Simultaneous Determination of Codeine and Pyridoxine in Pharmaceutical Preparations by First-Derivative Spectrofluorimetry

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A method for the simultaneous determination of codeine and pyridoxine was developed, based on the measurement of their native fluorescence signals, by using first-derivative spectrofluorimetry to resolve the mixture. Codeine was measured at $\lambda_{\text{em}} = 309$ nm, and pyridoxine was measured at $\lambda_{\text{em}} = 450$ nm. Instrumental parameters were optimized, and the emission spectra were recorded between 275 and 475 nm, at $\lambda_{\text{ex}} = 255$ nm and excitation and emission slit widths of 2.5 and 10 nm, respectively. Systematic studies on the influence of species usually present along with the analytes (such as caffeine, ascorbic acid, paracetamol, and thiamine) were also performed. The calibration graphs were linear over the ranges of 0.5–7.0 and 0.1–1.0 $\mu$g/mL for codeine and pyridoxine, respectively, and the relative standard deviations ($n = 10$) were about 3%. The method was successfully applied to the determination of codeine and pyridoxine in solutions of synthetic mixtures and in synthetic and semisynthetic pharmaceutical formulations.

Codeine (Cod) is an alkaloid from an opiate group that has important effects on the central nervous system (1–3), including analgesic and mood-altering effects. It is a narcotic whose effects, although less potent, resemble those of morphine. Thus, one of the main limitations of its clinical use is its addictive nature. It is used alone and in combination products to treat mild-to-moderate pain and as a cough suppressant, because it has good antitussive properties and raises the stimulus threshold of the cough center.

Pyridoxine (Pyr) is a water-soluble vitamin (vitamin B₆) that plays an important role in many cellular reactions involving carbohydrates, fats, and proteins (it is the master vitamin in the processing of amino acids), in the synthesis of hemoglobin, and in the secretion of the adrenal glands. It also aids in the formation of several neurotransmitters; it is, therefore, an essential nutrient in the regulation of mental processes and possibly mood. Although very rare, vitamin B₆ deficiency causes, among other symptoms, impaired immunity, skin lesions, and mental confusion. Pyr is used to improve these symptoms and also to prevent heart disease.

Few procedures have been described for the determination of Cod (4–8). Most of them use liquid chromatography (LC) to determine Cod in the presence of other active principles (paracetamol, morphine, etc.). Nevertheless, even though Cod is a fluorophor, there are no fluorimetric procedures based on measurement of its native fluorescence, and only a few luminescence methods have been reported (9–11).

Few procedures have been developed for the determination of Pyr. Electrochemical (12); spectrophotometric (13, 14), including the use of a photometric flow-through sensor (14); and chemiluminescence (15) procedures have been described. Some fluorescence procedures have also been described for the simultaneous determination of B₆ vitamins other than Pyr after derivatization (16, 17).

Nevertheless, even though Pyr is a fluorophor, the scarcity of procedures based on its native fluorescence (18) should be noted. A spectrofluorimetric flow-through sensor has been developed that is based on the transient retention of Pyr on a cationic gel placed in a flow-through cell and used to monitor its native fluorescence (19).

Several LC procedures have been described for the determination of Pyr along with other B vitamins in pharmaceutical preparations (20, 21) and in other samples (22, 23). In some cases, an LC procedure has been described for the determination of vitamins B along with Cod and central nervous system stimulants (e.g., Cod, caffeine, and thiamine; 24). Recently, we proposed an LC method to determine Cod and Pyr together with other analgesics (caffeine and paracetamol) and another water-soluble B vitamin (thiamine) in pharmaceuticals (8). Apart from that method, there are no alternative methods described for resolving the binary mixture Cod–Pyr. Only a fluorimetric method is available to determine the 2 compounds simultaneously by using their native fluorescence [a partial least squares (PLS) multivariate calibration-based procedure] to resolve a ternary mixture (25).
In this paper, a simple first-derivative spectrofluorimetric procedure is presented for the rapid resolution of Cod–Pyr mixtures. Because an interaction between the analytes could be proved, a nonadditive behavior in the analytical signals was found. Consequently, the working wavelengths are not the optimum wavelengths, but the mixture could be satisfactorily resolved, and the procedure was successfully applied to pharmaceuticals.

