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Simultaneous Determination of Harman, Harmaline and Norharman by Synchronous Fluorescence

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The simultaneous determination of harman, harmaline and norharman has been described using synchronous fluorescence technique. The method has been based on their natural fluorescence. It is difficult to analyze and determine their contents by conventional fluorescence method because of their similar molecular structures. The synchronous spectrum, maintaining a constant wavelength difference of $\Delta\lambda$ =185 nm between the excitation and emission monochromators, was selected as optimum to perform the determination. The method was also performed in aqueous medium at pH 4.0 and in presence of sodium dodecyl sulfate (SDS), 1×10^{-5} M. Under the optimum conditions, each analyte has the linear determination range of 1×10^{-7}M- 1×10^{-4} M.

Keywords: Synchronous fluorescence, β-carboline alkaloids, Harman, Harmaline, Norharman

INTRODUCTION

A number of tremorogenic β-carboline alkaloids like harman, harmaline, harmine, norharman (Figure 1) and others are found in common plant-derived foodstuffs [1] such as wheat, rice, corn, barley, soybeans, rye, grapes, mushrooms, vinegar; plant-derived beverages like wine, beer, whisky, brandy, sake and plant-derived inhaled substances like tobacco [2]. Because of their presence in the food chain, it is understandable that the route of exposure in humans would be from dietary sources, smoking, and consumption of alcoholic beverages. At high concentration harman and norharman are also reported to have cytotoxic and carcinogenic effects causing renal toxicity in male F344 rats at the dietary level of 1000 ppm [3]. The development of efficient and sensitive analytical procedures for detection and quantitation of β-carboline alkaloids is highly desirable to researchers involved in studies as diverse as pharmacology, toxicology and analytical chemistry. High performance liquid chromatography for norharman [4] and harmol [5] with fluorescence detection and high performance capillary electrophoresis with diode-array detection [3] for six alkaloids have been established important analytical tools due to their high resolving power, speed, automation and reproducibility, but synchronous fluorometry to determine alkaloids simultaneously has not been reported.

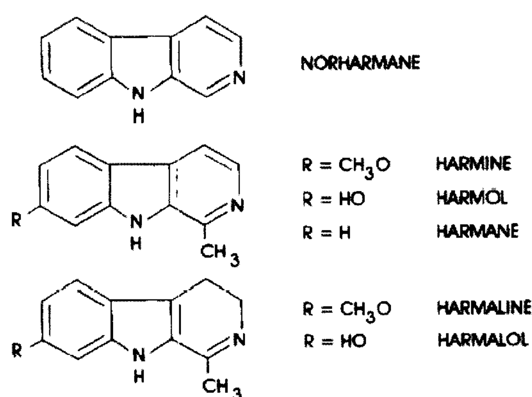

Figure 1. Chemical structures of β- carboline alkaloids.

Fluorescence spectroscopy is widely used in different fields of the chemical analysis owing to its high sensitivity and selectivity, and relatively low instrumental cost. However, as is usual in other spectroscopic techniques, when analyzing mixtures of components that show overlapping spectra, they often cannot be successfully resolved and a prior separation technique is usually required. As a consequence, analysis costs are increased and the procedure moreover, becomes more time consuming.

In recent years multicomponent analysis has appeared of great importance in resolution of mixtures of components in many fields including biomedical, clinical, environmental and drug analysis. Increasing attention has been paid to the synchronous fluorescence spectroscopy which has been found to have several advantages such as simple spectra, high selectivity and low interference effect. Synchronous fluorescence spectrometry first developed by Lloyd [6], is known to be a useful method for simultaneous determination of compounds in multi-component organic mixtures [7-10]. Synchronous fluorescence is classified into constant-energy, constant-wavelength, variable -angle and constant energy difference [11] as per scanning modes of the monochromators. At present, constant-wavelength method which maintains a

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constant wavelength difference between emission and excitation monochromators is extensively used. The fluorescence spectra (emission) of β-carboline alkaloids like harman, harmaline and norharman overlap considerably so that the conventional fluorimetric method does not allow simultaneous determination of these compounds.

