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Fluorescence quenching high-performance thin-layer chromatographic analysis utilizing a scientifically operated charge-coupled device detector

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A scientifically operated charge-coupled device detector combined with fluorescence quenching high-performance thin-layer chromatographic plates was employed for the detection of organic compounds, The plates were excited with 254 nm light from a mercury lamp, and quantitative information was obtained from organic compounds that absorbed the optimum conditions for detection. The linear dynamic range, sensitivity, and reproducibility of the system were evaluted by quantitative analysis of famotidine, acetaminophen, caffeine, and acetylsalicylic acid. The detection limits of the system were found to be in the nanogram range.

Introduction

Thin-layer chromatography (TLC) is a high-throughput technique used for the separation of organic compounds. TLC has several desirable characteristics as a chromatographic technique, including parallel separation ability and low solvent consumption. Additionally, liquid chromatographic (LC) techniques often require extensive sample clean up and pretreatment, because the column is used repeatedly and may be damaged for future separations by irreversibly absorbed materials. For TLC, however, the chromatographic surface is used only once, significantly simplifying extraction procedures for foods or pharmaceuticals. TLC also provides total sample accountability, because the analyte is bound to the chromatographic material during detection. In samples analyzed by LC methods, components may bind strongly to the stationary phase, avoiding detection by the on-line detector.

In the past, TLC demonstrated limited sensitivity and reproducibility because of inadequate techniques for sample application and detection. High-performance thin-layer chromatography (HPTLC) employing slit scanning densitometers has proven to be an accurate quantitative method;[1] unfortunately, the necessity for sequential scanning of each lane of the TLC plate limits throughput.

Imaging HPTLC using charge-coupled device detectors (CCDs) has been shown to achieve fast and efficient TLC analysis.[2–12] By coupling CCD detection with HPTLC, the entire TLC plate can be imaged in a single exposure yielding rapid quantitation in less analysis time than that of slit scanning densitometers. When used in 'scientific mode' (cooled, slow-scan), CCDs have extremely low read noise and dark current, excellent sensitivity from the UV to the near-IR, exceptional linearity, and a dynamic range of up to 16 bits. The 14-bit, scientifically operated CCD used for this work provides 16 000 levels of gray scale. In contrast, the 8-bit systems employing digital or video cameras only offer 256 levels of gray scale. As a result, the scientifically operated CCD provides much better sensitivity than can be obtained with a digital or video camera. Using a scientifically operated CCD camera system as a quantitative detector for fluorescence TLC has been demonstrated.[3] This paper explores the feasibility of using a scientifically operated CCD camera system as a quantitative detector for fluorescence quenching HPTLC.

Fluorescence quenching HPTLC employs a special chromatographic surface that fluoresces when illuminated with ultraviolet. Manganese-activated zinc silicate is used as a short-wavelength (254 nm) UV indicator for silica gel phases.[13] Although the technique is known as 'fluorescence quenching', in actuality, 254 nm light is absorbed by the analyte before it reaches the fluorescent/phosphorescent stationary phase material, resulting in a decrease in light emitted from the TLC plate—note that this is not a true quenching effect but an absorption or 'shadowing' of the excitation wavelength.[14,15] The measurement is analogous to an absorption technique, as the signal quantitated is differentiated from a very bright background (the HPTLC plate). Fluorescence quenching detection is commonly used to qualitatively determine impurities in syntheses or extractions and can be applied to most organic molecules, as it detects those that absorb 254 nm light. Because it is widely applicable to most organic molecules (especially in the pharmaceutical industry), it would be advantageous to develop parallel, high-throughput quantitative techniques for quality control purposes.

This paper will demonstrate the use of a scientifically operated CCD for fluorescence detection of HPTLC plates. The system was evaluated with several organic compounds found in over-the-counter pharmaceuticals: famotidine, acetaminophen, acetylsalicylic acid, and caffeine. Famotidine, an H_2 antagonist, is commonly used as an acid blocker in pharmaceutical formulations. Several determination methods have been employed for detection and quantitation of famotidine, including titrimetry,[16] colorimetry,[17] and HPLC.[18–22] None of these methods, however, allows for multiple detection and quantitation of the analyte, which the HPTLC method explored in this work should provide, along with excellent sensitivity and flexibility over other analytical techniques.

