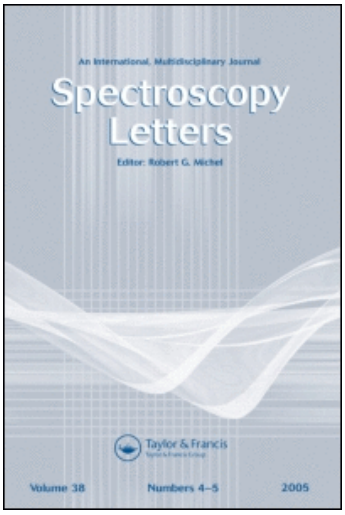


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# Micro-Scale UV/Vis Spectrometric Batch Procedures by Use of an Internal Standard-A Green Chemistry Approach

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# Micro-Scale UV/Vis Spectrometric Batch Procedures by Use of an Internal Standard–A Green Chemistry Approach

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**ABSTRACT** One approach to minimize environmental impact of laboratory-based activities is the reduced use of potentially harmful substances. Regarding UV/Vis spectrophotometric procedures, they typically consist of several steps and may include solvent extraction or evaporation, dilution, chemical derivatization, and so on. To assure reliable analytical results, classical batch procedures are performed in volumetric vessels and require considerable amounts of different reagents and solvents. Quite importantly, many official analytical methods are based on UV/Vis spectrophotometric measurements and, to the best of our knowledge, no micro-scale batch procedure has been accepted so far. In order to enhance the performance of such micro procedures, the use of internal standard method (IS) had been proposed. The most important feature of IS in UV/Vis spectrophotometry is that less rigorous procedures become possible and, from the green chemistry point of view, the micro-scale batch procedures can be used without sacrificing the results quality. In this article, the general requirements for an IS in UV/Vis spectrophotometry and its applications in the procedures involving many-fold dilution or extraction/preconcentration of the analyte are presented, highlighting two aspects: (i) analytical performance and (ii) environmentally friendly protocols.

**KEYWORDS** internal standard, mirco-scale procedures, UV/Vis spectrophotometry

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## INTRODUCTION

The green character of analytical chemistry relies on the importance analytical results may have in assessing pollution, in evaluating possible toxicological risks, and in designing the remediation strategies. On the other hand, however, performing the analytical procedures involves generation of potentially harmful wastes. According to the principles of Green Chemistry (GC), the adverse environmental impact of analytical methodologies can be reduced by choosing non-toxic reagents and solvents, by minimizing the amount of solvents employed in sample pretreatment or by developing alternative, direct procedures with minimum requirements

of chemicals.[1–4] In the advent in nanotechnology, miniaturization, and automation, down-scaling the analytical procedure is a general trend.[5,6] Within this context, the entire multi-step procedures can be now performed by designing appropriate integrated lab-on-a-chip devices.[7,8] In spite of considerable progress in instrumentation and methodology, such an approach is mainly used for specific applications, for example if the amount of sample is a limiting factor.[7] Otherwise, traditional analytical procedures are still in use, since they meet the performance requirements in terms of accuracy and precision. In particular, there is a wide range of UV/Vis spectrophotometric procedures designated as official analytical methods in food and drug analyses.[9–11] Owing to moderate sensitivity and selectivity of spectrophotometric measurements, these procedures typically involve sample pretreatment by means of solvent extraction, pre-concentration or dilution, chemical derivatization, and so on. Classical batch procedures are performed in volumetric vessels and require considerable amounts of different reagents and solvents, in disagreement with the GC concept.[12] In this particular case, down-scaling is not straightforward, because handling small sample and reagent volumes enhances the risk of analytical errors, which manifests in poorer analytical performance.