In our present work, harman, harmaline and norharman in a mixture were directly determined by synchronous fluorescence spectroscopic principle. Under optimum conditions of pH and surfactant concentrations the three alkaloids of interest were successfully separated using a constant difference of $\Delta\lambda$=185 nm.

EXPERIMENTAL

Materials

All chemicals used were of analytical-reagent grade. Harman, harmaline and norharman were supplied by Sigma-Aldrich USA. Surfactant triton® X-100 was purchased from sigma, sodium dodecyl sulfate (SDS) from fluka and dodecyl trimethylammonium bromide (DDTAB) from sigma. Stock standard solutions of harman, harmaline and norharman were prepared by dissolving the pure materials in ethanol and working standard solutions were made by appropriate dilution with deionized water. A buffer solution of pH 4.0 (0.2 M sodium acetate/acetic acid) was also used. The water used was obtained from a Milli-Q water purification system.

Instrumentation

In Figure 2, we show a schematic diagram of the experimental set up. Spectrofluorimetric measurements were made on SPEX (Edison, NJ, USA) FL 111 spectrofluorometer equipped with a 450-W xenon lamp, quartz cells having a 1cm optical path, 0.50 mm slits on monochromators. Fluorescence was collected and detected by a Hamamatsu R928 photomultiplier tube. All spectral data were obtained by SPEX DM 3000F spectroscopy computer. A pH meter (Model Orion 520A USA) was used for monitoring pH adjustment. Origin version 6.0 professional soft ware was used for obtaining calibration data.

Procedure

Using the synchronous fluorescence spectroscopic method described earlier a known concentration of 1×10^{-4} M of harman or harmaline or norharman, buffer solution of pH 4.0 (0.2 M sodium acetate/acetic acid) and SDS medium (1×10^{-5} M) (1:1:1) were mixed and excitation spectra (intensity as response) of harman, harmaline and norharman were recorded with the emission wavelengths at 427 nm, 481 nm and 444 nm respectively and emission spectra were obtained maintaining the excitation wavelengths at 242 nm, 365 nm and 247 nm for harman, harmaline and norharman respectively. The synchronous fluorescence spectrum of each analyte and their mixture were recorded by simultaneous scanning the excitation and emission monochromators with a constant wavelength difference, $\Delta\lambda$=185 nm between them. At the same time, the fluorescent intensity of harman, norharman and harmaline at 242, 290 and 350 nm were recorded.

RESULTS AND DISCUSSION

Spectral characteristics

Harman exhibits two excitation maxima at 242 and 292 nm and one emission maximum at 427 nm (Figure 3). Harmaline showed excitation maxima at 255 and 365 nm and its emission maximum was at 481 nm (Figure 4). Norharman showed three excitation maxima at 247, 295 and 365 nm with emission maximum at 444 nm (Figure 5). Considering the maximum intensities, the excitation wavelengths for harman, harmaline and norharman were 242, 365 and 247 nm and emission wavelengths recorded were 427, 481 and 444 nm respectively. The fluorescence spectra of harman, harmaline

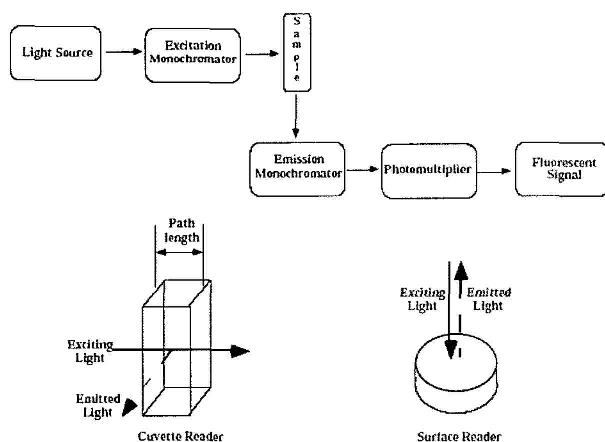

Figure 2. Schematic diagram of the synchronous fluorescence determination of β- carboline alkaloids.