**Experimental**

**Reagents**

(a) Stock solutions at 100 µg/mL.—Prepared by dissolution of appropriate amounts of Pyr hydrochloride (Fluka, Madrid, Spain) and Cod (Abelló, Barcelona, Spain) in deionized water. Solutions stored in a refrigerator at 4°C are stable for ≥4 weeks. Working solutions are prepared daily by suitable dilutions.

(b) NaH$_2$PO$_4$/Na$_2$HPO$_4$ buffer solution.—0.01M, pH 7.0.

(c) Deionized water.—Used throughout.

**Apparatus**

(a) Spectrofluorimeter for fluorimetric measurements.—Perkin-Elmer LS 50 (Beaconsfield, Buckinghamshire, UK), equipped with a xenon discharge lamp (20 kW), Monk-Gillieson (Beaconsfield) monochromators, a Quantic Rhodamine 101 counter to correct the excitation spectra, and a gated photomultiplier; connected via an RSC232 interface to a MITAC mpc 3000F-386 microcomputer running Fluorescence Data Manager Software (FLDM; Beaconsfield), Version 2.50, to control the instrument. The computer was also connected to an Epson (Suwa-Shi, Nagano-Ken, Japan) LX-800 printer for delivery of results. To compare all spectrofluorimetric measurements and ensure reproducible experimental conditions, the LS 50 spectrometer was checked daily with a sample of the fluorescent certified water standard. Smoothed spectra were calculated by using the Savitzky-Golay method (26), and contour plots in the excitation–emission plane were produced, linking points of equal fluorescence intensity. All measurements were made with a quartz cell of 1 cm pathlength at 20°C; the temperature was controlled to within ±0.1°C with the aid of a Selecta (Barcelona, Spain) Frigiterm 6000-382 ultrathermostat.

(b) Nuclear magnetic resonance (NMR) spectrometer.—Bruker Instruments (S. Fernando de Henares, Madrid, Spain) Advance DPX300, used to obtain the NMR spectra.

(c) Digital pH meter.—Crison 2000 (Barcelona, Spain), furnished with a combined glass/saturated calomel electrode assembly and used for pH measurements. The pH meter was calibrated with 2 buffers at pH 4.00 and 7.00. An Ultrasons Selecta (Barcelona, Spain) ultrasonic bath was also used.

(d) Liquid chromatograph.—Model HP 1050 (Hewlett-Packard Co., Avondale, PA), used for the reference measurements.
Figure 4. $^1$H-NMR spectrum of Cod in D$_2$O.

Figure 5. $^1$H-NMR spectrum of Pyr hydrochloride in D$_2$O.
An aliquot of the sample solution containing Cod at 0.5–7 μg/mL and Pyr hydrochloride at 0.1–1 μg/mL was transferred to a 50 mL volumetric flask; 5 mL phosphate buffer, pH 7.0, was added, and the solution was diluted to volume with deionized water. Calibration graphs were constructed in the same way by using Cod and Pyr standard solutions of known concentrations. The spectra (275–475 nm) were recorded at 20.0 ± 0.1°C (λex = 255 nm) and processed with the aid of the spectrofluorimeter’s bundled software, by using excitation and emission slit widths of 2.5 and 10 nm, respectively, and a scan speed of 240 nm/min. The spectra were then stored on a disk file and corrected for the blank signal, and the first-derivative spectra were obtained from the FLDM software by using 5 as the width factor and 5 as the number of points. Analytical signals were measured from the first-derivative spectra as the vertical distance at 309 (Cod) and 450 nm (Pyr).

**Treatment of Samples**

**Tablets.**—Twenty tablets were ground to a fine powder and dissolved in water by sonication.

**Capsules.**—The contents of 20 capsules were carefully transferred and dissolved in water by sonication.

The solutions were filtered through a 0.45 μm pore size Millipore filter, and the filtrates were diluted to an appropriate volume with deionized water. Suitable dilutions were performed in all cases.

