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Quantitative two-dimensional thin-layer chromatography

MIRKO PROŠEK*, IRENA DRUSANY and ALENKA GOLC-WONDRA
Boris Kidric Institute of Chemistry, Hajdrihova 19, 61000 Ljubljana (Yugoslavia)

ABSTRACT

Two-dimensional thin-layer chromatography (TLC) is seldom used as a quantitative method in spite of its enormous potential, the main reasons being a single sample, a complicated solvent system optimizing procedure, and lack of adequative evaluation procedures. A special set of computer programs which enable scanning and evaluation TLC scanner is described. A whole TLC plate is scanned in linear mode with normal slit shape, for instance 0.2 × 8 mm, with as small as 0.5 to 1.0 mm steps between scans. Each run is processed as for a normal densitogram, the baseline is constructed, substracted, and the eventual peaks are located and integrated. In the next step a special program selects the correct positions of peaks, according to the biggest peak height, of all the scans. The selected scans are reintegrated and peak heights and areas are reported. Manual mode results were prepared in addition to the automatic mode. The operator selects the most convenient scan and integrates it using the cursors and chromatograms on the screen. The practical application of the scanning and evaluation programs is presented with quantitative determination of some phenolic components in propolis. Plates are developed with the solvent system: toluene–ethyl acetate–formic acid (75:15:3) and in the perpendicular direction with the solvent system: chloroform–methanol–formic acid (75:5:4). Identification and quantification of some characteristic flavonoids is performed with the 2-aminoethyldiphenylborinate/polyethylene glycol 4000 derivation reagent and external standards.

INTRODUCTION

Modern analytical chemistry strongly supports the development of procedures which yield the greatest output of information in the shortest possible time. One of these methods can also be an uncomplicated, multidimensional thin-layer chromatography (TLC) separation. Without the use of sophisticated instruments it is possible to separate complicated mixtures using different mobile phases in different directions on normal TLC plate. The real problem arises when the quantitative evaluation of two-dimensional chromatograms has to be performed [1–4]. TLC and two-dimensional TLC are commonly used in our laboratory, therefore we prepared a group of programs under the name "td/QTLC-pack".

This software pack consists of programs for scanning, automatic or manual integration, documentation and post-run calculation. The program is prepared for a computer-controlled scanner, CAMAG TLC Scanner II equipped with an AD converter and RS232 serial interface. The construction of the "td/QTLC-pack" is shown in Fig. 1. The densitometer is connected to a personal computer: IBM PC/XT or AT, with 640 kb RAM, serial interface, and CGA, EGA or Hercules graphics.

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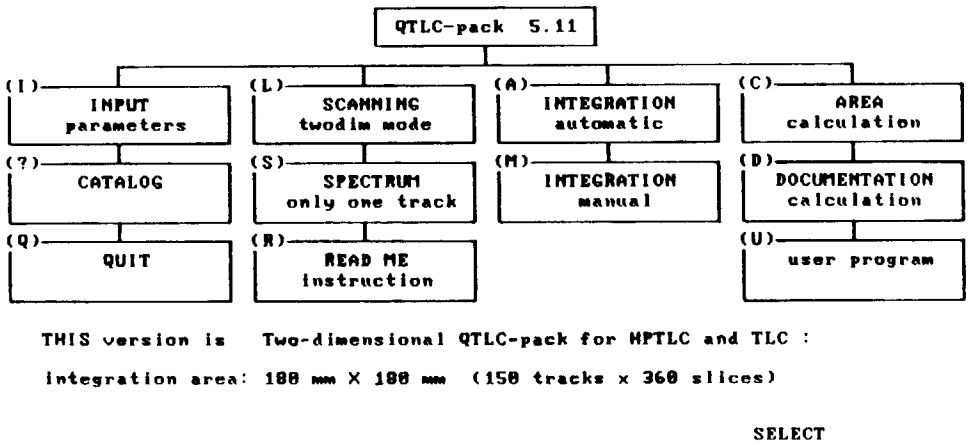

Fig. 1. Main programs in the td/QTLC-pack used in this work.

It is clear that only an image processing system which is able to collect at least 512 × 512 data points (pixels) from a plate is the correct answer to multidimensional quantitative TLC. As such systems are not commercially available, the only possible solution is data acquistion with a TLC densitometer.