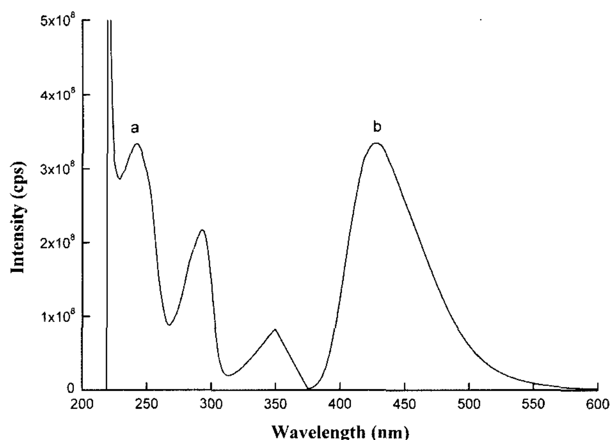

Figure 3. (a) Excitation (λ_{em} = 427 nm), (b) emission (λ_{ex} = 242 nm) spectra of harman. Conditions: Harman, 1×10^{-4} M; SDS, 1×10^{-5} M; Buffer, 0.2 M HOAc-NaOAc (pH 4.0).

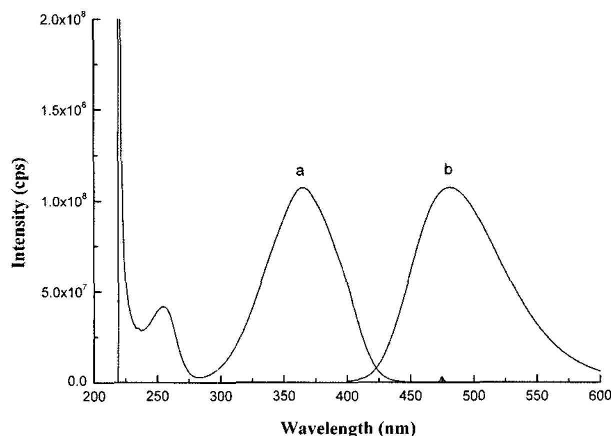

Figure 4. (a) Excitation (λ_{em} = 481 nm), (b) emission (λ_{ex} = 365 nm) spectra of harmaline. Conditions: Harmaline, 1 × 10^{-4} M; SDS, 1 × 10^{-5} M; Buffer, 0.2 M HOAc-NaOAc (pH 4.0).

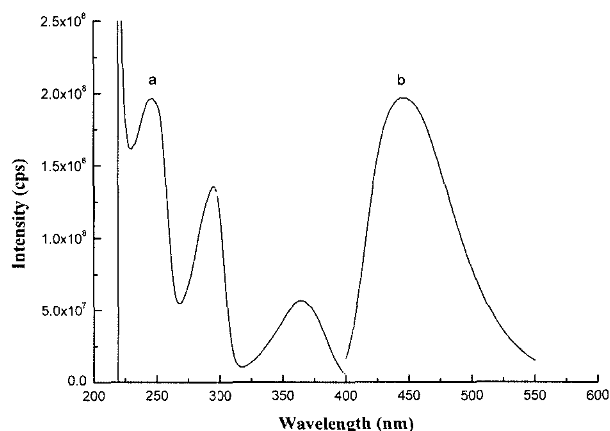

Figure 5. (a) Excitation (λ_{em} = 444 nm), (b) emission (λ_{ex} = 247 nm) spectra of norharman. Conditions: Norharman, 1 × 10^{-4} M; SDS, 1 × 10^{-5} M; Buffer, 0.2 M HOAc-NaOAc (pH 4).

and norharman overlap considerably because of their similar structures. Figure 6 shows synchronous spectra of harman, harmaline and norharman and synchronous spectrum of their mixture in Figure 7 was obtained by scanning the excitation wavelength, λ_{ex} and the emission wavelength λ_{em} with a fixed off-set, $\Delta\lambda$= (λ_{em} - λ_{ex})=185 nm. Figure 7 consists of three exceptionally well-resolved peaks. Bands 1, 2 and 3 correspond to harman, norharman and harmaline in the mixture and are correlated perfectly with each individual synchronous spectrum as shown in Figure 6. The determination of the mixture is sufficiently sensitive because the order of fluorescence of the alkaloids studied is as follows: harman>norharman> harmaline.

