THREE-DIMENSIONAL SYNCHRONOUS FLUORESCENCE SPECTROMETRY FOR THE ANALYSIS OF THREE-COMPONENT ALKALOID MIXTURES

F. GARCIA SANCHEZ, A. L. RAMOS RUBIO and C. CRUCE BLANCO
Department of Analytical Chemistry, Faculty of Sciences, The University, E-29071 Málaga, Spain

R. SUAU SUAREZ
Department of Organic Chemistry, Faculty of Sciences, The University, E-29071 Málaga, Spain

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Summary A study of a simple and sensitive method for the determination of berberine, luguine and sanguinarine in mixtures by normal and synchronous derivative spectrofluorimetry is described. The influence of solvents and other experimental variables is discussed. A three-dimensional plot of both emission-excitation and synchronous spectra obtained by a new software program provides additional information for optimizing instrumental parameters. Linear, normal and derivative calibration graphs are established in the ng/ml range. A statistical analysis of the results and their application to synthetic and natural samples is given.

Synchronous derivative spectroscopy offers an elegant approach to the problem of resolving spectral overlap. However, when multicomponent samples with overlapping spectral shapes are analysed, several problems related to pre- and post-filter effects can arise, as recently pointed out.

The information contained in a conventional excitation-emission matrix (EEM) is insufficient for analysis of complex mixtures of such components. However, the additional information, wider scope and cross-dependence of response (λexc; λem) that a three-dimensional EEM offers, makes it feasible to detect features that are normally hidden in conventional fluorescence scanning.

Careful observation of the EEM allows optimization of the conditions for obtaining a synchronous excitation matrix (SEM). The software currently offered by the intrument makers does not permit recording of this SEM as a three-dimensional plot. We have therefore developed a program for this purpose, which gives a simple system for optimizing the instrumental parameters affecting synchronous scanning fluorimetry.

Its application to the synchronous derivative approach for analysis of multicomponent samples prevents cross-interferences arising from absorption and/or emission by the other components; this is done by manipulation of the solvent composition and careful selection of the wavelength increment (Δλ).

Alkaloids and other compounds which produce physiological effects in animals are widespread. The alkaloids berberine, luguine and sanguinarine are structurally related compounds which have overlapping excitation spectra and thus cannot be determined in mixtures by conventional fluorescence spectroscopy, but are amenable to analysis by the derivative technique with the aid of our program.

EXPERIMENTAL

Reagents
Analytical-reagent grade materials and solvents were used unless otherwise indicated. Luguine and sanguinarine were obtained from the Department of Organic Chemistry, University of Málaga, and were used without further purification.

Standard 1 \( \times 10^{-3} \)M solutions of the alkaloids were prepared in 1:1 v/v ethanol–dioxan mixture and diluted with the same solvent, as required.

Spectrofluorimeters
A Perkin–Elmer model MPF-43A instrument with an Osram XBO 150-W lamp and an O23 recorder was used for quantitative analysis. The instrument was adjusted daily with a standard
bar of Rhodamine B ($1 \times 10^{-7} M$) to give a reading of 64 units with the sensitivity set at 10 coarse and 7 fine, the slit band-pass at 5 nm, and the temperature at 25°. Derivative spectra were obtained with the Perkin–Elmer Model DCSU-2 differential corrected spectra unit connected to the MPF-43A spectrorfluorimeter.

To obtain three-dimensional plots in both the excitation–emission and synchronous spectra modes, a Perkin–Elmer LS-5 spectrorfluorimeter was used with a 9.9-W xenon discharge lamp pulsed at line frequency, F/3 Mont–Gillieson type monochromators and 1 x 1 cm silica cells. For both monochromators the slits were set to give a band-pass of 5 nm. A ratio-recording system with a reference photomultiplier was used. The spectrorfluorimeter was computer-controlled through an RS232C serial interface with a Perkin–Elmer Model 3600 Data Station microcomputer. Instrumental control and data collection were achieved with the Perkin–Elmer Computerized Luminescence Software (PECLS-II). For graphical recording an Epson FX-85 printer-plotter was connected to the spectrorfluorimeter.

**Procedures**

*Extraction.* Roots of *Glaucium flavum* were left to dry for 20 days at room temperature, then triturated. Weighed samples (ca. 15 g) were extracted with methanol under reflux. The methanolic extract was evaporated to dryness, and the residue dissolved in 10% v/v sulphuric acid. The aqueous phase was made alkaline with sodium hydroxide solution and extracted with 50 ml of methylene chloride. The extract was evaporated to dryness and the residue dissolved in 50 ml of 1:1 v/v ethanol/dioxan mixture. This solution was diluted further as required.