Our routine work has revealed that scanning with a small square slit (0.5 × 0.5 mm), which is moved over the whole plate, is not the best solution. Scanning produces a large amount of noisy data which must be treated with a special smoothing program prior to being integrated. Since data acquisition is a long procedure (45 min), it is practically impossible to improve the signal-to-noise ratio with multiple scans. Thus small pixels can be used only with a video cameras.

We decided to try a different solution which yielded very good results in a relatively short time. The basic idea of our scanning is that a plate is scanned normally, as in linear TLC, with a slit the dimension of which is determined from the width of the spot, the width being slightly bigger than the measured spots [5,6]. Scans are repeated with small steps in the x direction, much smaller than the slit itself: for instance, the slit width is 5 mm and steps between scans are 0.5 mm (Fig. 2). In this mode the collected signal from the plate is already integrated in the x direction (slit length) during the scan in an analogous mode with improved signal-to-noise ratio. After data acquisition, the intergration program takes each lanc, finds the cardinal points of the possible peaks present, constructs the baseline, substracts it from the signal and integrates areas under the curve, as with normal one-dimensional TLC. The correct position of each peak on the two-dimensional plate is located according to the greatest peak height. The integration program records peaks heights of each lane and compares them with the corresponding values from other lanes. The position of the biggest peak height is taken as the correct peak position in the y direction, and a corresponding raw data set is used for quantitative evaluation of this peak. This quantitative two-dimensional scanning mode gives good signal-to-noise ratio because the first part of integration is performed during the scan and only the second part of data processing is done by the computer in post-run data processing.

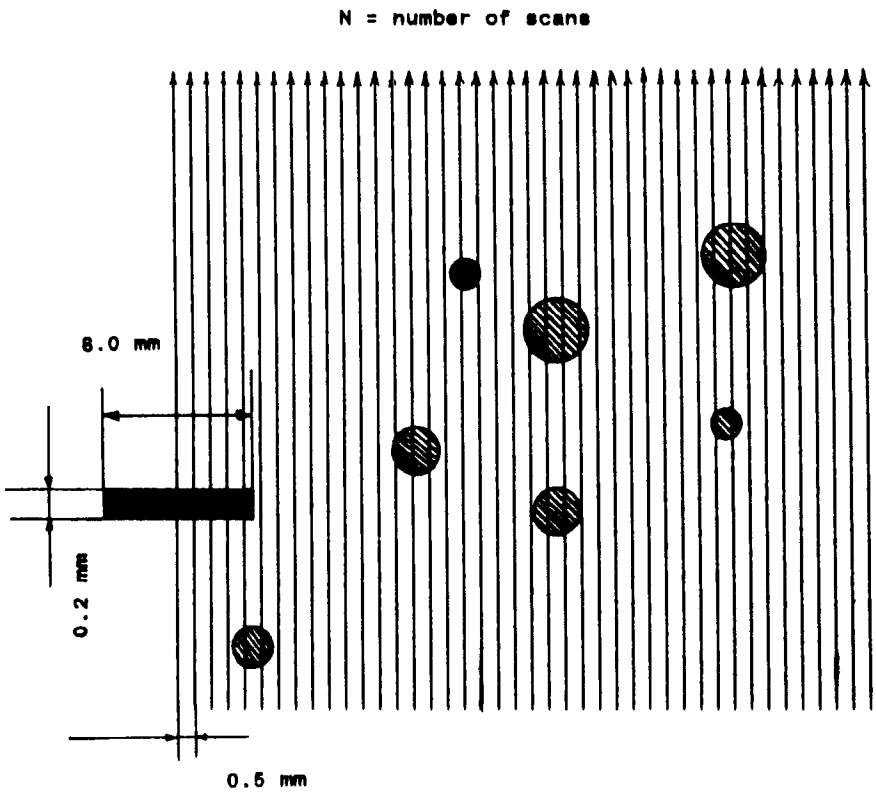

Fig. 2. The scanning principle of two-dimensional TLC, using a normal linear scanning mode.

td/QTLC-pack

Our interest in computer-controlled data acquisition and processing in TLC started nearly 20 years ago. Since then our own software system for computer controlled densitometry, the "QTLC-pack" has been developed. It was used on many different computers: HP9380, PDP 8, Apple II and IBM PC and also on different types of TLC separations: linear, circular, anticircular.

