An Image Analysis System for Thin-Layer Chromatography Quantification and Its Validation

Tang Tie-xin and **Wu Hong***

Center for Medicinal Plants Research, South China Agricultural University, Guangzhou 510642,China

Abstract

Quantitation of thin-layer chromatography (TLC) using image analysis is attractive for its low cost and convenience. The image analysis is investigated by designing a digital imaging system with simple equipment, developing an image analysis software based on our algorithm, and validated the system in the TLC quantitative assay of cichoric acid present in *Echinacea purpurea* (L.) Moench. TLC used a polyamide thin-layer plate with chloroform–methanol formic acid–water (3:6:1:1) as the mobile phase and 3% (m/v) aqueous aluminum chloride solution as the visualization reagent. Images are acquired with a standard digital camera under a UV viewing lamp (365 nm) in a dark room. The three-dimensional grayscale digital image dataset (x, y, gray) is reduced to two-dimensional dataset (distance, accumulative gray) and then plotted as a curve. The area under the peak corresponding to the cichoric acid spot is integrated and used for quantitation. The whole method was validated by the assay tests of detection limit, calibration curve, repeatability, reproducibility, and recovery. The results showed that our digital imaging method and image analysis algorithm were applicable for the quantification of TLC. The whole method is convenient, efficient, and moderately accurate for the quantitative assay of cichoric acid present in *Echinacea purpurea* (L.) Moench.

Introduction

Thin-layer chromatography (TLC) is a low cost, rapid method for quantitative analysis. However, compared with high-performance liquid chromatography (HPLC), its shortcomings of low efficacy, low sensitivity, and poor precision are obvious. Nowadays, a slit-scanning densitometry system is commonly used for the quantitation of TLC, but the equipment does not cost less than that of HPLC. Therefore, the application of TLC to quantitative analysis is limited (1,2).

At the end of the 20th century, due to development of digital electronics, digital imaging and analyzing were applied to qualitative and quantitative analysis in gel electrophoresis (3), and also to the quantitation of TLC (2). Though the commercial digital imaging and analyzing system cost much less than TLC slit-scanning densitometry, it is not used widely. This is because of the existence of other competitive technologies, as well as the immaturity of the technology itself.

Recently, the performance of the digital camera improved greatly, and its price reduced to an acceptable level, which made the use of such a system a very good prospect for TLC

Table I. Part of the Source Codes of the Software

```
…
//accumulate the gray value of a lane and store in arrraygray[]
        for i:=0 to Form1.Image1.height-1 do
        begin
            for j:=startp.X to endp.X do
            begin
            //get the gray value of a pixel
                gray:=getgray(form1.image1.Canvas.Pixels[j,i]);
                total:=total+gray;
            end;
            arraygray[i]:=total;
            total:=0;
        end;
…
//display the curve of a lane
        for i:=1 to Form1.Image1.height-1 do
        begin
            //restrict the image height of the curve within 480 pixels
            tem:=trunc((arraygray[i]-mingray)*480/(maxgray-mingray));
            Form2.Image1.Canvas.Pen.Color:=clblack;
            Form2.Image1.Canvas.LineTo(i,tem);
        end;
…
//integrate the accumulative gray value under a peak
        for i:=StartPoint.X to EndPoint.X do
        begin
            peakarea:=peakarea+arraygray[i];
        end;
        //minus the area under base line
        peakarea:=peakarea-0.5*(arraygray[StartPoint.X]
        + arraygray[EndPoint.X])
        *( StartPoint.X - EndPoint.X);
…
```

* Author to whom correspondence should be addressed: email wh@scau.edu.cn.

quantitation. Therefore, we investigated the image analysis system, employing a personal camera to acquire the images of the TLC plates with other simple equipment, designed an algorithm, developed test computer software, and validated the system with many tests in the quantitative TLC assay of cichoric acid present in *Echinacea purpurea* (L.) Moench.

Experimental

Apparatus

An 8890E-MT ultrasonic cleaner (Col-Parmer, IL) was used for sample extraction. Sample solution was applied to the plates with micropipettes (CAMAG, Switzerland). The plates were developed in a 100 × 100 mm (length × height) twin trough glass chamber (Xinyi, Shanghai, China.). ZF-1 UV viewing lamp (Gu Cun, Shanghai, China) was used for visualization. The images were acquired with DX7590 digital camera of 5 million pixels resolution, 10× optical zoom (Kodak). The test software was developed using Delphi 6.0 IDE software (Borland).

