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Combining standard addition method and second-order advantage for direct determination of salicylate in undiluted human plasma by spectrofluorimetry

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Abstract

Second-order advantage turns possible a determination in the presence of unknown interferences. This work presented an application of the second-order advantage provided by parallel factor analysis (PARAFAC). The aim was the direct determination of salicylic acid (SA), the main product of aspirin degradation, in undiluted human plasma by spectrofluorimetry. The strategy of this analysis combined the use of PARAFAC, for extraction of the pure analyte signal, with the standard addition method, for a determination in the presence of a strong matrix effect caused by the quenching effect of the proteins present in the plasma. For each sample, four standard additions were performed, in triplicates. A specific PARAFAC model was built for each triplicate of each sample, from three-way arrays formed by 436 emission wavelengths, 7 excitation wavelengths and 5 measurements (sample plus 4 additions). In all the cases, the models were built with three factors and explained more than 99.90% of the total variance. The obtained loadings were related to SA and two background interferences. The scores related to SA were used for a linear regression in the standard addition method. Good results were obtained for determinations in the SA concentration range from 3.0 to 24.0 $\mu g\,ml^{-1}$, providing errors of prediction between 0.7 and 6.3%.

Keywords: PARAFAC; Salicylic acid; Molecular fluorescence; SOSAM; Blood

1. Introduction

Spectrofluorimetry is an analytical technique that has been used as a quantitative tool in several areas, such as chemistry, medicine, environmental and food science. Nevertheless, the analyses of multi-component mixtures can be hindered when the measurements are carried out in only one excitation or emission wavelength. The use of spectrofluorimetry in clinical analysis is made difficult by the complexity of matrices, such as blood and urine, which show a great variety of natural fluorescent compounds, whose spectra often overlap the analyte signal. This situation demands tedious separation steps to enable the analyte determination.

When the fluorescence of a sample is measured at several emission wavelengths for several excitation wavelengths, an excitation–emission matrix (EEM) is obtained. When a sample set is measured at these same conditions, a three-way array is obtained, which must show a trilinear behavior in spite of the presence of noise, scatter (Rayleigh and Raman) and other phenomena, such as *n*-order diffraction. This behavior turns chemometric three-way methods, such as PARAFAC [1] and N-PLS [2], suitable for fluorescence data analysis. PARAllel FACtor analysis (PARAFAC) is a generalization of PCA to higher order data, which presents unique solution independent of rotation (uniqueness), a great advantage for modeling spectroscopic data [1,3,4]. In particular, the mathematical model of PARAFAC is coherent with the nature of fluorescence data and its structural basis can be given by the following equation:

$$x_{ijk} = \sum_{f=1}^{F} a_{if} b_{jf} c_{kf} + e_{ijk} \quad (1)$$

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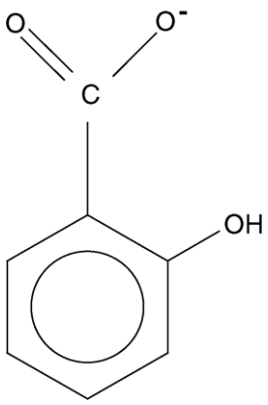

Fig. 1. Chemical structure of salicylate ion.

where x_{ijk} can represent the fluorescence intensity measured for the *i*th sample at the excitation wavelength *j* and emission wavelength *k*; a_{if} can be the concentration of the *f*th fluorophore in the sample *i*; b_{jf} can be the molar absorption coefficient of the *f*th fluorophore at the excitation wavelength *j*; c_{kf} can be the relative emission coefficient of the *f*th fluorophore at the wavelength *k* and e_{ijk} represents the residues of the model. A crucial step in the PARAFAC analysis is the choice of the appropriate number of the factors, for which there is no absolute criterion. This choice can be made based on the variance accounted for the model, the chemical knowledge of the system, split-half methods [5] or the recently proposed CORe CONsistency DIAgnostic (CORCONDIA) [6]. Due to the easiness of generating trilinear data, molecular fluorescence has probably been the subject of the major number of PARAFAC applications found in the literature. A recent review has discussed practical aspects related to this type of analysis, among them, the correct choice of the number of factors, the use of missing values to correct trilinearity deviations in the scattering spectral regions and the outliers' detection [7].

