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# APPLICATION OF SYNCHRONOUS EXCITATION SPECTROFLUORIMETRY TO DRUG ANALYSIS

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## Summary

Among the analytical techniques available for the determination of toxic products, fluorimetry is, at present, one of the most frequently employed. But in many cases, measurements at low concentrations are interfered with by Rayleigh and Raman scattering. We propose to eliminate, in great part, this interference and hence increase the sensitivity of the method, by using the new synchronous excitation spectrofluorimetry. Application of this technique to the identification and determination of drugs during recent forensic investigations demonstrates its advantages over classical spectrofluorimetry.

## Introduction

The toxicologist faced with a forensic problem now has at his disposal numerous physico-chemical methods for the separation, identification and determination of toxic organic products (thin-layer, gas, and high pressure liquid chromatography, UV and IR spectrophotometry, fluorimetry, phosphorimetry, mass spectrography...) [1].

His special contribution consists in extracting as much information as possible from the different techniques which he chooses to solve his particular problem, since in general it is difficult for him to apply them all rapidly.

Given the legal and/or medico-legal implications of drug analysis, the two mandatory factors guiding his choice will be, on the one hand, specificity of identification of the toxic product and, on the other hand, sensitivity of determination. Gain in sensitivity becomes primordial in the case of certain illicit

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drugs, taking LSD as an example, the concentrations involved may be very low; a few tenths of a microgram in very varied substrates.

Fluorimetry is one of the most sensitive of the spectroscopic techniques in use at present: the fluorescence spectrum of a substance is often characteristic of it and the intensity of the fluorescence emitted at a given wavelength depends on the concentration in a manner which is practically linear [2].

Thus, curves 1 and 2 in Fig. 1 are the emission, or analysis and excitation spectra obtained by us for three substances frequently found nowadays in toxicological analysis: amphetamine sulphate, morphine chlorohydrate and LSD. Our results are in agreement with those published recently by other authors [3—9].

Although, in theory, it would appear possible to lower the detection limit of a fluorescent substance by increasing the intensity of the light coming from the exciter lamp (directly, by changing the lamp, or indirectly, by increasing the slit width of the excitation monochromator), in practice, below a certain concentration which depends on the nature of the fluorescent substance being studied, numerous factors intervene to limit the sensitivity of the apparatus used. These factors are:

the photoreaction of the substance being studied;

the light scattered by the solution and the light reflected by the measuring cell;

the luminescence of the analysing cell itself;

the imperfections in the construction of the optical system of the apparatus (parasitic light);

the fluorescence due to impurities present in the solution or solvent.

These different effects, particularly that of light scattering by the solution * and that of reflection of light by the measuring cell, can be seen in Fig. 2, which shows the emission spectra at concentrations close to the detectable concentration limit of the three toxic products with which we are concerned: amphetamine, morphine and LSD.

The emission spectra shown are appreciably perturbed by Rayleigh and Raman scattering **. The intensity I, measured at the maximum emission wavelength $\lambda_a$, corresponds to the sum of the fluorescence and diffusion intensities. Rayleigh scattering intensity varies with cell and solvent quality; consequently, it is very difficult to eliminate these effects by running a blank. Raman scattering intensity however is much more stable and can be allowed for by subtraction.

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* It will be recalled that scattering terminology follows particle size: one speaks of Rayleigh scattering in the case of solvents such as water and of Tyndall scattering in the case of colloids. In Rayleigh scattering, the scattered light has the same wavelength as the incident light. Raman scattering, involving an "inelastic collision" between photon and solvent, is emitted at wavelengths greater than that of the incident light. Rayleigh and Raman scattering intensities are proportional to $1/\lambda^4$, $\lambda$ being the wavelength of excitation [2].

** The half-height width of the scattering band depends on the characteristics of the apparatus used. In particular, the half-height width for monochromatic excitation is equal to the slit width (slits assumed to be identical) of the analysing monochromator.