In order to control undetermined changes in analyte concentration and also fluctuations of instrument response, the method of internal standard (IS) is often used in a number of techniques as chromatography, optical emission spectrometry, mass spectrometry, and so on. The principle requirement for IS method is the multi-component ability of the instrumental technique applied. Even though UV/Vis had long been considered one-component technique, the application of different chemometric methods for spectral data treatment has enhanced its potential for the resolution of two or more sample components. In particular, if components of interest do not interact chemically and their absorption spectra are not totally overlapped, satisfactory resolution of the mixture can be obtained using derivative spectra.[13,14]

The feasibility of UV/Vis spectrophotometry for multi-component analysis has encouraged us to use the method of internal standard in micro-scale multi-step spectrophotometric procedures and thus, enhance their analytical performance and reduce adverse environmental impact.

## INTERNAL STANDARD METHOD IN UV/Vis SPECTROPHOTOMETRY

Among different quantification methods, internal standard is specially well suited if the amount of sample reaching analytical cell or the instrument response varies slightly from run to run for reasons that are difficult to control. As general rule, IS is a chemical species other than analyte, which is added in a constant and known amount to all samples, calibration, and blank solutions. Analytical signals for analyte ($S_{analyte}$) and IS ($S_{IS}$) are then registered and the ratios of analyte response versus internal standard response ($S_{analyte}/S_{IS}$) are used for calibration and quantification. If the amount of IS is kept constant in all solutions, $S_{analyte}/S_{IS}$ value remains directly proportional to analyte concentration. The internal standard should match closely the physicochemical characteristics of analyte(s), which would assure similar behavior of these species during sample preparation and signal registration. If so, variations in analytical conditions such as imprecision related to the use of small volumes, incomplete extraction, dilution errors, or uncontrolled fluctuation of instrumental response would have no effect on $S_{analyte}/S_{IS}$ value, thus enabling for elimination of major sources of experimental error. In other words, the ratio of analyte to IS signals for a given sample should remain constant, independently of changes in its composition derived from analytical protocol and also independently of the instrument performance.

As already mentioned, batch spectrophotometric procedures often involve a sample pretreatment step, which in a micro-scale assays would increase a risk of analytical errors. A great majority of procedures actually in use are based on the chromogenic reaction, in which the analyte is converted to a species presenting relatively intense absorption band in UV/Vis region. If the active component of pharmaceutical formulation is to be determined, many fold dilution is typically required. For lower analyte concentrations, the separation and pre-concentration may be needed. In order to control possible changes of analyte concentration in the aforementioned steps, IS should be added at the beginning of analytical

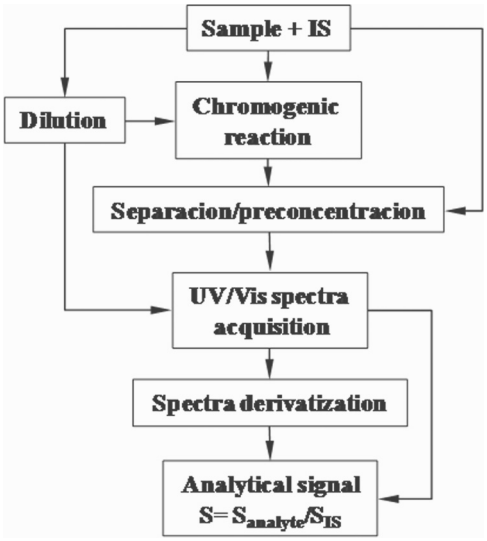


**FIGURE 1** General scheme of micro-scale UV/Vis spectrophotometric procedure with the application of internal standard method.

procedure, as shown schematically in Fig. 1. Considering the general requirements for an IS in different instrumental techniques,[15,16] in UV/Vis spectrophotometry the following parameters should be taken into account: (1) similar physicochemical properties of the IS and the analyte (polarity, complex formation, etc., depending on the pretreatment procedure); (2) IS should present chemical stability under experimental conditions applied in the procedure; (3) no interactions between the IS and the analyte, nor with other sample components; (4) the IS should not be a natural component of the sample; (5) the IS should be soluble in the assay medium; (6) low absorption coefficient of the IS in the region of the absorption band of the analyte; (7) relatively intense absorption band of the IS in the region of low absorption of the analyte.