Materials and methods

Standards, solvents and reagents

The famotidine, acetaminophen, acetylsalicylic acid, and caffeine used for stock and standard solutions were obtained from Aldrich Chemical Company (Milwaukee, WI, USA) (Structures shown in Fig. 1). All stock and standard solutions were prepared

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weekly. Standard solutions were prepared daily from the stock solution. Famotidine tablets were obtained and analyzed to verify the quality control applicability of the system.

Chromatographic analysis

Whatman (Clifton, NJ, USA) (10 × 10 cm) HP-KF high-performance TLC plates with a 254 nm fluorescent/phosphorescent coating were used for the chromatographic analysis. The plates were chromatographically washed with methanol prior to analysis in order to remove fluorescent impurities. A solution of ethyl acetate, methanol, toluene and ammonium hydroxide (40 : 25 : 20 : 2) was used as the mobile phase for the qualitative analysis of famotidine, as prescribed by the US Pharmacopeia. The mobile phase for the analysis of acetaminophen, caffeine, and acetylsalicylic acid consisted of ethyl acetate and acetic acid (95 : 5), as demonstrated by Sherma and Rolfe.[23] The developing tank was equilibrated with the mobile phase prior to each development. Samples were spotted 1 cm from the bottom of the plate and developed to a distance of 7.5 cm. The plates were then dried in a fume hood and analyzed by the HPTLC camera system.

HPTLC camera system

The camera system employed for this work was provided by Photometrics, Ltd. (Tucson, AZ, USA) and included a CC200 camera controller, a CE200 camera electronics unit and a PM512 scientific-grade CCD. A personal computer using National Instruments LabVIEW 5.1 software controlled the camera system. The CCD was mounted in a cryogenic evacuated dewar cooled to −100 °C with liquid nitrogen to decrease dark current generation. The camera used an AF Nikkor 35–70 mm focal length zoom lens (f3.3–4.5), which was attached to the camera system with an electro-mechanical shutter that controlled the exposure time. The shutter assembly also provided mounting for a CVI 1″ 550 nm bandpass filter (50 nm bandwidth) to reject any unwanted light from the UV source. The plates were illuminated with a mercury lamp from Spectronics Corp. (Westbury, NY, USA). The camera system and illumination components are illustrated in Fig. 2.

Results and discussion

Flat-field correction

It is critical to have an even background signal for analysis, as changes or gradients across the plate surface will affect the signal of interest. The CCD camera system is sufficiently sensitive to see small changes in fluorescence/phosphorescence intensity caused by illumination gradients from the UV light source. Therefore, an even signal must be achieved across the plate by flat-field correction. The background can be flat-field corrected by taking an image of the plate prior to sample application and separation and then employing the following mathematical calculation:

$$\text{Corrected image} = \frac{\text{Adjusted raw image}}{\text{Adjusted background image}} \times \text{Average value}$$

Adjusted images are obtained by subtracting a bias image—an image taken when the camera shutter is closed—which eliminates fixed pattern responses in the CCD detector resulting from differing pixel offsets and responses. Fig. 3a shows the raw image of a plate of famotidine samples after development on which a large intensity spot appears at the center with a gradient out towards the edges. A profile of the intensity of the samples compared to the background is shown beneath the image. It is obvious that no useful analytical data can be obtained from this image, as non-uniform illumination across the plate drastically warps the baseline. A lower intensity emission line on the left side of the image, from a plate defect, is also observed. The same plate after flat-field correction can be seen in Fig. 3b. The image is more uniform, as the gradient from the light source has been eliminated by the flat-field correction technique. The famotidine sample baseline has been corrected, the low fluorescence/phosphorescence line caused by the plate defect has been removed, and analytical information can now be obtained from the plate.

