A “chromatospectroscopic” technique has been developed to quantitate two compounds that coelute in high-performance liquid chromatography. The method uses a diode-array detector with Millenium 32 software to extract spectra at regular time intervals during the elution of the unique peak and recover spectral data (absorbance versus wavelength), which can then be processed using the Excel software package. The method is applied to mixtures of two coeluting UV filters. Both could be accurately quantitated even when the mixture consisted of 99.5% of one and only 0.5% of the other.

Introduction

The development of the diode-array detector for high-performance liquid chromatography (HPLC) has considerably broadened the scope of this analytical technique in that the UV spectrum of the eluate can be recorded at any point, thereby enabling the capacity to detect a given substance by observing the chromatogram recorded at a specific wavelength. Not only is the compound detected, but its UV spectrum is simultaneously recorded.

The software packages designed for this kind of apparatus offer various possibilities—the simultaneous detection at several different wavelengths; recovering the UV spectra at the apex of the peak and at its inflections (either increasing or decreasing); and comparing the recorded UV spectrum with one stored in the memory (i.e., a control substance).

These functions have already been exploited to quantitate two different compounds that happen to elute at the same time either by detecting at two different wavelengths (1) or by means of analysis coupled with the semiempirical processing of the UV spectra recorded at the inflections of the peak (2). Other mathematical methods based on the analysis of the shape of the peak have been developed, but these are somewhat difficult for the nonspecialist chemist to understand (3,4).

The fact that it is now possible to record UV spectra at any time has made it possible to develop another method, which is referred to as “chromatospectroscopy”. In brief, this consists of recording UV spectra at regular intervals throughout the emergence of the peak (as opposed to only at the three time points corresponding to the apex and the two inflections). The software used (the Waters Millenium 32 package) (Waters, Saint-Quentin-Yvelines, France) allows for the recovery of the spectral data (SD) (the absorbance readings over a range of wavelengths) for all the extracted spectra. All of these data can then be transferred into a simple Excel spreadsheet in which they can be mathematically manipulated (i.e., spectra can be added and subtracted, areas under the curve calculated, and different spectra matched).

The sum of all the UV spectra generated throughout the elution of the peak corresponds to the spectrum of all the compounds that crossed the measuring cell, and the operations that can be performed with this summed spectrum are the same as those that can be performed using a modern spectrophotometer that records SD in digital form. In other words, these mathematical operations are equivalent to recovering the eluted peak and subjecting it to conventional spectrophotometric analysis. This is why this technique is called “chromatospectroscopy”.

First, the new method is applied to the processing of spectra generated with a pure compound, octyl p-methoxycinnamate, or Parsol MCX (which is commonly used as a UV filter in the cosmetics industry). Next, the method is applied to the quantitation of two coeluting compounds, Parsol MCX and another UV filter, t-butyl-4 methoxy-4’ dibenzoyl methane, or Parsol 1789. Both compounds are shown in Figure 1. This problem is of industrial interest because these compounds are often included together in sunscreen products, but because they coelute in standard chromatographic conditions, quantitation is a problem (5). Finally, another possible application of the method is proposed that would allow for the quantitation of a known compound coeluting with an unknown compound (this is investigated with the same pair of UV filters, but one of them will be considered as an unknown).
Experimental

Reagents and materials
Parsol MCX and Parsol 1789 are commercially available products that were kindly supplied by DIPTA SA (Aix-en-Provence, France). The Waters HPLC apparatus was equipped with an E600 pump and a PDA 996 diode-array detector. Waters Millenium 32 software was used to control the unit and process chromatographic data.

HPLC conditions
The analysis of the UV filters was performed using a 100-5 C18 column (Chromcart, Macherey Nagel, Hoerdt, France) (12.5 × 4.8 cm). A linear MeOH–water (70:30 to 100:0) gradient was used for approximately 3 min and then pure MeOH for 12 min, followed by the reestablishment of the initial conditions and stabilization for 8 min. The constant flow rate was 0.8 mL/min. Solutions were injected using a Rheodyne valve with a 20-µL injection loop.