The td/QTLC software system was developed from this basic programing concept. It consists of four groups of programs. In the first group, there is an INPUT program. It is used for definition of all necessary parameters for scanning, integration, and calculation which are under the direct control of the operator. The programs for data acquisition are in the second group. The TDSCAN program collects raw data: 80–160 scans are taken from one plate, each scan being stored under the name RSCAN.X (x from 1 to 160). After collection, raw data are treated with the REDUCE program which reduces the number of measurements. In each scan the number of raw data points is reduced by a factor (N). The reduction results in less than 360 slices in one scan, regardless of the original number of data points. Each slice is the mean value of N original points. A new data set with 300 points is obtained from a chromatogram with scan length 90 mm and 900 measurements (0.1 mm steps).

Reduced data are stored on a disk under the name RSAMPLE.*Y*, where *Y* is a plate number.

A two-step scanning procedure has been selected in order to optimalize the use of a disk space (340 Kbyte disk on PC-XT). As separation on the TLC plate is not very large it is sufficient to have 360 points to succesfully handle all the separated peaks in a scan. Reduction helps in elimentation of the noise problem, where slices are used instead of points. The same integration algorithm can be used for data processing of all types of plates in TLC (150 × 150 mm) and high-performance (HPTLC) (70 × 70 mm), as there is always the same number of slices in a scan. In addition it helps to speed up the data processing. The PC, especially the XT version, is capable of performing all the calculations, but is slow when a lot of data are involved.

In the third group there are programs for automatic and manual intergration. The automatic integration program finds positions and relative intensity of peaks as a function of peak height. In a scan a baseline is constructed and subtracted before peak positions are set. After automatic mode determination, an operator can set additional, non-integrated peaks, or eliminates those already found.

The program for manual integration is prepared for situations where it is necessary to analyse only few spots among many substances on a plate. It is possible to set a baseline, peak height and area for each individual spot on the plate.

Post-run calculation is done in the fourth group. The TDAREA program reintegrates lanes with the selected spots. The TDDOC program documents the spots already integrated on a screen and printer. A short description of the practical operation of the td/QTLC-pack is given. With this we want to stress the importance of individual programming in the analytical laboratory. We hope that other analysts will start describing their approach to computer control of individual steps in chromatography. It is strange that most useful papers about digital data acquisition and integration are from a peroid when lab computers were rare. It seems that any interest in further implementation of computer power to analytical methods is nowadays the concern of producers of the instrument only – this is unfortunate as the developement of new, original computer-supported analytical procedures is possible only with the analysts who have computer knowledge and are capable of developing their own programs.

Our group is one of few groups still using their own programs for data processing in chromatography. These programs are not on the same professional level as some commercially available ones, but are very flexible and have original algorithms regarding laboratory manipulation. The software is started with the QTLC program. The computer prepares a shell for a pack and calls up the START program which connects all other programs (every other program is selected and started by it).

The INPUT program enters and corrects all the parameters used in QTLC-pack. The parameters are loaded from a disk with key (1); key (2) changes the PATH command, key (3) selects scanning parameters, key (4) displays integration parameters. Some of these parameters are:

Spot width: used for recognition of spots on a plate; too narrow peaks are rejected and too broad peaks are used as a baseline.

Threshold: determines a noise limit and mode of integration. Integration is performed by calculation of the first derivative, peaks positions are found and the baseline is constructed. Baseline construction can be done with two different modes:

