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New concept for HPTLC peak purity assessment and identification of drugs in multi-component mixtures

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ABSTRACT

Simple methods for HPTLC peak purity assessment and identification of the HPTLC peaks were presented. The spectrodensitograms – selected at different time intervals across the elution time of the HPTLC peak – were extracted and digital algorithms for manipulating the data were carried out in the wavelength domain. Three different methods were developed for testing the HPTLC peak purity using the mathematically transformed data of the spectrodensitograms. These included the method of relative absorption, the method of $\log A$ versus the wavelength plots and the derivative (first, second, third and fourth) method. The identification of the HPTLC peaks was based on the use of the derivative profile of the spectrodensitogram and the derivative ratios as fingerprints for the compounds. The wavelengths of absorbance and derivative (first, second, third and fourth) optima of the extracted spectrodensitograms were allocated. The data were compared with those obtained using the corresponding reference standard. The validity of the proposed methods was performed by chromatography of a mixture containing mebendazole and methylparaben as a model versus the winCATS® spectral correlation method as a reference method. The study indicated that the proposed concept is a reliable non-confusing valuable tool for testing the purity and identity of the HPTLC peaks as the results are easily and rigorously interpreted.

1. Introduction

In all chromatographic procedures, peak purity assessment has become a crucial step in the analysis to ensure the reliability of the results. High performance thin layer chromatography (HPTLC) is a sophisticated instrumental technique based on the full capabilities of thin layer chromatography. The advantages of automation, scanning, full optimisation, minimum sample preparation and the hyphenation of HPTLC makes it a powerful analytical tool for chromatographic information of complex mixtures of inorganic, organic and pharmaceutical compounds [1]. In HPTLC, two closely related compounds may not be sufficiently resolved and hence migrate as a single peak. This situation would result into seriously misleading results in quantitative analysis, especially if one of the non-resolved compounds is an unknown impurity or a degradation product. Two-dimensional TLC is a classical approach that has been used to improve the resolution of closely related compounds and separate them into distinct pure peaks [2]. The literature included some techniques to confirm the identity of the resolved HPTLC peaks of the tested compounds. These included the comparison of the UV-absorption spectra obtained by direct solid-phase spectrodensitometry with those obtained from solutions of their reference standards [3]. Another technique was the identification of the separated compounds using the online coupling of TLC and FTIR spectroscopy [4]. TLC coupled to high-speed counter-current chromatography provided useful information about the peak purity and the separation achievement [5].

Recent advances in spectrodensitometric instruments have allowed simultaneous measurements at several wavelengths using multi-channel scanners or diode-array scanners, hence allowing chemometric treatment of the results for peak purity monitoring [6]. The CAMAG TLC scanner 3 allows the recording of the UV-absorption spectra of the resolved compounds at several points across each peak, among which the point of peak start slope, the point of zero slope (peak maximum) and the point of peak end slope. The spectral data is handled through winCATS® software in order to evaluate the purity of the resolved spots through two processes. The first process is the calculation of the correlation coefficients between the spectra extracted at the peak start slope and the peak maximum ($r_{s,m}$) and the correlation coefficient between the spectra extracted at the peak maximum and the peak end slope ($r_{m,e}$). The equation used to calculate $r_{s,m}$ and $r_{m,e}$ is given below:

$$r = \frac{\sum_N (x_i - \bar{x})(y_i - \bar{y})}{\sqrt{\sum_N (x_i - \bar{x})^2 \sum_N (y_i - \bar{y})^2}}$$

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where N is the number of the measured data points of the two spectra to be correlated, x_i is the data point of the first spectrum at wavelength i, $\bar{x}$ is the mean of all data points of the first spectrum, y_i is the data point of the second spectrum at wavelength i and $\bar{y}$ is the mean of all data points of the second spectrum.

The second process is to interpret mathematically the significance of the correlation values in order to assess the purity of the peak. The software provides two mathematical approaches to compare the extracted spectra and their correlation data. The first approach is to set a predetermined correlation limit, so that if both $r_{s,m}$ and $r_{m,e}$ exceed this limit, the peak is considered spectrally pure. The second approach is to apply the null hypothesis with two-sided significance and an error probability of 1%. The correlated spectra are considered identical, and hence the peaks spectrally pure, only if the calculated z value is lower than the theoretical one (2.567). The z value is calculated by the following equation:

$$z = \frac{|\ln((1 + r_{s,m})/(1 - r_{s,m})) - \ln((1 + r_{m,e})/(1 - r_{m,e}))|}{2\sqrt{2/(N-3)}}$$

where $r_{s,m}$ is the correlation of the peak start spectrum to the peak maximum spectrum, $r_{m,e}$ is the correlation of the peak maximum spectrum to the peak end spectrum and N is the number of the measured data points of the two spectra to be correlated.

A serious limitation of the two approaches is encountered when two compounds are completely non-resolved from each other and constituting a single homogeneous spot of both compounds. In this case, both peak start and peak end spectra are well correlated to the peak maximum spectrum and hence the spot is judged to be spectrally pure. However, if the two components are heterogeneously distributed throughout the single spot, the peak start and peak end spectra are different and show poor correlation to the peak maximum spectrum. An opposite situation – in which spectrally pure spots are judged impure – may be also encountered with the two approaches of the spectral correlation method; the correlation limit and the null hypothesis. In case of the correlation limit approach, the peak start or peak end spectra may show good correlation to the peak maximum spectrum but fail to exceed a too high correlation limit (0.9999 for example). On the other hand, lowering the correlation limit is accompanied by higher risk of ignoring significant spectral differences. Therefore, the choice of the correlation limit should be the subject of a balanced compromise depending on the optimum experimental conditions of each assay. In case of the null hypothesis, the better the correlation of the start to maximum spectra, the narrower the range allowed for the correlation of the maximum to end spectra to conclude the spectral purity of the spot. This means that the spot may be judged spectrally impure just because the peak end spectrum failed to correlate to the peak maximum spectrum as well as did the peak start spectrum, although its spectrum is individually well correlated to the peak maximum spectrum. For this reason, the manual of the software warns the users that: "*if the purity results show 'fail' for a very good correlation, this is not caused by a calculation error in winCATS, but by the statistical interpretation of the available data*".

