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QUANTITATIVE ANALYSIS OF ANABOLICS ON THIN-LAYER CHROMATOGRAPHIC PLATES USING IMAGE ANALYSIS TECHNIQUES

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SUMMARY

A new detection system is introduced for the quantitative analysis of thin-layer chromatographic plates, which is based on a relatively simple, cheap but advanced image analysis system. Both one- and two-dimensional plates can be analysed. Recording and analysis can also be performed from photographs or even slides. Applications are shown for a number of samples containing anabolic compounds.

INTRODUCTION

Thin-layer chromatography (TLC) is a very useful method in the multicomponent analysis of anabolic compounds [1–4]. Both the two-dimensional R_F values and the specific detection (fluorescent or colorimetric) give a high specificity, which is necessary for unambiguous identification. However, quantitative analysis is rather difficult and is often performed visually or with densitometers using a lightbeam scanner. Limitations of this kind of scanner are the long recording and analysis times, the fact that scanning is only one-dimensional and the cost of the equipment. Another way that detection and quantitation can be performed is with a camera system coupled to an image system or video-densitometer. Such systems using rather simple hardware [5,6] or an advanced computer system [7] have been described recently.

In the present paper a new detection system is introduced for the qualitative

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and quantitative analysis of anabolics after one- and two-dimensional separations on thin-layer plates. The method is based on an image analysis system, which in addition has a high level of image processing software that can be used on a normal XT personal computer. Practical applications to a number of samples containing anabolic compounds are reported.

EXPERIMENTAL

Image detection and analysis

The image analysis system consisted of the following components (Fig. 1): a CCD camera (Model MX, High Technology Holland, Eindhoven, The Netherlands) equipped with a 1.7/17 mm lens (Model Xenoplan, Schneider-Kreuznach, High Technology Holland), which has been adjusted for close-up measurements by Mr. M.A. van Ingen (Department of Fine Mechanics of our Institute, Bilthoven, The Netherlands), a colour video monitor (Model Trinitron PVM-1271Q/1371QM, Sony, Badhoevedorp, The Netherlands), a personal computer (Model M24 with a 20 MB hard disc, Olivetti, Ivrea, Italy) equipped with a frame grabber (Model PCvision-plus, Imaging Technology, Difa Measuring Systems, Breda, The Netherlands) using a software package for image analysis (TIM version 3.02, Difa Measuring Systems/TEA, Dordrecht, The Netherlands) and a matrix printer (Epson, Model FX-800, Difa Measuring Systems). The system has two kinds of memory where image data can be stored: display memory, residing on the display card (frame grabber), and standard computer memory, which is also at your disposal when performing other functions. The image memory card adds two extra display images to this. These are called X and Y and can act as both source and destination of operations. Images can be of three different formats: standard images, standard sub-images and variable (cursor) sub-images. Routinely, TIM op-

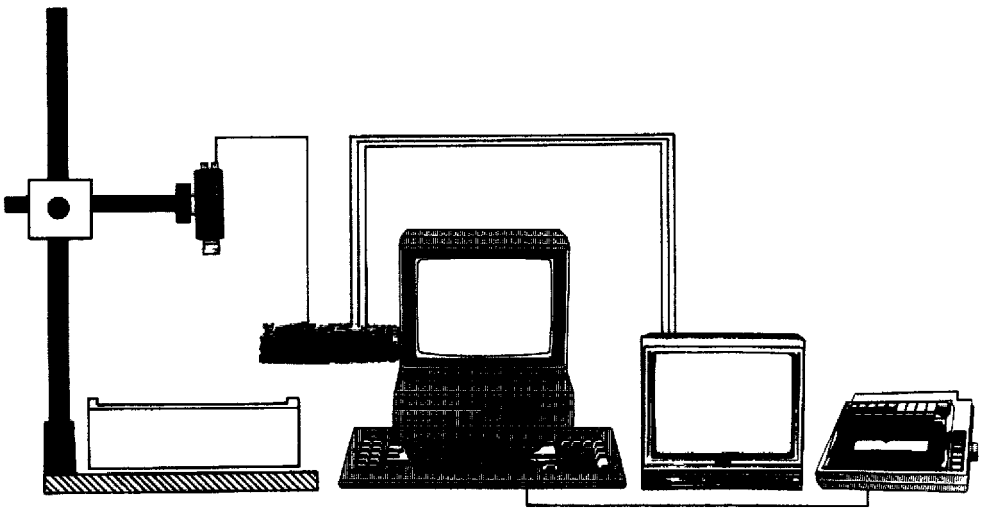

Fig. 1. Equipment for recording and image analysis of TLC plates.