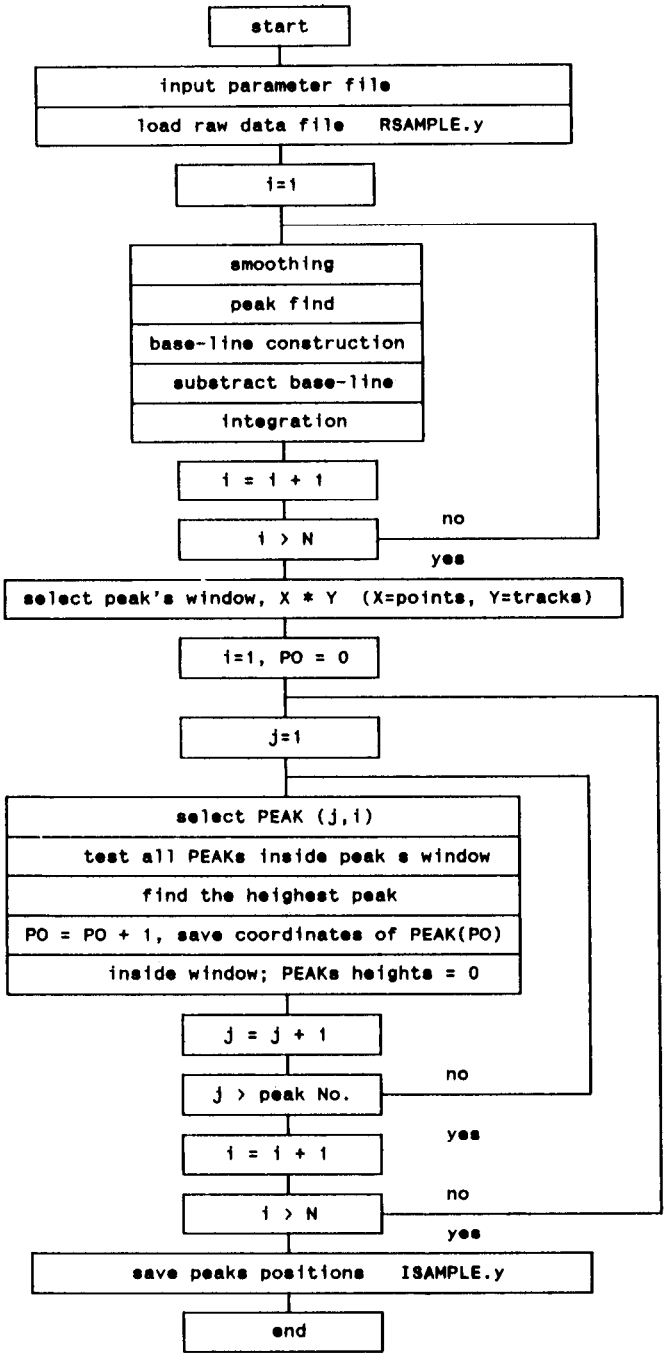

Fig. 3. The flow cart of the scanning program, TDSCAN.COM.