During the last ten years, HPTLC – as an analytical tool – has been applied to the development of stability-indicating methods for determination of drugs in their pharmaceutical preparations [7]. The technique has been also applied to the determination of drugs in bio-matrices [8]. Introduction of the modern fibre optic TLC scanner with a DAD has several advantages including that the scanner could measure TLC plates simultaneously at different wavelengths without destroying the plate surface and permits parallel recording of chromatograms and in situ UV spectra in the range of 191–1033 nm [9]. The aim of the present study is to introduce a new concept for assessment of the HPTLC spectral peak purity and identification of the compounds due to the pure peaks. The new concept is based on the comparison of the spectral features of the spectrodensitograms extracted at five points across the HPTLC peak. The concept included mathematical transformation of the data of the spectrodensitograms in the wavelength domain. Mathematical transformation included the development of three proposed methods; the relative absorbance method, the log A versus the wavelength plots method and the derivative spectrodensitograms method. Identification of the compound of an HPTLC peak is carried out through allocation of wavelengths of derivative optima and derivative ratios of the extracted spectrodensitograms. The new concept is applied to a mixture containing mebendazole and methylparaben as a model versus the winCATS® spectral correlation method as a reference method.

2. Experimental

2.1. Materials and reagents

Mebendazole (MBZ) and methylparaben (MP) were of USP reference standard grade. Methanol, ethyl acetate, formic acid (85%) and ammonia solution (27%) were of analytical reagent grade (Merck, Darmstadt, Germany).

HPTLC silica gel precoated aluminum Plate 60 F_{254} plates (20 cm × 20 cm with 250 μm thickness; E. Merck, Darmstadt, Germany) were used. The plates were prewashed with methanol and activated at 110 °C for 15 min prior to chromatography.

2.2. Instrumentation

TLC-spectrodensitometric measurements were carried out using a CAMAG Linomat TLC-Applicator IV. A CAMAG Linomat syringe (100 μL) was used for application of solutions onto the silica gel plates. The plates were developed in CAMAG chromatographic tanks (15 × 20 × 30), and scanned densitometrically using CAMAG TLC-Scanner 3 (Version 4.06), supported with UV-lamp and interfaced to an IBM computer loaded with CAMAG-TLC-Software (winCATS®) and connected to a Hewlett-Packard laser jet 1100 printer.

2.3. Preparation of solutions

MBZ stock standard solution was prepared by weighing 75 mg of MBZ reference standard into a 25 mL volumetric flask. The powder was dissolved in 5 mL of formic acid (85%, w/v) and diluted to volume with methanol. MBZ working standard solution (300 μg mL^{-1}) was prepared by diluting a 1 mL aliquot of the stock standard solution to 10 mL with methanol (MBZ standard solution).

MP stock standard solution (1 mg mL^{-1}) and MP standard solution (100 μg mL^{-1}) were prepared in methanol.

MBZ standard mixture was prepared to contain 300 μg mL^{-1} of MBZ and 100 μg mL^{-1} of MP by suitable dilution of their stock standard solutions.

2.4. Procedure and treatment of data

A 5 μL aliquot of each of the prepared solutions was automatically applied onto HPTLC plates (20 cm × 20 cm) using CAMAG-Linomat TLC applicator IV as 5 mm wide bands. A constant application rate of 0.1 μL s^{-1} was used and the space between two bands was 15 mm. The slit dimension was kept at 5 mm × 0.45 mm and the scanning speed was 10 mm s^{-1}. The monochromator bandwidth was set at 20 nm. Two chromatographic systems were used for the development of the HPTLC plates. The first system (system I) consists of a mobile phase composed of ethyl acetate–methanol–27% ammonia solution 23:1:1