erates on images of 512×512 pixels, which is sufficient resolution for most TLC applications. Quantification is performed by integration of the grey values of the pixels concerned. A pixel can have 256 different grey values between white (value 255) and black (value 0).

TLC experiments

The TLC experiment as described in Fig. 2 was performed as follows: samples and standards were spotted on the left and on the right side of a high-performance TLC (HPTLC) plate (No. 5631, Merck, Darmstadt, F.R.G.) as indicated in Fig. 2. The spotted amount was 0.75 μl using microcapillaries with a manual applicator (Merck). Solvent system 1 consisted of chloroform–acetone (9:1, v/v) and solvent system 2 consisted of cyclohexane–ethyl acetate–ethanol (77.5:20:2.5, v/v). The chromatographic direction is indicated in Fig. 2A by arrows. After chromatography the plate was dried with compressed air and sprayed with 10% sulphuric acid in methanol (v/v). After heating at 95 °C for 10 min the spots became visible. Colour development occurred after spraying with 10% sulphuric acid in methanol (v/v).

The TLC experiment shown in Fig. 3 was performed as follows: two identical TLC plates (DC Fertigplatten, No. 60, Merck) were spotted with 25 μl of a chloroform extract of the sample. The urine extract was purified by high-performance liquid chromatography prior to TLC analysis [8]. On one plate (the B plate), 1

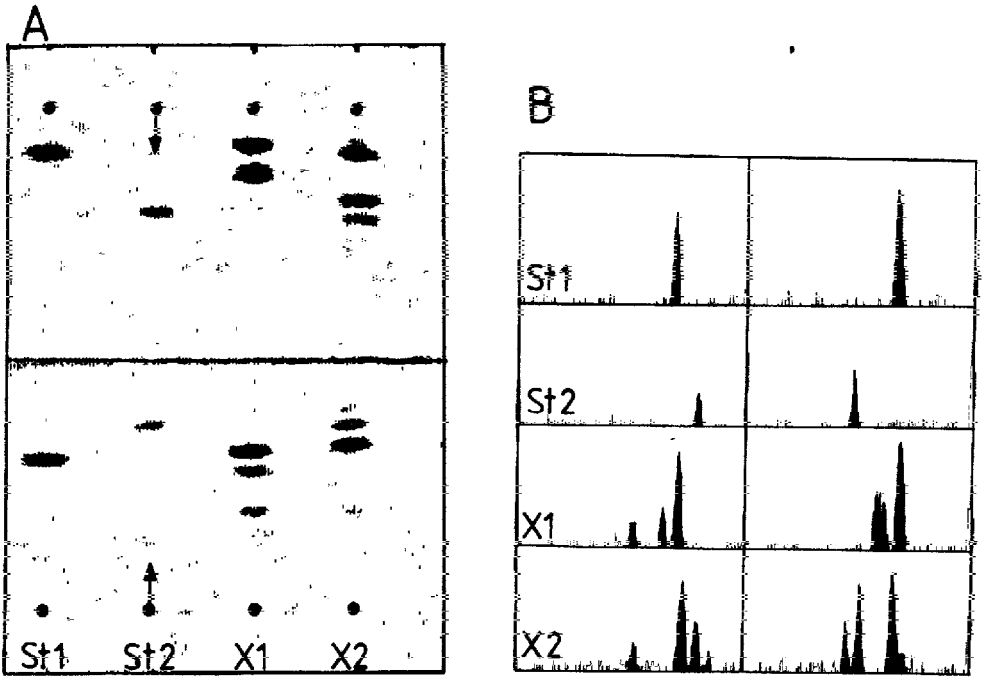

Fig. 2. Example of line analysis (integration) in a double one-dimensional TLC system. (A) Representation of the TLC plate by image recording and threshold filtering to obtain a binary image. As indicated, two standards and two samples have been analysed. (B) Integrals of the four samples by line analysis as described in the text.

