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Evaluation of different techniques for peak purity assessment on a diode-array detector in liquid chromatography

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Abstract

The efficiencies of nine different techniques implemented on a diode-array detector in liquid chromatography for peak homogeneity determination were compared at a concentration level of a drug that can be found in biological samples. Solutions of cefotaxime sodium (analyte) spiked with various amounts of a model impurity (theophylline) were injected under chromatographic conditions giving severe overlap of the peaks ($R_s = 0.14$). Under the conditions used, the most efficient techniques implemented in the instrument were spectral suppression (ca. 0.5%), derivative spectrum (<1%), followed by spectral overlay, absorbance ratio plot (<5%), multiple-wavelength chromatograms overlay and purity parameter (ca. 5%). The numerical value of the absorbance ratio could also be exploited statistically to detect 0.5% of impurity. Theophylline at levels up to 10% could not be detected using three-dimensional plots, contour diagrams and derivative chromatograms.

1. Introduction

Since the introduction of diode-array detectors (DAD), many approaches, the advantages and limits of which have been widely discussed in the literature ([1–6] and references cited therein), have been proposed for peak purity determination in liquid chromatography. Several techniques are generally used in conjunction to ensure the selectivity of an analytical procedure in research and development. Their selection is guided by their sensitivity for detecting the impurity and the spectral information available on both the analyte and impurity. A comparison of the performances, under the same chromatographic conditions, of several techniques implemented on a commercial instrument may be of interest to the analyst faced with the problem of peak homogeneity assessment.

In a previous study [7], we had compared the sensitivity of spectral suppression, spectral overlay and absorbance ratio techniques using a bench-top diode-array detector with a spectral bandwidth of 5 nm. We have now evaluated the efficiency of nine different techniques on a diode-array detector presenting a higher resolution and set at a spectral bandwidth of 1.3 nm. The study was carried out on the same compounds (cefotaxime sodium as analyte and theophylline as model impurity), under chromatographic conditions giving severe overlap of the peaks and at a concentration level of the analyte that can be found in biological samples (20 mg l^{-1}), with a view to biological applications.

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2. Experimental

2.1. Chemicals

Cefotaxime (Roussel UCLAF) and theophylline (Cetrane Unicet) were used as received. Other chemicals were of analytical-reagent or HPLC grade.

2.2. Solutions

Stock standard aqueous solutions (100 mg l^{-1}) of cefotaxime and theophylline were prepared. From these solutions, working standard solutions of theophylline (20 mg l^{-1}), cefotaxime (20 mg l^{-1}), and cefotaxime (20 mg l^{-1}) spiked with 10%, 5%, 1% and 0.5% of theophylline with respect to cefotaxime, were prepared by suitable dilution with the mobile phase.

2.3. Apparatus and experimental conditions

The chromatographic system consisted of a Waters Model 510 pump fitted with a Rheodyne injection valve provided with a 20-μl loop, a Waters Model 991 diode-array detector, a Waters Model 5200 printer and a Powermate SX Plus Nec information system. The cartridge used (25 cm × 4.5 mm I.D.) was packed with LiChrosorb RP-18 (7 μm) (Merck). The mobile phase was 0.025 *M* potassium dihydrogenophosphate (pH 4)–methanol (85:15, v/v). The flow-rate was set