## REPRESENTATIVE APPLICATIONS OF INTERNAL STANDARD IN THE ANALYSIS OF REAL-WORLD SAMPLES

Several applications of IS in the analysis of real-world samples have been proposed.[17–26] It has been shown that, in addition to lower consumption of the reagents and solvents, less rigorous protocols can be accepted without sacrificing the analytical performance. In particular, only the volumes of IS and sample have to be measured precisely, while the rest of operations do not need extreme care. Consequently, the micro-assays are typically performed in 0.5–1.5 ml plastic tubes, small volumes of volatile solvents can be handled, preconcentration by solvent evaporation can be used and absorption spectra are registered using quartz ultra-micro spectrophotometer cuvettes, minimum volume 50 µl (Sigma).

The first assay was developed for the determination of azinphos-methyl in commercial formulations.[17] This pesticide presents two absorption bands with maximum at 223.0 nm and 287.9 nm, respectively. Owing to its high concentration in the samples (about 35% w/w), no derivatization was needed. The sample was first dissolved in methanol and then diluted with the same solvent. In this case, the spectral resolution was the main selection criterion for IS. Based on the reversed phase chromatographic procedure, acetophenone ($\lambda_{max} = 230.4$ nm) was initially selected. However, partially overlaped absorption bands of pesticide and acetophenone could not be resolved by the application of derivative spectra. The addition of Erioglaucine A ($\lambda_{max} = 623.0$ nm) was proposed and the composition of mixed IS (blue dye and acetophenone) was optimized to obtain the best conditions for zero-crossing technique while using first derivative spectra. The analytical signal was defined as $^1D_{analyte(219.59nm)}/^1D_{IS(268.93\,nm)}$. To fit within the linear range, 100 mg of the commercial formulation was precisely weighed, 5.0 ml of IS (7.0 mmol $l^{-1}$ Eriogalucine A, 7.9 mmol $l^{-1}$ acetophenone in methanol) were added and the volume was brought to about 10 ml. Dilution was accomplished by adding 4 µl of the solution to 2 ml of methanol. The accuracy of the procedure was confirmed by reversed phase liquid chromatography and, in spite of non-rigorous protocol and small volumes handled, precision measured as relative standard deviation for five independent analytical runs of the sample was 0.6%.

In the pharmaceutical industry, quantification of the active compound in commercial formulations is an issue. Bismuth salts are currently used in treating diarhea, gastric, and duodenal ulcer diseases. Different batch spectrophotometric procedures are available for Bi-containing pharmaceuticals. Among them, the procedure based on the formation of tetraiodobismuthate (III) in aqueous acidic medium

(sulfuric acid), ion pair formation with benzyltributylammonium cation (BTAB) and extraction to chloroform has been selected and down-scaled.[21] In search of suitable IS, a series of blue compounds were examined and Blue Nile was finally selected. The spectral bands of bismuth complex ($\lambda_{max} = 491$ nm) and IS ($\lambda_{max} = 623$ nm) did not present any overlapping, so the analytical signal was measured from zero-order spectra ($S = A_{491\,nm}/A_{623\,nm}$). Another important selection criterion was similar extraction yield of analyte and IS to chloroform. The effect of reagents and ion pairing agent concentrations on the IS and Bi signal was systematically studied, which enable for extraction yields $99.5 \pm 0.6\%$ and $99.8 \pm 0.5\%$ for the two species. In Table 1, the experimental parameters and some figures of merit evaluated for classical batch procedure and micro assay proposed are compared. According to Green Chemistry (GC) principles, the micro procedure offered the reduced consume of reagents and consequently, lower waste generation. On the other hand, similar detection limits and precision were obtained in the two procedures, which should be ascribed to effective elimination of signal variation by the method of standard addition.