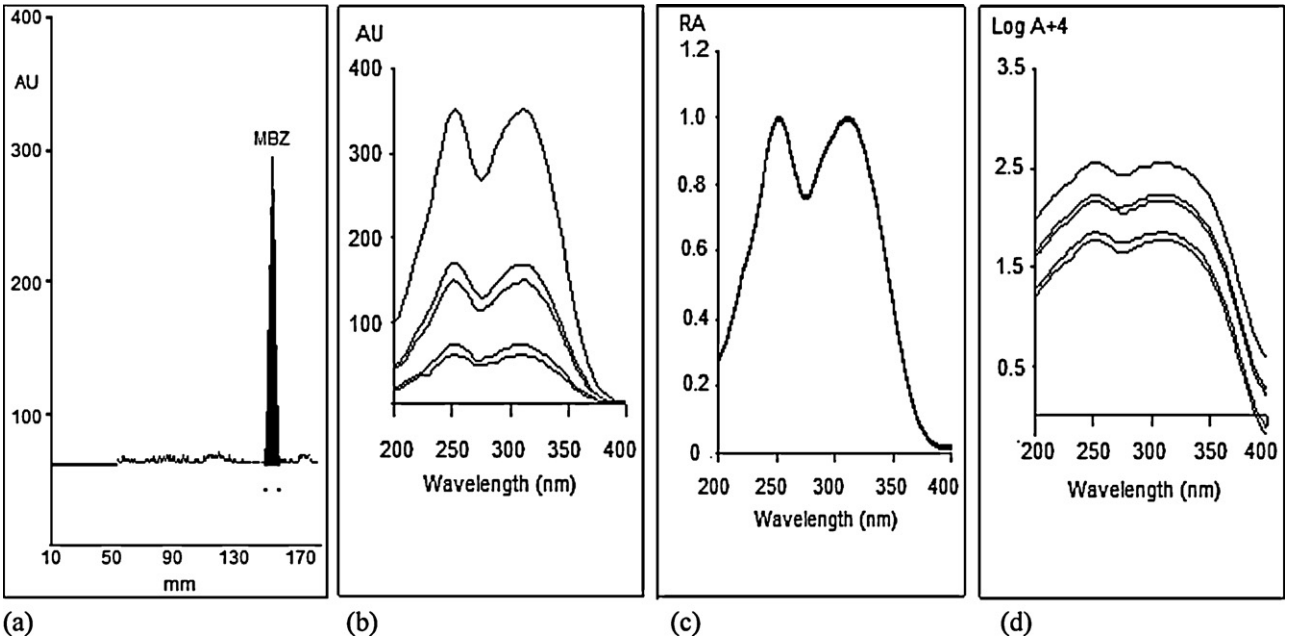

Fig. 1. (a) HPTLC densitogram obtained from MBZ [the mobile phase was ethyl acetate–methanol–27% ammonia solution 23:1:1 (v/v)], (b) its spectrodensitograms at the five points, (c) their RA spectra and (d) their log *A* versus the wavelength plots.

(v/v). The mobile phase of the second system (system II) is composed of ethyl acetate–methanol–27% ammonia solution 22:2:1 (v/v). The plates were developed by ascending technique and dried. The TLC plates were scanned spectrodensitometrically in the absorption mode in the wavelength of 200–400 nm at a speed of 10 mm s^{-1}. The bandwidth was 5 nm and slit dimension was 5 mm × 0.45 mm. The spectrodensitograms extracted at five points throughout the retention distance of each peak were computed. The five points included the peak start, maximum ascending slope, zero slope, maximum descending slope and peak end. The data of the extracted spectrodensitograms i.e. absorbance readings and their corresponding wavelengths were filtered into a designed computer program directly attached to the data station. The program was designed to compute the relative absorption spectra, the log *A* versus the wavelength plots, the first, the second, the third and the fourth derivative spectra of the extracted spectrodensitograms. The first, second, third and fourth derivative spectra were constructed at 2 nm intervals. The wavelengths of derivative optima and their derivative values were filtered into a designed computer program directly attached to the data station where the derivative ratios at the selected optima were computed.

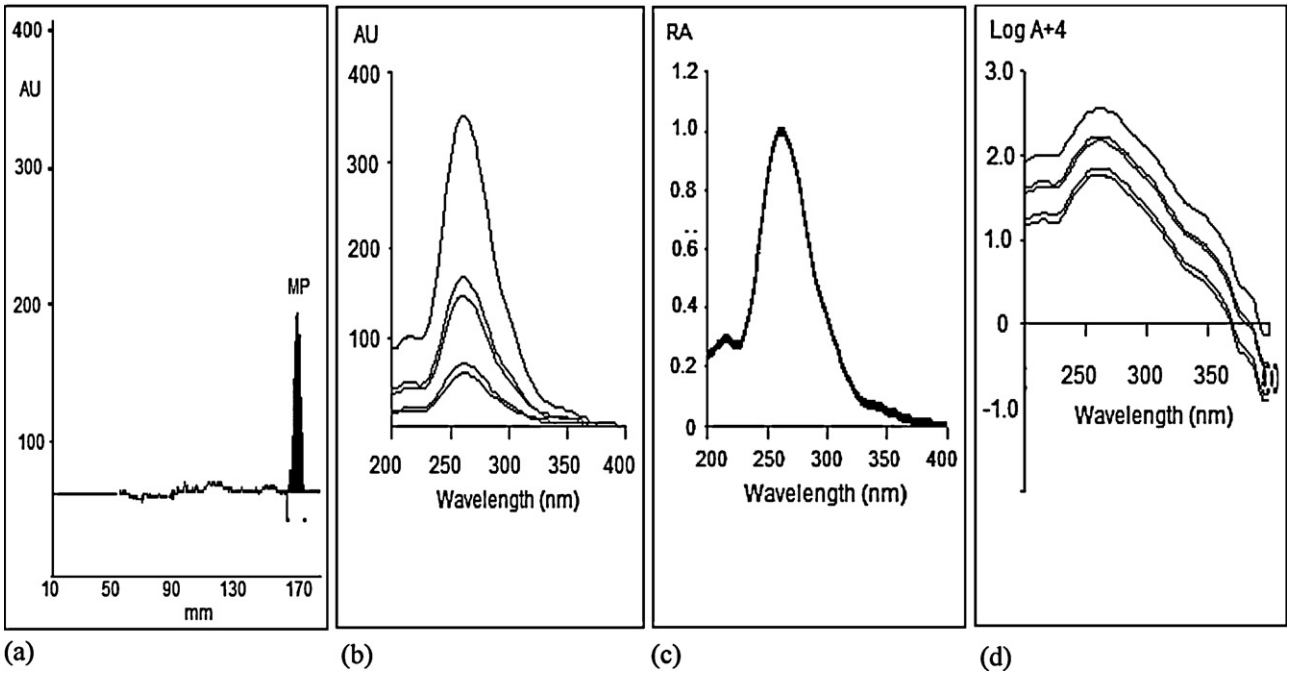

Fig. 2. (a) HPTLC densitogram obtained from MP [the mobile phase was ethyl acetate–methanol–27% ammonia solution 23:1:1 (v/v)], (b) its spectrodensitograms at the five points, (c) their RA spectra and (d) their log *A* versus the wavelength plots.