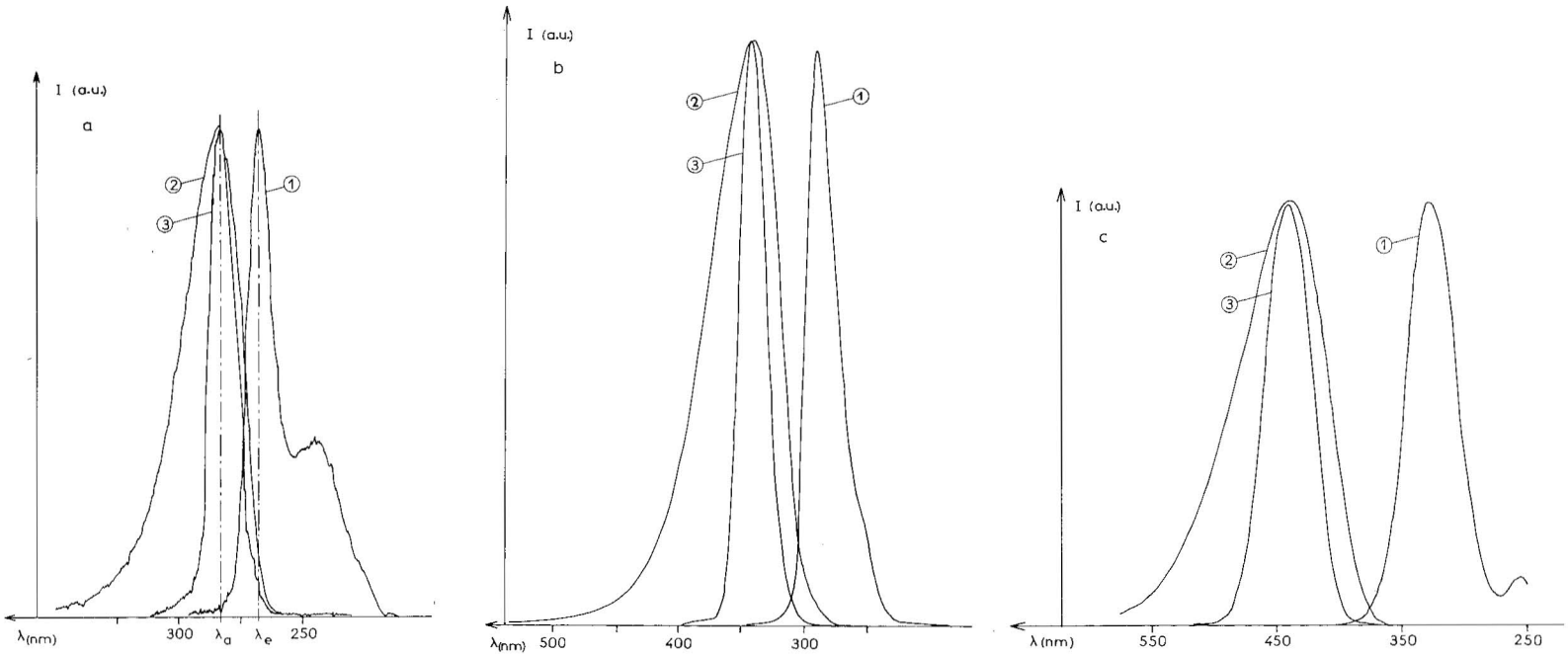


Fig. 1. Fluorescence spectra (curves: 1, excitation spectrum; 2, analysis spectrum; 3, "constant step" spectrum). (a) DL-Amphetamine sulphate (1 ppm) in b.d. water. 1, analysis wavelength 300 nm; 2, excitation wavelength 260 nm; 3, "constant step" of 25 nm, slit widths 5 nm. (b) Morphine chlorohydrate (10 ppm) in b.d. water. 1, analysis wavelength 350 nm; 2, excitation wavelength 285 nm; 3, "constant step" of 60 nm, slit widths 10 nm. (c) LSD tartrate (1 ppm) in 0.004 M HCl. 1, analysis wavelength 440 nm; 2, excitation wavelength 325 nm; 3, "constant step" of 110 nm, slit widths 10 nm.