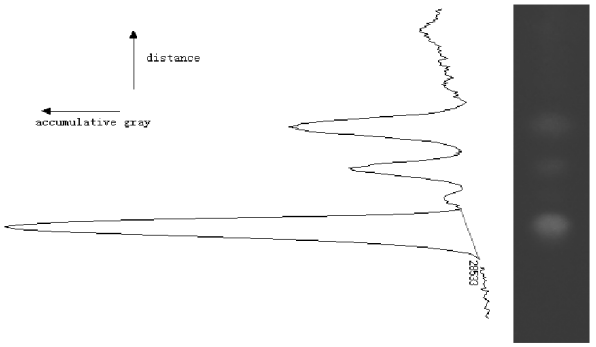

Figure 1. A lane of TLC image and image analysis results. Note: the gray value of a pixel recorded by computer in brighter region is greater.

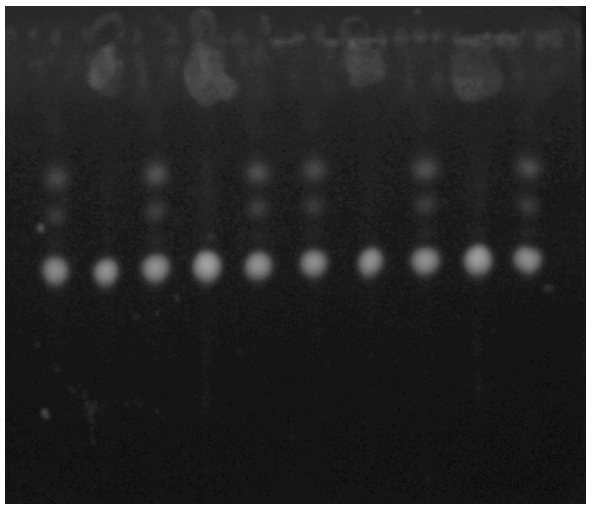

Figure 2. Chromatogram of cichoric acid on polyamide TLC plate: Lanes of 1, 3, 5, 6, 8, and 10 were *Echinacea purpurea* samples; Lanes of 2 and 7 were standard solution of 0.125 mg/mL; Lanes of 4 and 9 were standard solution of 0.25 mg/mL. Sample volume was 2 µL.

Reagents

Cichoric acid (purity > 98.5%) was purchased from Chemical Industry College of Hunan Normal University. *Echinacea purpurea* was harvested from the farm of South China Agricultural University. Polyamide (nylon 6) thin-layer (0.1 mm thickness) plates backed with polyester film were purchased from Lu Qiao Si-Jia biochemistry plastics company (Taizhou, China). All reagents were of analytical reagent grade.

Procedures

Standard solution

A standard solution of 0.50 mg/mL was prepared by dissolving cichoric acid in 95%(v/v) ethanol. The solution was diluted to give concentrations 0.25 mg/mL and 0.125 mg/mL.

Sample solution

Approximately 100 mg dry root powder of *Echinacea purpurea* was precisely weighed and mixed with 25 mL 75% (v/v) ethanol in a volumetric flask. The mixture was treated in ultrasonic bath (35 W, 47 KHz) for 45 min and filtered.

TLC procedure

Sample and standard solutions were applied to the polyamide plate. The plate was developed in the chamber using chloroform–methanol formic acid–water (3:6:1:1) as the mobile phase in ascending mode. The plate was removed when the mobile phase migrated 8 cm and was dried at room temperature. Then it was soaked in 3% (m/v) aqueous aluminum chloride solution, and dried. The development temperature was from 20°C to 30°C and relative humidity from 60% to 80% are recommended.

Digital imaging, test software, and image analyzing

In a dark room, the TLC plate was placed under the UV viewing lamp (365 nm) and made flat with edges fixed. The Kodak DX7590 camera was fixed on a tripod and set to SCN -> fireworks mode, self-timer, and black & white color. Then the digital image of the plate was acquired and transferred to the computer.

The image was properly cropped and saved in bitmap (.bmp) format, and then it was analyzed using our test software. The algorithm can be described as follows: the acquired grayscale digital image is actually a three-dimensional dataset (x, y, gray). The different lanes of the TLC image were treated separately, the gray values of pixels in a same lane in x direction (vertical to the

Table II. Recovery Assay Results*

Sample weight (mg)	Content (mg)	Cichoric acid added (mg)	Detected (mg)	Recovery rate (%)
49.0	0.647	0.65	1.39	114
49.4	0.652		1.48	127
48.9	0.645		1.39	115
48.2	0.637		1.22	89.7
49.6	0.655		1.43	119
50.2	0.663		1.31	99.5

*Average recovery = 110.7%, n = 6, RSD = 12.3%.

lane) were added up and a new two-dimensional dataset (distance, accumulative gray) was obtained, then it was plotted. We manually chose the start point and end point of the peak with the computer mouse on the plot corresponding to the cichoric acid spot and integrated the accumulative gray value under the peak. Thus we obtained the integrated gray value of the cichoric acid spots of samples and references for quantitative analysis (Figure 1).