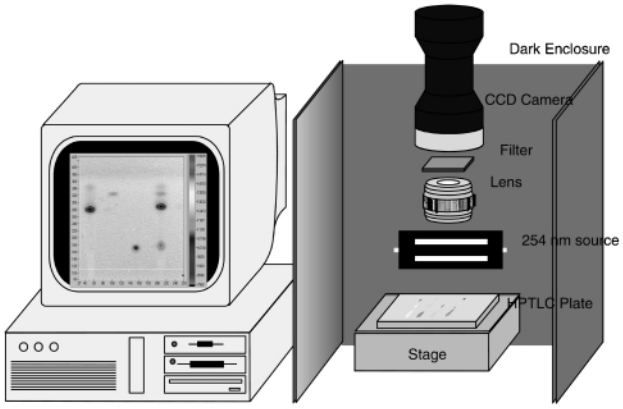

Fig. 2 Diagram of fluorescence quenching HPTLC setup.

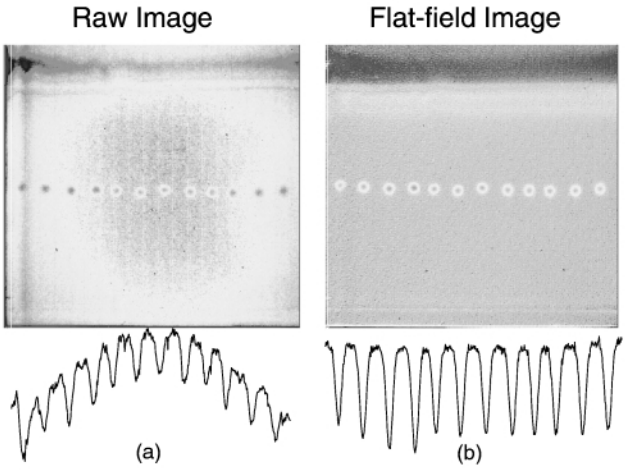

Fig. 3 Raw and flat-field images of fluorescence quenching HPTLC plate.

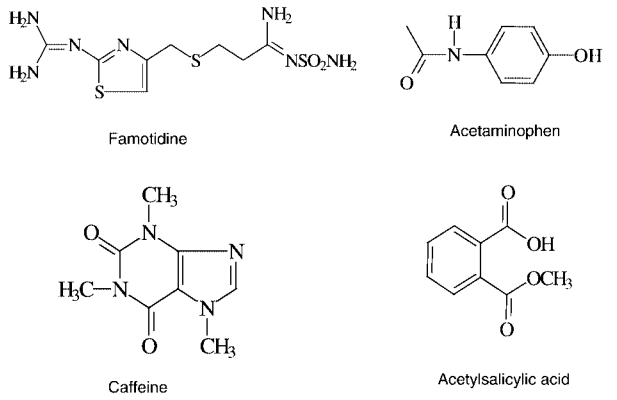

Fig. 1 Molecular structures of famotidine, acetaminophen, caffeine, and aspirin.

Integration time

The effect of integration time on CCD response was studied to obtain optimum conditions for fluorescence quenching HPTLC analysis. Fig. 4 shows four different concentrations of famotidine on a fluorescence quenching HPTLC plate at different integration times. With a short integration time, the ratio of analyte signal-to-background noise was low, as shown in Fig. 4a. As the integration time increases, so does the signal-to-noise ratio for the samples (Table 1), because more of the CCD's pixel well is occupied (Fig. 4b and 4c). When the CCD pixels reach full well capacity, the CCD detector is saturated and the baseline goes flat. Charge then overflows from full pixels into adjacent ones, diminishing sensitivity because the extra charge must flow into pixels where reduced signal from the plate (dim spots) is found (Fig. 4d). At this time, the signal-to-noise ratio is decreased. Optimum signal-to-noise ratio can be achieved just before signal from the fluorescence quenching plate saturates the CCD detector. Because of the dramatic effect on the baseline noise, it is critical to optimize the time conditions to ensure maximum system sensitivity

Quantitation

Quantitation is achieved by detecting and digitally summing the pixels of a reduced (negative) signal at the location of the analyte of interest on the fluorescent quenching plate. Following development, the position of the analyte is identified by linearly scaling the digital image of the TLC plate to highlight the best digital dynamic range. In this manner, the analyte spots are easily distinguished from the background. Once the analyte spots have been identified, cursors are positioned to bracket the top and bottom of the largest analyte spot in a row across the plate. Then each vertical column of pixels is summed so that a numerical value proportional to the total intensity is obtained. By plotting column number against its intensity value, the absorbance of the same analyte across different lanes can be compared. Alternatively, a chromatogram can be generated by bracketing the left and right side of a lane and digitally summing pixels in a horizontal direction. For accurate quantitation, a series of standards is spotted along with the unknown samples on each plate to account for variability between runs.