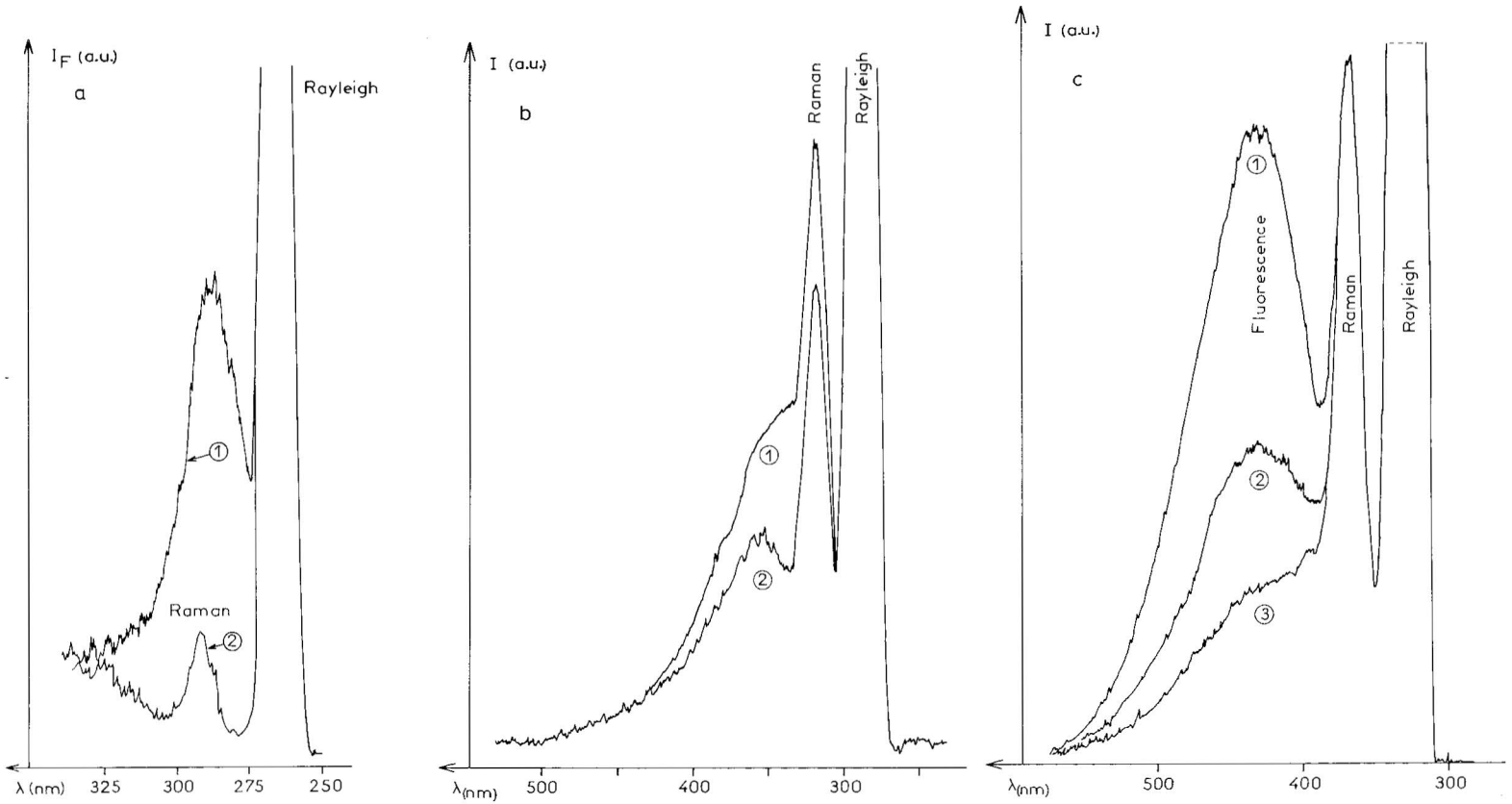


Fig. 2. Pertubation of emission spectra by Rayleigh and Raman scattering (fluorescence spectra recorded with classical method). (a) DL-Amphetamine sulphate (excitation 265 nm, scanning of the analysis wavelength, slit widths of 5 nm). 1, DL-Amphetamine sulphate ($1.8 \times 10^{-8}$ g/ml) in b.d. water; 2, b.d. water. (b) Morphine chlorohydrate (excitation 290 nm, scanning of the analysis wavelength, slit widths of 10 nm). 1, morphine chlorohydrate ($4 \times 10^{-7}$ g/ml) in b.d. water; 2, b.d. water. (c) LSD tartrate (excitation 325 nm, scanning of the analysis wavelength, slit width of 10 nm). 1, LSD tartrate ($10^{-9}$ g/ml in 0.004 M HCl; 2, LSD tartrate ($3 \times 10^{-10}$ g/ml) in 0.004 M HCl; 3, b.d. water.

## Experimental

*Synchronous excitation spectrofluorimetry or "constant step" method*

Having occasion to employ fluorimetry regularly in toxicological analysis, we have often encountered these difficulties. Thus, when the slit widths of the excitation and analysing monochromators are greater than the separation between the excitation and analysing wavelengths, the emission spectrum observed consists, at low concentrations of fluorescent material, of an intense scattering band with a shoulder due to the fluorescent emission band. It is then no easy matter to define the spectrum of the substance whose identity is sought, a fortiori to determine low concentrations of toxic products (see Fig. 2 for example).

We have developed the synchronous excitation spectrofluorimetric method as we were the first to notice that it eliminates interference from scattering light and permits determination of trace quantities of fluorescent dyes [10, 14]. The method was described by Lloyd in 1971 but was only used for qualitative comparison of spectra of polyaromatic materials [11,12]. John and Soutar have employed it for qualitative analysis of crude oils [13].