The test software was edited and built with Delphi 6.0 IDE software under Windows 2000. The software has three forms. User can click on a command button on form 1 to choose the image file from a popup dialog. The image will display on form 2. After choosing the left and right boundary of a lane on the TLC image by mouse click, the accumulative gray curve of the lane will appear on Form 3. The peak area will show up beside the peak of the curve after selecting the start point and end point of the peak.

Part of the source codes of the software is shown in Table I.

Detection limit

The sample solution assayed to be 1.35 mg/mL was diluted to 1:20; 2 and 1 µL of the solution were applied onto the same plate. The plate was developed and its digital image was analyzed for the signal-to-noise ratio (S/N).

Calibration curve

Five spots were applied on the plate with 1 µL micropipette. The standard solutions were applied to the plate with 1 µL micropipette as follows: 2 µL 0.50 mg/mL , 1 µL 0.50 mg/mL +1 µL 0.25mg/mL, 2 µL 0.25mg/mL, 1 µL 0.25 mg/mL + 1 µL 0.125 mg/mL, and 2 µL 0.125 mg/mL, and then assayed.

Repeatability

Six parallel spots of the sample solution were applied on the same plate with a 2 µL micropipette and assayed. Repeatability was expressed as the relative standard deviation (RSD).

Reproducibility

Six samples of the same batch were prepared and assayed. Every three sample solutions and standard solutions of 0.25 mg/mL and 0.125 mg/mL were alternately applied to the same plate with two replicates. Reproducibility was expressed as RSD of the assay results.

Recovery

Six samples of the same batch (cichoric acid content: 1.32%) together with 1.30 mL standard solution of 0.50 mg/mL were prepared and assayed. The amount of *Echinacea purpurea* dry root powder in the samples for recovery assay was 50% of that for the normal assay. The accuracy of the whole method was expressed as average recovery rate of the assay results.

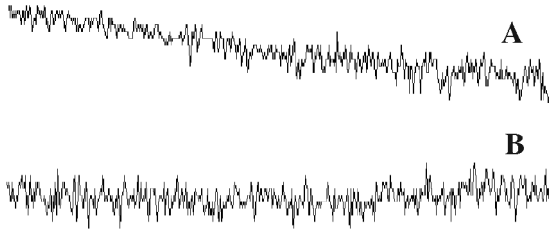

Figure 3. Comparison of vertical baseline (A) and horizontal baseline (B) in the background.

Result and Discussion

TLC assay of cichoric acid

In the literature, silica gel plates were used in the TLC assay of cichoric acid present in *Echinacea purpurea*, and the fluorescence of the spots under 365 nm UV viewing lamp was enhanced with spraying methanolic 2-aminoethyl diphenyl borate solution and methanolic 5 % PEG 400 solution (4). A new TLC method is developed for the assay of cichoric acid.

On studying the TLC of flavonoids on a polyamide plate, we also applied a standard solution of cichoric acid. However, the results showed that the mobile phases for flavonoids: ethanol–water (1:1), ethanol–water (7:3), and acetic acid–water (4:6), could not develop cichoric acid, but 3% ethanol (95%) solution of aluminum chloride could change the fluorescence of the cichoric acid spot from pale blue to bright blue. A number of other mobile phases, such as 95% ethanol, chloroform–methanol–water (4:6:1), acetic acetate–methanol–water (3:6:1), were also tried. Our observations revealed that the chloroform–methanol–water system could develop cichoric acid, but, unfortunately, the tailing was bad. Therefore, we tried adding formic acid to reduce tailing and adjusted the mobile phase constitution to chloroform–methanol–formic acid–water (3:6:1:1), which led to a satisfactory separation (Figure 2).

The extraction solvent (75% ethanol) used could not develop cichoric acid, so the applied sample spot on the plate would not be enlarged, which facilitated the sample application operation and improved the parallelism.