Trapezium method calculation of the area under the peak and derivation of the resultant spectrum
The SD of the spectra that were extracted from a chromatographic peak every 3 s were transferred into an Excel spreadsheet. Wavelengths were entered in column A, and columns B to M were used for the SD of the twelve extracted spectra. Column N contained the following equation:

\[
3(B^2) + 3(C^2 - B^2)/2 \quad \text{Eq. 1}
\]

which gives the area of a trapezium drawn between points \(t = n\) and \(t = n - 3\) (i.e., 3 s). The same equation was repeated across the spreadsheet until column X, thus giving a total of eleven trapezia. The same line was recorded for each wavelength (down to 396 nm). Column Y was used to display the sum of columns N to X, which was then plotted against column A to give the resultant spectrum (RS) of the peak being analyzed. The area under the curve (AUC) of the RS was taken as the sum of all the figures in column Y. The number of columns in the spreadsheet could be adjusted for the number of spectra extracted in any given analysis.

Matching the reconstructed spectrum to the RS using the Excel “Solver” function
The calculation sheet was set up in the following manner.

Line 1 was used for the column titles; column A for the wavelength; column B contained the SD of the RS for the mixture of the two different filters; column C contained the SD of the normalized spectrum (NS) for Parsol MCX; and column D contained the SD of the NS for Parsol 1789. Columns E and F were used for the coefficients \(P\) and \(Q\); the first cells (E2 and F2) contained the initial value (0) and the cells below contained the equation “= E2” and “= F2”, respectively. Column G contained the following equation (and all of the subsequent cells below) for the reconstruction of the RS:

\[
(E2 \times C2) + (F2 \times D2) \quad \text{Eq. 2}
\]

Column H contained the equation \(A2 - G2\), which gave the difference between the SD of the RS (column A) and those of the reconstructed spectrum. Cell I2 contained the equation “= sum(H2:H159)”, which was the sum of the differences between the SD of the RS and those of the reconstructed spectrum. Cell J2 was the standard deviation of the values in column H.

Perfect matching of the reconstructed spectrum to the RS resulted in cells I2 and J2 having the value of zero. Matching was actually performed by the “Solver” function of the Excel program by varying the values in cells E2 and F2 (i.e., coefficients \(P\) and \(Q\)) with the stipulation that the value in cell J2 should be zero. This yielded the values for \(P\) and \(Q\) that came closest to giving a perfect match with J2 fixed at zero. The value of the standard deviation gives an objective measurement of the closeness of the fit.

Results and Discussion

Preliminary experiments
Initial settings for the diode-array detector were determined without bearing any particular application in mind. Spectral resolution was set at 1.2 nm (the highest resolution possible), the recording frequency was one spectrum per second, the range was from 210 to 400 nm, and the observation wavelength was set at 300 nm.

Six solutions containing known concentrations of Parsol MCX in methanol were run. The areas under all the peaks were proportional to the concentration so that a conventional standard curve could be plotted. The chromatospectroscopic method involved commencing to record spectra at the beginning of the elution of the peak (whose point is determined manually) and then repeating the recording every 3 s until the peak has completely eluted. Figure 2 shows the window displayed by the software. In this example, the first spectrum was recorded at 8.45 min (\(t = 0\)) and the last at 9.05 min. Extracted spectra were displayed in the upper-right portion of the window. The “Spectrum Points” function (lower right) gave access to the SD of the selected spectrum (in this case, that recorded at 8.75 min). These data were then cut and pasted into an Excel spreadsheet.

