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Integrated derivatization–chemiluminescence detection system for the determination of β-carboline alkaloids by high-performance liquid chromatography

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Abstract

An integrated derivatization–chemiluminescence detection system was used for the high-performance liquid chromatographic determination of β-carboline alkaloids such as harmol, harmalol, norharmane, harmane, harmine and harmaline by the chemiluminescence produced in their reaction with the bis(2,4-dinitrophenyl)oxalate (DNPO)–hydrogen peroxide system. The ability of the proposed detection system based on its zero-dead-volume to overcome the problems associated with the use of this oxalate ester in peroxyoxalate chemiluminescence detection in HPLC was exploited to develop a sensitive chromatographic method for the determination of the above-mentioned hallucinogens. The method excels over other alternatives based on different detection techniques, such as UV and fluorescence spectroscopy, in terms of limits of detection. The proposed method was validated by determining the alkaloids (the identification and quantification of some are reported for the first time) in *Heliconiini* butterfly specimens with good results.

Keywords: Derivatization, LC; Detection, LC; Chemiluminescence detection; Alkaloids; β-Carboline alkaloids; Harmol; Harmalol; Norharmane; Harmane; Harmine; Harmaline

1. Introduction

The use of chemiluminescence (CL) reactions has grown considerably in recent years as a result of the high sensitivity and speed, and the fairly simple instrumentation required [1–3]. Actually, CL-based techniques are also highly selective compared to many other spectroscopic techniques such as UV–visible and fluorescence techniques; however, the relatively low selectivity achieved in some CL-based determinations restricts the scope of their application. In order to overcome this problem, several CL reactions have been used as detection systems in combination with high-performance liquid chromatography (HPLC). Initially, the oxidation of luminol by hydrogen peroxide in aqueous alkaline solutions (one of the best known examples of CL processes) and related systems were used with this purpose for the identification and quantification of metal ions by their catalytic effect on these reactions [4]. This

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system is restricted to metal trace analysis, so in order to expand the scope of HPLC–CL determinations, peroxyoxalate chemiluminescence (PO-CL) reactions are widely used, as they afford the measurement of the fluorescence (whether native or produced after derivatization) of the organic compounds with better limits of detection (typically two orders of magnitude) than that of conventional fluorescence methods [5,6]. The use of PO-CL reactions as detection systems in HPLC, however, is restricted by some practical shortcomings arising from their intrinsic nature. Thus, the instability of the oxalate ester in the presence of hydrogen peroxide [7–9], the typical background emission observed [10] and the need for critical adjustment of pH [11] decisively influence the choice of the detection system configuration. Recently, our group addressed these shortcomings by developing a simple zero-dead-volume PO-CL detection system for liquid chromatography that exhibited quite good performance in terms of sensitivity and band broadening [12]. The system was evaluated in the HPLC determination of polycyclic aromatic hydrocarbons (PAHs) using the bis(2,4,6-trichlorophenyl)oxalate (TCPO)–hydrogen peroxide system.

This paper extends the scope of the original zero-dead-volume PO-CL detection system and reports the results of a study of the potential of the DNPO–hydrogen peroxide system for the HPLC–PO-CL determinations. The special features of the proposed integrated derivatization–CL detection system widens the scope of the application of this oxalate ester in LC–CL systems, since it is not limited by its fast reaction kinetics. In addition, this detection system resolves the above-mentioned shortcomings arising from the intrinsic nature of PO-CL reactions. The proposed detection system was tested in the HPLC determination of β-carboline alkaloids, namely harmaline, harmine, harmalol, harmol, harmane and norharmane. The good results achieved were exploited for the determination of these hallucinogenic alkaloids in *Heliconiini* butterfly specimens. Various analytical techniques (particularly chromatographic ones) have been used for the determination of β-carboline alkaloids [13–16]. Specifically, HPLC is one of the most frequent choices for the determination of these compounds in various samples, especially with fluorimetric detection [17,18].

2. Experimental

2.1. Standards and reagents

All chemicals used were of analytical-reagent grade. Harmaline and harmine were supplied by Sigma, whereas harmalol, harmol, harmane and norharmane were purchased from Aldrich. Standard solutions containing 1000 μg ml^{-1} of each were prepared in methanol (chromatographic grade, Romil Chemicals) and stored at 4°C in a refrigerator. All dilute solutions were prepared in 55:45 (v/v) methanol–0.01% (m/v) triethylamine (TEA). A $4.75 \cdot 10^{-3}$ *M* DNPO solution was made by dissolving 200 mg of the chemical (Aldrich) in 100 ml of ethyl acetate (Merck). The oxidant/pH solution was prepared by mixing 46 ml of concentrated hydrogen peroxide (Merck) and 4 ml of 0.1 *M* sodium hydroxide (Merck) and diluting the mixture to 100 ml with 2-propanol (Merck). TEA was supplied by Merck and bidistilled water was used throughout.