**Results and Discussion**

**Spectral Characteristics and Effects of Experimental Variables**

At pH 7.0, both Cod and Pyr show 3 excitation maxima: at 215, 240, and 283 nm and 220, 255, and 325 nm, respectively. Emission maxima were at 350 nm for Cod and 395 nm for Pyr.

We studied the influence of the variables potentially affecting the fluorescence intensity or the position of the emission maxima in order to optimize measurement conditions. Fig-

Table 1. Values obtained by the proposed method for various analytical parameters

<table>
<thead>
<tr>
<th>Analytical parameter</th>
<th>Cod (μg/mL)</th>
<th>Pyr (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept on y-axis</td>
<td>1.22</td>
<td>0.36</td>
</tr>
<tr>
<td>Slope, mL/μg</td>
<td>3.00</td>
<td>14.83</td>
</tr>
<tr>
<td>Correlation coefficient (r)</td>
<td>0.997</td>
<td>0.999</td>
</tr>
<tr>
<td>LDR, μg/mL</td>
<td>0.5–7</td>
<td>0.1–1.0</td>
</tr>
<tr>
<td>LOD, μg/mL</td>
<td>0.11</td>
<td>0.02</td>
</tr>
<tr>
<td>LOQ, μg/mL</td>
<td>0.35</td>
<td>0.06</td>
</tr>
<tr>
<td>RSD, %</td>
<td>2.94</td>
<td>3.12</td>
</tr>
</tbody>
</table>

* LDR = linear dynamic range.
* LOD = limit of detection (criterion, 3σ).
* LOQ = limit of quantitation (criterion, 10σ).
* RSD = relative standard deviation; n = 10.
Figure 1 shows the influence of pH on the fluorescence intensity. This influence was studied by adding small volumes of diluted solutions of HCl and NaOH to adjust this variable. pH values over the range 1.5–12 had no appreciable effect on the fluorescence intensity of Cod; however, Pyr showed a maximum fluorescence at pH 7.0, with a strong decrease in the signal for the pH values above and below this value; therefore, we chose pH 7.0 as the optimum value. We found NaH2PO4/Na2HPO4 buffer solution, pH 7.0, had no effect on the fluorescence intensity of the analytes in the concentration range assayed, 0.005–0.025M. We chose a 0.01M buffer concentration.

Increasing temperature in the range of 0–70°C decreased the fluorescence intensity; therefore, we used a thermostat to maintain the temperature of solutions at 20.0±0.1°C in subsequent experiments. The fluorescence signal remained constant for several hours and no photodecomposition was observed.

Figure 2 shows the conventional emission spectra of the analytes and a mixture of the analytes. The extensive spectral overlap does not allow a satisfactory resolution of the mixture.

### Table 2. Determination of Cod and Pyr in commercial formulations

<table>
<thead>
<tr>
<th>Drug</th>
<th>Proposed method</th>
<th>Reference method (LC)</th>
<th>Label claim</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cod</td>
<td>Pyr</td>
<td>Cod</td>
</tr>
<tr>
<td>Benadomb</td>
<td>—</td>
<td>279±15</td>
<td>—</td>
</tr>
<tr>
<td>Conductasa</td>
<td>—</td>
<td>29±1</td>
<td>—</td>
</tr>
<tr>
<td>Serfoxided</td>
<td>—</td>
<td>284±3</td>
<td>—</td>
</tr>
<tr>
<td>Codeisánd</td>
<td>27.6±0.3</td>
<td>—</td>
<td>27.3±0.3</td>
</tr>
<tr>
<td>Codeine Perduretasd</td>
<td>48.5±0.5</td>
<td>—</td>
<td>47±1</td>
</tr>
</tbody>
</table>

a Results are given in mg/unit.
b Expressed as pyridoxine hydrochloride.
c Expressed as pyridoxine α-cetoglutarate.
d Expressed as anhydrous pyridoxine phosphoserinate.
e Expressed as codeine phosphate.