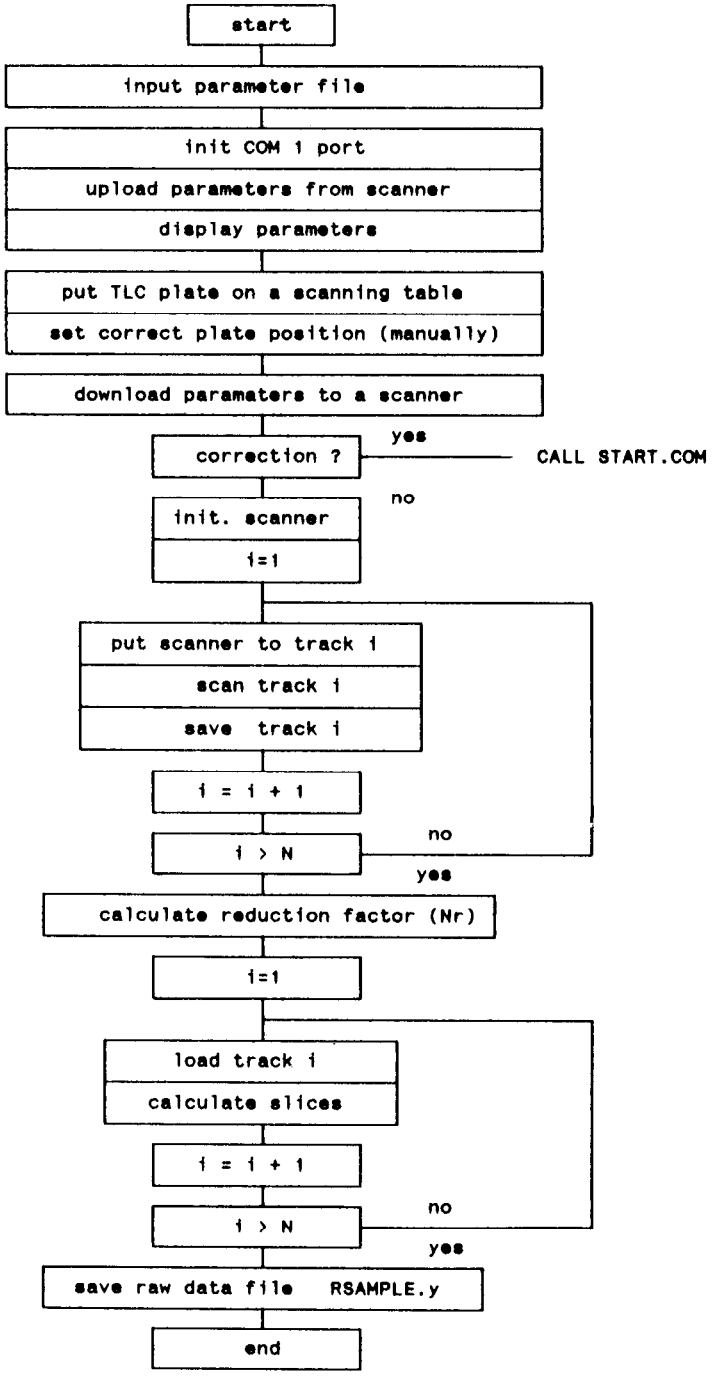

Fig. 4. The flow cart of the integration program, TDINT.COM.

the lowest slope is taken as baseline or a special INTAL algorithm [7] which tests the shapes and positions of peaks beside the slope in order to select the right baseline.

Plot each lane: during the intergration each chromatogram and peak positions can be displayed. This option is useful for examination of integration, but is time consuming.

Print report: can be generated.

Plot: can be generated.

Attenuation: determines the signal level which is used in plotting routine on a screen. If the signal is higher than attenuation a colored point is plotted on the CRT.

Integ. start/from lane: sets the point (mm) from which integration starts in the chromatogram and the lane from which the chromatograms are processed.

Integ end/to lane: sets the point (mm) where the integration is concluded, and selects which track is the last one to be processed.