Aluminum ion will chelate with the two adjacent phenolic hydroxyls of cichoric acid molecule and increase its rigidity and co-planarity, which results in the enhancement of the fluorescence. Moreover, aluminum ion is soluble in water (the weakest eluant for polyamide stationary phase). Therefore, we have used aqueous 3% aluminum chloride solution to substitute methanolic 2-aminoethyl diphenyl borate solution for visualization, in consequence, avoiding the enlargement and elution effect of the organic solvent on cichoric acid, and facilitating the plate soaking operation in order to uniformly visualize the plate.

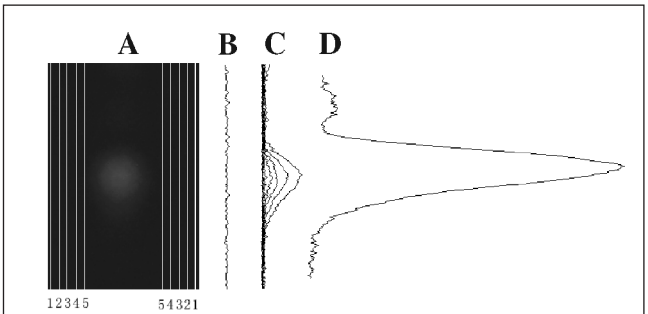

Figure 4. A spot in a lane (A), a gray value curve of a line in the background (B), several overlapped gray value curves of the lines in the background and in the spot (C), accumulative gray value curve of the spot (D).

The indicated chromatographic conditions guarantee reproducible operation, appropriate separation, uniform visualization, and sensitive detection. These are prerequisites to high quality imaging and accurate image analysis.

Digital imaging and image analysis

Enough sensitization to light is very important for digital imaging. Insensitivity of charged-coupled device (CCD) camera in the UV spectral region was regarded as a problem in video scanning (2). The problem was resolved in our method by setting the camera to the fireworks mode, mounting it on a tripod, and using the timer instead of pressing the shutter button. Setting the camera to fireworks mode will prolong the exposure time (2 s in our camera), which multiplies the sensitivity to the fluorescence spots because the camera will take many shots in the period and add up the signals. We mounted the camera on the tripod and used the timer in order to avoid shaking during the relatively long exposure time. In addition, to minimize the background noise, images were taken in a dark room to eliminate heterogeneous light.

Another problem in image analysis is that a UV lamp will not illuminate the whole plate uniformly (2,5,6). We used a 15 cm long UV lamp, which was much longer than the plate length (10 cm) to minimize the problem. To study the problem, a plot of a lane was prepared in the background with our software (Figure 3A), then the image was rotated by 90°, and a second plot of a lane in the background was prepared (Figure 3B). The baseline of Figure 3A had a downward drift trend, which showed that the illumination gradually weakened, while the baseline of Figure 3B maintained on a horizontal line, indicating that the illumination did not vary significantly. The repeatability result (RSD = 2.0%) of the validation tests also supported that the illumination of the plate is satisfactory uniform. The gradient illumination of the plate in vertical direction will not greatly affect the quantitation of image analysis just like regular baseline drifting in an HPLC chromatogram due to gradient elution and in gas chromatography (GC) due to gradually increasing the temperature. In addition, the replicate spot of the sample was applied to a different half region of the plate, and an average value was used for calculation to further reduce the system error.

At present, most of the published algorithms for gel electrophoresis image and TLC image analysis are designed to remove, first, the background or separate the spots from the background by edge recognition, then integrate the value of the spots (3,5,7). These algorithms are very effective, but they are also relatively complicated and used much computer running time. We separated the different lanes of the chromatogram at first, then accumulate the values vertically to the developed direction in the same lane, which changes the data set from three-dimensional to two-dimensional, and finally plot a curve resembling the chromatogram of HPLC or GC with the new dataset, integrating the area under the curve of the peaks corresponding to the spots.

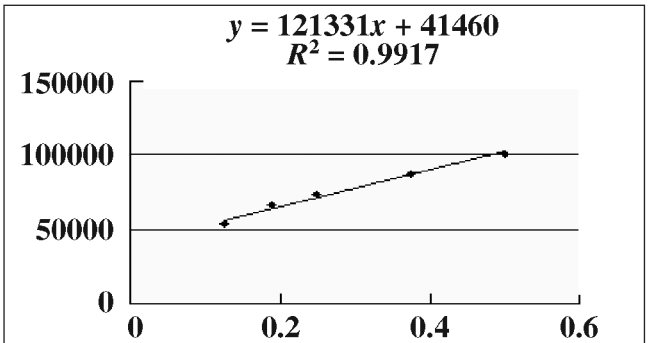

Figure 5. Calibration plot for cichoric acid.