### Table 3. Determination of Cod and Pyr in semisynthetic formulations

<table>
<thead>
<tr>
<th>Drug</th>
<th>Pyr (hydrochloride)</th>
<th>Cod (phosphate)b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Labeled concn, µg/mL</td>
<td>% of label found ± SDa</td>
</tr>
<tr>
<td>Benadom</td>
<td>0.4</td>
<td>94 ± 1</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>94.4 ± 0.8</td>
</tr>
<tr>
<td>Conductasa</td>
<td>0.78</td>
<td>95 ± 1</td>
</tr>
<tr>
<td></td>
<td>0.96</td>
<td>93 ± 1</td>
</tr>
<tr>
<td>Serfoxide</td>
<td>0.38</td>
<td>96 ± 1</td>
</tr>
<tr>
<td></td>
<td>0.96</td>
<td>95 ± 2</td>
</tr>
</tbody>
</table>

| Codeisán      | 4.60 | 94 ± 1 | 0.52 | 103 ± 1 |
|               | 5.74 | 96 ± 2 | 1.00 | 101 ± 2 |
| Codeine Perduretas | 4.00 | 98 ± 2 | 0.3 | 97 ± 2 |
|               | 6.00 | 96.1 ± 0.4 | 1.0 | 98 ± 3 |

a SD = standard deviation.
b Active principle added.
c Expressed as pyridoxine α-cetoglutarate.
d Expressed as anhydrous pyridoxine phosphoserinate.
Table 4. Composition (mg) of synthetic drugs

<table>
<thead>
<tr>
<th>Compound</th>
<th>Drug 1</th>
<th>Drug 2</th>
<th>Drug 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Codeine</td>
<td>50</td>
<td>40</td>
<td>50</td>
</tr>
<tr>
<td>Pyridoxine</td>
<td>10</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Sodium saccharine</td>
<td>10</td>
<td>220</td>
<td>225</td>
</tr>
<tr>
<td>Saccharose</td>
<td>220</td>
<td>285</td>
<td>280</td>
</tr>
</tbody>
</table>

by using conventional spectrofluorimetric measurements. Therefore, derivative spectrofluorimetry was evaluated. Nevertheless, the system showed a very interesting nonadditive signal behavior, as shown in Figures 2 and 3. Thus, the regions in which signal additivity is achieved are restricted to those wavelengths below 312 nm and above 445 nm. Although sensitivity is strongly decreased in these regions, it is still appropriate for the determination of these compounds in pharmaceuticals.

Because previous separation of the analytes is unnecessary, the analysis is simple and quick; 309 and 450 nm were chosen as the appropriate wavelengths to determine Cod and Pyr, respectively. In fact, the height $h_1$ ($\lambda_{ex}/\lambda_{em} = 255/309$ nm) is proportional to the Cod concentration, whereas $h_2$ ($\lambda_{ex}/\lambda_{em} = 255/450$ nm) is proportional to the Pyr concentration (Figure 3). Other wavelengths at which the “zero crossing” technique apparently could be used (i.e., 330, 348, and 395 nm; see Figure 3) failed for this purpose because the lack of additivity made these wavelengths vary, depending on the Cod:Pyr ratio in the mixture, and led to significant errors. This nonadditive signal behavior could also be proved by means of ultraviolet spectrophotometry.

In order to know the possible interaction between the 2 analytes, the high-resolution $^1$H NMR spectra of Cod, Pyr hydrochloride, and a mixture of Cod and Pyr hydrochloride in D$_2$O (concentrations of about 1 g/L) with tetramethylsilane as an internal standard were obtained (Figures 4–6). Moreover, a direct heterocorrelation $^1$H–$^13$C heteronuclear multiple quantum correlation (HMQC) was performed for both compounds individually, as well as for the mixture. The spectrum of the mixture showed positive and strongly significant variations in the chemical shifts, $\Delta\delta$, of the $\alpha$ (7, 9, and 20) and $\beta$ (6, 10, and 18) H atoms in Cod with respect to N ($\Delta\delta$, about +0.60 ppm). On the one hand, the shift values were consistent with the protonation of the N atom by the H$^+$ from Pyr hydrochloride. On the other hand, the variations in the chemical shifts observed in H atoms 2 and 12 ($\Delta\delta = -0.39$ and $-0.14$, respectively) in Pyr hydrochloride were consistent with deprotonation of the H bound to aromatic N. Therefore, a chemical acid–base interaction between Pyr hydrochloride and Cod could explain the nonadditive signal behavior shown by the system.