The difficulty experienced with the classical method of measurement is related to the fact that the underlying "scattering spectrum" has a very considerable slope, as can be seen in the schematic example in Fig. 3. In the "constant step" scanning method, diffusion is not suppressed, but recorded continuously and this results in it taking the form of an almost horizontal base line (see Fig. 4, curve 2).

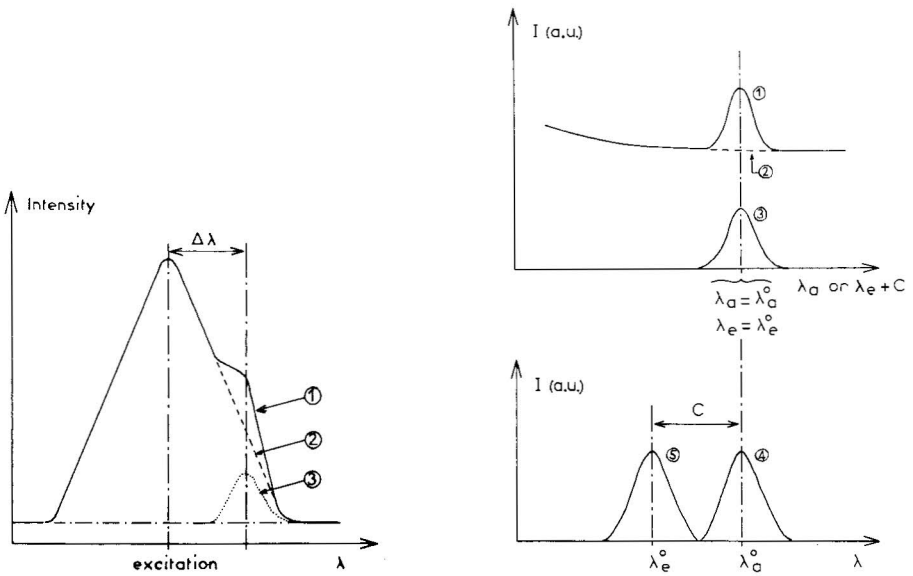


Fig. 3. Schematic spectra in classical fluorescence. 1, actual spectrum; 2, "scattering" spectrum; 3, theoretical fluorescence spectrum.

Fig. 4. Schematic interpretation of the "constant step" spectrum. 1, experimental spectrum; 2, "scattering" spectrum; 3, true emission spectrum; 4, emission spectrum; 5, excitation spectrum.

TABLE I

RESULTS OF TOXICOLOGICAL ANALYSES CARRIED OUT USING SYNCHRONOUS EXCITATION

| | Sample confiscated | Substance suspected | Substance identified * | Limit of measurable concentration (g/g) | % substance (g/g) | Remarks |
|---|---|---|---|---|---|---|
| First case | White powder | ? | Amphetamine | $\sim 10^{-9}$ | 93% | Measurement limited by Raman scattering |
| Second case | White powder | Morphine | Morphine and quinine | $\sim 10^{-7}$ | 23% morphine, 7% quinine plus excipient | Separation necessary |
| Third case | Minute whitish-coloured tablet | LSD? | LSD | $\sim 10^{-10}$ | 1.4% plus excipient | |

* Identification confirmed by other techniques.

In the classical technique, the excitation wavelength ($\lambda_e$) is fixed and the emission spectrum trace is produced by scanning at the analysing wavelength ($\lambda_a$). With "constant step" scanning, excitation and analysing wavelengths are scanned simultaneously, a "constant step" C being maintained between $\lambda_e$ and $\lambda_a$. The value chosen for the step is arbitrary, but maximum sensitivity is obviously obtained when the value corresponds to the separation between the maxima of the excitation and emission spectra (at $\lambda_a^0$). (See Fig. 4, curves 4 and 5).

At any wavelength then, a signal due to scattering is obtained. This signal does not vary greatly with wavelength. Over an appropriate range of wavelengths (when excitation and reception of emission are taking place at the same time), the fluorescence signal is obtained. This time, the latter is much more clearly visible (see Fig. 4, curves 1 and 3). On the practical side, the stepper-type motors for wavelength scanning by the two excitation and analysing monochromators were driven simultaneously. The tests were carried out for the most part on an "Farrand" MK I apparatus which had been modified electronically.

*1. Application to qualitative analysis in toxicology*

To illustrate not only the advantages, but also the limitations, of this new fluorimetric technique, we have selected three very different examples from the toxicological analyses carried out recently in our laboratory (see Table I). In all three cases the identity of the toxic products was confirmed by other techniques: TLC, GC, UV, and mass spectrography for LSD.