In the last years, the association of spectrofluorimetry and PARAFAC has provided new ways for direct determination of drugs in complex biological samples, without needing a protein precipitation step, which is common in traditional analysis. The blood proteins use to interact with drugs and to quench their fluorescence (quenching). Since 2002, the first articles have appeared in the literature describing the use of spectrofluorimetry/PARAFAC for determination of drugs, such as salicylic acid and naproxen [8,9], piroxicam [10], fluoroquinolone antibiotics [11], doxorubicin [12] and carbamazepine [13], in matrices, such as plasma, serum and urine. However, in the most of these articles the determination has performed only after a dilution step (500–2000 times), which sometimes leads to the need of an auxiliary complexing agent to improve the performance of the method. Specifically, the direct determination of a drug without a previous matrix dilution step has been performed in only one of these applications [12].

Salicylic acid (SA) is the product of hydrolysis and the main metabolite of acetylsalicylic acid (ASA) [14]. In the human blood (pH $\cong$ 7.4), it is present as the deprotonated form, salicylate ion (Fig. 1). Besides, SA is also used in ointments for their analgesic and antiseptic effects. The biological half-life of SA, between 3 and 19 h, is much greater to ASA half-life, between 15 and 20 min. Healthy volunteers, who have taken a dose of 500 mg of ASA, have presented a maximum concentration of 30 μg ml^{-1} of SA in the first 3–4 h [15]. The determination of SA in biological samples has been performed by titrimetry (Trinder method) [16], HPLC [17,18], capillary electrophoresis [19] and using an amperometric biosensor [20]. The most of these methods have demanded protein precipitation and extraction steps. Other methods have been based on fluorescence spectra obtained at a single excitation wavelength and first-order chemometric methods, such as PLS [21,22]. However, these determinations have also required solvent extraction steps.

The goal of this article was the direct determination of SA in undiluted human plasma, aiming at the maximum simplification of the sample manipulation. Two different strategies were tested. The simplest strategy tried to utilise a calibration curve for this determination. Nevertheless, the determination in undiluted whole plasma, in the presence of a strong matrix effect, was only possible by means of a second strategy, which includes the second-order advantage provided by PARAFAC combined with the standard addition method. More than this specific SA determination, the main objective of this article was to demonstrate the potential of this combination of PARAFAC, trilinear spectrofluorimetric data and standard addition method in the analysis of complex biological matrices of clinical interest.

1.1. Standard addition method and second-order advantage

The standard addition method is well known among analytical chemists and its description can be found in many books of instrumental and quantitative chemical analysis [23,24]. It is applied to univariate data (from zeroth-order instrumentation, such as a pHmeter) as a means of overcoming matrix effects that change the analyte signal. This method requires that the instrumental response be under two constraints:

(1) It must depend linearly on the increase of the analyte concentration.
(2) It must be zero when the analyte concentration is zero.

The estimation of the analyte concentration in the sample can be obtained through a plot of the instrumental response as a function of the amount of standard added by fitting a line to the data and finding its intercept on the abcissa.

In 1979, Saxberg and Kowalski published an extension of standard addition method to multivariate data (from first-order instrumentation, such as a diode array spectrophotometer), named generalized standard addition method (GSAM) [25]. GSAM requires that the analyte and the interferences be sequentially added in the sample. This relaxes the constraint that the analytical method must be fully selective to the analyte of interest. However, reliable results cannot be obtained if an uncalibrated source of instrumental signal is present. Therefore, in the absence of every species included in the calibration model, the instrumental response must be zero at all channels.

In 1995, Booksh et al. published another extension of standard addition method, in this case for multi-way data (from second-order instrumentation, such as HPLC-UV), named second-order standard addition method (SOSAM) [26]. They treated kinetic-spectrophotometric second-order data aiming at determining trichloroethylene and utilised direct trilinear decomposition (DTD) [27] as data decomposition method. SOSAM can be summarized in three steps:

(1) DTD is applied for decomposition of a third-order data array constructed by gathering the second-order data of the sample and of each successive addition. The correct number of factors used in the decomposition should correspond to the number of analytes plus the interferences.
(2) The loading matrix of the mode corresponding to the sample composition should contain in their columns the information related to analyte and interference concentrations. To identify which column corresponds to the analyte of interest, the loadings of the spectral mode (or other type of signal) can be compared with the spectrum of the pure analyte.
(3) The values of the identified column, corresponding to the analyte concentrations in the sample and after each addition, are used in a linear regression, in the same way as in univariate standard addition.

A key aspect of SOSAM is the use of the second-order advantage, defined as the ability to perform a determination in the presence of unknown interferences [4,28,29]. The use of second-order advantage requires that two assumptions be fulfilled:

(1) The data must be trilinear.
(2) The utilised method must be of second-order and decompose the data set to be predicted simultaneously with the calibration set.