It seemed interesting to examine the feasibility of IS in the analysis of complex biological samples. For this purpose, the environmentally friendly micro extraction-spectrophotometric procedure for the determination of malondialdehyde (MDA) in blood serum has been proposed.[22] MDA is a widely accepted biomarker of lipid peroxidation, an important indicator of oxidative injury. The most common analytical approach to the determination of MDA is the formation of its adduct with thiobarbituric acid (TBA). The reaction occurs at low pH and elevated temperature yielding a red, fluorescent 1:2 MDA:TBA adduct.[27] Other fatty peroxide–derived decomposition products and nonlipid-related compounds also form adducts with TBA and that is why the procedure is often referred to as the test for TBA-reactive substances (TBARS).[28] It should be noted however, that TBARS levels were shown to be directly correlated with other parameters characteristic of oxidative damage.[29] For enhanced selectivity of the micro assay, extraction of MDA–TBA was carried out and the analytical signals were obtained from the first derivative spectra. To control possible errors committed during sample manipulation (extraction, measuring of small volumes, solvent evaporation, etc.), the method of internal standard was applied. To the aliquot of serum (20 µl), 200 µl of IS ($6.25 \times 10^{-4}$ Erioglaucine A), 20 µl butylated hydroxytoluene (2% in methanol) and 200 µl of TBA (0.6% in acetic acid, pH 2.5) were added and the sample was incubated at 95°C for 45 min. The MDA adduct and IS were extracted with 300 µl of ethyl acetate containing 0.06% aliquat 336 (ion pairing agent for neutralization of IS electrical charge). Absorption spectrum of organic phase was registered for each sample against aliquat-ethyl acetate solution, and the analytical signal was

**TABLE 1** **Extraction–Spectrophotometric Determination of Bismuth in Pharmaceutical Formulations: Comparative Characteristics of Classical Batch Procedure versus Micro-Scale Procedure**

| | Batch procedure | Micro-scale procedure |
|---|---|---|
| **Aqueous Phase** | | |
| Sample volume | 3 ml | 100 µl |
| Acidification (sulfuric acid) | 3 ml (1.0 mol $l^{-1}$) | 100 µl (1.6 mol $l^{-1}$) |
| Chromogenic agent: 5% KI + 5% ascorbic acid (v/v) | 5 ml | 400 µl |
| Cationic surfactant (BTAB, 1% (v/v)) | 3 ml | 100 µl |
| IS (Nile blue) | No | 100 µl ($1.6 \times 10^{-3}$%) |
| Final volume | 25 ml | 800 µl |
| **Organic Phase** | | |
| Chloroform | $2 \times 4$ ml | 400 µl |
| Final volume | 10 ml | No need to adjust |
| **Analytical parameters** | | |
| DL, mg $l^{-1}$ | 0.5 | 0.6 |
| CV (N = 5) | 0.6% (20 mg $l^{-1}$ Bi) | 1.0% (20 mg $l^{-1}$ Bi) |

DL—detection limit expressed as mg of Bi in 1 ml of sample digest; CV—variation coefficient for 5 replicates.

obtained from first-derivative spectra using a zero-crossing principle ($S = {}^1D_{543.1\,nm}/{}^1D_{644,4nm}$), as can be observed in Fig. 2. The main features of the micro assay encompass operational simplicity, non-rigorous protocol (only the precise addition of sample and of IS needed), small amounts of sample, reagents, and solvent (ethyl acetate) as well as high precision (<1%), which makes this procedure readily adopted for the analysis of a large number of clinical samples. The utility of assay was demonstrated by analyzing diabetic sera in three groups of patients, classified according to the development of typical diabetic complications.[22]