Curves 3 in Fig. 1 show the "constant step" spectra of the three products studied *.

* Reference substances were: LSD tartrate (Sandoz), morphine chlorhydrate (Prolabo), DL-amphetamine sulphate (Koch and Light purissimum), quinine sulphate (Fluka, purissimum). All solutions are made with bidistilled water: b.d. water.

The half-height width $\Delta\lambda_{1/2}$ obtained from "constant step" spectra is less than that obtained under classical conditions. If $\Delta\lambda_e$ and $\Delta\lambda_a$ represent the half-height widths of the excitation and analysis spectra, respectively, then the half-height width obtained using the method we propose is nearly equal to [10]:

$$\Delta\lambda_{1/2} \# \Delta\lambda_e + \Delta\lambda_a - \sqrt{(\Delta\lambda_e)^2 + (\Delta\lambda_a)^2}$$

The results obtained are shown in Table II.

The reduction in half-height width of the emission curve improves measurement quality. At low concentrations it is possible to obtain, in a single experiment, qualitative information about an unknown substance by:

comparison of the half-height width $\Delta\lambda_{1/2}$ obtained with that of a reference substance;

comparison of the "constant step" spectra of the unknown substance and the reference substance by a graphical method or by optimisation using a computer.

During chromatographic analysis of confiscated samples we detected the presence of other fluorescent substances such as quinine. This rendered necessary the carrying out of "constant step" spectrofluorimetric studies of such mixtures.

It can be seen from Fig. 5 that it is possible to detect morphine in the presence of quinine as long as the morphine to quinine ratio is greater than or equal to 10; this problem cannot be solved using spectra given by the classical analytical method.

On the other hand, mixtures of LSD and quinine cannot be resolved by any fluorimetric technique. Resolution of morphine-quinine mixtures with the "constant step" method is possible because the emission maxima are separated (Table II) and because the spectrum is better defined. In the case of LSD-quinine mixtures, the maxima are too close together to permit separation. However, as long as the LSD-quinine concentration ratio is $\geqslant 0.1$, the presence of quinine does not interfere with the determination of LSD (see Fig. 6). The

TABLE II

SPECTROSCOPIC CHARACTERISTICS OF THE PRODUCTS STUDIED

| Substance | Analysis | | Excitation | | Constant step | | |
|---|---|---|---|---|---|---|---|
| | $\lambda_{max}$ (nm) | $\Delta\lambda_{1/2}$ (nm) | $\lambda_{max}$ (nm) | $\Delta\lambda_{1/2}$ (nm) | $\Delta\lambda_{1/2}$ expected (nm) | $\Delta\lambda_{1/2}$ calculated (nm) | Optimum step (nm) |
| Amphetamine sulphate | 282 | 28 | 260 | 12 | 12, with a step of 25 | 10 | 30 * |
| LSD tartrate | 435 | 86 | 325 | 52 | 44 | 38 | 110 |
| Morphine chlorohydrate | 345 | 66 | 285 | 28 | 25 | 22 | 60 |
| Quinine sulphate | 450 | 80 | 350 | 56 | 40 | 38.5 | 100 |

* The optimum step is not necessarily the same as the difference between the excitation and maximum emission wavelengths; one may in certain cases, use a slightly different value which does not greatly change the fluorescence intensity, but considerably decreases the "blank" intensity.