The use of a second-order method is a necessary but not sufficient condition for second-order advantage. For example, N-PLS [2] is a second-order method, but not provides second-order advantage because it firstly decomposes the calibration set and then projects the samples to be predicted. Among the methods that allow the use of second-order advantage, PARAFAC, DTD and generalized rank annihilation method (GRAM) [30] can be cited, since they satisfy the second assumption mentioned above.

Nevertheless, the number of applications of SOSAM found in the literature is relatively small. Herrero et al. have applied SOSAM in spectroelectrochemical data (UV/visible spectra obtained at different potentials of a cyclic voltamogram) [31]. Wu et al. [32] have used a variant of SOSAM, in which the data is decomposed with a new algorithm called alternating trilinear decomposition (ATLD) and developed by the authors [33]. They have determined organo-chlorinated compounds by using HPLC-DAD. Comas et al. have combined standard addition method and GRAM in the determination of aromatic sulfonates in water [34] and polycyclic aromatic compounds in marine sediments [35], both by HPLC-DAD. Since GRAM is restricted by one mode having maximally dimension two (i.e., two samples), these last two applications have been limited to only one addition for each sample. More recently, Sinha et al. combined PARAFAC, standard addition and data generated by coupling GC × GC to time-of-flight mass spectrometry (TOFMS) to determine chlorobenzene in an environmental sample [36].

In the present application, instead of DTD originally proposed in SOSAM, PARAFAC was the method used for data decomposition. By comparing these two methods, PARAFAC presents the advantage of being more robust to data noise, what sometimes prevents the use of DTD in real noisy data [4].

2. Experimental

2.1. Reagents and plasma samples

A 300 $\mu g\,ml^{-1}$ stock solution of sodium salicylate (Merck) was prepared from deionised water (Milli-Q) and from this, two dilute solutions of concentrations 120 and 75 $\mu g\,ml^{-1}$ were also prepared. Human plasma samples (fresh frozen plasma) from 10 healthy volunteers were obtained at the Hemocentro of State University of Campinas and were kept in the freezer at $-8\,^{\circ}C$. It was assumed that the SA concentration of all the plasma samples is zero.

2.2. Apparatus and software

The spectra were obtained in a Perkin-Elmer LS 55 Spectrofluorimeter with the FL Winlab Software and using a 10.00 mm quartz cuvette. The data were imported using a home-made program and treated in MATLAB Version 6.1 (The MathWorks, Natick, USA). The PARAFAC calculations were carried out with the N-way toolbox for Matlab Version 2.10 [37].

2.3. Procedure

Firstly, an attempt to determine salicylate through a calibration curve was performed. Thirty undiluted plasma samples were prepared in 10.0 ml volumetric flasks, in the range from 1.5 to 30.0 $\mu g\,ml^{-1}$, by spiking appropriate amounts of 75 or 300 $\mu g\,ml^{-1}$ salicylate solutions. The volumetric flasks were completed with plasma from different individuals. For the second tested method, 10 undiluted samples were prepared in the range from 1.5 to 24.0 $\mu g\,ml^{-1}$. The plasma from a different individual was employed for each concentration level. For each measurement, 2.5 ml of sample were added in the cuvette. In the sequence, four successive additions of 50 μl of a 120 $\mu g\,ml^{-1}$ salicylate solution were performed and measured. After each addition, the samples were homogenized with the aid of a micro magnetic bar. All the determinations were carried out in triplicate.

All the spectral surfaces were obtained in the excitation range from 280 to 340 nm (step 5 nm) and in the emission range from 360 to 580 nm (step 0.5 nm). The excitation and emission monochromator slit widths were both 4.0 nm and the scanning rate was 1200 nm min^{-1}.

3. Results and discussion

3.1. Preliminary studies: spectra resolution and an attempt to determine SA in plasma through a calibration curve

Excitation–emission matrices obtained for a pure plasma sample and for the same plasma spiked with SA 9.0 μg ml^{-1} are plotted in Fig. 2. The most remarkable difference between these two spectral surfaces is the appearance of a strong band with maximum wavelength of excitation/emission at approximately 320/400 nm, due to SA fluorescence.