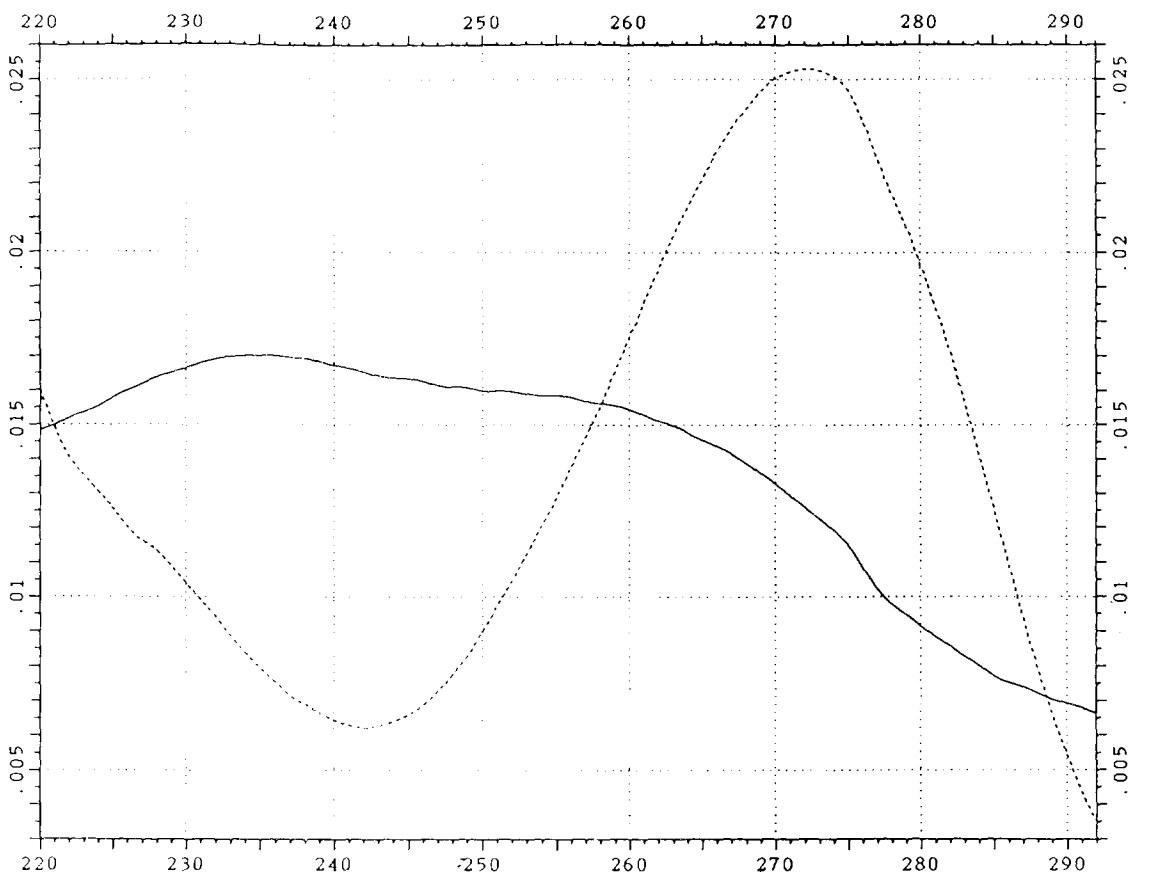

Fig. 1. Spectra of cefotaxime (20 mg l^{-1}) and theophylline (20 mg l^{-1}) recorded with the diode-array detector. Solid line, cefotaxime; dashed lines, theophylline. For operating conditions, see text.