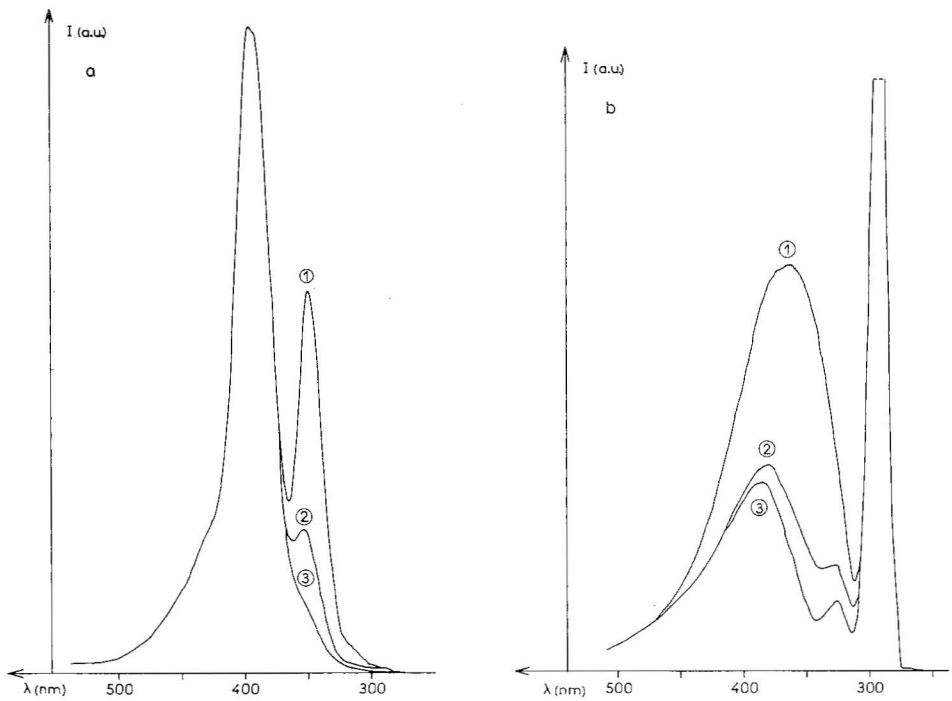


Fig. 5. Fluorescence spectra of mixtures of morphine and quinine. (a) "Constant step" spectra ("constant step" of 60 nm, slit widths of 10 nm). (b) Classical analysis spectra (excitation 285 nm, slit widths of 10 nm). Curves: 1, morphine chlorohydrate ($8 \times 10^{-6}$ g/ml) and quinine sulphate ($10^{-7}$ g/ml) in b.d. water; 2, morphine chlorohydrate ($1.6 \times 10^{-6}$ g/ml) and quinine sulphate ($10^{-7}$ g/ml) in b.d. water; 3, quinine sulphate ($10^{-7}$ g/ml) in b.d. water.

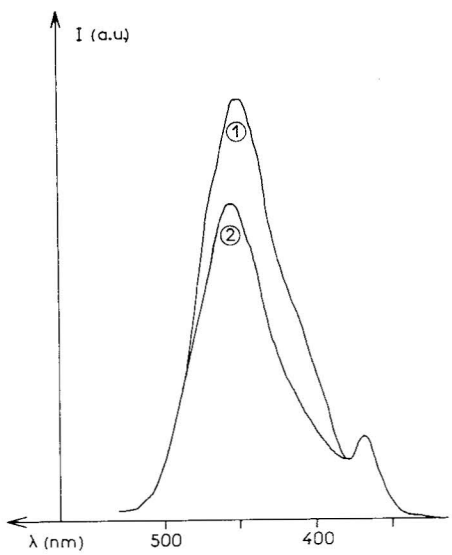


Fig. 6. "Constant step" spectra: mixture of LSD and quinine ("constant step" of 120 nm, slit widths of 10 nm). Curves: 1, LSD tartrate ($10^{-9}$ g/ml) and quinine sulphate ($10^{-7}$ g/ml) in b.d. water; 2, quinine sulphate ($10^{-7}$ g/ml) in b.d. water.