**Normal (non-derivative) spectrofluorimetry.** The fluorescence intensities were measured for alkaloid concentrations between 0.5 and 250 ng/ml in 1:1 v/v ethanol/dioxan. The wavelengths used were $\lambda_{em}$ 533 nm, $\lambda_{ex}$ 350 nm for berberine; 440 nm, 340 nm for luguine and 410 nm, 330 nm for sanguinarine.

**Synchronous scanning derivative spectrofluorimetry.** The samples were the same as for normal spectrofluorimetry. The first and second derivative synchronous spectra were recorded with a derivative wavelength interval ($\Delta \lambda_{d}$) of 10 nm, a response time of 1.5 sec and a scan-speed of 240 nm/min, with a synchronized difference between $\lambda_{em}$ and $\lambda_{ex}$ ($\Delta \lambda_{s}$) of 98, 47 and 121 nm for berberine, luguine and sanguinarine, respectively. The first and second derivative signals were measured from peak to trough.

**RESULTS AND DISCUSSION**

**Influence of experimental variables**

The excitation and emission spectra of the individual alkaloids in 1:1 v/v ethanol/dioxan

Fig. 1. Three-dimensional emission spectra (EEM) of berberine (B), 1.25 x 10⁻⁵ M; luguine (L), 5 x 10⁻⁷ M; sanguinarine (S), 1.5 x 10⁻⁵ M and their ternary mixture: 50 scans, excitation wavelength increments 5 nm.
Table 1. Spectral characteristics of several alkaloid/solvent solutions

<table>
<thead>
<tr>
<th>Compound</th>
<th>Solvent</th>
<th>( \lambda_{\text{abs}} )</th>
<th>( \lambda_{\text{em}} )</th>
<th>( \Delta\lambda^* )</th>
<th>( \log \varepsilon )</th>
<th>R.F.I.$</th>
<th>R.E.‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Berberine</td>
<td>Chloroform</td>
<td>352</td>
<td>525</td>
<td>173</td>
<td>4.26</td>
<td>100</td>
<td>54.1</td>
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<tr>
<td></td>
<td>Dioxan-ethanol</td>
<td>350</td>
<td>533</td>
<td>183</td>
<td>4.38</td>
<td>91</td>
<td>37.9</td>
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<tr>
<td></td>
<td>Acetone</td>
<td>349</td>
<td>540</td>
<td>191</td>
<td>4.36</td>
<td>88</td>
<td>38.9</td>
</tr>
<tr>
<td></td>
<td>Ethanol</td>
<td>349</td>
<td>534</td>
<td>185</td>
<td>4.33</td>
<td>82</td>
<td>38.0</td>
</tr>
<tr>
<td></td>
<td>Acetonitrile</td>
<td>345</td>
<td>427</td>
<td>82</td>
<td>4.19</td>
<td>92</td>
<td>59.4</td>
</tr>
<tr>
<td>Luguine</td>
<td>Dioxan</td>
<td>342</td>
<td>427</td>
<td>85</td>
<td>4.37</td>
<td>83</td>
<td>35.5</td>
</tr>
<tr>
<td></td>
<td>Dioxan-ethanol</td>
<td>342</td>
<td>440</td>
<td>98</td>
<td>4.40</td>
<td>86</td>
<td>34.4</td>
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<tr>
<td>Sanguinarine</td>
<td>Dioxan-ethanol</td>
<td>325</td>
<td>410</td>
<td>85</td>
<td>4.13</td>
<td>93</td>
<td>68.9</td>
</tr>
<tr>
<td></td>
<td>Acetone</td>
<td>330</td>
<td>420</td>
<td>90</td>
<td>4.11</td>
<td>89</td>
<td>68.5</td>
</tr>
<tr>
<td></td>
<td>Ethanol</td>
<td>327</td>
<td>405</td>
<td>118</td>
<td>4.26</td>
<td>83</td>
<td>46.1</td>
</tr>
<tr>
<td></td>
<td>Acetonitrile</td>
<td>328</td>
<td>540</td>
<td>212</td>
<td>4.33</td>
<td>92</td>
<td>42.6</td>
</tr>
</tbody>
</table>

*Stokes shift, \( \Delta\lambda = \lambda_{\text{em}} - \lambda_{\text{abs}} \).
†\( \varepsilon \) expressed in \( 1\,\text{mole}^{-1}\,\text{cm}^{-1} \).
§Relative fluorescence intensity.
‡Relative efficiency = R.F.I./\( \varepsilon_{\text{max}} \).

mixture are shown in Fig. 1. Each compound is characterized by three well-resolved excitation maxima and a single emission peak (at 533, 440 and 410 nm for berberine, luguine and sanguinarine, respectively). There is serious overlap of the excitation spectra of the three alkaloids, which precludes determination of the individual components in mixtures by normal spectrofluorimetry.