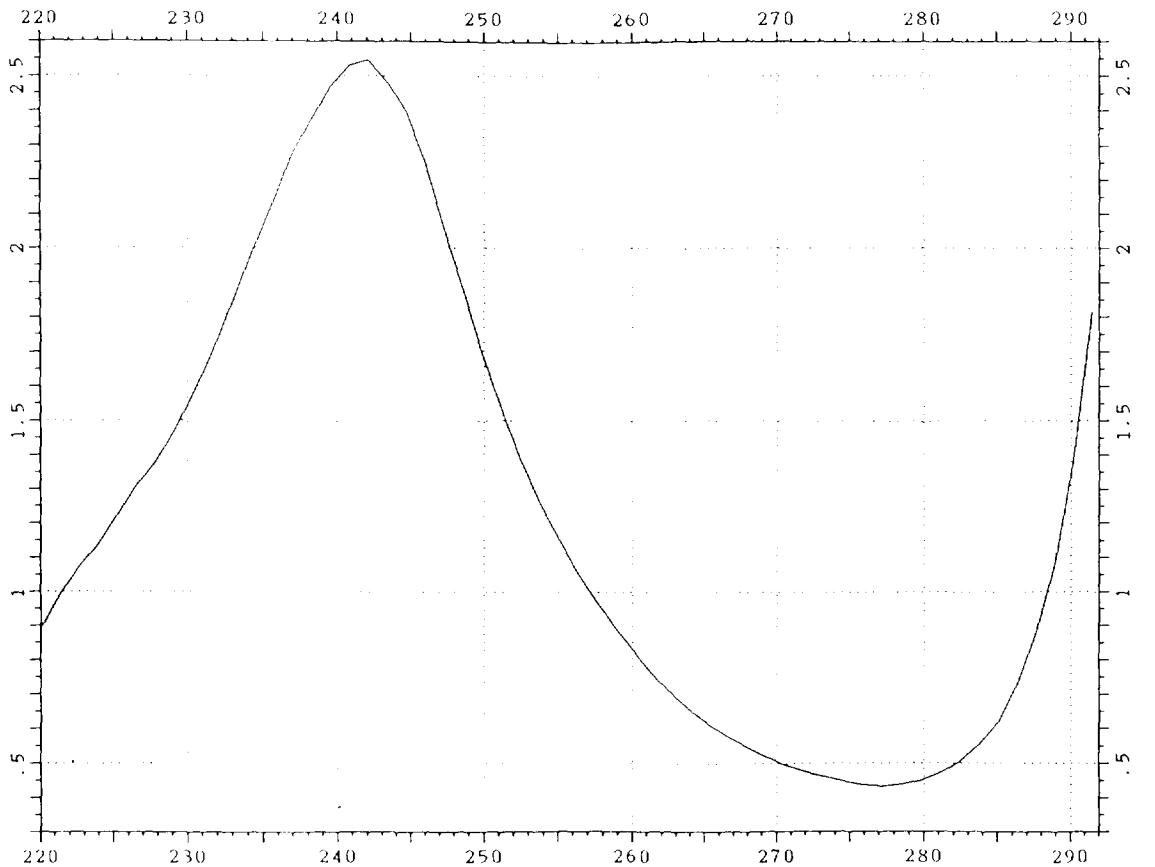

Fig. 2. C/T absorbance ratio as a function of wavelength.

at 1.3 ml min^{-1}. Each solution was injected six times successively in order to assess the repeatability of the results given by the different techniques. The spectral information was recorded in the range 220–300 nm with a spectral resolution of 1.3 nm. The sampling time was 26.64 ms.

The three-dimensional (3-D) plot of absorbance–time–wavelength was examined under 45° and 90° right and left viewing angles. The contour plot was analysed with 0.01 and 0.05 absorbance steps. Chromatograms were overlaid at 236.0, 242.2, 261.0, 276.2 and 290.0 nm. The derivative chromatograms (first to fourth) in the time domain were examined at the same wavelengths. Absorbance ratio and spectral suppression were investigated using 242 and 276 nm as selected wavelengths. For the absorbance ratio, the threshold values were set at 0.005 $\mu V\ s^{-1}$. Spectral overlay was carried out by collecting two spectra upslope, one at the apex and two downslope. The peak purity function was used with three threshold values (900, 950 and 980).

The first- and second-derivative spectra in the wavelength domain were recorded at the apex of the chromatographic peaks for the spiked and unspiked solutions. The derivative spectrum of the "impure" peak was overlaid with that of "pure" cefotaxime. Derivative spectra were recorded with an average number of eleven and fifteen points, respectively.

All the spectra were corrected for background absorbance using an interpolated baseline correction between peak start and peak end spectra.

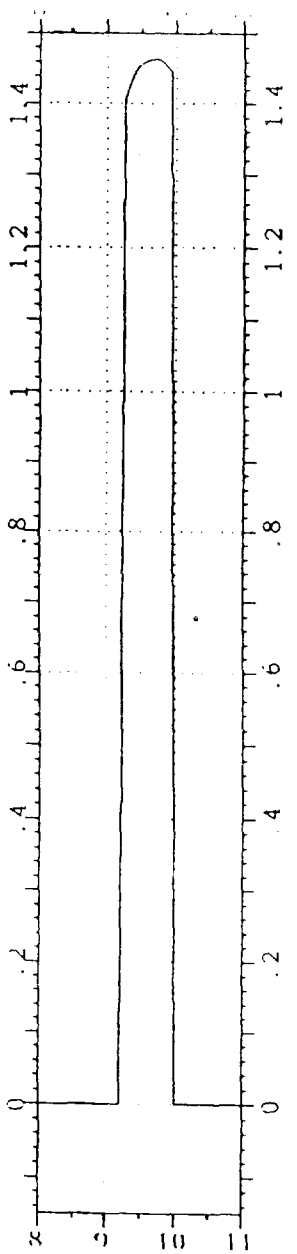

Fig. 3. Plot of absorbance ratio at 242 and 276 nm for a solution of C spiked with 5% of T (with respect to C).