2.2. Apparatus

A simplified scheme of the instrumental assembly used [12] is depicted in Fig. 1. The HPLC system consisted of a Waters W-600E multisolvent pump, a Rheodyne 7125 injector (20 μl loop) and two coupled 4 μm C_{18} Nova-Pak (15 cm×3.9 mm) cartridge columns. The CL detection system con-

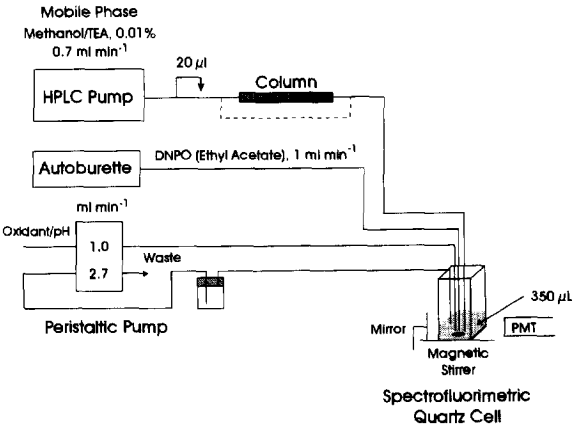

Fig. 1. Set-up used for the HPLC determination of β-carboline alkaloids with the proposed integrated derivatization–CL detection system.

sisted of a Gilson Minipuls-3 peristaltic pump, a 50-ml Metrohm Dossimat autoburette and a displacement bottle leading to waste, which ensured constancy in the volume of the reaction mixture in the cell. The autoburette was used to deliver the DNPO solution because the use of a displacement bottle resulted in gradual decomposition of the DNPO on contact with water across the interface. Poly(vinyl chloride) pumping tubes were used for carrying all reactants, and all other connections were established through stainless steel tubing. The CL detection was monitored with a Perkin-Elmer 650 10S spectrofluorimeter with its light source off. The sample compartment of the spectrofluorimeter was replaced by a small magnetic stirrer which supported the 1.0 cm quartz cell and an Oriel 441321 1 in. diameter mirror, in order to acquire as much emitted light as possible (see Fig. 1). Retention times, peak heights and peak areas were provided by a Waters Maxima 820 chromatographic workstation interfaced to a NEC PC-AT 33-MHz compatible computer.

2.3. Procedure

In the HPLC system described above, the mobile phase was delivered at 0.7 ml min^{-1} in a staircase eluent composition gradient from 55:45 (v/v) methanol–0.01% (m/v) TEA initially, to pure methanol; the ramp was applied 20 min after the injection. Peak heights for standard compounds (2.5–3600 pmol in 20 μl of sample, depending on the hallucinogen) were used to determine sample concentrations. Retention times were used to identify compounds.

2.4. Determination of β-carboline alkaloids in butterfly specimens

A whole insect specimen was dried to constant weight at 40°C and accurately weighed samples of between 50 and 70 mg were ground and extracted with methanol for 5 min. The extract was evaporated to dryness at 50°C at a low pressure. The residue was dissolved in 3.0 ml of 1.2 *M* hydrochloric acid and the resulting solution was filtered. The soluble fraction was adjusted to pH 8 with sodium hydroxide and then was passed through a C_{18} minicolumn (HPLC sorbent with 21% carbon content and an average particle size of between 15 and 40 μm, purchased from Sigma) that had previously been conditioned with a stream of 0.1 *M* ammonia chloride–ammonium buffer (pH 8) at 0.5 ml min^{-1}. The hallucinogens were eluted with 5 ml of methanol flowing at 0.5 ml min^{-1}. The eluent was evaporated to dryness at 50°C at a low pressure and dissolved in 100 μl of methanol. Aliquots (20 μl) were subsequently used for the HPLC determination of the hallucinogenic alkaloids.