3. Results and discussion

3.1. Theoretical background of the proposed concept

Collecting complete spectral data and mathematically comparing spectra within an HPTLC peak can be an effective way to assess peak purity and identity of the compound of such peak using an HPTLC instrument equipped with multi-channel or DAD detectors. The proposed method for testing the spectral purity of the HPTLC peaks of the separated compounds was based on the comparison of the spectral features of the spectrodensitograms extracted at the five selected points throughout the peak. The points included the peak start point, the peak maximum ascending slope, the peak zero slope, the peak maximum descending slope and the peak end point. The extracted spectra were compared with each other and with that of a reference standard of the same compound. The spectral peak purity assessment was carried out through further treatment of the spectral data using three different methods.

3.1.1. The relative absorption spectra method

The method depended on construction of the relative absorption spectra (RAS) for the spectrodensitograms in the wavelength domain. The relative absorption spectrum was obtained according to the relationship:

$$RA_{\lambda_i} = \frac{A_{\lambda_i}}{A_{\lambda_{max}}}$$

where RA_{λ_i} is the relative absorbance at wavelength i, and A_{λ_i} is the absorbance reading at wavelength i, $A_{\lambda_{max}}$ is the absorbance reading at maximum wavelength.

The spectrodensitograms i.e. the absorption spectra extracted at different intervals throughout the HPTLC peak are dependent on concentration while the RAS are independent of concentration. Consequently, the superimposed RAS was considered as an evidence for the spectral purity and homogeneity of the HPTLC peak. The co-migration of an absorbing impurity and/or compound with the main compound as a single sharp peak would result into distortion of the RAS. The RAS would not be superimposed and hence the corresponding HPTLC peak would be impure. Generally, on chromatography of a multi-component mixture using HPTLC equipped with a multi-channel detector or DAD, a selected peak is considered pure if the RAS obtained from its spectrodensitograms are superimposed on each other and on the RAS of the spectrodensitogram of the corresponding reference standard. It should be mentioned that the RAS method has advantages over the absorbance ratio method commonly applied in UV-spectrophotometry and known as spectrophotometry purity index [10]. These advantages could be summarized in the following points:

a. The spectrophotometry purity index depends on calculations using the absorbance readings at only two wavelengths of the absorption spectrum but in RAS method, the ratios are calculated at each individual wavelength.
b. For compounds with one absorption maximum or in case where the value of absorbance at λ_{min} is small, the possibility of error in calculation of the spectrophotometry purity index is high.
c. The possibility that the irrelevant absorbance due to the impurities would not contribute to analyte absorbance at λ_{max} and λ_{min} – although it is a rare case – would result in to misleading results. The situation is avoided by application of the RAS method.

3.1.2. Plots of log A versus the wavelength method

The principle of the plots of log A versus the wavelength in UV spectrophotometry was discussed [10]. The application of the method to HPTLC depended on construction of the log A versus the wavelength plot for each of the extracted spectrodensitograms. Superimpose of traces of the constructed plots was taken as an evidence that the HPTLC peak was pure and homogenous. The presence of absorbing impurities that co-migrate with the main compound as a single sharp peak would result into plots whose traces are not superimposed and hence the HPTLC peak would be impure. Generally, on chromatography of a multi-component mixture using HPTLC equipped with a multi-channel detector or DAD, a peak is considered pure when the traces of log A versus the wavelength plots of its spectrodensitograms are superimposed on each other and on that obtained from the spectrodensitogram of the corresponding reference standard.

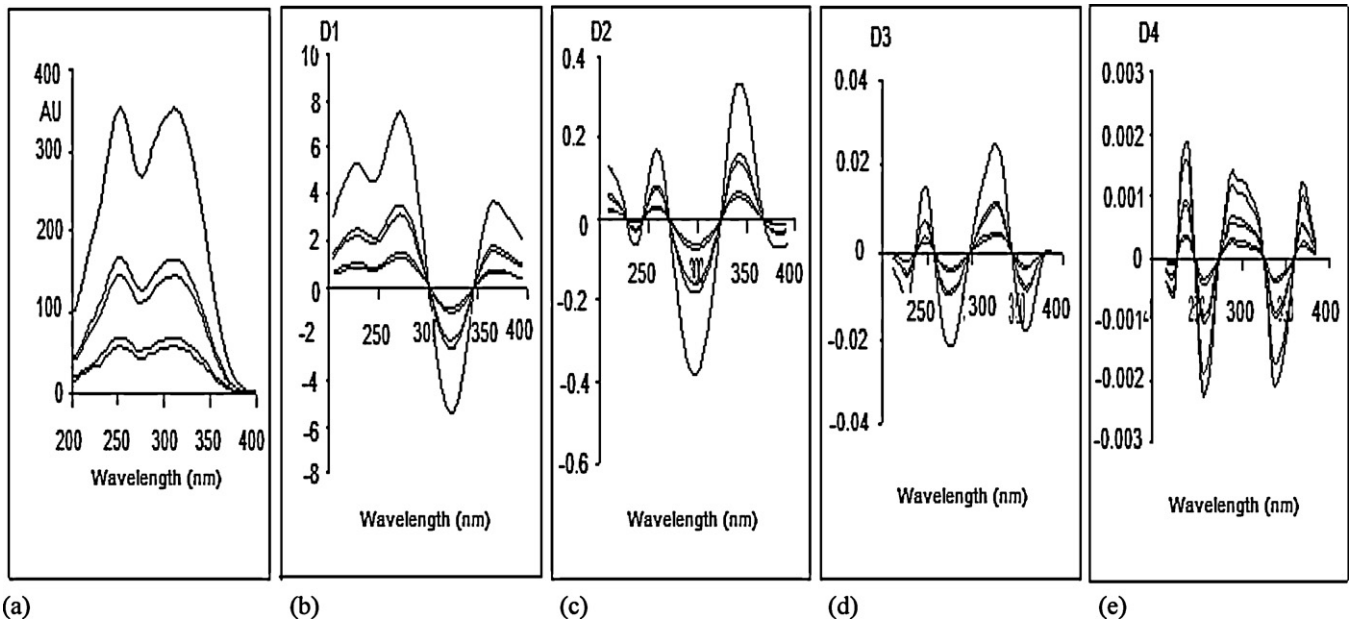

Fig. 3. (a) Spectrodensitograms of MBZ at the five points, (b) their first derivative, (c) their second derivative, (d) their third derivative and (e) their fourth derivative spectra.