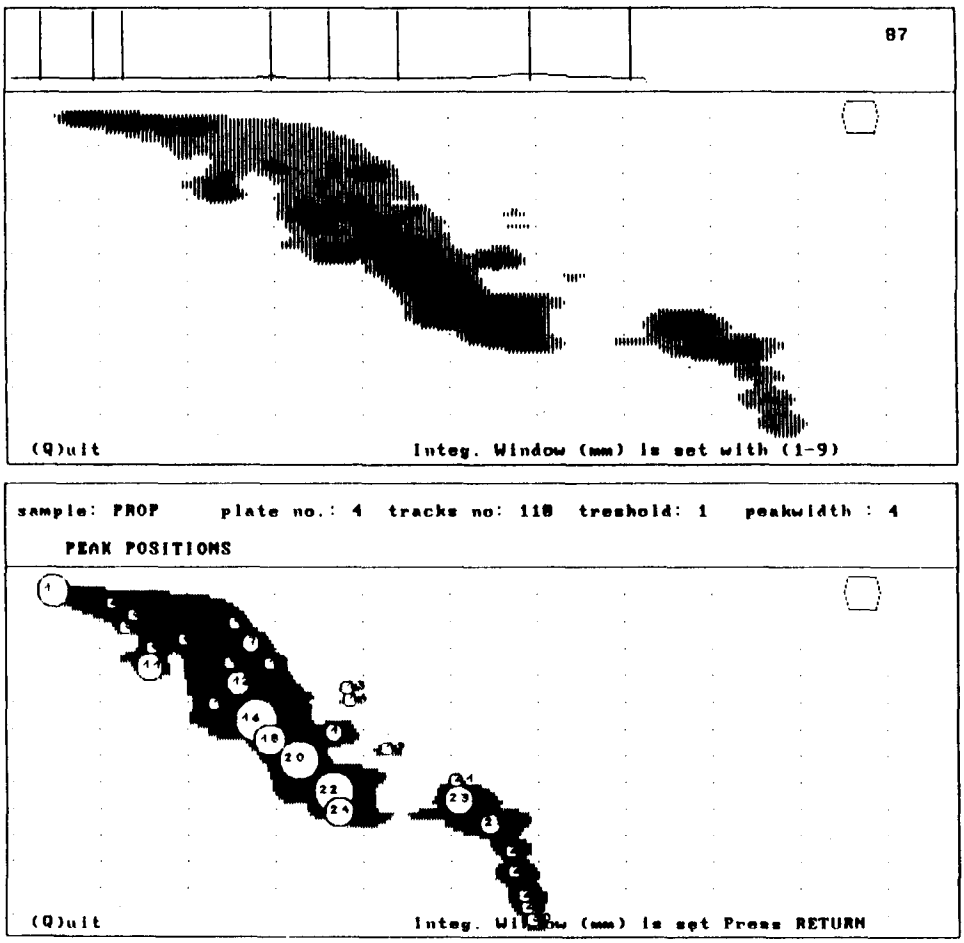

Fig. 5. Presentation of results after integration. The sample is anethanolic extraction of propolis.

Smoothing: during integration, the smooting algorithm can be used according to the selected value.

With key (5) the user program can be linked into the pack.

With key (6) data are stored. It is not possible to store empty files or leave this program without saving data.

The SCAN program collects data from a plate and stores them on a disk; the flow chart for this is shown in Fig. 3. Raw data are stored under RSCAN.X, where *X* represents a scan number from 1 to the selected the number of scans. This program controls a scanner through the RS232 communication protocol. Commands are English words in ASC format, like START:, RUN:, LOCAL:, INLINE:, etc. Some of these commands are publicly available and documented, some are used only by CAMAG. Scanning parameters are taken from the QLTC.PAR file, and are displayed. The operator loads a TLC plate on a scanning table and sets the plate in the correct starting position using commands on the scanner. The table coordinates are unloaded from a scanner to a PC and the scanning parameters are downloaded. After initializing, the densitometer returns all active parameters which are displayed on a screen.

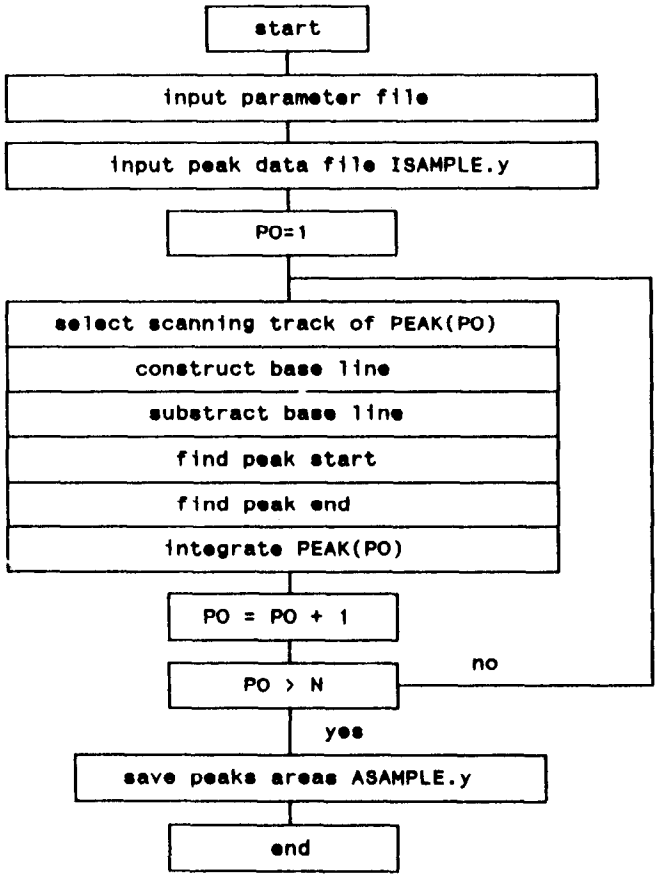

Fig. 6. The flow chart of the reintegration program, TDAREA.COM.

If an error is located, a message describes the type of the error on a screen. The error must be corrected and the initializing of the scanner repeated. After the correct set up a scanning procedure is started. Data are scanned and stored on a disk. After scanning, raw data points are collected into slices. The number of measurements is determined up to a total number of slices less than 360. All slices and plate parameters are stored in one data file.