3. Results and discussion

The aim of this work was to evaluate the potential of the DNPO–hydrogen peroxide system using a simple, flexible and inexpensive integrated derivatization–CL detection system for the HPLC–PO-CL determinations, in order to resolve the problems associated with the well-known CL postcolumn detection flow systems. To test the performance of the proposed approach, it was used for the HPLC determination of β-carboline alkaloids (harmaline, harmine, harmalol, harmol, harmane and norharmane), the structural formulae of which are shown in Fig. 2. In addition, the proposed method is the first report on the HPLC determination of these hallucinogenic alkaloids with CL detection.

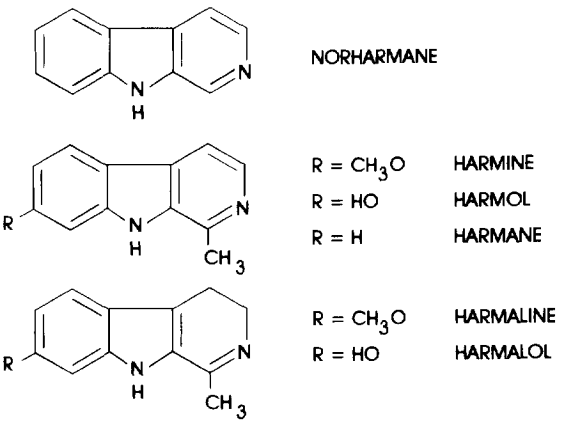

Fig. 2. Structure of β-carboline derivatives.

3.1. Influence of experimental variables

In order to ensure the most suitable conditions for the HPLC determination of the hallucinogenic alkaloids, the effect of experimental variables potentially affecting the detection and separation processes was investigated using the univariate method. Because harmaline exhibited the highest response in the DNPO–hydrogen peroxide reaction [19], we chose this β-carboline alkaloid to study the effect of chemical and flow variables affecting the integrated derivatization–CL detection system. Experiments were carried out using the instrumental setup of Fig. 1, minus the column (see dashed line in the figure).

One important variable markedly affecting the performance of this detection system is the solvent composition of the mobile phase, which must ensure efficient CL production. In many cases, an additional cosolvent is required to improve miscibility of the solvent in which the DNPO is generally dissolved, ethyl acetate, with the water from hydrogen peroxide and buffer, and the mobile phase (which also usually contains some water). The composition of the mobile phase used in this work for optimization of the CL detection system was 70:30 (v/v) methanol–0.03% (m/v) TEA, which provided sufficient methanol to ensure quite good miscibility in the reaction medium, so no additional cosolvent was needed.

The optimization of the essential ingredients of the peroxyoxalate CL reaction used as the detection system in the HPLC determination of hallucinogens, viz. hydrogen peroxide and DNPO, entailed adopting a compromise between several factors in order to ensure maximum sensitivity with minimal band broadening in the chromatographic response. In order to maximize the sensitivity, the water content in the reaction medium must be as low as possible [20] and the ratio of hydrogen peroxide to DNPO concentration must be as high as possible, to diminish the typical background emission observed in this type of CL reaction [10]. Under these conditions, the reaction half-life is dramatically shortened and the peak width is decreased as a result. On the other hand, the use of low concentrations of DNPO is recommended provided they do not detract from sensitivity, due to the high cost of this reagent.

The influence of the hydrogen peroxide and DNPO concentrations and their flow-rates is reflected in Fig. 3. The highest concentration tested for both reagents was the maximum allowed; concentrated [33% (v/v)] hydrogen peroxide and $7.1 \cdot 10^{-3}$ *M* for DNPO (imposed by its solubility in ethyl acetate). The concentrations and flow-rates chosen were 10.8 *M* hydrogen peroxide at 0.4 ml min^{-1}, and $4.75 \cdot 10^{-3}$ *M* DNPO at 1.0 ml min^{-1}. Higher flow-rates for the hydrogen peroxide stream could not be used due to the formation of two immiscible phases in the reaction mixture the methanol content in the mobile phase was inadequate for appropriate mixing of water and ethyl acetate. At this point it is worth

