for target and multitarget compound analysis. Since the many chromatographic separations on the TLC plate are frozen, they can be investigated with various non-destructive spectroscopic methods and additionally with subsequent chemical or biological assays.

Until now the lack of access to the software necessary to control the transfer of the effluent from the column separation in fractions to the TLC plate in a way that the transfer can be freely defined by the analyst has been considered to be the main obstacle for widespread application of this valuable analytical tool. The new dedicated software for a personal computer running under MS Windows and interfaced to commercially available equipment enables the analyst to spray the effluent from the HPLC column in fractions onto the plate in a freely definable

manner. This significant step forward is expected to help promote the widespread use of this analytical technique of great potential.

Acknowledgements

The authors would like to thank Mr. B. Hatton for his help in preparing this manuscript.

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