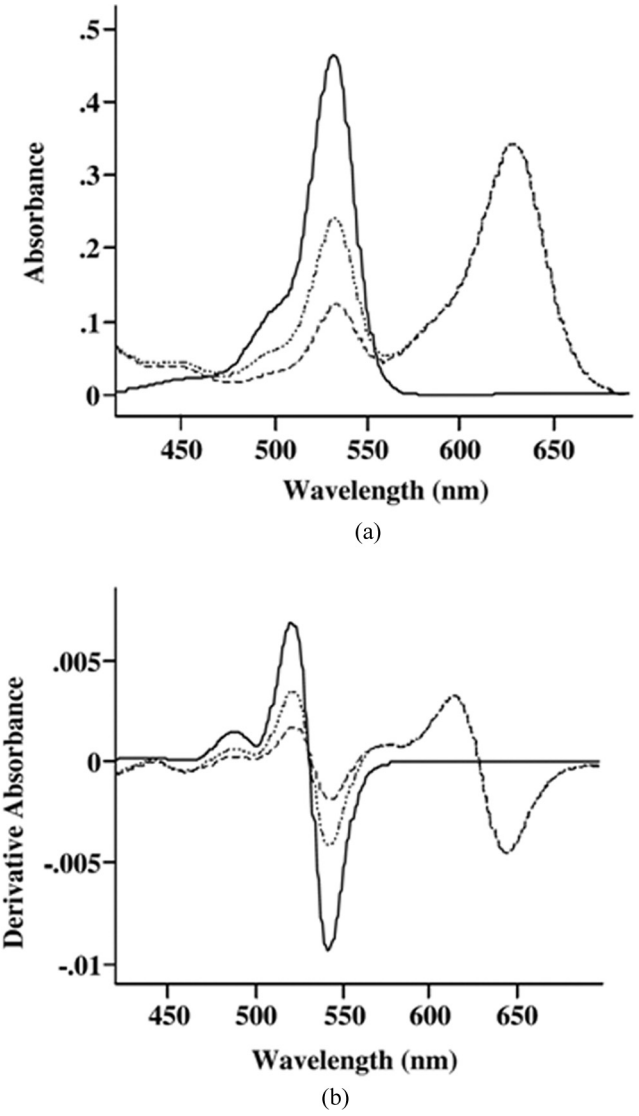


**FIGURE 2** **Absorption spectra obtained by the proposed micro-assay for malondialdehyde: (—) MDA standard, 4 μmol $l^{-1}$; (- - -) serum sample + IS (Erioglaucine A, $2.5 \times 10^{-4}$%); (- - -) serum sample spiked with 1 μmol $l^{-1}$ MDA + IS. (a) zero order spectra, (b) first derivative spectra (reproduced from Ref. 30 with the permission of Springer-Verlag).**

The determination of metallic species in different types of real-world samples is another application field of UV/Vis spectrophotometry. The feasibility of internal standard method has been explored aiming the improved performance of few batch micro assays (Table 2). As an example, the environmentally relevant procedure for hexavalent chromium in natural waters is presented here.[18] Diphenylcarbazide reaction with Cr(VI) was used, owing to its high selectivity and relatively good sensitivity ($\lambda_{max} = 546$ nm, $\varepsilon = 41700\,l\ mol^{-1}\,cm^{-1}$).[30] However, the quantification limit of 41.5 μg $l^{-1}$(1 cm cuvvete)[30] is not always satisfactory. In particular, direct batch procedure is of limited use for the analysis of drinking water, since WHO regulates Cr(VI) under the 50 μg $l^{-1}$ maximum contaminant level.[31] In the micro procedure proposed, IS wad added to the aliquot of sample (100 ml) and approximately 400-fold preconcentration was achieved in two steps: (1) by ion pairs formation with cationic surfactant followed by extraction to methylene chloride and (2) solvent evaporation and re-disolution in micro volume of methylene chloride (200 μl) (Table 2). In search of adequate IS, the spectral criterion had been considered. Furthermore, the IS should present negative electric charge and form ion pairs with this same cationic surfactant as Cr(VI). Several blue compounds were tested and the best results in terms of spectral resolution and comparable extraction yields of analyte and IS ion pairs, were obtained using Eriogalucine A. The zero-crossing conditions were found using first derivative spectra normalized against ${}^1D_{653.5\,nm.}$ The analytical signal was defined as $S = {}^1D_{analyte(591.3\,nm)}/{}^1D_{IS(653.5nm).}$ In Table 2, the experimental conditions and the analytical parameters of micro assay are presented. As can be observed, the quantification limit was 0.2 μg $l^{-1}$ and the precision expressed as variation coefficient for five repetitive measurements for 10 μg $l^{-1}$ of Cr(VI) was 3.2%, respectively. Acceptable recoveries (96–110%) were obtained in the analysis of tap water spiked with 2–8 μg $l^{-1}$ Cr(VI), which confirm the suitability of the procedure for the analysis of natural water matrices containing trace levels of this important contaminant.[18]