The first purpose of this work was the determination of SA through a calibration curve constructed with plasma samples from different individuals. The adopted strategy aimed at simplifying the sample handling by eliminating the need of plasma dilution. In a preliminary study, 30 plasma samples spiked with SA (18 for calibration and 12 for validation) were prepared in the concentration range from 1.5 to 30.0 μg ml^{-1} and their EEMs were recorded. From a three-way array formed with 442 emission wavelengths, 13 excitation wavelengths and 30 samples, a PARAFAC model was built under non-negativity constraint in the spectral modes. The best model was built with three factors and accounted for 99.2% of the total data variance. The trilinear consistency of this model was evaluated by a CORCONDIA of 95.3%, which indicates its suitability. A value of CORCONDIA above 80% indicates the trilinear consistency of a PARAFAC model; a value around 50% means trilinearity deficiency and a value close to zero or negative means trilinear inconsistency [6]. The PARAFAC factors were associated with SA and two possible plasma background interferences.

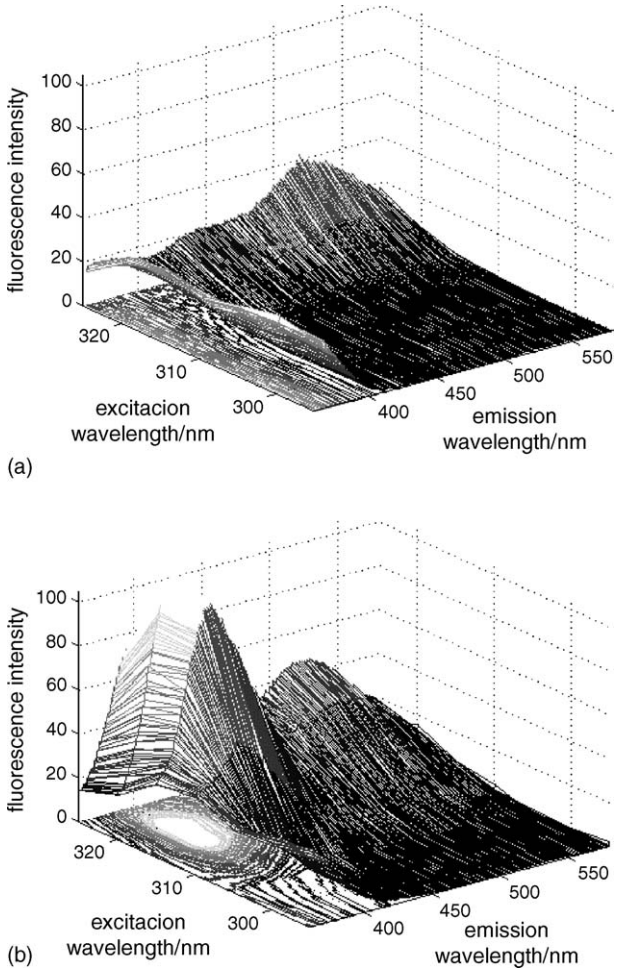

Fig. 2. Excitation–emission matrices of: (a) a pure plasma sample and (b) a sample of this same plasma spiked with SA 9.0 μg ml^{-1}.

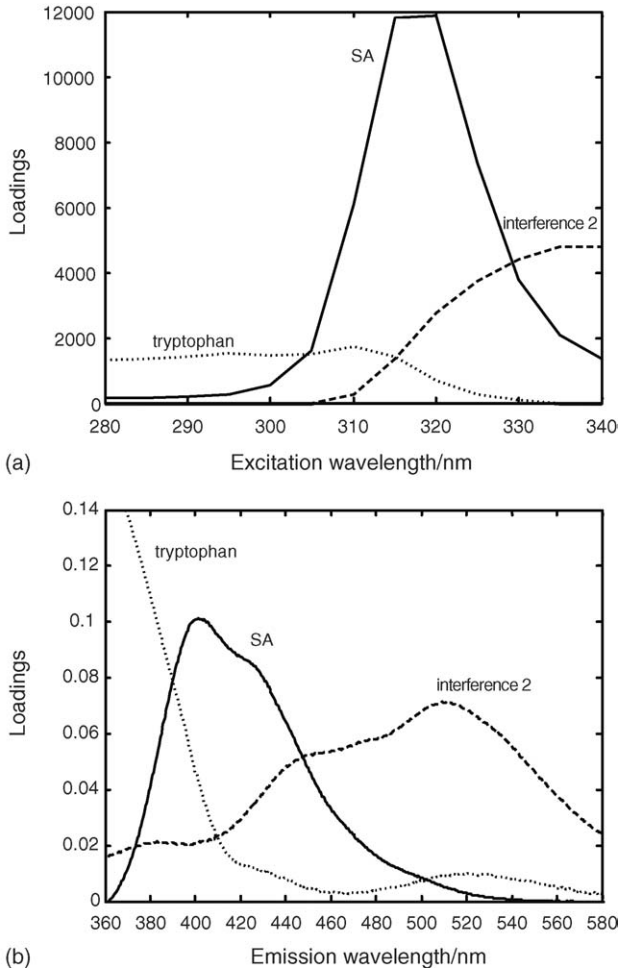

Fig. 3. Deconvoluted fluorescence spectra obtained from the loadings of the PARAFAC model for the (A) excitation and (B) emission modes.