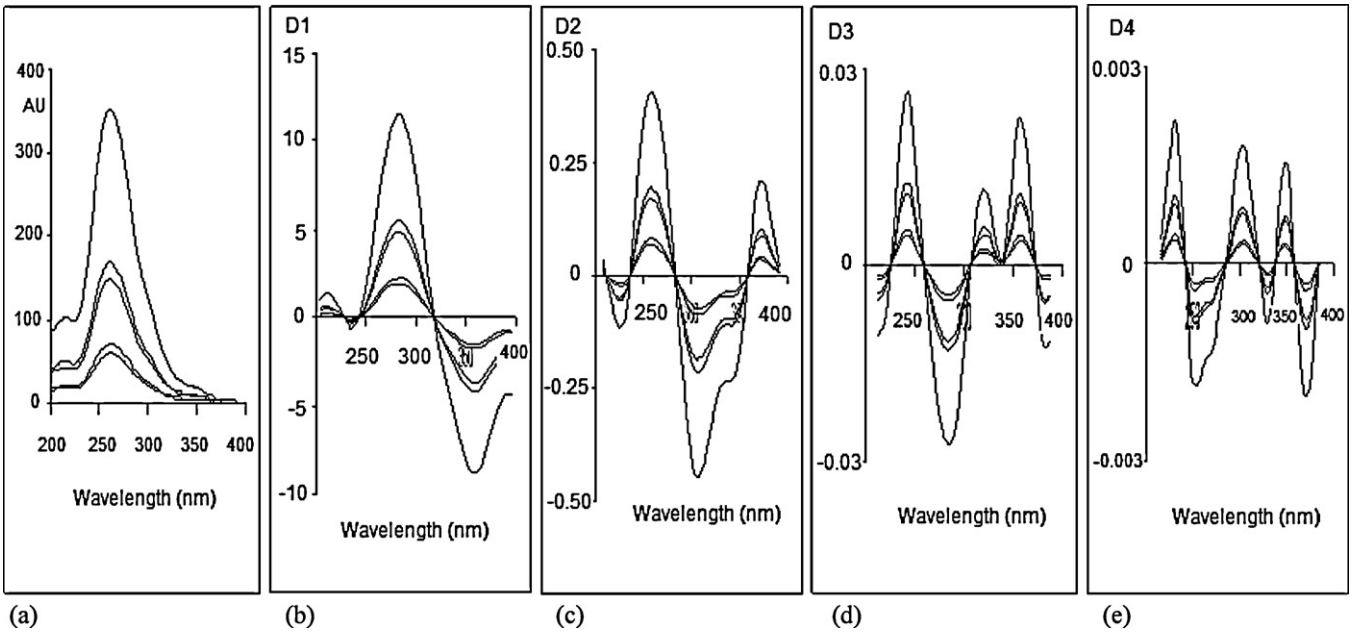

Fig. 4. (a) Spectrodensitograms of MP at the five points, (b) their first derivative, (c) their second derivative, (d) their third derivative and (e) their fourth derivative spectra.

3.1.3. The derivative – in the wavelength domain – method

The method depended on construction of the first, second, third, and fourth derivative spectra with respect to wavelength for the spectrodensitograms extracted at different time intervals throughout the HPTLC peak. Generally, the purity of an HPTLC peak obtained from chromatography of a multi-component mixture using the proposed derivative – in the wavelength domain – method is indicated by the following characteristic features:

a. The spectrodensitograms of the investigated HPTLC peak are similar to each other and to that obtained from the spectrodensitogram of the corresponding reference standard.
b. The wavelengths of absorption and derivative (^{1}D, ^{2}D, ^{3}D, ^{4}D) optima of the extracted spectrodensitograms of the investigated HPTLC peak are identical to each other and to those of the spectrodensitogram of the corresponding reference standard.
c. The derivative (^{1}D, ^{2}D, ^{3}D, ^{4}D) spectra of the same order of the extracted spectrodensitograms of the investigated HPLC peaks intersected at the same wavelengths.
d. The relative standard deviations values of the calculated derivative ratios of the same order of the reference standard spectrodensitograms must not exceed 2%.
e. The calculated derivative ratios values for each spectrodensitogram of the investigated HPTLC peak must not deviate from the corresponding mean derivative ratio of the reference standard by more than 3%.

The presence of co-eluting absorbing impurities with the HPTLC peak could be easily detected as the extracted spectrodensitograms and their derivative spectra would not fulfil the aforementioned requirements.

An additional proof of the identity of the resolved peaks was based on the use of derivative profile and derivative ratios as fingerprints for each compound [11]. The method depended on the allocation of the wavelengths of absorption and derivative (first, second, third and fourth) optima of the UV-absorption spectra extracted at the five selected points throughout the peak. The ratios of the derivative (first, second, third and fourth) optima of the UV-absorption spectra extracted at the five selected points across the HPTLC peak on the chromatogram were calculated. The allocated derivative optima and the calculated derivative ratios for each individual peak in the mixture were compared with those obtained from the corresponding reference standards treated similarly.