Effect of pH

The fluorescence of β- carboline alkaloids with basic ring substituents is usually pH dependent. In order to obtain the optimum pH, the experiment was run between the pH ranges

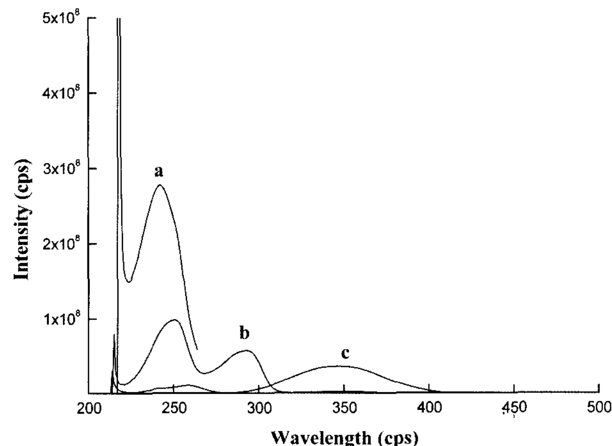

Figure 6. Synchronous fluorescence spectra of (a) harman ($\Delta\lambda$=185 nm), (b) norharman ($\Delta\lambda$= 197 nm) and (c) harmaline($\Delta\lambda$= 116 nm). Conditions: Harman, 1 × 10^{-4} M; harmaline, 1 × 10^{-4} M; norharman, 1 × 10^{-4} M; SDS, 1 × 10^{-5} M; Buffer, 0.2 M HOAc-NaOAc (pH 4.0).

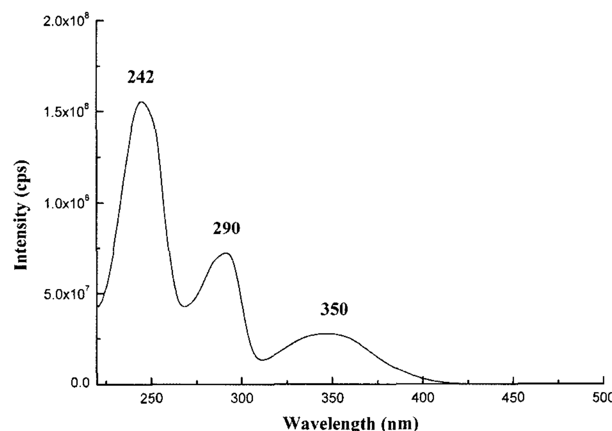

Figure 7. Synchronous fluorescence spectrum of a mixture of harman, harmaline and norharman. Conditions: Harman, 1 × 10^{-4} M; harmaline, 1 × 10^{-4} M; norharman, 1 × 10^{-4} M; SDS, 1 × 10^{-5} M; Buffer, 0.2 M HOAc-NaOAc (pH 4.0); Wavelength interval, $\Delta\lambda$= 185 nm.

of 3.6-7.6. Studies on the fluorescence intensity of harman, harmaline and norharman in various pH showed that pH of the medium had a significant effect on the fluorescence intensity (Figure 8). The results showed the optimum pH range for harman, harmaline and norharman was between 3.8 and 4.2.Therefore, a pH of 4 was fixed with the use of acetate buffer solutions. The difference in the effect of pH on the fluorescence of harman, harmaline and norharman owe to their chemical structures. The fluorescence intensities of alkaloids were not affected by the concentration of the buffer. A 0.2 M of HOAc-NaOAc buffer was selected to obtain suitable buffering capacity.