Two types of graphs can be plotted from these data. First, spectra extracted at different time points were represented as absorbance versus wavelength (Figure 3). Because the product was injected in a pure form in this case, none of these spectra

![Figure 1. The molecular structures of (A) Parsol MCX and (B) Parsol 1789.](https://example.com/image.png)
were compound. Secondly, a plot of absorbance at each different wavelength versus time (Figure 4) represented the chromatographic peaks that would have been obtained by measuring the chromatographic profile at each different wavelength (i.e., every 1.2 nm in this case). The area under each of the peaks was calculated using the trapezium method, and by summing the areas, an RS could be reconstructed. This represented the UV spectrum of all the material that passed through the measuring cell. Again, because the material tested in this case was pure, the RS had the same proportions as the spectra obtained at each different time point.

Proportionality was maintained between the RS and the concentration of the solution injected using the chromatospectroscopic processing method. The AUC that was derived by summing the RS absorbance values between 210 and 400 nm for each of the six RS was directly proportional to the concentration of Parsol MCX. Figure 5 shows a comparison of a conventional standard curve (based on the area of the peak) and the chromatospectroscopic method (based on the AUC of the RS) together with the corresponding linear regression values. Dividing the SD of each RS by the concentration gave an NS for 1 mg/mL. Therefore, this represents a novel calibration system that is based on the UV spectrum of all the material injected rather than the area of a peak obtained at a given wavelength. This method was also applied to Parsol 1789, and the NS for both UV filters are shown in Figure 6.

**Quantitation of two coeluting compounds**

This method is useful for the analysis of mixtures of different compounds. By way of example, we have applied it to the problematic quantitation of a mixture of the two compounds mentioned previously that coelute in standard HPLC conditions (a methanol–water gradient in an RP-C18 column). These two UV filters are often combined in sunscreen products (Parsol MCX filters out UVB and Parsol 1789 UVA); therefore, a method that would allow for their simultaneous quantitation in a mixture containing both would be useful in the cosmetics industry.

This work was based on such a mixture (M1) containing 48 mg/L of Parsol MCX and 37 mg/L of Parsol 1789 in methanol (these proportions are typical of sunscreen products). In addition, in order to investigate the sensitivity of the assay, we also tested another mixture (M2) that contained 99.3% Parsol MCX and 0.7% Parsol 1789.

Spectra that were extracted from the peak given by the M1 mixture are shown in Figure 7, and the peaks at each different...
wavelength are shown in Figure 8. It is clear that the two compounds did not elute at exactly the same instant with the Parsol 1789 maximum located at 9 s after the beginning of the peak, and for the Parsol MCX maximum not until 6 s later. The observed retention time varied to a certain extent with the wavelength, having a value of 8.614 min recorded at 360 nm and 8.721 min at 310 nm. For this reason, any quantitation method based on the spectra that were automatically extracted at the apex and the inflections of the peak would be inaccurate because these points are evidently wavelength-dependent.

As shown in Figure 9, the RS of the mixture was based on all the material that passed through the measuring cell, and it was reconstructed by summing the NS of the two compounds after attributing to them the coefficients P and Q that correspond to their concentration (in mg/L) in the mixture. The reconstructed spectrum was then matched to the RS by varying P and Q using the Excel “Solver” function. This function begins with multiplying the SD of the two NS by P and Q, and the results are then summed to give the reconstructed spectrum. Then, the SD of this reconstructed spectrum is subtracted from the SD of the mixture's RS, and when the differences, their sum, and the standard deviation are zero, then the values of P and Q are correct. The software calculates P and Q by imposing a minimum value for the sum of the differences and a value of zero for the standard deviation. In the example shown in this study, this calculation gave values of 47.85 and 36.63 for real concentrations of 48 and 37 mg/L. The sum of differences was 1.85 and the standard deviation 2.29 x 10^-2. In Figure 9, the mixture’s RS was superimposed onto the reconstructed spectrum, showing how close the match actually was. The main deviation was located between 210 and 230 nm. This...
could be attributed to the near instability at such low wavelengths. Similar accuracy was obtained for a series of ten mixtures containing different proportions of the two Parsol compounds, thereby confirming the validity of this chromatographic method for quantitating two distinct but coeluting compounds.