When summing pixels for peak quantitation, the number of summed pixels must be consistent across the plate in order to compare the spots objectively. This is especially important for fluorescence quenching analysis, as signal from spot-free areas of the plate is high. The analyst must not crop part of the analyte spot, as this results in a decreased signal. Conversely, when the area summed is larger than the analyte spot, the noise is increased. Fig. 5 shows the difference in signal when the number of digitally summed pixels is changed. Maximum signal-to-noise ratio is achieved when the number of pixels summed is equal to the diameter of the largest analyte spot. For the second peak (98.5 ng), the signal-to-noise ratio decreased from 11.6 to 4.9 as the number of pixels summed changed from 40 to 200. Although this is a large difference in the number of summed pixels, it demonstrates the change in sensitivity. As more pixels are summed, the lowest concentration spot is lost in the noise.

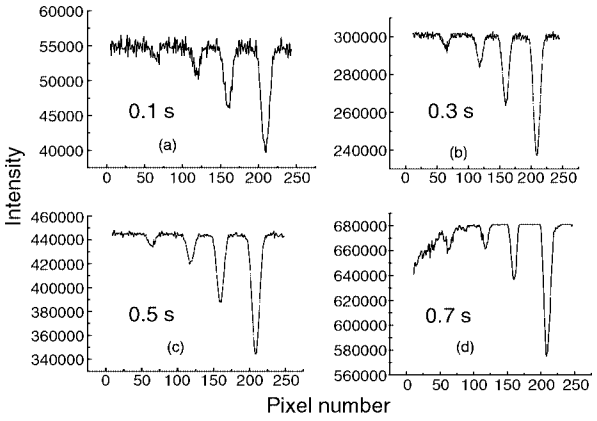

Fig. 4 Influence of integration time on signal in fluorescence quenching HPTLC.

Table 1 Time dependence of signal-to-noise ratio with HPTLC system using fluorescence quenching plates

Time/s	Signal-to-noise
0.1	3.6
0.2	5.9
0.3	7.2
0.4	8.1
0.5	8.9

Analysis of pharmaceuticals

Famotidine, acetaminophen, caffeine, and acetylsalicylic acid were used to test the sensitivity and reproducibility of the HPTLC system. Detection limits, linearity, and quantitation were determined for famotidine with the HPTLC camera system. Two different sample application techniques were employed. In the first technique, a modified HPLC sample injector loop with a nebulizer head deposited the sample onto the chromatographic surface in semi-dry form.[24] Eight samples were applied singly over 10 min for a total sample application time of 80 min. Unfortunately, famotidine has several degradation products,[25–27] and the samples applied first show significant amounts of degradation compared with the samples applied last. In the second sample application technique, micropipettes (0.5 μL) were used to deposit the samples onto the plate. This procedure is more rapid and eliminates the degradation problem associated with sample application by nebulization. However, the micropipette applies the samples over a larger area than those samples applied by nebulization, so detection limits measured on samples applied with micropipettes are higher than those measured on samples applied with a nebulizer. A micropipette was used to apply samples for all subsequent famotidine analyses.