3. Results and discussion

Under the chromatographic conditions used, the retention times were 9.37 min for theophylline (T) and 9.52 min for cefotaxime (C). The chromatographic resolution, calculated [8] from separate injections of the reference solutions of C and T, was found to be 0.14.

In all the techniques applied, the detection limit for the impurity in the analyte peak was evaluated by comparison to the results obtained on a pure peak of analyte.

The spectra of C and T (Fig. 1) were recorded with the diode-array detector from reference solutions of C and T. The plot of their ratio as a function of wavelength (Fig. 2) showed a maximum and a minimum at 242 and 276 nm which corresponded to a maximum spectral difference between the two compounds; these results are similar to those obtained previously [7]. This pair of wavelengths was selected for spectral suppression and absorbance ratio techniques [9].

The 3-D plots and the contour plots showed no difference between solutions of C spiked with T (even at a 10% level) and a reference solution of C. The first to fourth derivatives of the chromatograms also showed no difference. These results were as expected, owing to the extent of co-elution [2,4] and the low concentrations of T used in this study.

The overlay of chromatograms recorded at selected wavelengths showed a slight shift in the retention times of the chromatographic peaks for a solution of C spiked with 10% of T.

An absorbance ratio plot at 242 and 276 nm allowed 5% of T to be easily detected visually (Fig. 3) but failed for a 1% concentration. The insensitivity we found for this technique using a graphical format has been mentioned elsewhere [4,7]. When the chromatographic peaks of the analyte and an impurity are strongly overlapped and a pure standard is available, a numerical format that allows a statistical objective comparison between the absorbance ratio values for the "pure" and "impure" peaks should be preferred. It was not implemented in the instrument used. However, we collected the ratio values for the "pure" and "impure" peaks across the chromatographic peaks and performed a statistical comparison of their mean. The ratio values were collected every 4 s across the chromatographic peaks. Statistical evaluation showed that the variances between injections for the same solu-