The PARAFAC loadings related to excitation and emission modes are shown in Fig. 3. The factor loadings associated with SA presented excitation and emission maxima between 315 and 320 nm, and 400 and 405 nm, respectively. These loadings were compared with SA spectra acquired in aqueous solution buffered with phosphate at pH 7.4 (Fig. 4), reproducing the pH of human blood. The SA spectra extracted by PARAFAC slightly differed from the ones acquired in aqueous solution: in plasma, a blue shift of the emission maximum were observed and a shoulder appeared at 425 nm; the excitation spectrum also showed a small shift presenting a maximum at 300–310 nm; besides, SA fluorescence in plasma presented a strong quenching caused by the proteins. All these spectral changes were considered as consequences of the strong interaction of SA with the proteins of the plasma.

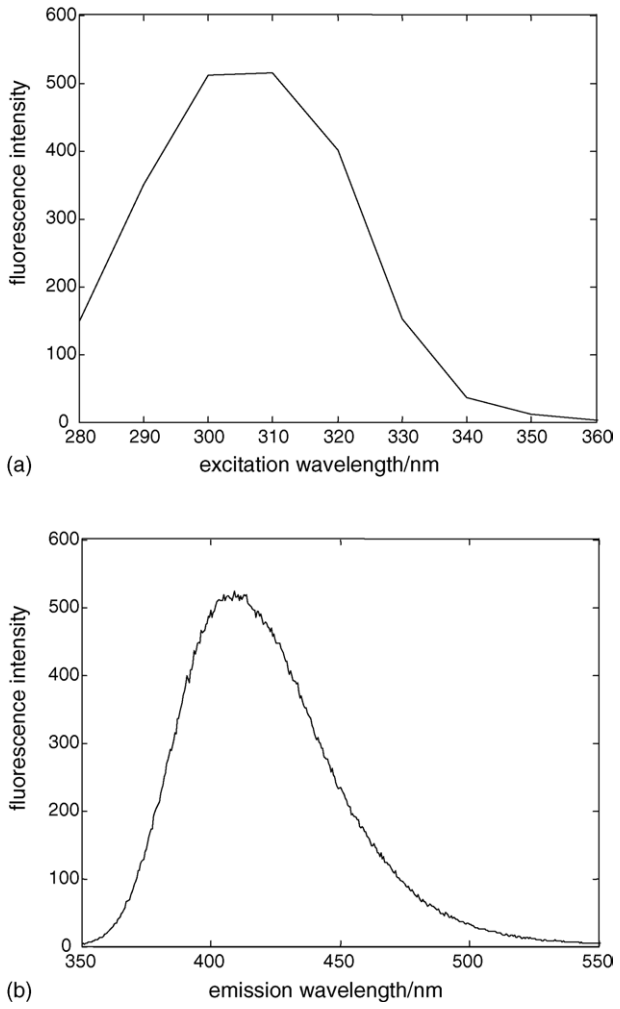

Fig. 4. Fluorescence spectra of a 10.0 μg ml^{-1} SA in phosphate buffer (pH 7.4) solution. (a) Excitation spectra obtained at λ_{em} = 410 nm. (b) Emission spectra obtained at λ_{exc} = 300 nm.

The attempt to identify the modeled interferences was carried out based on the article of Wolfbeis and Leiner, who have mapped the total fluorescence of human blood serum [38]. The interference 1, whose spectrum is present in shorter wavelengths, was ascribed to tryptophan (excitation/emission maximum at 278/348 nm). The interference 2, whose spectrum is present in longer wavelengths, was more difficult to be characterized and among the possibilities, the following substances can be suggested: nicotinamide adenine dinucleotide (NADH) and its phosphate, and riboflavin (Vitamin B_2), free or in the form of their coenzymes, such as flavine mononucleotide (FMN) and flavine adenine dinucleotide (FAD). The authors of this article have highlighted that the fluorescence profile of human serum in the UV region, where tryptophan fluoresces, does not use to show great variability for healthy individuals. On the other hand, near UV and visible regions, where interference 2 fluoresces, uses to present great variations depending on the sample, which turns difficulty the characterization of the species fluorescing in them.