3.2. Validity of the new concept

The validity of the new concept was tested by its application to the assessment of the spectral purity of the peaks obtained from the HPTLC of the standard solutions of MBZ, MP and a mixture of both using the two chromatographic systems. System I, using a mobile phase consisting of ethyl acetate:methanol:27% ammonia solution 23:1:1 (v/v), provided complete resolution of MBZ and MP as two distinct peaks. Application of the proposed method for peak purity assessment included the construction of reference spectra for MBZ and MP by chromatography of solutions of their corresponding reference standards separately (Figs. 1a and 2a). The spectrodensitograms i.e. the UV-absorption spectra were extracted at the five selected points across each peak (Figs. 1b and 2b). The relative absorption spectra (Figs. 1c and 2c) and the log$A$ versus the wavelength plots of the extracted spectrodensitograms were computed (Figs. 1d and 2d). The derivative (first, second, third and fourth) spectra (Figs. 3 and 4) of the extracted spectrodensitograms were constructed and the wavelengths of absorption and derivative optima were recorded (Table 1). The first, second, third, and fourth derivative ratios were computed (Table 2). The results obtained upon application of the method to the peaks obtained from chromatography of a mixture containing MBZ and MP proved that the peaks due to the resolved compounds (Fig. 5a) were spectrally pure as indicated by the following observations:

a) The spectrodensitograms extracted at the five selected points across each of the resolved peaks were similar to each other and to that extracted from the peak of the corresponding reference standards (Fig. 5b and c).
b) The RAS obtained from the spectrodensitograms extracted at the five selected points across each of the resolved peaks were superimposed on each other and on that obtained from the corresponding reference standard. The spectra were similar to those of the reference standards (Figs. 1c and 2c).

Table 1
Wavelengths of the absorption and derivative (first, second, third, and fourth) optima of the spectrodensitograms extracted at the five selected points across the peak obtained from the HPTLC densitograms of mebendazole and methylparaben reference standards.

Compound	Wavelengths of absorption optima (nm)	Wavelengths of the main derivative optima (nm)			
		First	Second	Third	Fourth
Mebendazole	252	+228	+264	−228	+240
	312	+276	−296	+248	−256
		−324	+344	−276	+288
		+364		+330	−366
				−364	+368
Methylparaben	262	+284	−228	+244	+232
		−356	+256	−284	−256
			−304	+324	+304
			−344	+356	−328
			+376	−380	+348
					−368

+, optimum on the positive side of the spectrum; −, optimum on the negative side of the spectrum.

Table 2
Ratios of the first, second, third, and fourth derivative optima of the spectrodensitograms of mebendazole and methylparaben reference standards extracted at the five selected points across the migration distance of the HPTLC peaks.

Selected point	Compound							
	Mebendazole				Methylparaben			
	$^{1}D_{276/324}$	$^{2}D_{296/344}$	$^{3}D_{276/330}$	$^{4}D_{240/256}$	$^{1}D_{284/356}$	$^{2}D_{256/304}$	$^{3}D_{244/284}$	$^{4}D_{348/368}$
Peak start slope	1.343	1.041	0.875	0.864	1.437	0.909	0.969	0.758
Maximum ascending slope	1.339	1.037	0.860	0.860	1.433	0.900	0.978	0.750
Peak maximum	1.331	1.045	0.879	0.854	1.451	0.913	0.962	0.763
Maximum descending slope	1.329	1.048	0.882	0.873	1.439	0.917	0.957	0.753
Peak end slope	1.322	1.030	0.870	0.869	1.429	0.902	0.972	0.765
Mean	1.333	1.040	0.873	0.864	1.438	0.908	0.968	0.758
SD	0.008	0.007	0.009	0.007	0.008	0.007	0.008	0.006
RSD (%)	0.600	0.673	1.031	0.810	0.556	0.773	0.826	0.792

c) The traces of log *A* versus the wavelengths of the extracted spectrodensitograms were superimposed with each other and with that extracted from the peak of the corresponding reference standards. The plots were exactly similar to those of the reference standards (Figs. 1d and 2d).

d) The derivative spectra (first, second, third and fourth order) of the extracted spectrodensitograms were intersected with each other and with that of the corresponding reference standard at the same wavelengths.

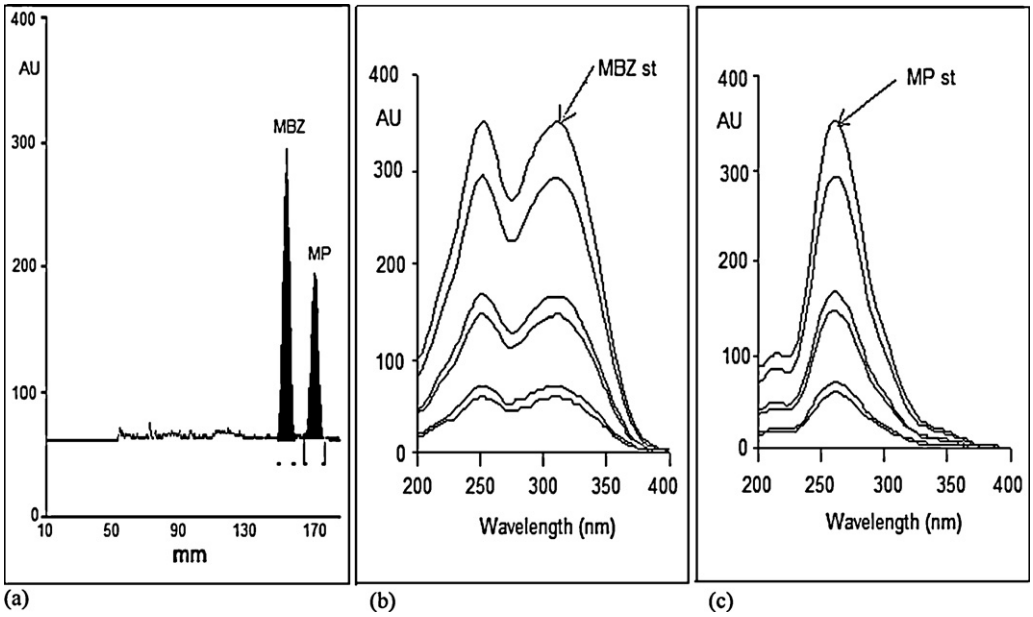

Fig. 5. (a) HPTLC densitogram obtained from a mixture containing MBZ and MP [the mobile phase was ethyl acetate–methanol–27% ammonia solution 23:1:1 (v/v)], (b) the spectrodensitograms of the separated peak correspond to MBZ overlaid with its reference standard spectrum (MBZ st) and (c) the spectrodensitograms of the separated peak correspond to MP overlaid with its reference standard apex spectrum (MP st).