The sensitivity of the method was investigated using the M2 mixture. In this mixture, Parsol 1789 eluted after Parsol MCX, although this might have been because of some minor variations in the run conditions. In light of previous experience, spectra were only recorded between 250 and 400 nm. The reconstructed spectrum is shown in Figure 10; the value derived for P was 93.92 and for Q 0.60. The closeness of the match is shown in the expanded section (which represents a magnification of the portion of the UV spectrum between 350 and 400 nm) in which it can be seen that the reconstructed spectrum coincided perfectly with the real measurements. The lower line represented the reconstructed spectrum with values of P = 95 and Q = 0.

In this example, the chromatographic method detected and accurately quantitivered one small quantity of Parsol 1789 in an excess of Parsol MCX. However, this case was somewhat particular because the spectra of the two compounds were so different. This is obviously a key consideration when it comes to trying to apply this method to other coeluting compounds.

**Quantitation of a known compound eluting at the same time as an unknown compound**

The chromatographic method can be applied to the quantitation of a known compound eluting at the same time as an unknown compound. For example, it is worthwhile to imagine the M1 mixture consisting of one known and one unknown compound.

An analysis of the SD of the UV spectra extracted at 3 s and 6 s (Figure 7, spectra in bold) showed that they were homothetic by a factor of 2.8 with a standard deviation of 0.06, meaning that they can be attributed to a single pure compound.

In the first alternative, the extracted spectra correspond to the known compound (in this case Parsol 1789). In these conditions, the RS of the mixture cannot be reconstructed because the SD of the unknown compound cannot be determined.

In the second alternative, the extracted SD are those of the unknown compound. In this case, the RS can be reconstructed because the coefficient P is always attributed to the SD normalized for the known compound (in this case Parsol MCX). In contrast, the coefficient Q is applied to the SD pertaining to the unknown compound; therefore, the latter cannot be quantitated. Matching (as described previously) gave a concentration of 47.75 mg/L for Parsol MCX.

**Conclusion**

This method was developed with a specific problem in mind, namely the quantitation of two UV filters that coelute in standard chromatographic conditions. The method is particularly suitable for these two compounds for two reasons. First, they both absorb UV strongly (as would be expected), and secondly, they are designed to absorb at different wavelengths, thus their respective spectra are easy to distinguish. However, the method does not depend on this difference (which happens to be so marked in this case), and in fact, Parsol 1789 can be quantitated purely on the basis of the readings taken at 360 nm and higher, because Parsol MCX hardly absorbs at all at these longer wavelengths. However, this method is only suitable if the spectra of the coeluting compounds are sufficiently different, because the sensitivity threshold (in this case 0.5% of one compound with respect to the other) is expected to be strongly dependent on this factor. Obviously, if the two compounds had identical spectra, the method would not work.

The lag between the actual time of the elution of the first and the second “coeluting” compounds does not affect the method, which works whether both elute at exactly the same time or at significantly different times. However, the quantitation of a known compound that elutes at exactly the same time as an unknown compound would not be possible, because at no point would it be possible to determine its SD.

The software used in this study is widespread and user-friendly, and all the special operations necessary are easy to program in macros. Using the “Solver” function does not necessitate any particular expertise, neither in mathematics nor in computing.

The most delicate and tedious step in using this method is recovering the SD. It is clear that the accuracy of the method is largely dependent on both the number of spectra recorded per second and the time interval between the recovery of sequential spectra. In this work, the interval was 3 s for spectra recorded every second. Because our aim was to investigate the feasibility of the method rather than make it as accurate as possible, we only recovered one out of every three spectra extracted. Furthermore, all the recorded spectra were stored in the machine’s memory; it would be easy for a specialist to design software to automatically perform all the necessary operations.

**References**


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