This result is similar to that previously described by us (27) for the system Cod–acetylsalicylic acid, in which an acid–base interaction could also explain the nonadditive signal behavior of the system.

Instrumental Parameters

The most appropriate parameters to register conventional derivative spectra were selected. A scan speed of 240 nm/min and a luminescence spectrometer response time of 1 s were selected after we verified that these parameters did not affect the signals. To reduce the noise levels in the spectra, we used a software smoothing function based on the Savitzky-Golay method (26); we selected 7 as the smoothing width and 49 as the number of points.

Analytical Parameters

Under the optimal conditions described above, $h_1$ and $h_2$ were linearly related to the concentrations of Cod and Pyr hydrochloride, respectively, for the ranges 0.5–7.0 µg/mL (Cod) and 0.1–1.0 µg/mL (Pyr hydrochloride). The values for the analytical parameters are shown in Table 1.

In order to test the mutual independence of the analytical signals for Co and Pyr, i.e., to show that $h_1$ and $h_2$ are independent of the Pyr and Cod concentrations, respectively, we obtained 4 calibration graphs from height measurements for standards containing Cod concentrations of 1–10 µg/mL, in the presence of Pyr hydrochloride concentrations of 0.2, 0.4, 1.0, and 1.5 µg/mL. The values of the slopes of the calibration lines for Cod, obtained with Pyr hydrochloride concentrations of up to 1.0 µg/mL, were found to be included in the confidence limits for the slope of the calibration line obtained in the absence of Pyr, as evaluated according to ref. 28.

By following the same procedure, 5 calibration graphs were prepared for standards containing Pyr hydrochloride concentrations of 0.1–1.0 µg/mL in the presence of Cod concentrations of 1, 2, 5, 7, and 10 µg/mL. Cod concentrations of up to 7 µg/mL could be tolerated (the confidence limits for the slope of the calibration line obtained in the absence of Cod were evaluated according to ref. 28).

Therefore, it can be concluded that the amplitude of the derivative signals at the working wavelengths is a function of only one of the components of the mixture.

Effect of Foreign Species

A systematic study was made to determine the effect of foreign species on the determination of Cod at 5 µg/mL and Pyr

Table 5. Determination of Cod and Pyr in synthetic drugs

<table>
<thead>
<tr>
<th>Drug</th>
<th>Cod/Pyr (w/w)</th>
<th>Conc added, mg/L</th>
<th>Recovery ± SD, %</th>
<th>Conc added, mg/L</th>
<th>Recovery ± SD, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>5</td>
<td>98 ± 1</td>
<td>1</td>
<td>104 ± 1</td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>4</td>
<td>98 ± 2</td>
<td>0.5</td>
<td>105 ± 3</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>5</td>
<td>97 ± 2</td>
<td>0.5</td>
<td>105 ± 2</td>
</tr>
</tbody>
</table>

* SD = standard deviation.
Ascorbic acid, caffeine, and paracetamol concentrations of ≤0.5 μg/mL, as well as thiamine concentrations of ≤1 μg/mL, were tolerated in the determination of cod. Also, in the determination of Pyr, ascorbic acid at 0.5 μg/mL, caffeine and paracetamol each at 1 μg/mL, and thiamine at 5 μg/mL were tolerated.

Ascorbic acid, thiamine, and paracetamol can sometimes be found together with Pyr in commercial pharmaceuticals. Thiamine is tolerated at the level in which it is found with Pyr. However, ascorbic acid and paracetamol are found in commercial pharmaceuticals at levels higher than those tolerated in the procedure. Their interference can be suppressed by pretreating the sample solution (10 mL) with 0.5 g Sephadex QAE-A-25 anion-exchange gel, equilibrating for 5 min at pH 8.0 for ascorbic acid (29) or pH 11.0 for paracetamol, filtering the equilibrated solution, and then applying the proposed procedure to the filtrate.