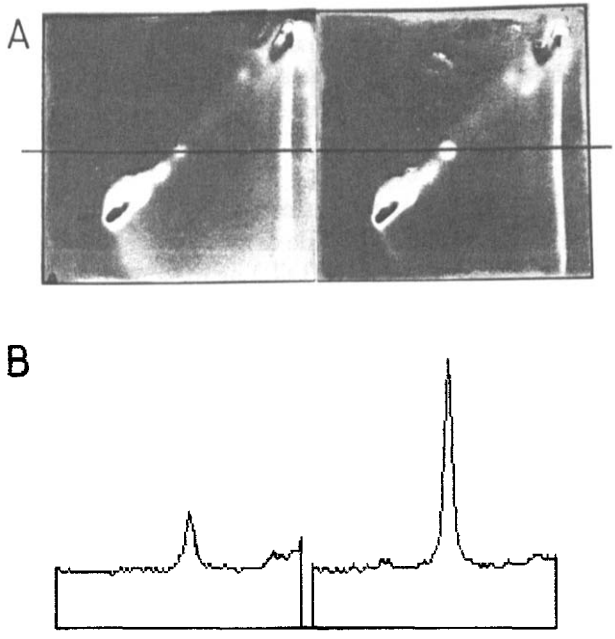

Fig. 3. (A) Representation of so-called A and B TLC plates in the analysis of 19-nortestosterone taken from a 36×24 mm colour slide. (B) Plot of the grey density value pattern of the image line in (A).

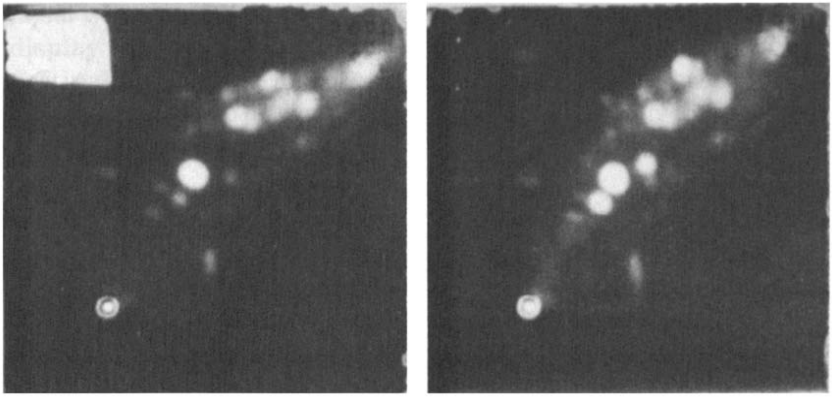

Fig. 4. Representation of so-called A and B TLC plates in the analysis of ethinylestradiol taken from a black and white photograph.

μl of a standard of 19-nortestosterone in chloroform was spotted in addition. Both plates were chromatographed under identical conditions. Solvent system I consisted of chloroform–*n*-propanol (95:5, v/v). The TLC plate was dried at room temperature. Then the plates were developed a second time perpendicular to the first direction. Solvent system II consisted of *n*-hexane–diethyl ether–dichloromethane (4:3:2, v/v). The plates were dried at room temperature and sprayed

with a solution of 20 ml of phosphoric acid (75%) and 30 ml of water. After heating at 110–120°C for 15 min the spots were visible.

The TLC experiment shown in Fig. 4 was performed under similar conditions.

RESULTS

The specificity of the TLC analysis of anabolics in urine samples can be increased using two-dimensional or two independent one-dimensional systems. In Fig. 2A an example of such a double one-dimensional system is shown. Components in the sample are compared with anabolic standards. Both the R_F values of the two solvent systems and the colour of the spots must be identical. In Fig. 2B the grey density patterns are shown of the four lanes in Fig. 2A. With this kind of analysis the relative distances between the start and end positions can be determined very accurately. In this example neither of the two standards was present in the two samples. The information of the colour of the spots is, however, lost.