The TDINT program finds the peak height of each spot on a two-dimensional chromatogram (Fig. 4). On CRT two windows are constructed. In the upper, smaller window, the chromatogram with the constructed baseline is plotted. In the bigger window, the two-dimensional chromatogram is shown. Signals in the x and y directions are shown in different colors, according to the level of the selected attenuation parameters. During the plot the computer calculates the maximum peak of each detected spot and shows it on a screen in the form of circles (Fig. 5). It is possible to add new spots or to delete some already detected peaks with a cursor which is shown on a screen. With left, right, up, and down arrows a cursor is moved across the plate on CRT. With the key (F1) a new spot is fixed, with key (F2) one spot is eliminated, and with (F10) the program is ended.

A special program named TDMAN can be used along with manual correction of the plate already integrated. This program shows a picture of TLC plate on a screen and with a cursor and selected keys it is possible to select one peak, set base line, peak start and peak end. Peak area and position of a peak are shown on the screen and stored on a disk for further processing.

Peaks detected with TDINT are reintegrated with the program TDAREA; the flow chart is shown in Fig. 6. Data are taken from the file ISAMPLE.y. From R_F (x) the successive number of chromatogram in which the maximal peak response is found, and from R_F (y) values the precise positions of the peaks in the selected chromatogram are obtained. The program calculates baseline and finds the start and end of the selected peak (Fig. 7). In this operation the peak width parameter is very important because integration cannot be performed outwith a range of three times

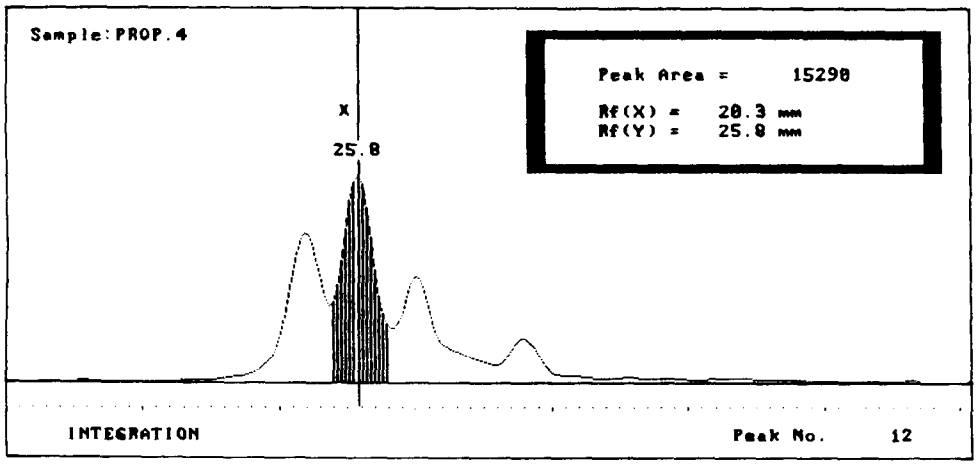

Fig. 7. Reintegration of lanes in which peaks positions were recognised with integration program.

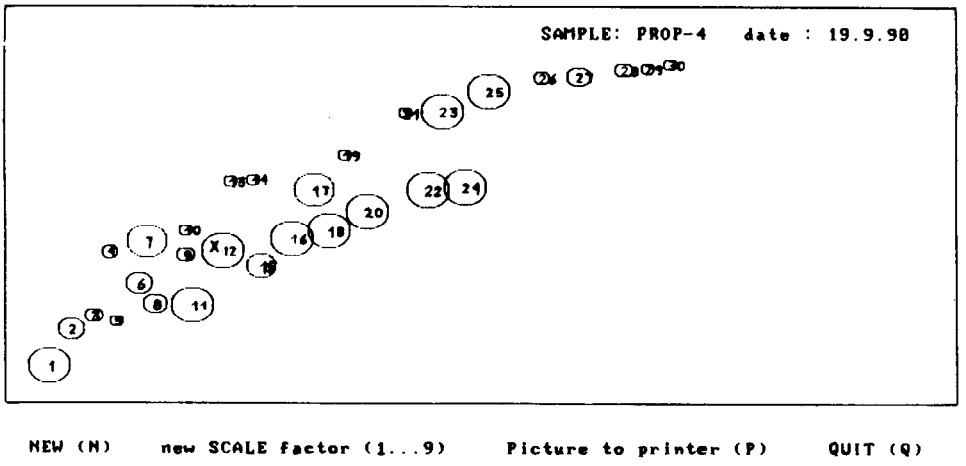

Fig. 8. Documentation of integrated peaks in a two-dimensional chromatogram.

larger than the peak width selected. Integration is documented and printed out. Results are stored in a file named ASAMPLE.y.