To find the most appropriate solvent for simultaneous determination of the three compounds, both the fluorescence and absorption

*Fig. 2. Three-dimensional synchronous spectra (ESM) of three-component mixture of the alkaloids. Optimum \( \Delta\lambda \) (89, 47, 121 nm) to determine berberine, luguine and sanguinarine, respectively: 50 scans, excitation wavelength increment 3 nm.*
spectra of $1 \times 10^{-5}M$ solutions of the compounds in each solvent tested were obtained. From the results shown in Table 1, it was deduced that the highest relative fluorescence intensities and good Stokes shifts were obtained with ethanol/dioxan mixtures having a medium dielectric constant. The best mixture was found to be 1:1 v/v. The effect of sunlight, temperature and time on the relative fluorescence intensity was examined and indicated that the samples could be left in sunlight and at 25° provided the measurements were made within 3 hr of preparation.

**Synchronous scanning derivative spectrofluorimetry**

In synchronous scanning spectrofluorimetry, the choice of an appropriate synchronized wavelength difference, $\Delta \lambda_s$, for the determination is critical, especially for quantitative multicomponent analysis. The selection of $\Delta \lambda_s$ is usually empirical and must be made for each component. Generally, the recommended value provides the greatest spectral resolution and minimal half band-width, and avoids Rayleigh scatter. The parameters affect-

![First derivative](image1)

![Second derivative](image2)

Fig. 3. Synchronous first and second derivatives of ternary mixtures with $\Delta \lambda_s = 98$ nm (---), sensitivity (0.3–3.0), detail (1.0–3.0, scale expansion x 5); $\Delta \lambda_s = 47$ nm (---), sensitivity (0.3–3.0); 121 nm (-----), sensitivity (0.3–3.0).
ing the synchronous spectra are optimized to minimize or eliminate the spectral interference of other compounds present.

A set of sequential scans of the synchronous spectra, with $\Delta \lambda$, starting at 13 nm and increasing by 3 nm for each scan (Fig. 2), provides a full picture of the synchronous profile of the mixture. The selection of $\Delta \lambda$ values suitable for determination of each component is easily done by simple inspection of the plot (see Fig. 2).

The information in Fig. 1 indicates that the scans should all start from an excitation wavelength of 209 nm, the emission wavelength starting at 222 nm in the first scan and 369 nm in the 50th. The slits should be set to give a band-pass of 5 nm, the scan-speed should be 240 nm/min and the sensitivity factor 0.13.

As indicated in Fig. 2, the appropriate $\Delta \lambda$ values for the determination of each component in the ternary mixture are 98, 47 and 121 nm for berberine, luguine and sanguinarine, respectively. It may be emphasized that the program designed to obtain this sequential synchronous scanning avoids the incomplete trial and error search traditionally used for this type of technique.

Despite the advantages of the synchronous technique for separating overlapping bands, it is not sufficient when the interfering spectral band is stronger than that of the analyte. For such cases, the derivative technique can be used to eliminate the interfering band in the synchronous spectrum by recording the synchronous derivative spectrum at a wavelength where only the analyte of interest gives a signal. This signal may be amplified as much as the signal-to-noise ratio permits. This was done for the three synchronous spectra chosen from Fig. 2, with $\Delta \lambda$ = 98, 47 and 121 nm for berberine, luguine and sanguinarine, respectively, the result being the synchronous derivative spectra in Fig. 3.

Parameters affecting the derivative spectra, such as scan-speed, response time and wavelength range over which the derivative is averaged were selected.\textsuperscript{13,14} A combination of scan-speed 240 nm/min, response time 1.5 sec and wavelength range 10 nm provides the best conditions for good sensitivity and selectivity in determination of the alkaloids.

Peak-height measurements (as vertical distance from peak to trough), were made for different concentrations to establish the calibration graph. The distances $D_0$, $D_1$ and $D_s$ shown in Fig. 3 were found to be proportional to the berberine, luguine and sanguinarine concentrations and were the least affected by spectral interferences from the other alkaloids. The 5 x magnification clearly shows the otherwise hardly detectable shoulder characteristic of berberine.

\textbf{Quantitative analysis}

To obtain calibration graphs for each alkaloid in ternary mixtures, concentrations in the range between 50 and 250 ng/ml for the species to be determined were introduced into five solutions, with a total constant concentration of 300 ng/ml for the two other alkaloids.

Each calibration graph was repeated five times with solutions containing the same constant total concentration of the other two alkaloids but in different ratios ranging from 1:5 to 5:1. Statistical analysis of the slope and intercept of the regression curves obtained for each case shows a small standard deviation for the slopes, suggesting that no multiplicative interference exists. On the other hand, the intercepts are more affected by the matrix composition, because energy transfer occurs as a consequence of the overlapping spectral profiles. To reduce this problem, the regression equations have been corrected by making the intercepts zero.