Our algorithm is relatively simple and easy to implement, and it will enhance the signal-to-noise ratio (S/N). The gray values in the background vary randomly (naturally, they will respond to the imperfect plane of the plate), and the gray values in the spots vary regularly (Figure 4). Accumulation of the values vertically to the developed direction in the same lane will accumulate the signal and minimize some irregular noise, then enhance the S/N. We estimated the deviation error of the different separation width of a lane by accumulating the gray values between white line pairs of number 1, 2, 3, 4, and 5 in Figure 4A. Their integrated gray values (or peak area) were 145901, 145736, 145483, 144371, and 142269, respectively. The RSD value is 1.04%, indicating that the integrated gray value will not change significantly when more background pixel lines are included (slightly widen lane width). However, there is a decrease trend with the reduction of lane width, indicating that we must separate the different lanes with the same width like the fixed slit length for different lanes of the same plate in slit-scanning densitometry.

Validation of the method

Quantification studies of TLC with image analysis often used pure substances and validated the method only by a calibration plot (5,6,7). We validated our method with many additional items according to the Validation of Analytical Procedures of International Conference on Harmonization (ICH) (8).

The detection limit is below 0.067 µg because the S/N of the spot of 1 µL, 1/20 diluted sample solution (1.35 mg/mL), was over 3. The calibration plot showed that there was a good linearity for cichoric acid in the range of 0.25 µg~1.0 µg (R^2 = 0.9917) (Figure 5).

Assaying the repeatability, the integrated gray value of the cichoric acid spots were: 145781, 146944, 140813, 143788, 140338, 139597, and 140874 (RSD = 2.0%).

Assaying for reproducibility, the contents of six samples were: 0.135, 0.128, 0.122, 0.130, 0.143, and 0.135 mg/mL (RSD = 5.5%). The results of recovery are given in Table II.

In the validation study, results for the detection limit, repeatability, and reproducibility were satisfactory, but the deviation of the recovery results was too large. Because the accuracy of the method is evaluated by the recovery result, the large deviation indicated that the accuracy was unsatisfactory. The reasons might be that the weighing error of the half amount dry root powder in the recovery samples was larger than that of normal samples; addition of the standard solution would increase the operation error, and because the divisor in the recovery calculation was the added amount of cichoric acid, which was half of the detected amount, and the assay error did not change much, the error of the recovery would be doubled. If an autosampler would be used, the repeatability, reproducibility, and recovery might be improved.

Conclusion

It is convincing that we can do digital imaging with simple equipments, and our algorithm is suitable for image analysis of TLC. However, the accuracy of the whole method was unsatisfactory, and more research is needed to improve the precision of the TLC method, digital imaging, and image analysis. The TLC image analysis system used by us is not applicable for quantitative assay requiring high accuracy and high confidence, but considering its simplicity, low-cost, convenience, and moderate accuracy, it can benefit some studies such as agricultural cultivation test, parallel comparison studies, and studies that don't require absolute content accuracy, making better use of the low cost, rapid TLC.

Acknowledgments

The authors would like to thank the Science and Technology Bureau of the Guangzhou Municipal Government for financial support (200423-ES021).

References

1. C.F. Poole. Planar chromatography at the turn of the century. *J. Chromatogr. A* **85:** 6399–427 (1999).
2. C.F. Poole. Thin-layer chromatography: challenges and opportunities. *J. Chromatogr. A* **1000:** 963–84 (2003).
3. W.F. Patton. Biologist's perspective on analytical imaging systems as applied to protein gel electrophoresis. *J. Chromatogr. A* **698:** 55–87 (1995).
4. C. Bodinet, R. Mentel, U. Wegner, et al. Effect of oral application of an immunomodulating plant extract on Influenza virus type A infection in mice. *Planta Medica* **68:** 896–900 (2002).
5. L. Zhang and X.G. Lin. Quantitative evaluation of thin-layer chromatography with image background estimation based on charge-coupled device imaging. *J. Chromatogr. A* **1109:** 273–78 (2006).
6. I. Vovk and M. Prosek. Quantitative evaluation of chromatograms from totally illuminated thin-layer chromatographic plates. *J. Chromatogr. A* **768:** 329–33 (1997).
7. A. Akbari and F. Albregtsen. Normalizing the background and removing the trend in one-dimensional DNA fingerprint images. *J. Chromatogr. A* **1014:** 11–19 (2003).
8. ICH. Validation on analytical procedures. Commission of the European Communities. Proceedings of the International Conference on Harmonization (ICH) [C]. 1996.

Manuscript received July 9, 2006;
revision received November 25, 2006.