Effect of surfactant

In order to investigate the influence of surfactants on the

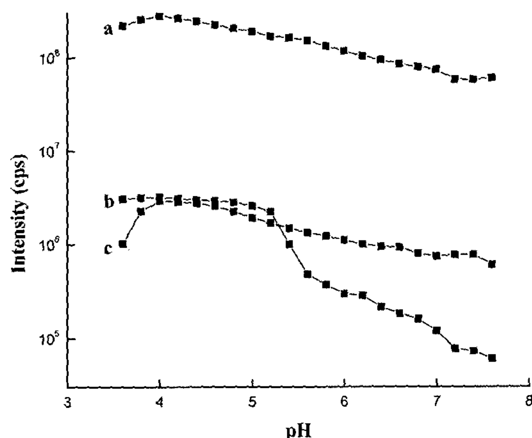

Figure 8. Effect of the buffer on the intensities of (a) harman, (b) norharman and (c) harmaline. Conditions: Harman, 1×10^{-4} M; harmaline, 1×10^{-4} M; norharman, 1×10^{-4} M ; SDS, 1×10^{-5} M; wavelength interval, $\Delta\lambda$= 185 nm.

synchronous fluorimetric determination of β-carboline alkaloids like harman, harmaline and norharman, firstly SDS (anionic) was chosen to determine whether it had effect on the intensity. The effect of other surfactants such as DDTAB (cationic) and Triton X-100 (non-ionic) was also studied but had a poor enhancement effect. The results as shown in the Figure 9 indicate that anionic surfactant SDS has a stronger effect than the system in which no surfactant is present. According to our experiments the order of effectiveness is SDS>Triton-X> DDTAB. We studied the concentration range of SDS from 1×10^{-7}M to 1M. The optimum intensity was recorded at 1×10^{-5} M of SDS. This phenomenon was proposed that SDS formed a micelle and the fluorescent molecules was absorbed into the centre of the micelle where they could be protected from collision with solvent molecules and more particularly, oxygen molecules and causes a increase in fluorescence intensity. But high concentrations of SDS cause the occurrence of bubbles in the solution which results in poor reproducibility and instability of baseline. Thus 1×10^{-5} M of SDS was used in the following experiments.

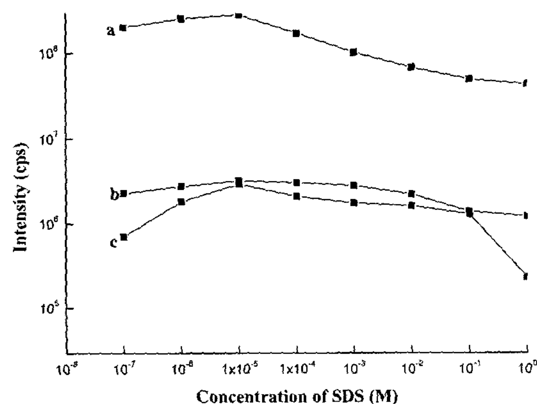

Figure 9. Effect of the SDS concentrations on the intensities of (a) harman, (b) norharman and (c) harmaline. Conditions: Harman, 1×10^{-4} M; harmaline, 1×10^{-4} M; norharman, 1×10^{-4} M ; Buffer, 0.2 mol/l HOAc-NaOAc (pH 4); wavelength interval, $\Delta\lambda$= 185 nm.