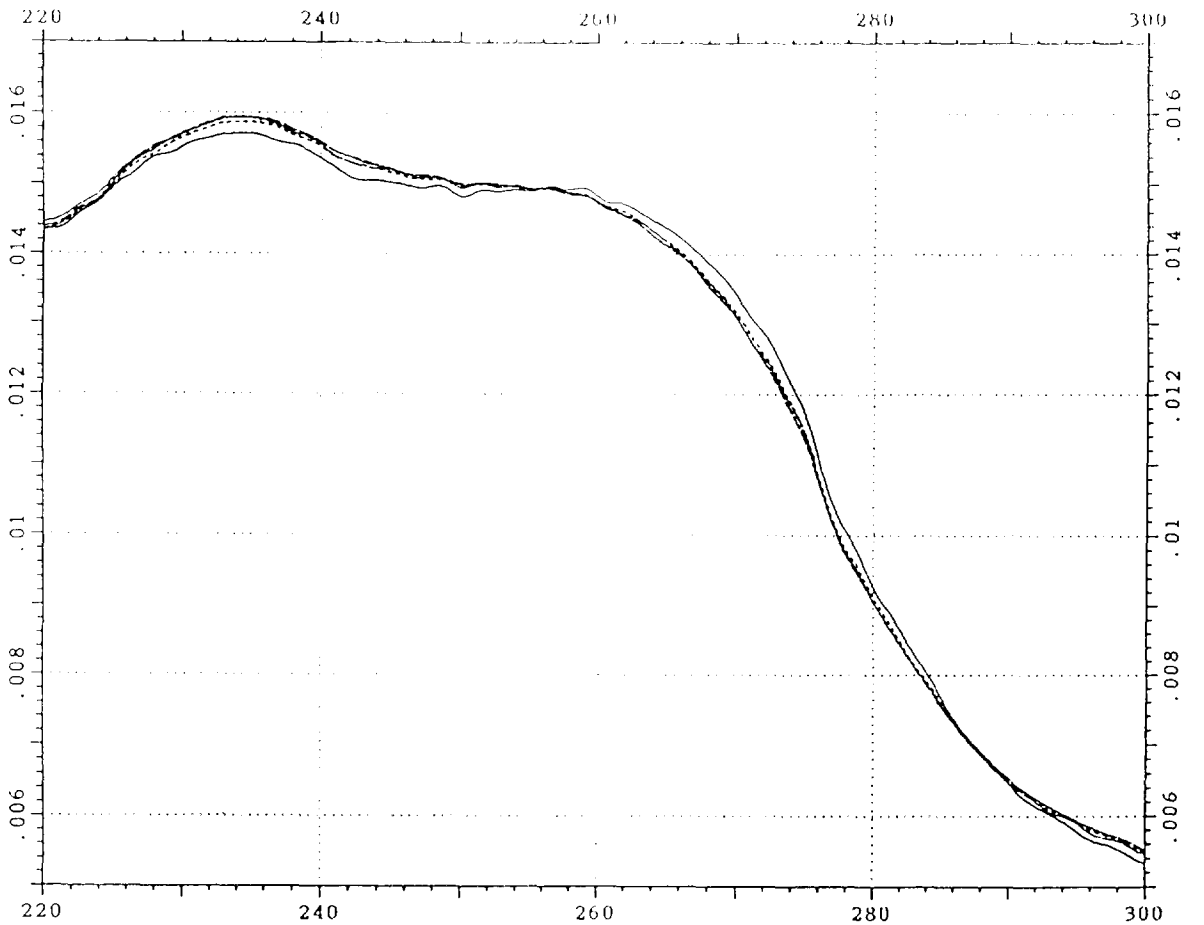

Fig. 4. Spectral overlay (apex, upslope, downslope) for a solution of C spiked with 5% of T (with respect to C).

Table 1
Evaluation of peak homogeneity by the peak purity function for a cefotaxime solution (C) and cefotaxime solutions spiked with different concentrations of theophylline (T)

No. of injection	T concentration added (%)[a]				
	0	10	5	1	0.5
1	99.41	88.89	97.82	99.54	99.55
2	99.31	88.99	98.05	99.47	99.47
3	99.38	87.67	96.54	99.39	99.44
4	99.93	88.16	97.72	99.32	99.35
5	99.50	87.41	97.97	98.90	98.79
6	99.52	86.42	96.94	99.61	99.02
Average	99.34	88.01[b]	97.51[b]	99.37	99.27

[a] Results are expressed as match percentage and are given for six successive injections of each solution.
[b] Significantly different from 0% ($\alpha = 0.05$).