Table 3
Ratios of the first, second, third, and fourth derivative optima of the spectrodensitograms of the HPTLC peaks obtained from chromatography of a mixture containing MBZ and MP using system I extracted at the five selected points across the peak.

Selected point	Compound							
	Mebendazole				Methylparaben			
	$^1D_{276/324}$	$^2D_{296/344}$	$^3D_{276/330}$	$^4D_{240/256}$	$^1D_{284/356}$	$^2D_{256/304}$	$^3D_{244/284}$	$^4D_{348/368}$
Peak start slope	1.349 (1.200)	1.016 (2.308)	0.855 (2.062)	0.844 (2.315)	1.480 (2.921)	0.923 (1.652)	0.939 (2.996)	0.778 (2.639)
Maximum ascending slope	1.321 (0.900)	1.022 (1.731)	0.860 (1.135)	0.880 (1.852)	1.421 (1.183)	0.897 (1.211)	0.984 (1.653)	0.745 (1.715)
Peak maximum	1.339 (0.450)	1.054 (1.346)	0.879 (0.687)	0.854 (1.157)	1.451 (0.904)	0.913 (0,551)	0.962 (0.620)	0.763 (0.660)
Maximum descending slope	1.354 (1.575)	1.027 (1.025)	0.888 (1.718)	0.851 (1.504)	1.449 (0.765)	0.917 (0.991)	0.953 (1.550)	0.769 (1.451)
Peak end slope	1.311 (1.650)	1.061 (2.019)	0.891 (2.062)	0.887 (2.662)	1.411 (1.878)	0.927 (2.093)	0.944 (2.479)	0.739 (2.507)

The figures between parentheses are the percentage relative deviation of the test ratios at the selected points from the mean of reference standard ratios.

e) The wavelengths of the absorption and derivative optima of the extracted spectrodensitograms were identical to each other and to that of the corresponding reference standard.
f) The calculated test ratios (Table 3) of the first, second, third and fourth derivative optima of the extracted spectrodensitograms were not deviated from the mean ratios of the corresponding reference standard (Table 2) by more than 3%.

The results were in good agreement with those obtained from the application of the winCATS® software spectral correlation methods. The winCATS® software spectral correlation method depended on the correlation of the peak start spectrum to the peak maximum spectrum ($r_{s,m}$) and the correlation of the peak maximum spectrum to the peak end spectrum ($r_{m,e}$). The correlation values for the peak due to MBZ were $r_{s,m} = 0.999748$ and $r_{m,e} = 0.999805$. The correlation values for the peak due to MP were $r_{s,m} = 0.999824$ and $r_{m,e} = 0.999798$. Both peaks were judged as spectrally pure using both the null hypothesis at 99% confidence limit and the correlation limit approach at 0.999 correlation limit.

The second HPTLC system included the use of a mobile phase consisting of ethyl acetate–methanol–27% ammonia solution 22:2:1 (v/v). Using such a mobile phase, MBZ and MP were completely non-resolved from each other, migrating simultaneously and resulting into a single spot as if they were a single component (Fig. 6a). The results obtained upon application of the method to the peak obtained from chromatography of the mixture containing MBZ and MP proved that the peak due to the non-resolved compounds (Fig. 6a) was spectrally impure as indicated by the following observations:

a) The UV-absorption spectra extracted at the five selected points across the peak were not similar to each other (Fig. 6b).
b) The relative absorption spectra of the extracted UV-absorption spectra were not superimposed on each other (Fig. 6c).
c) The traces of log A versus the wavelengths of the extracted UV-absorption spectra were not superimposed with each other (Fig. 6d).
d) The derivative spectra (first, second, third and fourth order) of the extracted UV-absorption spectra were intersected with each other but at different wavelengths (Fig. 7).

Upon application of the winCATS® spectral correlation method, the correlation values for the investigated peak were $r_{s,m} = 0.988578$ and $r_{m,e} = 0.987468$. In the context of the winCATS® spectral correlation method, such correlation values could be interpreted differently using the two mathematical data handling approaches. According to the null hypothesis at 99% confidence limit, such correlation values were not significantly deviated from