Selection of optimum $\Delta\lambda$

The optimum $\Delta\lambda$ is a vital parameter in synchronous scanning for the best quantification of each component in a mixture. It has direct effect on spectral appearance, bandwidth and intensity. In synchronous scanning or dual wavelength spectroscopy, spectral profile simplification, reduction of spectral overlap and high selectivity are always desirable. For the selection of the optimum $\Delta\lambda$ value in the synchronous spectra for the resolution of the mixture, the following considerations were taken into account. The $\Delta\lambda$ for the individual determination of harman, without losing sensitivity, would be $\Delta\lambda = \lambda_{em(max)} - \lambda_{ex(max)}$ = 427-242 nm=185 nm. Similarly, for harmaline and norharman determinations: $\Delta\lambda = \lambda_{em(max)} - \lambda_{ex(max)}$ = 481-365 nm=116 nm and $\Delta\lambda = \lambda_{em(max)} - \lambda_{ex(max)}$ = 444-247 nm=197 nm respectively (Figure 6). But for the selection of the optimum $\Delta\lambda$ value in the synchronous spectra for the resolution of the mixture, the following $\Delta\lambda$ values were taken into account: 150 nm, 160 nm, 165 nm, 175 nm, 185 nm, 186 nm, 190 nm, 197 nm and 200 nm. If $\Delta\lambda$ is less than 165 nm distinct peaks cannot be obtained and the spectra shape is irregular and fluorescence intensity is very weak. In case of the value of $\Delta\lambda$ more than 190 nm, the three peaks cannot be separated completely. When $\Delta\lambda$ is between 165 nm and 190 nm, it appeared three peaks. The value chosen 185 nm is intermediate between 165 nm and 190 nm. This selection gives a well- defined resolution of the overlapping emission spectra of the analytes investigated (Figure 7). So $\Delta\lambda$ = 185 nm was taken as an optimum parameter.

Statistical analysis of results

Calibration curve was prepared from the fluorescence signal of harman at 242 nm under the optimized conditions such as $\Delta\lambda$= 185 nm, SDS, 1×10^{-5}M, pH, 4.0 in the presence of harmaline, 1×10^{-4}M and norharman, 1×10^{-4}M. Similarly, the concentration of harmaline and the fluorescence intensity measured at 350 nm are linearly related in the presence of harman, 1×10^{-4}M and norharman, 1×10^{-4}M and norharman concentration and the fluorescence measured at 290 nm are linearly related in the presence of harman, 1×10^{-4}M and harmaline, 1×10^{-4}M. Linear regression equations (Y=A+Bx) for harman, harmaline and norharman were obtained. The slopes, intercepts, errors, correlation coefficients, standard deviations, linear ranges and detection limits obtained for determination of harman, harmaline and norharman by constant wavelength synchronous spectra are summarized in the Table 1.

Application of the method

The proposed method was applied to the determination of

Table 1. Statistical analysis of results of calibration curves of β-Carboline alkaloids

Parameters	Harman	Harmaline	Norharman
Regression equations	$Y_{242\,nm} = A + Bx$	$Y_{350\,nm} = A + Bx$	$Y_{290\,nm} = A + Bx$
Intercepts, A	8.83761	8.74465	8.38599
Errors	0.04553	0.04647	0.0178
Slopes, B	0.16797	0.35323	0.13092
Errors	0.00811	0.00828	0.00317
Linear range	1×10^{-7}M- 1×10^{-4}M	1×10^{-7}M- 1×10^{-4}M	1×10^{-7}M- 1×10^{-4}M
[a]LOD	1.5×10^{-8}M	3×10^{-8}M	2.5×10^{-8}M
[b]R	0.99768	0.99945	0.99941
[c]SD	0.01814	0.01851	0.00709

[a]Lower limit of detection
[b]Correlation coefficient
[c]Standard deviation

Table 2. Analysis of the synthetic mixture of harman, harmaline and norharman

	Harman	Harmaline	Norharman
Mixture (μg/ml)	0.182	0.214	0.168
[a]Found, (μg/ml)	0.179, 0.182, 0.179, 0.175, 0.170	0.209, 0.195, 0.214, 0.204, 0.199	0.169, 0.160, 0.157, 0.160, 0.159
Mean ± SD	0.177 ± 0.004	0.204 ± 0.008	0.161 ± 0.005

[a]Five measurements

three alkaloids in synthetic mixtures containing different ratios of the analytes and the results are given in the Table 2.

CONCLUSIONS

The results obtained in this work allow us to demonstrate that three components of a tertiary mixture were successfully determined using synchronous fluorescence spectroscopy. The narrowing of the emission band produced by synchronous scanning significantly improved the resolution of the overlapping spectra of the analytes investigated. The application of synchronous fluorimetric method to the analysis of β-carboline alkaloids has yet to be reported. The method seems interesting for broader applications in drug analysis as a rapid, simple alternative to conventional fluorescence methods.

ACKNOWLEDGEMENT

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