Integrated values are documented as two-dimensional chromatograms with the TDDOC program. Peak heights from ISAMPLE.y file or peak areas from ASAMPLE.y file are presented. According to the observed values of R_F (x) and R_F (y) circles are plotted. The diameters of circles correspond to the calculated peak heights or areas (Fig. 8).

TWO-DIMENSIONAL SEPARATION OF PROPOLIS

A good example of integration of td/QTLC-pack is quantitative evaluation of

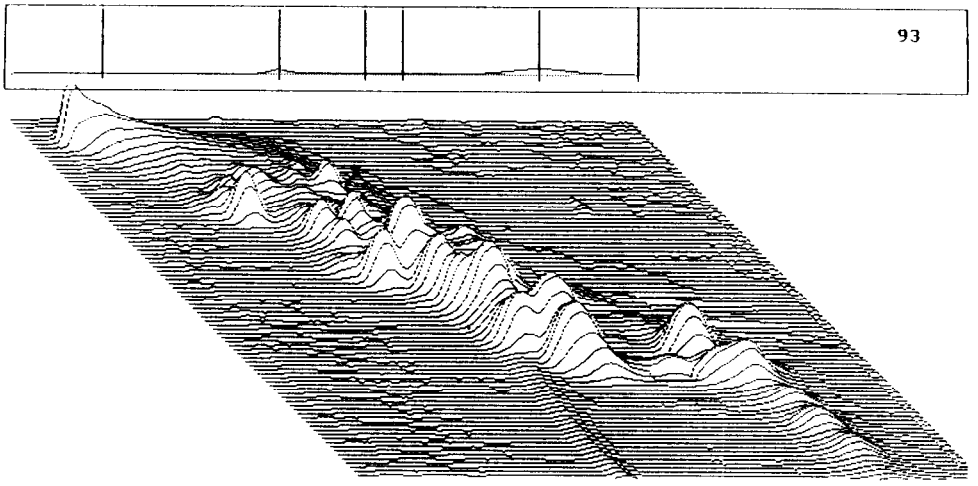

Fig. 9. Three-dimensional presentation of collected data, after substraction of the base line in each lane.

some components in propolis (Fig. 9). Propolis is a very complex mixture of various closely related natural compounds and is therefore an ideal sample for two-dimensional separation. It has been suggested that its biological activity depends mainly on the presence of flavonoids and their content.

Separation of the main phenolic components of the ethanolic extract of propolis was performed on silica 60 F_{254} HPTLC plates. The plates were first developed with the solvent system toluene–ethyl acetate–formic acid (75:15:3), and the development was repeated with the solvent system chloroform–methanol–formic acid (75:5:4) in the perpendicular direction.

Identification and quantification of some characteristic flavonoids was performed using external standards and the 2-aminoethyldiphenylborinate/polyethylene glycol 4000 derivative reagent. Identification and quantitative results of propolis will be given in another paper. Standard deviation of measurements using internal standard and the manual integration mode were $< 5\%$ for substances not separable with linear TLC.

CONCLUSIONS

More information in the quickest possible time is the demand of modern analytical methods. In future TLC will disappear if it cannot provide more information to the user. The main problem in TLC is not application and separation but data collection. Skilled analysts are capable of obtaining much useful information from a plate quite radiply but this information is only qualitative; when they require quantitative results they must use obsolete scanning densitometers in order to obtain values which are only a poor reproduction of data stored on a plate. A computer-controlled scanner is a small step forward, which gives the analyst access to a bigger amount of information, yet such data acquisition is slow. It is useful for two-dimensional TLC, two-dimensional electrophoresis and a combination of both in quantitative determination of amino acids. All these techniques were tested in our laboratory with good results. The described scanning and evaluation system is merely a tool more or less suitable for certain samples. It provides a link between classic linear densitometry and video-oriented data acquisition and image processing of TLC plates.

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