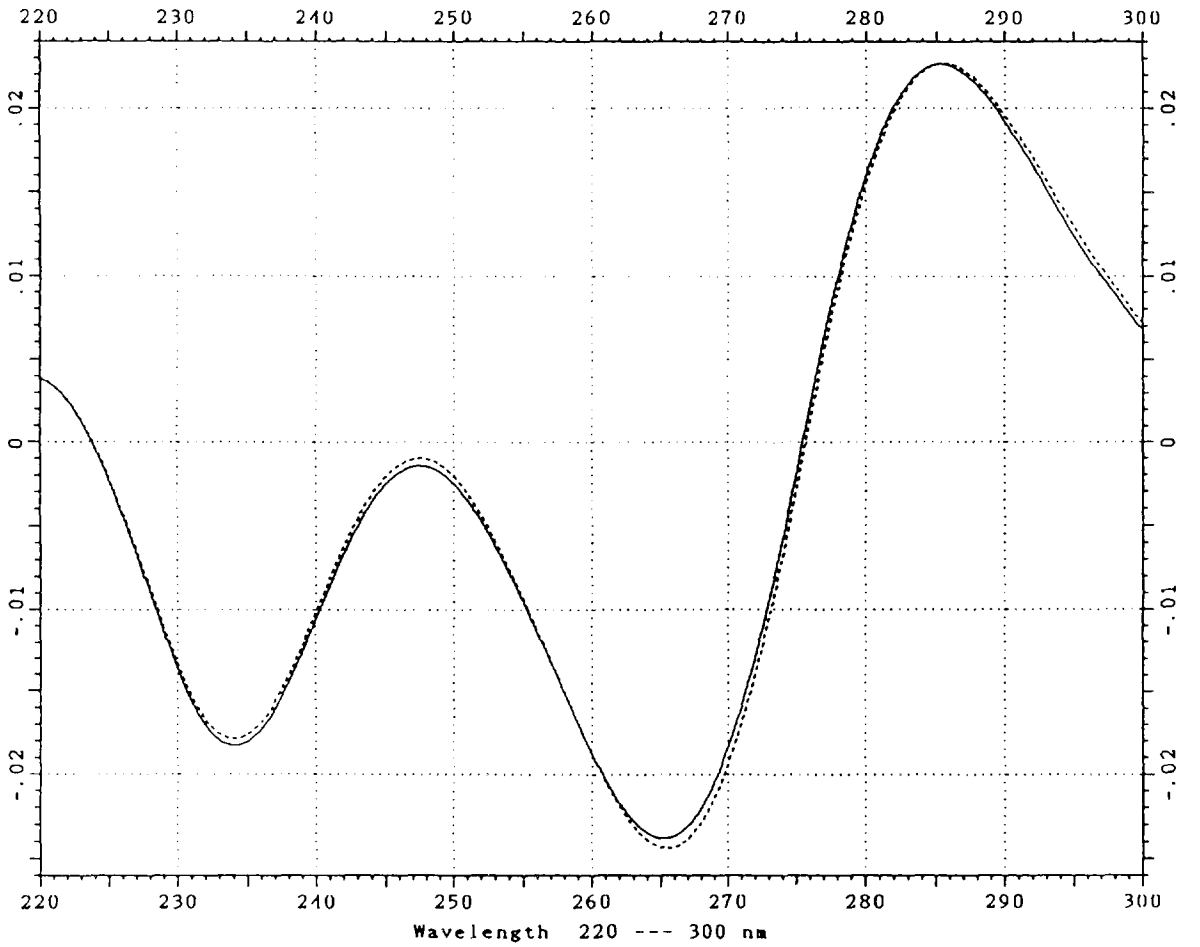

Fig. 5. Overlay of the second-derivative spectra recorded at the apex of the chromatographic peak for C spiked with 1% of T (with respect to C) and reference C.

tion were not significantly different ($\alpha = 5\%$). Therefore, the ratio values from a set of injections could be pooled for pure and impure peaks. The mean values for a peak spiked at the 0.5% level and an unspiked peak were significantly different ($\alpha = 5\%$). This shows that a statistical analysis of a large number of numerical ratio values across the peaks is a time-consuming but sensitive technique.

The overlay of spectra captured at the apex, upslope and downslope could detect easily less than 5% of impurity (Fig. 4), but failed at a 1% concentration. This low sensitivity, compared with the results obtained in previous studies [7], is due to the wavelength range used (220–300 nm instead of 190–300 nm). At about 210 nm, T presents a dramatic difference in absorptivity relative to C (see Fig. 1 in Ref. [7]), which explains the lower detection limits previously obtained. However, wavelengths higher than 220 nm should be adopted in routine use, owing to the absorbance of the mobile phase at low wavelengths.