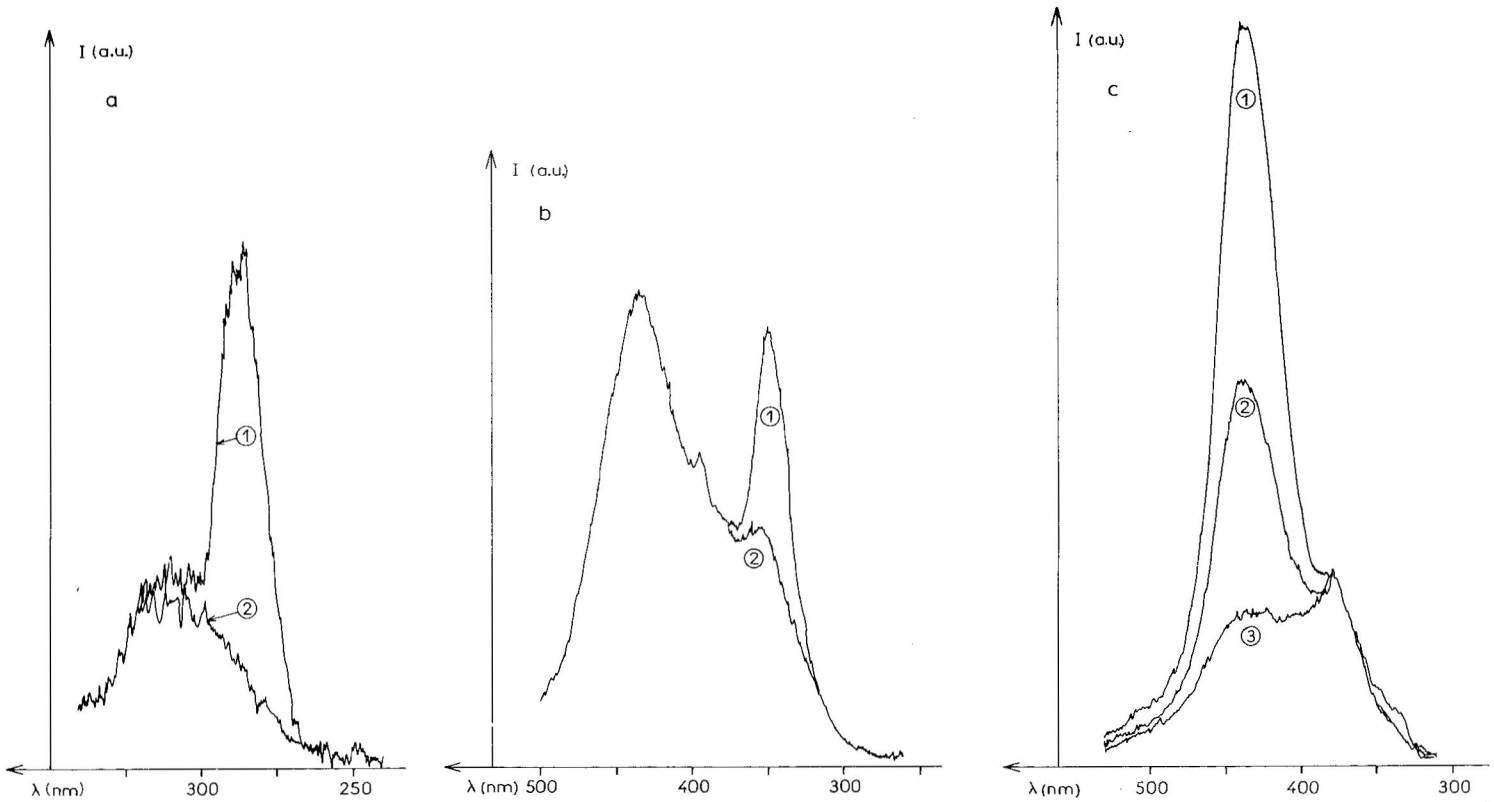


Fig. 7. "Constant step" spectra of amphetamine sulphate (a), morphine chlorohydrate (b) and LSD tartrate (c) (concentration close to the detectable concentration limit). (a) "Constant step" of 30 nm, slit widths of 5 nm, curves: 1, amphetamine sulphate ($1.8 \times 10^{-8}$ g/ml) in b.d. water; 2, b.d. water. (b) "Constant step" of 60 nm, slit widths of 10 nm, curves: 1, morphine chlorohydrate ($4 \times 10^{-7}$ g/ml) in b.d. water; 2, b.d. water. (c) "Constant step" of 110 nm, slit widths of 10 nm, curves: 1, LSD tartrate ($10^{-9}$ g/ml); 2, ($3 \times 10^{-10}$ g/ml) in b.d. water; 3, b.d. water.

results obtained by fluorimetric analysis of products confiscated by the police are shown in Table I. Preliminary separation of quinine and morphine was, of course, necessary in the case of the second sample.

*2. Application to quantitative analysis*

Fig. 7 shows "constant step" spectra for the three substances studied under the same concentration conditions as those in Fig. 2. Comparison of these two figures reveals the marked improvement in measurement from both qualitative and quantitative standpoints. This is important in our toxicological analyses for confirmation of the identity of substances and for their assay. (We choose fluorimetry whenever we can because of its ease of application.)

## Discussion

### *Limits of the technique*

In fact, the hypotheses advanced earlier are only valid as a first approximation. As can be seen in Fig. 8, there is a slight variation with wavelength of the "constant step" spectrum of the solvent. However, it is possible to compensate electronically for the greater part of the signal arising from scattering. The resultant signal can be amplified and allows measurements to be made under conditions where fluoresence is masked by scattering. The analytical limit depends, varying with the nature of the emitting substance, on the slope of the "constant step" base line (optical limitation) or on the amplifier noise (electronic limitation).

*1. Effects of Raman scattering*

As can be seen in Fig. 2, in the cases studied, interference from both Rayleigh and Raman scattering takes place. These effects are considerably reduced when the "constant step" method is used. However, as a result of the fixed wave number gap between Rayleigh and Raman scattering, the "constant step" spectrum of a solvent shows a maximum when the gap between excitation and analysis corresponds exactly with that between Rayleigh and Raman scattering. An effect of this sort is shown in Fig. 8.