The loadings of PARAFAC model related to sample mode could be used for calibration through a linear regression. However, the loadings obtained for this model did not show a linear behavior as a function of SA concentration, turning impossible the construction of a calibration curve. Further on, it was verified that replicates spiked with the same SA concentration in plasmas from different individuals showed up to 30% of difference in their loadings, while samples spiked with the same SA concentration and from the same individual shown differences lower than 1%. The conclusion extracted from this fact was that is impossible to construct a calibration model for SA in different plasmas, because the intensity of their fluorescence quenching depends on the plasma composition of each individual. In other words, a strong individual matrix effect was observed. Since it was obviously not feasible to construct a calibration curve specifically for each individual, another strategy for SA determination was adopted.

3.2. SA determination in plasma using standard addition method

The strategy adopted for SA determination was based on the use of the standard addition method, recommended for situations such as this, in which the matrix effect turns impossible the construction of a robust calibration curve. One aspect to be highlighted in the methodology used here is the utilisation of PARAFAC loadings for plotting the standard addition line, instead of the pure instrumental signal. These loadings are equivalent to the filtered signal free of interferences. The determinations were carried out in a situation of variable total volume with continuous variation of standard and the consequent analyte dilution was taken into account according to reference [39].

For this new analysis, the initial excitation wavelength range was restricted between 295 and 325 nm, around the SA band. It was verified that the absence of the eliminated region did not significantly affect the models. The PARAFAC models were built from three-way arrays formed with 442 emission wavelengths, 7 excitation wavelengths and 5 measurements (the original sample plus 4 standard additions). A specific model was built for each triplicate of each sample, totalising 30 models. In all cases, three factors and no constraints were used and the models accounted for more than 99.90% of the total data variance. It was verified that models without constraints provided results with lower errors than models under non-negativity constraints. The obtained CORCONDIA values were between 25 and 80%, meaning in some cases trilinearity deficiency. Models built with two factors presented 100% of trilinearity, but provided prediction errors significantly larger than the models built with three factors. Besides, models with two factors showed systematic and significant residual variance, indicating the need of extracting one more factor. In all cases, the obtained loadings were very similar to the ones shown in Fig. 3.

The results for the determination of the samples are shown in Table 1, together with the errors in relation to the expected values. In most of the cases, the correlation coefficient (r) of the standard addition line was higher than 0.990; in a few cases, r values were between 0.970 and 0.990. The worst result was obtained for the sample at the lowest concentration level, 1.5 μg ml^{-1}, showing an error of prediction higher than 20%. This result may indicate a possible limit of the method, taking into account that

Table 1
SA determination in human plasma by using PARAFAC and standard addition method

Amount added ($\mu g\,ml^{-1}$)	Amount predicted ($\mu g\,ml^{-1}$)[a]	Error (%)
1.5	1.8 ± 0.2	21.3
3.0	3.2 ± 0.3	6.3
4.5	4.6 ± 0.3	3.3
6.0	6.2 ± 0.3	4.5
7.5	7.7 ± 0.3	3.5
9.0	8.9 ± 0.2	−0.7
12.0	12.4 ± 0.4	4.1
15.0	15.6 ± 0.5	4.4
21.0	20.7 ± 0.4	−1.4
24.0	24.3 ± 0.4	1.5

[a] Mean and standard deviation of three determinations.

in this concentration level the SA signal is completely superimposed by the signal of the plasma background. The predictions of the other samples showed errors of at maximum 6.3%, being that the most of these errors were lower than 5%.

4. Conclusion

This work presented an example of the use of the second-order advantage. The results showed that the proposed method was robust enough to determine SA in undiluted human plasma (in the concentration range from 3 to 24 $\mu g\,ml^{-1}$) with a minimum of sample handling. The potential advantages of this determination, such as rapidity and low cost, can even more highlighted by considering the possibility of automating the proposed method. The determination could be performed in a lower scale, the errors of manipulation could be decreased and the time of analysis could be optimised. Since the applications combining spectrofluorimetry, PARAFAC and standard addition are scarce in the literature, one of the objectives of this work was also to divulge the potential of this methodology as a new alternative for determining fluorescent species in complex matrices. Beyond the possible applications in clinical analysis, this alternative can be applied on another matrices, such as food, plants, soils, etc.

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