The values of the purity parameter (expressed as match percentage) obtained from six successive injections of spiked and unspiked solutions (Table 1) were statistically compared. A one-way analysis of variance (ANOVA) and a Dunnett test showed that the mean value of the purity parameter calculated for 5% of T at a

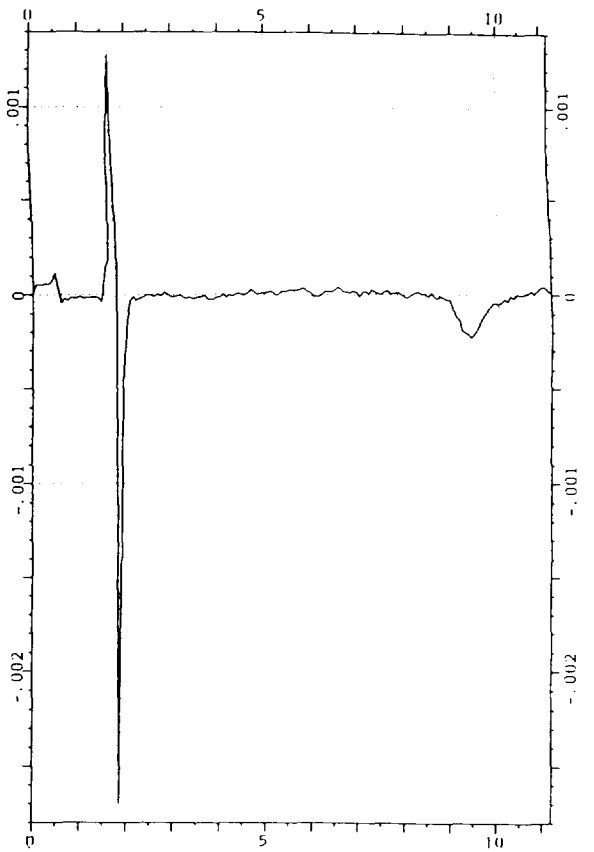

Fig. 6. Signal of T after suppression of C. Chromatogram from a solution of C spiked with 1% of T (with respect to C).

threshold value of 980 was significantly different ($\alpha = 0.05$) from that of C. No significant difference was found for 1% of theophylline.

The presence of 1% of T in the peak of C could easily be detected by recording the second-derivative spectrum captured at the apex of the peak. The derivative spectrum presents a significant difference to that of C (Fig. 5), showing the efficiency of the second derivative to enhance small spectral differences.

Spectral suppression was applied by measuring the difference absorbance ΔA at 242 and 276 nm and using the equation $\Delta A_{242,276} = A_{242} - 1.550A_{276}$. The constant for the suppression of the signal of C (1.550) was calculated from the ratio of the absorbances at 242 and 276 nm for a pure solution of C (100 mg l^{-1}). The efficiency of the analyte suppression was checked by injecting this solution and using the above equation. No signal was observed. Under the same conditions, the detection limit for T in C (signal-to-noise ratio = 2) was close to 0.5% and 1% of T in the peak of C could easily be detected (Fig. 6). Such a low detection limit could be obtained because the pair of wavelengths was fully optimized. An advantage of this technique over the absorbance ratio is that it can be successfully applied even if peaks are totally co-eluted and the response is related to the concentration [3,7,9].

4. Conclusions

The purpose of this work was to compare the efficiency of different techniques to evaluate the homogeneity of a chromatographic peak of an analyte at a concentration level of biological interest. The importance of performing an adequate background correction for the techniques comparing spectra or using ratios across the chromatographic peaks should be emphasized. An unspiked solution of the analyte should give a perfect match of the spectra collected at different time intervals across the chromatographic peak if the correction is properly carried out.

Among the different techniques investigated which have been shown to be sensitive under the selected conditions and with the specific instrument used, some of them (spectral overlay at the apex, upslope and downslope, peak purity parameter, multiple-wavelength chromatogram overlay) do not require any spectral information; others require a knowledge of the analyte spectra (spectral suppression, overlay of derivative spectra from the test and reference solutions). Some techniques cannot be fully optimized without a knowledge of the spectra of both the analyte and the impurity (spectral suppression and absorbance ratio); the performances obtained for the latter two in this study have been fully optimized with respect to the selection of the wavelengths used. The use of the peak purity function under the conditions used was not a very efficient technique, in contrast to the results

reported on some commercial instruments [6,10], which use different algorithms.

It should be noted that in our study we used a low concentration of analyte (20 mg l^{-1}), corresponding to a level of analyte that can be found in biological samples; lower detection limits of impurity should be obtained by using higher concentrations of analytes, e.g., for peak purity assessment for raw materials.

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