The image system is also very suitable for analysing two-dimensional TLC plates. Quantitative analysis is even possible from slides or photographs taken from TLC plates, as is shown in Fig. 3 for the detection of 19-nortestosterone in urine after purification with high-performance liquid column chromatography [8]. In Fig. 3A a TLC analysis using the so-called A and B plates is shown. The A plate (left) contained an extract of the sample whereas the B plate (right) contained the same sample extract plus the expected anabolic standard. In Fig. 3B line analysis has been performed on an image line through the centre of the suspected spot (left) and the standard spot (right) as indicated in Fig. 3A. The unknown spot can now be quantified relative to the standard. Both two-dimensional chromatograms can be visualized in the form of a three-dimensional plot. The computer program can set up the plot from different points of view: from the left side, from the right side, from a front view, from a back view and also from various heights from which the plot can be observed. Fig. 5 shows the three-

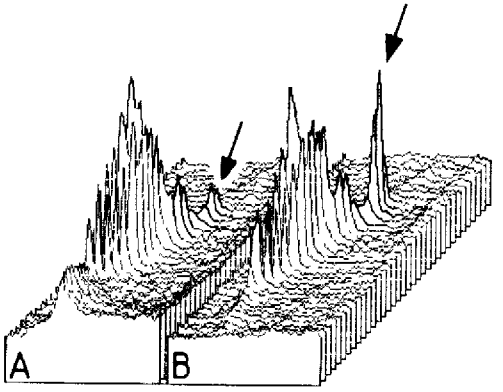

Fig. 5. Three-dimensional representation of the image taken from the slide shown in Fig. 3A. The 19-nortestosterone spots are indicated with arrows.

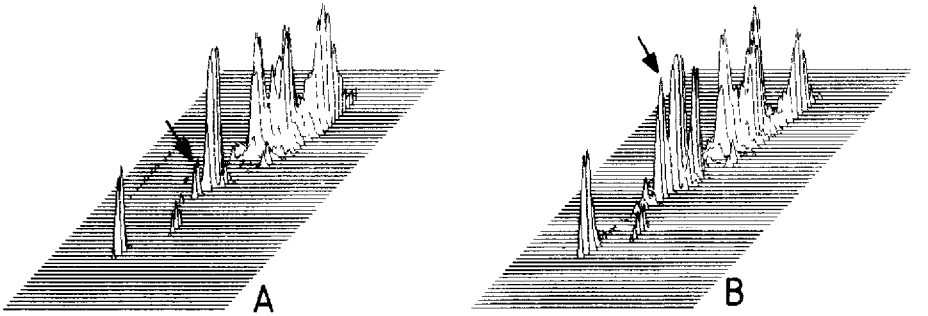

Fig. 6. Three-dimensional representation of the image taken from the photograph shown in Fig. 4. The ethinylestradiol spots are indicated with arrows.

A

11	10	9	6	4	4	7	13	23	40	62	83	99	106	105
13	12	9	6	5	6	10	12	17	25	39	53	66	75	72
5	8	6	6	8	8	12	15	15	17	22	33	42	46	43
5	8	8	8	11	15	23	26	26	22	19	21	24	22	21
0	2	5	10	14	22	28	29	29	26	20	16	12	9	9
0	0	2	10	20	29	36	40	37	29	19	11	6	3	2
0	0	0	6	18	30	40	45	43	35	22	13	5	1	0
0	0	0	5	17	28	40	47	44	35	22	10	2	0	0
0	0	0	0	9	23	35	43	43	35	22	12	2	0	0
0	0	0	0	5	16	27	35	37	30	19	8	0	0	0
0	0	0	0	3	9	16	22	22	18	10	2	0	0	0
0	0	0	0	0	1	5	11	12	10	7	4	0	0	0
0	0	0	0	0	0	3	5	5	5	1	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0