In the three cases studied, this phenomenon results in the "constant step" fluorescence appearing as a shoulder on the "constant step" Raman spectrum of the solvent.

*2. Comparison between the proposed technique and commercially available apparatus*

Raman radiation is polarized. Hence, it is possible to eliminate it by using the classical system of vertically polarized light for excitation and horizontally polarized light for analysis. Under these conditions, the curve shown in Fig. 8 becomes 'practically horizontal", which allows easy extrapolation when measuring fluorescence.

However, if these optical effects are easy to eliminate, one has, nevertheless, to take into account the fact that the polarizers used absorb at least 75% of the light energy (the absorption proper of the polarizers also has to be taken into

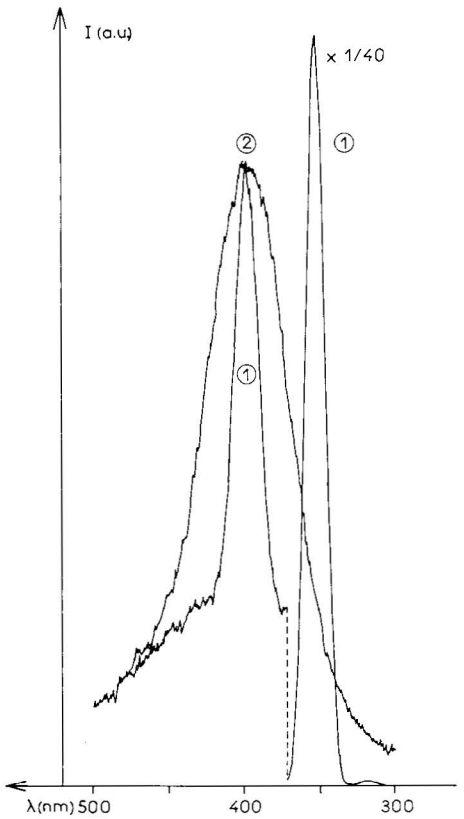


Fig. 8. "Scattering" spectra of bidistilled water. Curves: 1, excitation at 350 nm, scanning of the analysis wavelength (classical conditions) slit widths of 10 nm; 2, scanning of both excitation and analysis wavelength with a "constant step" of 50 nm, slit widths of 10 nm. Elimination of Rayleigh scattering practically complete. The shape of Raman scattering spectrum is broadened (while any fluorescence spectrum is better defined).

account) and that in the majority of cases measurement is limited by the "electronic noise" of the apparatus.

In addition, this technique is inapplicable when the solution to be investigated is very viscous or when the emitted fluorescence is strongly polarized.

The various advantages and disadvantages of classical spectrofluorimetrical instruments are collected together in Table III.

Thus, it seems at the moment that, for the reasons presented in Table III, the proposed technique is very well suited to the determination of trace quantities.

## Conclusion

We have used the new technique of synchronous excitation fluorimetry and we have demonstrated that in addition to its high resolution, which increases the probability of identification of the substance under investigation, it improves the sensitivity of analysis by reducing the interference from scattering light.

TABLE III

COMPARISON OF DIFFERENT SPECTROSCOPIC TECHNIQUES

| Type of apparatus | Advantages of the technique | Disadvantages |
|---|---|---|
| Classical apparatus (spectrofluorimeter) * | | Measurement limited by Rayleigh and Raman scattering |
| Apparatus fitted with polarizers | Raman scattering eliminated | Measurement limited by Rayleigh scattering. Loss of energy >75% |
| Commercial differential apparatus ** | Raman scattering eliminated | Cost.<br>Rayleigh scattering not eliminated. Loss of light energy due to presence of chopper |
| Spectrofluorimeter equipped with simultaneously scanning excitation and analysis monochromators (constant step) † | Elimination of Rayleigh scattering practically complete.<br>Shape of Raman scattering spectrum modified (broadened). "Constant step" spectrum better defined. Technique easy to work | Partial elimination of Raman scattering |
| Double differential apparatus without chopper †† | Rayleigh and Raman scattering completely eliminated without loss of light energy | Cost |

* Tests carried out on Farrand MK I, Jobin and Yvon JY 3, Aminco-Bowman and Hitachi MPF 4 spectrofluorimeters.

** Test carried out on Fica 55 000 and Perkin-Elmer differential spectroflurorimeters.

† Perkin-Elmer MPF-3L, this appararus was originally designed for recording absorption spectra of highly fluorescent solutions but may be used for synchronous excitation fluorimetry.

†† This apparatus is in the course of construction in our laboratory.

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