The linearity range was tested to locate useful regions in which to perform quantitative analysis. Detection analytes on a fluorescence quenching plate is analogous to obtaining an absorption measurement, as the technique indirectly detects the

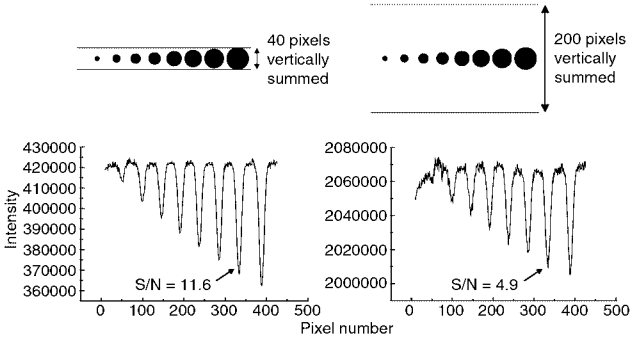

Fig. 5 Effect of pixel summation on signal-to-noise ratio in fluorescence quenching HPTLC.

absorption of 254 nm light. Fig. 6 depicts a plot obtained on an HPTLC fluorescence quenching plate spotted and developed with famotidine. The linear region ranges from the detection limit to more than 400 ng, supplying over one order of magnitude linear range for famotidine. At quantities greater than 450 ng of sample, the plot begins a roll-off similar to those found in other types of absorption analysis. This roll-off is due to the limitation of the absorption process at high concentrations. However, the roll-off is not as drastic as that encountered in conventional absorption analyses because the signal generated results from the absorption of light and spot size. Even at high concentrations, when the plate becomes saturated (*i.e.*, no light is emitted from the center of the chromatographic peak), peaks can be quantitated according to the size of the saturated area, which continues to enlarge with increasing amounts of analyte on the plate.

The detection limit of famotidine was determined at a signal-to-noise ratio of three, with the noise determined at two times the standard deviation of the baseline signal. The experiment was run four times, with an average detection limit of 36.0 ng. The detection limit could be lowered by devising a nebulizer system capable of simultaneously applying multiple samples, eliminating the degradation problem observed when samples were sequentially applied.

Pharmaceutical tablet analysis

Famotidine tablets were analyzed to test the reproducibility and quantitation ability of the system. Quantitation was achieved by plotting known calibration standards on a HPTLC plate with the extracted famotidine samples. Extraction of famotidine from excipients is usually accomplished using a phosphate buffer or glacial acetic acid.[28] Due to the low volatility of aqueous solutions, it is undesirable to use an aqueous phase for an extraction because they produce larger spot sizes when samples are applied to the chromatographic surface. Therefore, several extraction schemes with a significant amount of organic character were attempted. Because famotidine is highly soluble in methanol, a pure methanol extraction was attempted. The extraction by methanol, however, yielded a recovery of only 80.3%, probably because some excipients in the pharmaceuticals did not dissolve completely, binding or entrapping famotidine. Water was then added to the extraction solution to provide an aqueous character. An 80:20 methanol:water extraction yielded a recovery of 93.3%. Extraction efficiency was maximized by using an extraction solution of 80% methanol and 20% 0.2 M phosphate buffer (pH 4.5), yielding recoveries of 98.9%.

The reproducibility of the system was tested by running multiple plates. Each plate contained 7–8 spots of the sample and three calibration standards. The system was found to have a pooled relative standard deviation (RSD) of 2.5%. The results are summarized in Table 2.

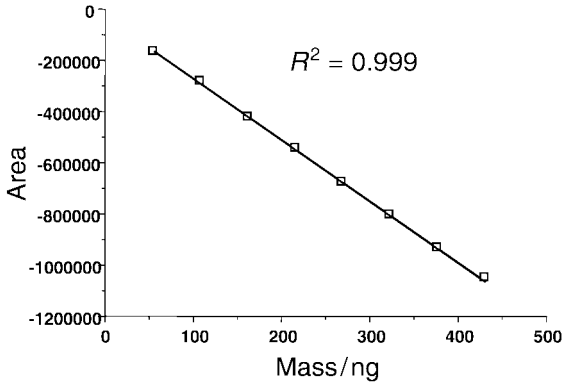

Fig. 6 Linearity of famotidine when analyzed with HTPLC system.

Quality control applications

Ten tablet samples were analyzed to demonstrate quality control applications. Ten individual extractions were performed, with one tablet per extraction, after which two TLC plates were prepared, each plate containing five tablet extracts and three calibration standards. The three-point standard calibration curve consisted of points at 75, 100, and 125% of total famotidine content per tablet (Table 3). With the methanol/phosphate extraction, a total percent recovery of the ten tablets was 98.9% with an RSD of 2.5%.