The mean regression curves applied for the simultaneous determination of the three alkaloids in ternary mixtures by normal, and synchronous first and second derivative spectrofluorimetry are as follows:

\textbf{Berberine}

\begin{align*}
I_f &= (0.134 \pm 0.120) \times [\text{berberine}] + (19.3 \pm 23.9) \\
D^1 &= (0.035 \pm 0.01) \times [\text{berberine}] - (0.02 \pm 0.43) \\
D^2 &= (0.026 \pm 0.006) \times [\text{berberine}] + (0.96 \pm 0.52)
\end{align*}

\textbf{Luguine}

\begin{align*}
I_f &= (0.515 \pm 0.334) \times [\text{luguine}] + (23.8 \pm 34.1) \\
D^1 &= (0.079 \pm 0.002) \times [\text{luguine}] - (0.13 \pm 0.32) \\
D^2 &= (0.066 \pm 0.002) \times [\text{luguine}] + (0.35 \pm 0.47)
\end{align*}
Table 2. Application of the methods to synthetic mixtures

<table>
<thead>
<tr>
<th>Mixture*</th>
<th>First derivative</th>
<th>Second derivative</th>
</tr>
</thead>
<tbody>
<tr>
<td>B(250) + L(250) + S(250)</td>
<td>71.4 ± 0.4</td>
<td>92.0 ± 2.0</td>
</tr>
<tr>
<td>B(50) + L(50) + S(50)</td>
<td>85.8 ± 2.1</td>
<td>-</td>
</tr>
<tr>
<td>B(210) + L(130) + S(90)</td>
<td>87.1 ± 0.4</td>
<td>123.6 ± 0.6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Mixture*</th>
<th>First derivative</th>
<th>Second derivative</th>
</tr>
</thead>
<tbody>
<tr>
<td>B(250) + L(250) + S(250)</td>
<td>94.9 ± 0.2</td>
<td>95.4 ± 0.1</td>
</tr>
<tr>
<td>B(50) + L(50) + S(50)</td>
<td>104.4 ± 0.6</td>
<td>106.3 ± 0.1</td>
</tr>
<tr>
<td>B(210) + L(130) + S(90)</td>
<td>90.3 ± 0.2</td>
<td>101.9 ± 0.3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Mixture*</th>
<th>First derivative</th>
<th>Second derivative</th>
</tr>
</thead>
<tbody>
<tr>
<td>B(250) + L(250) + S(250)</td>
<td>70.8 ± 0.2</td>
<td>79.5 ± 0.2</td>
</tr>
<tr>
<td>B(50) + L(50) + S(50)</td>
<td>74.8 ± 0.6</td>
<td>88.5 ± 0.7</td>
</tr>
<tr>
<td>B(210) + L(130) + S(90)</td>
<td>70.1 ± 0.2</td>
<td>82.1 ± 0.5</td>
</tr>
</tbody>
</table>

*Concentrations (ng/ml) in parentheses.
†Five determinations.

Table 3. Determination of the alkaloids in *Glaucium flavum*

<table>
<thead>
<tr>
<th>Compound</th>
<th>Alkaloid ± S.D., mg/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Berberine</td>
<td>0.77 ± 0.03, 0.76 ± 0.04</td>
</tr>
<tr>
<td>Luguine</td>
<td>0.77 ± 0.03, 0.80 ± 0.03</td>
</tr>
<tr>
<td>Sanguinarine</td>
<td>0.24 ± 0.01, 0.26 ± 0.004</td>
</tr>
</tbody>
</table>

Sanguinarine

\[ I_t = (0.404 ± 0.072) \times [\text{sanguinarine}] + (11.4 ± 15.2) \]
\[ D^1 = (0.127 ± 0.022) \times [\text{sanguinarine}] \]
\[ D^2 = (0.136 ± 0.016) \times [\text{sanguinarine}] - (1.9 ± 3.4) \]

Synthetic mixtures containing the three alkaloids were prepared and measured by the normal, first and second derivative techniques to prove the utility of the regression curves proposed above. The results are shown in Table 2. Serious interferences occurred with the normal spectra, as was expected, but the recovery obtained with the first and second derivatives was much closer to 100%.

From the recoveries in Table 2, the following observations can be made. First, berberine is only accurately determined by the second-derivative method, whereas luguine and sanguinarine can be measured by either the first or second derivative method. Secondly, the best technique for measuring the three alkaloids in the proportions quoted is the second derivative, which gives good recoveries and the smallest R.S.D.

**Application of proposed methods to natural samples**

To confirm the usefulness of the proposed methods, they were applied to the determination of berberine, luguine and sanguinarine in root extracts of *Glaucium flavum* taken from Misericordia beach (Málaga). The procedure is detailed under “extraction procedure”. The results are given in Table 3, and good concordance was obtained between the quantities found by the first and second derivative technique by use of the mean regression curve established for each alkaloid in mixtures.

**REFERENCES**