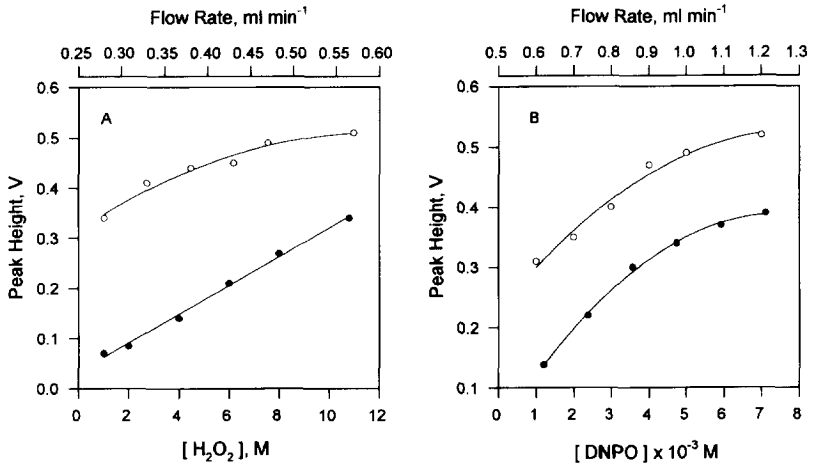

Fig. 3. Influence of (A) the concentration (●) and flow-rate (○) of hydrogen peroxide and (B) the concentration (●) and flow-rate (○) of DNPO on peak height. The injected amount of harmaline was 400 pmol in (A) and 200 pmol in (B).

noting that, under these selected experimental conditions, the peroxyoxalate CL reaction was unfavorable due to the high water content in the reaction medium (ca. 35%), according to the composition of the mobile phase required to ensure efficient separation. In order to work in the most favorable conditions for detection, an additional cosolvent can be incorporated into the reaction medium. This cosolvent was 2-propanol because it performs in a similar manner to the CL intensity as acetone [12]. For simplicity, a 50:50 (v/v) concentrated hydrogen peroxide–2-propanol solution delivered at 1.0 ml min^{-1} was used, which ensures the selected hydrogen peroxide concentration in the reaction medium (2.0 *M*) and decreases its water content with an appreciable increment in the CL intensity.

The effect of the composition of the mobile phase on the peroxyoxalate CL detection of hallucinogens was studied in order to elucidate the influence of its water content, pH and TEA concentration. As can be seen in Fig. 4, the detector signal decreased with increasing pH (i.e. with increasing TEA content in the mobile phase); however, the effect can be ascribed to the subsequent increase in the water content in the reaction medium rather than to the influence of pH itself. In fact, additional experiments using the same methanol–water proportions in the mobile phase and variable TEA concentrations of between 0.01 and 0.03% did not significantly affect the peak height. Variation of the pH of the mobile phase (shown in Fig. 4) does not practically affect the pH of the reaction medium in the integrated derivatization–CL detection system, which ranged from 3.3 to 3.8. As the maximum sensitivity for the PO-CL determination of β-carboline alkaloids using DNPO was achieved at pH 5.0 [19], the hydrogen peroxide–2-propanol solution was modified, incorporating enough sodium hydroxide to ensure a final pH in the reaction medium of ca. 5.0. So, the so-called oxidant/pH solution was prepared as indicated in Section 2. These results were used as a reference in the subsequent study of the effect of the composition and flow-rate of the mobile phase on the separation process.

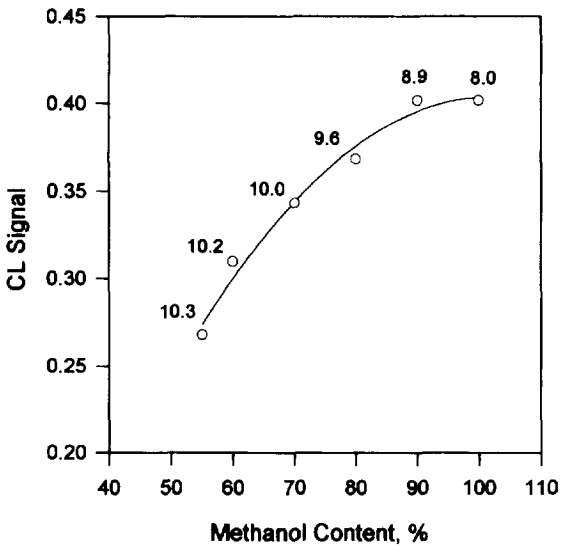

Fig. 4. Effect of the methanol content and pH of the mobile phase on the detector response. The injected amount of harmaline was 50 pmol. Numbers by the points are the pH values of the mobile phase based on its composition.