Sensitivity

The sensitivity of the system was further evaluated by determining detection limits for acetaminophen, caffeine, and acetylsalicylic aicd. Detection limits were determined at a signal-to-noise ratio of three, as in the famotidine determination. Each compound was dissolved in methanol and spotted on the plates by the nebulizer spotting technique. The detection limit was measured for each analyte, and the results were compared with those obtained by using a video framing CCD system (home video camera) with a frame grabber to obtain individual images.[24] This system differs from the scientific CCD system used in this study in that it has only 8-bit grayscale resolution (compared to 14) and is not cooled to decrease dark current generation. The detection limits of the scientifically operated CCD system were in the low nanogram range, as shown in Table 4, and the limits obtained for acetaminophen and caffeine were over one order of magnitude lower in mass than those obtained by the video framing system, which can also be used for HPTLC analysis. It should also be noted that the samples analyzed by the video CCD system were separated on 5 cm × 5 cm HTPLC plates, which have a shorter chromatographic distance and decreased peak width than that of the 10 cm × 10 cm HPTLC plates used with the current CCD system.

Table 2 Reproducibility of famotidine analysis using HPTLC (200 ng samples)

Trial	*N*	RSD (%)
1	7	1.37
2	8	1.88
3	7	2.87
4	7	2.78
5	7	2.27
Pooled	36	2.50

Table 3 Extraction of famotidine from tablets

Extraction	Percent recovery	RSD (%)
100% Methanol	80.5	2.0
80% Methanol + 20% water	93.5	5.0
80:20 Methanol:phosphate (pH 4.5)	98.9	2.5

Table 4 Detection limits for fluorescence quenching HPTLC

Compound	Video framing CCD[19]/ng	Scientific CCD/ng
Acetaminophen	50 (100)	4.2 (8.4)
Caffeine	200 (400)	12.5 (25.0)
Acetylsalicylic acid	300 (600)	71.1 (142.2)

Detection limits are based on the mobility of the analyte on the plate and on the amount of light absorbed (molar absorptivity at 254 nm and emitted light from the chromatographic surface). Fig. 7 illustrates the UV/Vis absorptions of famotidine, acetaminophen, caffeine, and acetylsalicylic acid. The spectra show that not all analytes have absorbance maxima at the wavelength of interest (254 nm). The detection limit should be maximized when the molar absorptivity of analytes at the wavelength of interest is maximized. The sensitivity of the system to each compound could be increased by incorporating fluorophores and/or phosphors with the same absorbance maxima as the compounds being analyzed into the chromatographic stationary phase.

Conclusions

Combining a scientifically operated charge-coupled device detector for the detection of fluorescence quenching high-performance thin-layer chromatographic plates has proven to be useful for the analysis of pharmaceuticals. The detection limit of famotidine established by this system was 36.0 ng, with a reproducibility of 2.5% for 200 ng samples. Detection limits were also determined for acetaminophen, caffeine, and acetylsalicylic acid, with values found in the low nanogram region. The use of a fluorescence quenching HPTLC system could save manufacturers time and money, as HPTLC coupled with CCD imaging detecting provides the high throughput, sensitivity, and reproducibility required for routine quality control analysis and has the added benefits of reduced solvent consumption and total sample accountability, not supplied by other separation techniques.

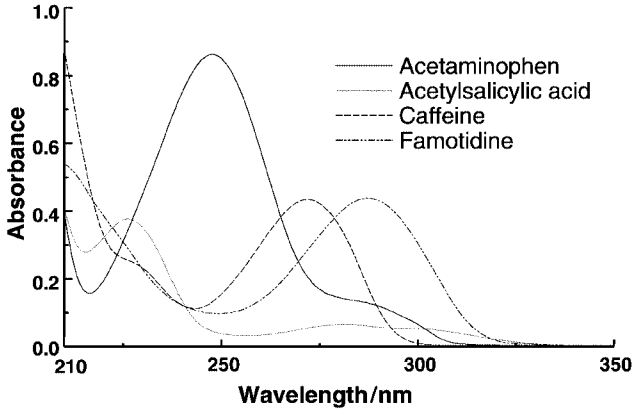

Fig. 7 UV spectra of studied compounds.