Applications

Pharmaceutical formulations.—The method was first applied to the determination of Cod and Pyr in pharmaceutical formulations. Samples were prepared and analyzed as described above, and the results were compared with those obtained by a reference reversed-phase LC method with diode-array UV detection that we developed previously (8). The 2 compounds were separated on a reversed-phase C₁₈ Nucleosil column with a mobile phase consisting of 2 successive eluants: water at pH 2.1 (5 min) and acetonitrile–water (75 + 25) at pH 2.1 (9 min); the pH of both eluants was adjusted with phosphoric acid, and Cod and Pyr were detected at 240 and 285 nm, respectively. The results obtained by both methods are shown in Table 2.

Semisynthetic mixtures from commercial formulations.—Next, the method was applied to the determination of Cod and Pyr in semisynthetic products prepared from all the assayed drugs. Because there was no pharmaceutical formulation containing both Cod and Pyr, we prepared semisynthetic products from the above assayed drugs to check the applicability of the procedure; therefore, the absent analyte was added in each case, respectively, at 3 different levels. Thus, a semisynthetic product was obtained by the addition of a component to a real pharmaceutical preparation. Consequently, in Benadom, Conductasa, and Serfoxide, the original content of Pyr and the added amounts of Cod were determined. In the same way, the content of Cod and the added amounts of Pyr were determined in Codeisán and Codeine Perduretas.

The results obtained, summarized in Table 3, show excellent agreement with those found by the reference method and the values indicated by the supplier; however, the amounts recovered are also in good agreement with the added amounts.

Synthetic drugs.—The proposed method was also applied to the analysis of several synthetic drugs that contained different Cod:Pyr ratios and were prepared in the proportions indicated in Table 4. Sodium saccharin and sucrose acted as excipients. Table 5 shows the results obtained.

Binary mixtures.—Finally, the proposed method was applied to the analysis of several synthetic mixtures of Cod and Pyr in solution at different ratios, inside the established linear range for each active principle. Table 6 summarizes the results that show very acceptable accuracy in all instances. Figure 7 shows the separation of the 2 compounds (Cod and Pyr) in a sample analyzed (Cod:Pyr = 2.5).

Conclusions

Because the first-derivative spectrofluorimetric procedure proposed for the simultaneous determination of Cod and Pyr is based on the measurement of the native fluorescence of the 2 analytes, it is fast and very simple. As a result of the nonadditive signal behavior of the system, the working wavelengths are not the optimum wavelengths but the mixture is satisfactorily resolved. The interaction between Cod and Pyr appears to be due to an acid–base reaction in which the N atom from Cod is protonated by the H⁺ from Pyr hydrochloride. No derivatization reaction is required, and the method is also inexpensive and useful for the determination of these compounds in pharmaceuticals.

Table 6. Determination of Cod and Pyr in binary mixtures (solution)

<table>
<thead>
<tr>
<th>Cod/Pyr (w/w)</th>
<th>Cod</th>
<th>Pyr</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Conc. added, μg/mL</td>
<td>Recovery ± SD, %</td>
</tr>
<tr>
<td>2.5</td>
<td>2.5</td>
<td>96 ± 2</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>101 ± 2</td>
</tr>
<tr>
<td>10</td>
<td>2</td>
<td>100 ± 4</td>
</tr>
<tr>
<td>17.5</td>
<td>7</td>
<td>98 ± 2</td>
</tr>
</tbody>
</table>

a SD = standard deviation.

hydrochloride at 1 μg/mL. A 50 μg/mL level of each potentially interfering species was tested first. If interference occurred, the ratio was reduced progressively until the interference ceased. The tolerance level was defined as the amount of foreign species that produces an error not exceeding ±5% in the determination of the analyte.

Ascorbic acid, caffeine, and paracetamol concentrations of ≤0.5 μg/mL, as well as thiamine concentrations of ≤1 μg/mL, were tolerated in the determination of cod. Also, in the determination of Pyr, ascorbic acid at 0.5 μg/mL, caffeine and paracetamol each at 1 μg/mL, and thiamine at 5 μg/mL were tolerated.

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The relative standard deviations obtained by the proposed method are slightly higher than those obtained by LC procedures; however, the proposed method has the advantages of being inexpensive, simple, and fast and of allowing the determination of Cod and Pyr at high Cod:Pyr ratios without previous separation.

Acknowledgment

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References