Hallucinogenic alkaloids were previously separated and determined by HPLC using a variable methanol–TEA gradient and fluorimetric detection [17]. In this work, we used a similar prepacked analytical reversed-phase C_{18} column (particle size 4 μm instead of 10 μm) and a different detection system, so it was advisable to re-optimize the separation. Several variables including the composition of the mobile phase in the isocratic and gradient mode, the flow-rate and the TEA concentration were studied. Using 55:45 (v/v) methanol–0.01% (m/v) TEA at a flow-rate of 0.7 ml min^{-1} ensured quantitative separation of the six hallucinogenic alkaloids studied; however, the elution of harmaline, the last in the sequence, occurred at a long retention time that must be shortened by using a simple gradient. Thus, the mobile phase was initially used with the above-mentioned composition (harmol, harmalol, norharman, harman and harmine were thus quite well resolved) and 20 min later, the composition was changed to pure methanol. Fig. 5A shows a typical chromatogram for a mixture of the analytes that is recorded in 30 min. The selected conditions were simpler than those recommended in the literature [17], namely a variable gradient with several changes in the composition of the mobile phase and the whole chromatogram recorded in about 40 min.

Finally, the effect of the cell volume (or, specifically, the volume of the reaction mixture in the

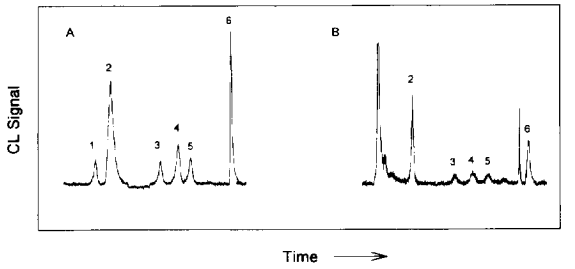

Fig. 5. Chromatograms obtained under the conditions described in Section 2.3 for (A) a standard and (B) a *Heliconiini eratohydara* extract. Peaks: 1=harmol, 2=harmalol, 3=norharmane, 4=harmane, 5=harmine and 6=harmaline.

detection cell), an important variable closely related to band broadening, was studied between 200 and 450 μl. As the likely result of the short half-life of the peroxyoxalate CL reaction used, this variable had virtually no effect on the detector response over the range assayed, so a cell volume of 350 μl was chosen. These settings endow the integrated derivatization–CL detection system with high flexibility, since the location of the waste tube is not critical, with a view to maintaining a constant cell volume (see Fig. 1). The peak width obtained under these conditions was only ca. 1.5–2 min.

3.2. Calibration of the system

Calibration graphs were obtained under the experimental conditions described in Section 2.3, by plotting peak height against the injected analyte mass. Table 1 gives the corresponding regression equations, limits of detection (calculated as the mass of analyte providing a signal equal to twice the peak-to-peak noise [21] and the precision, expressed as the relative standard deviation (R.S.D.) and obtained by analysing eleven samples containing an amount of each hallucinogen within the range of the calibration graph. The dynamic range shown in Table 1 can be expanded to greater amounts of β-carboline alkaloids by changing the instrumental response of the spectrofluorimeter used for CL detection.

Table 1
Features of the calibration plots and analytical figures of merit of the CL determination of hallucinogenic alkaloids

Hallucinogen	Dynamic range (pmol)	Linear regression equation	Correlation coefficient ($n=10$)	LOD (pmol)	R.S.D. (%)
Harmol	75–3000	$H=3.2\times10^{-4}+9.3\times10^{-5}\,C$	0.998	12.5	3.8
Harmalol	150–600	$H=1.3\times10^{-2}+2.3\times10^{-3}\,C$	0.993	18.5	4.7
Norharmane	90–3600	$H=2.4\times10^{-3}+6.9\times10^{-5}\,C$	0.996	13.5	4.8
Harmane	80–3000	$H=5.3\times10^{-4}+1.4\times10^{-4}\,C$	0.999	11.0	3.5
Harmine	70–2500	$H=2.1\times10^{-3}+1.1\times10^{-4}\,C$	0.998	11.5	5.0
Harmaline	2.5–125	$H=1.2\times10^{-3}+8.6\times10^{-3}\,C$	0.995	0.3	4.2

H, peak height (in volts); C, analyte mass (in pmol); LOD, limit of detection; R.S.D., relative standard deviation.

Table 2
Comparison of the analytical figures of merit for the HPLC determination of hallucinogenic alkaloids using chemiluminescence (CL) and fluorimetric (F) detection

Hallucinogen	Dynamic linear range (nmol)		Limit of detection (pmol)		Precision (R.S.D.) (%)	
	CL	F	CL	F	CL	F
Harmol	0.075–3.0	0.2–15.0	12.5	60	3.8	3.7
Harmalol	0.15–0.6	5.0–15.0	18.5	1500	4.7	4.4
Norharmane	0.09–3.6	0.6–12.0	13.5	185	4.8	4.0
Harmane	0.08–3.0	0.5–11.0	11.0	150	3.5	4.1
Harmine	0.07–2.5	0.5–9.5	11.5	145	5.0	4.1
Harmaline	0.0025–0.125	0.5–14.0	0.3	155	4.2	3.7