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References

1 *Densitometry in Thin-Layer Chromatography: Practice and Applications*, ed. J. Touchstone and J. Sherma, John Wiley and Sons, New York, 1979.
2 Y. Liang and M. B. Denton, *Recent Developments in Scientific Optical Imaging*, ed. M. B. Denton, R. E. Fields and Q. S. Hanley, The Royal Society of Chemistry, Cambridge, 1996, pp. 161–169.
3 Y. Liang, M. E. Baker, B. T. Yeager and M. B. Denton, *Anal. Chem.*, 1996, **68**, 3885.
4 J. Summanen, T. Yrjonen, R. Hiltunen and H. Vuorela, *J. Planar Chromatogr.—Mod. TLC*, 1998, **11**, 421.
5 M. Petrovic, M. Kastelan-Macan, K. Lazaric and S. Babic, *J. AOAC Int.*, 1999, **82**, 25.
6 M. Petrovic, M. Kastelan-Macan and S. Babic, *J. Planar Chromatogr.—Mod. TLC*, 1998, **11**, 421.
7 I. Vovk and M. Prosek, *J. Chromatogr. A*, 1997, **779**, 329.
8 I. Vovk and M. Prosek, *J. Chromatogr. A*, 1997, **768**, 329.
9 J. C. Guilleux, K. N. Barnouin, F. A. Ricchiero and D. A. Lerner, *J. Liq. Chromatogr.*, 1994, **17**, 2821.
10 F. G. Sanchez, A. N. Diaz and M. R. F. Correa, *J. Chromatogr. A*, 1993, **655**, 31.
11 S. M. Brown and K. L. Busch, *J. Planar Chromatogr.—Mod. TLC*, 1992, **5**, 338.
12 P. K. Aldridge, J. B. Callis and D. H. Burns, *J. Liq. Chromatogr.*, 1990, **13**, 2829.
13 *Handbook of Thin-Layer Chromatography*, ed. J. Sherma and B. Fried, Marcel Dekker, Inc., New York, 1996.
14 H. Nakamura, *J. Chromatogr.*, 1974, **96**, 195.
15 Z. Tamura, *Talanta*, 1972, **19**, 573.
16 *The United States Pharmacopeia*, The USP Pharmacopeial Convention, Rockville, 1990, XII revision, pp. 559–560.
17 B. Guverner and S. Ates, *Acta Pharm. Turc.*, 1998, **67**, 30.
18 B. A. Bidlingmeyer, J. K. Del Rios and J. Korpi, *Anal. Chem.*, 1982, **54**, 442.
19 W. C. Vincek, M. L. Constanzer, G. A. Hessey and W. F. Baye, *J. Chromatogr.*, 1985, **338**, 438.
20 S. E. Biffar and D. J. Mazzo, *J. Chromatogr.*, 1986, **363**, 243.
21 J. Parasrampouria and V. Dasgupta, *Drug Dev. Ind. Pharm.*, 1989, **15**, 1977.
22 N. Beaulieu, S. J. Graham, R. W. Sears and G. Lovering, *J. Pharm. Biomed. Anal.*, 1989, **7**, 1705.
23 J. Sherma and C. D. Rolfe, *J. Planar Chromatogr.*, 1993, **5**, 197.
24 B. T. Yeager, University of Arizona, USA, 1995.
25 G. Junnarkar and S. Stavchjansky, *Anal. Lett.*, 1992, **25**, 1907.
26 B. V. Kamath, K. Shivram, B. L. Newalker and A. C. Shah, *J. Liq. Chromatogr.*, 1993, **16**, 1007.
27 H. C. Hsu, C. F. Wang and C. S. Lo, *Chin. Pharm. J.*, 1994, **46**, 33.
28 *The United States Pharmacopeia*, The USP Pharmacopeial Convention, Rockville, 1990, XXII revision, USP 23-BF 18.