In Table 2, the application of IS method for micro-scale determination of other metal ions are also resumed. These applications encompass the determination of total copper and iron in blood

**TABLE 2** Some Applications of Internal Standard in Micro-Scale Spectrophotometric Determination of Trace Elements in Natural Water

| | Analyte | | | |
|---|---|---|---|---|
| | Cr(VI)[18] | Fe[20] | Cu[20] | As[32] |
| Chromogenic agent | Diphenylcarbazide | Batophenantroline | Bathocuproine | Silver diethyldithiocarbamate |
| Internal standard | Erioglaucine A | Bromophenol blue | Bromophenol blue | Erioglaucine A |
| Sample volume | 100 ml | 10 ml | 10 ml | 40 ml |
| Organic solvent | Methylene chloride 10 ml | Methylene chloride 2 ml | Methylene chloride 2 ml | Chloroform: Dimethylformamide: Diethanolamine (18:6:1), 1 ml |
| Concentration factor | 400× | 50× | 60× | 200× |
| Analytical signal | $^1D_{591.3\,nm}/^1D_{653.5\,nm}$ | $A_{490\,nm}/A_{602\,nm}$ | $A_{477\,nm}/A_{602\,nm}$ | $A_{525\,nm}/A_{623\,nm}$ |
| Quantification limit | 0.2 μg $l^{-1}$ | 9.0 μg $l^{-1}$ | 1.3 μg $l^{-1}$ | 1.0 μg $l^{-1}$ |
| CV (N = 5) | 3.2% (10 μg $l^{-1}$Cr(VI)) | 3.1% (40 μg $l^{-1}$Fe) | 1.6% (40 μg $l^{-1}$Cu) | 0.4% (40 μg $l^{-1}$As) |

serum and in natural waters[20] and the determination of total arsenic in aquatic samples.[32] In each case, the IS was selected aiming similar behavior of analyte and IS during separation/preconcentration as well as spectral resolution criterion. It was demonstrated that such an approach enables one to enhance the precision and the accuracy as referred to the micro assays without using IS.

## CONCLUSIONS

A number of official analytical methods still in use in pharmaceutical and food industries rely on traditional, batch UV/Vis spectrophotometric procedures. Down-scaling seems to be attractive, owing to lower quantities of reagents and solvents employed, in agreement with the principles of green chemistry. With the aim to enhance the analytical performance of spectrophotometric micro assays, the use of internal standard method had been proposed. Based on the results obtained in several applications to real-world samples, it can be concluded that the method of IS enables for controlling possible errors committed during sample manipulation. If the appropriate IS is applied, the procedures become operationally simple and not rigorous. There is no need to use volumetric vessels, small volumes of samples and reagents can be handled, and the unique precise operation required is the measurement of sample and IS volumes. It has been demonstrated that analytical errors committed in non-rigorous extraction, solvent evaporation, or dilution can be reduced or eliminated by defining the analytical signal as the ratio between the responses obtained for analyte and internal standard. In summary, the most important feature of spectrophotometric micro procedures with the use of internal standard is their low environmental impact, operational simplicity and low cost, without sacrificing the accuracy, and precision of the analytical results. In future development, the application of internal standard in different flow-based UV/Vis spectrophotometric methodologies would be of interest.

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