Table 3
Determination of β-carboline alkaloids in *Heliconiini* butterfly specimens using the proposed method

Specimen	Alkaloids									
	Harmalol		Norharmane		Harmane		Harmine		Harmaline	
	Found (μg)	Content (%)	Found (μg)	Content (%)	Found (μg)	Content (%)	Found (μg)	Content (%)	Found (μg)	Content (%)
H. eratohydara[a]	1.07±0.07	1.8×10^{-3}	0.56±0.04	9.5×10^{-4}	0.85±0.03	1.4×10^{-3}	0.52±0.01	8.9×10^{-4}	0.072±0.004	1.2×10^{-4}
H. eratohydara[b]	1.24±0.02	2.0×10^{-3}	0.45±0.03	7.5×10^{-4}	0.14±0.01	2.4×10^{-4}	0.13±0.01	2.3×10^{-4}	0.025±0.001	4.1×10^{-5}
H. charithonia	0.99±0.04	1.5×10^{-3}	0.29±0.05	4.3×10^{-4}	0.80±0.02	1.2×10^{-3}	1.05±0.05	1.5×10^{-3}	0.035±0.002	5.2×10^{-5}

[a,b] Different samples of *Heliconiini eratohydara*.

The analytical figures of merit of the proposed HPLC determination were compared with those obtained using fluorimetric detection, to date the most sensitive alternative for the determination of these β-carboline alkaloids. Although HPLC in conjunction with fluorescence detection had previously been used for this purpose [17], we repeated measurements in order to ensure accurate comparison with the instrumentation used. For this purpose, the integrated derivatization–CL detection system was replaced with a 200-μl spectrofluorimetric flow-cell and the fluorescence signal was measured at $\lambda_{ex}=370$ nm and $\lambda_{em}=425$ nm. Table 2 shows the results obtained with both chemiluminescence and fluorimetric detection. As can be seen, CL detection provides wider dynamic linear ranges and lower limits of detection (of between one and two orders of magnitude). The precision was only slightly lower with CL detection on account of the intrinsic configuration of the zero-dead-volume detector used; however, R.S.D. values were quite acceptable.

3.3. Determination of β-carboline alkaloids in Heliconiini butterfly specimens

The proposed method was applied to the identification and quantification of hallucinogenic alkaloids in several adult *Heliconiini* butterfly specimens. The butterflies were collected approximately 1000 m above sea level on the road from Estanques to Las Coloradas in Mérida (Venezuela) in 1994. For extraction of hallucinogens, the specimens were subjected to the procedure described in Section 2, which was developed for this purpose. Fig. 5B shows a representative chromatogram for *Heliconiini eratohydara*, as can be seen, five hallucinogenic alkaloids (harmalol, norharmane, harmane, harmine and harmaline) were identified and quantified by the proposed method. While harmol may have been present, it was difficult to quantify; also, an unknown substance (possibly another β-carboline alkaloid) that was eluted immediately before harmaline and did not interfere with its identification and quantification was also detected. The results obtained (Table 3) are consistent with the reported contents of β-carboline alkaloids in other *Heliconiini* specimens (for norharman, harman and harmine) [17]. In summary, the use of the proposed CL detection method

considerably enhances the determination of hallucinogenic alkaloids in this type of sample.

4. Conclusions

The integrated derivatization–chemiluminescence detection system proposed in this work was shown to be a useful means of using the DNPO reaction for HPLC–PO-CL determinations without the problems associated with its fast reaction kinetics. The evaluation of this detection system revealed two important features with respect to the flow systems: (1) higher hydrogen peroxide–DNPO concentration ratios can be used, which resulted in increased sensitivity in the PO-CL detection (these conditions favor suppression of background emission and increase the signal-to-noise ratio) and (2) there is no dead time between mixing DNPO and hydrogen peroxide and the reaction with the fluorophor (in contrast to typical flow systems), which increases the efficiency of the PO-CL reaction and avoids the typical instability of DNPO solutions in the presence of hydrogen peroxide.

The proposed approach was evaluated in the HPLC–PO-CL determination of hallucinogenic alkaloids, which is reported for the first time. The limits of detection (pmol range) and working ranges found allowed the identification and quantification of the hallucinogenic alkaloids in *Heliconiini* butterfly specimens (some of which are reported for the first time).

Acknowledgments

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