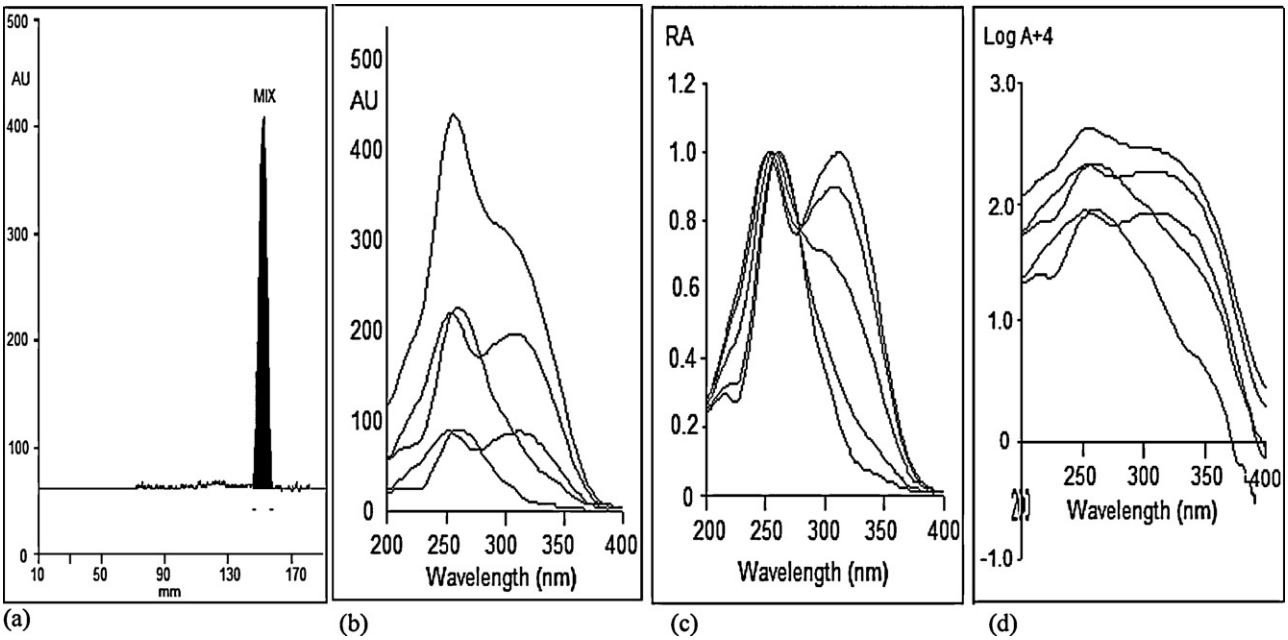

Fig. 6. (a) HPTLC densitogram obtained from a mixture containing MBZ and MP [the mobile phase was ethyl acetate–methanol–27% ammonia solution 22:2:1 (v/v)], (b) its spectrodensitograms at the five points, (c) their RA spectra and (d) their log A versus the wavelength plots.

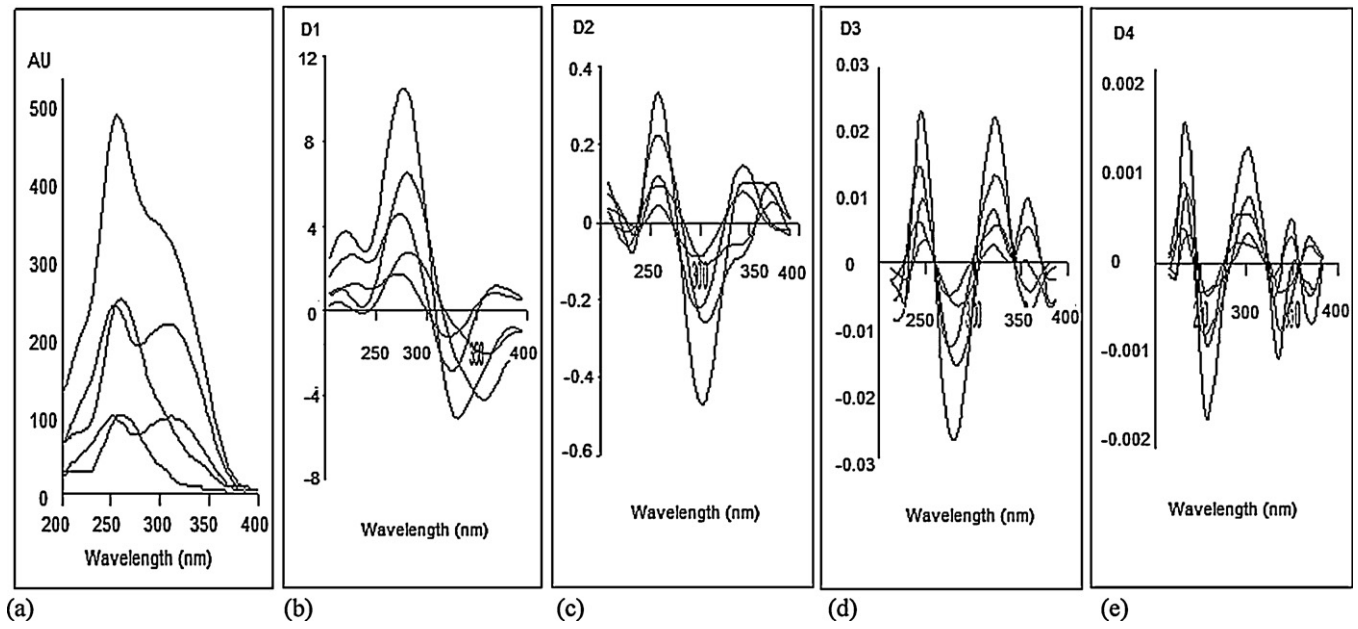

Fig. 7. (a) Spectrodensitograms of the HPTLC densitogram obtained from a mixture containing MBZ and MP [the mobile phase was ethyl acetate–methanol–27% ammonia solution 22:2:1 (v/v)] at the five points, (b) their first derivative, (c) their second derivative, (d) their third derivative and (e) their fourth derivative spectra.

each other, concluding that the HPTLC peak due to the non-resolved mixture is spectrally pure. This erroneous and misleading conclusion was in discordance with those obtained by the application of the new concept. Such correlation values, however, were less than the correlation limit 0.999. Contrarily to the null hypothesis approach, the HPTLC peak was considered spectrally impure according to the winCATS® spectral correlation method using the correlation limit approach.

4. Conclusion

The proposed concept could be successfully applied to the spectral purity assessment of HPTLC peaks. Unlike the winCATS® spectral correlation method, the proposed concept is non-confusing and its results are easily and rigorously interpreted. Even in situations where the purity results are free from sources of confusion, the proposed method remains superior to the correlation limit method as it does not only detect spectral interference and confirm the purity of the resolved peaks, but also constitutes a useful tool for the identification of the resolved components. The proposed identification concept makes TLC and HPTLC coupled with a scanner with a multi-channel detector or diode array detector (TLC-DAD) and (HPTLC-DAD) valuable tools for reliable and correct identification of compounds in multi-component mixtures providing characteristic fingerprints for each component.

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