B

6	7	12	15	21	29	37	43	54	68	86	104	115	121	124
12	15	20	27	35	46	57	63	66	71	79	89	98	106	106
14	16	21	29	44	61	74	81	84	81	75	71	74	77	76
22	22	26	41	61	81	97	103	103	94	79	68	59	55	51
16	22	32	48	71	92	108	116	115	109	95	76	56	42	34
16	25	38	59	83	104	118	122	124	117	104	81	57	38	26
12	20	33	55	83	106	120	124	127	122	110	87	59	38	23
8	17	33	59	87	109	121	127	130	125	113	89	60	36	20
4	13	26	49	76	101	117	124	124	124	114	92	63	38	20
6	12	23	45	72	95	111	120	123	122	111	88	58	34	18
1	9	18	35	57	79	97	109	114	112	103	82	57	34	18
8	8	13	26	43	64	82	97	103	100	88	67	41	22	10
12	11	11	16	29	46	63	75	81	80	71	53	33	17	8
15	12	11	15	21	31	42	50	57	55	44	32	20	10	3
22	18	15	13	14	19	24	28	30	30	24	16	10	4	2

Fig. 7. Print-out of the pixel values (grey density) and localization of the areas of the ethynylestradiol spots indicated with arrows in Fig. 6.

dimensional plots of the TLC plates from Fig. 3A from the right side in order to get a clear view of the 19-nortestosterone spots (indicated by arrows).

The same analysis can be performed from photographs of TLC plates, as shown in Figure 4 for the analysis of ethinylestradiol in bovine urine. The three-dimensional plots in Fig. 6 show clearly the presence of ethinylestradiol (indicated by arrows) added as standard (Fig. 6B) and in the sample (Fig. 6A). From the surrounding areas of both ethinylestradiol spots (in Fig. 6) a print-out of the grey values of the pixels concerned can be made. This is shown in Fig. 7 with an indication of the localization of the spots. As can be seen, the surface area of the spot of the standard (Fig. 7B) is much more extended than the spot in Fig. 7A because of the higher intensity. With these data qualitative confirmation can be accomplished by comparison of the coordinates of both spots and quantitative analysis can be performed by integration of the grey values concerned.

DISCUSSION

The image analysis and processing system introduced here is an excellent scanning system for TLC plates and also for slides and photographs of TLC plates. Compared with conventional light beam scanners, image analysis systems provide a number of advantages, such as speed of recording and detection, image enhancement, image filtering techniques, the possibility of storing the original recordings on disk, etc. In addition the system uses the relatively cheap XT personal computer, and the high level of software makes it easy to handle. Even technicians unexperienced with computer use can soon handle this image system. The system is equally suitable for processing and analysis of both one- and two-dimensional plates.

In this report emphasis is put on the quantitative analysis of one- and two-dimensional TLC plates with sample extracts of anabolics. Since image analysis of TLC plates is currently under development, the applications and possibilities of the system that have been demonstrated here using an actual TLC plate, a photograph and a slide of TLC plates are still preliminary. Yet the examples have shown the excellent prospects for quantitative analysis and three-dimensional representations. Quantitation can be achieved via the grey value of a pixel. The range of grey values is limited, however, to 256 units. Since a spot consists of a large number of pixels (67 pixels in Fig. 7) the mean value of the grey values of all pixels of a TLC spot will increase the accuracy of quantitation substantially. Since the present report only indicates the extended possibilities of the system, no attention is paid to the statistical evaluation of the method.

Further possible uses of the image analysis system that have not been described here include local background filtering using a combination of minimum and maximum filtering, image enhancement by automatic contrast stretching, resolution enhancement by deconvolution or Laplace filtering and automatic localization and integration of all spots present in the image. All kinds of illumination such as normal light conditions, background illumination, and UV or fluorescence detection, can be used. Although the camera used for the present study has its optimal sensitivity from 400 to 800 nm, UV-sensitive cameras can be obtained for detection in the UV region. The camera can also be used under reduced light conditions since it has a good sensitivity in the order of 1 Lux. If necessary the light sensitivity of the system can be increased by image averaging or by the use of a camera equipped with an image intensifier, which decreases the detection level by a factor 100.

At present the system is meant to be used as a research instrument. For routine operations, general user modules have to be developed consisting of a number of software commands in sequence. These so-called command files are under development for most routine analysis procedures, such as the integration of one-dimensional patterns, difference patterns between two samples, etc. These command files can be easily set up by the user, or existing command files can be adjusted by users to meet their own requirements or applications.

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