Multivariate curve resolution applied to sequential injection data. Analysis of amoxicillin and clavulanic acid

Doctoral thesis

ROVIRA I VIRGILI UNIVERSITY

ROVIRA I VIRGILI UNIVERSITY
Department of Analytical Chemistry and Organic Chemistry

Multivariate curve resolution applied to sequential injection data. Analysis of amoxicillin and clavulanic acid

Thesis presented by

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While writing this thesis I was supervised by Dr. Pilar Callao, and the work was carried out in the Chemometrics, Qualimetrics and Nanosensors research group led by Prof. F. Xavier Rius at the Department of Analytical Chemistry and Organic Chemistry of the Rovira i Virgili University in Tarragona, Spain.

It included a research stay in the laboratory of Prof. Jaromir Ruzicka at the CPAC Center of the Department of Chemistry of the University of Washington, Seattle (USA).

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The Doctoral thesis entitled: "**MULTIVARIATE CURVE RESOLUTION APPLIED TO SEQUENTIAL INJECTION DATA. ANALYSIS OF AMOXICILLIN AND CLAVULANIC ACID**", presented by ALBERTO PASAMONTES FÚNEZ to receive the degree of Doctor of the Rovira i Virgili University, has been carried out under my supervision, in the Department of Analytical Chemistry and Organic Chemistry at the Rovira i Virgili University, and all the results presented in this thesis were obtained in experiments conducted by the above mentioned student.

Tarragona, October 2005

Dr. Pilar Callao Lasmarías

Esta tesis esta dedicada con todo mi cariño a mi familia y en especial a Vicente,
Dolores, Ignacio y Àngels por su ayuda y por estar siempre a mi lado.

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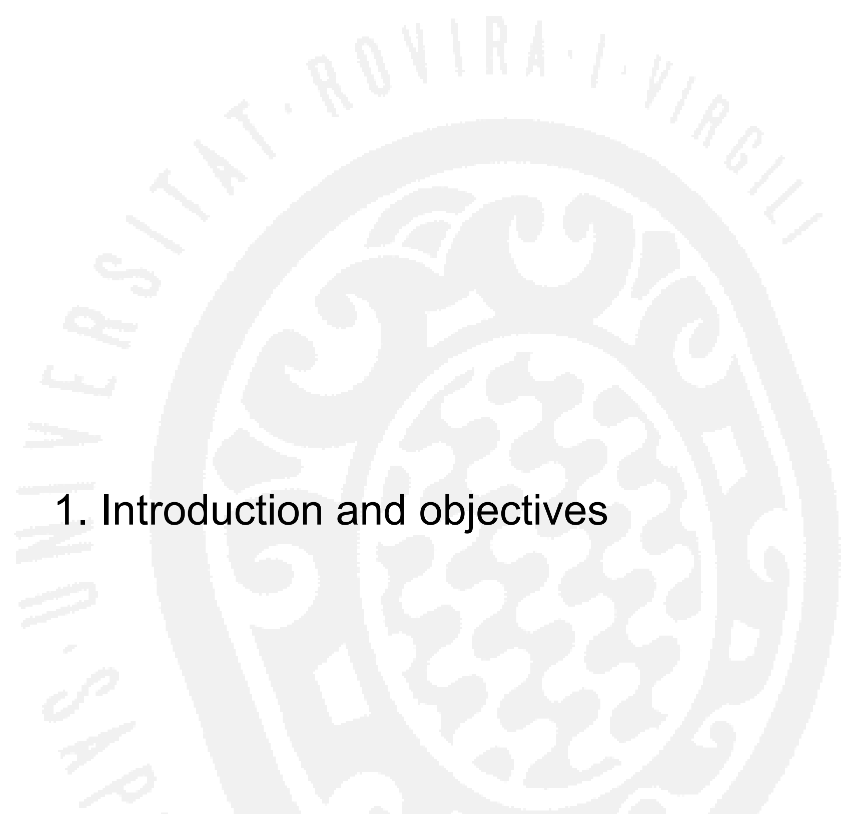

1. Introduction and objectives

1.1. INTRODUCTION

Since the Chemometrics, Qualimetrics and Nanosensors research group of the Rovira i Virgili University was set up in the 1990s, several chemometrics applications have been developed within analytical chemistry. This has led to the defence of 14 doctoral thesis and numerous scientific publications [1].

A few years ago, our group began the development and application of calibration methods for data matrices (second-order data), which allow any analyte to be quantified or qualified, even in the presence of unknown and uncalibrated interferents [2]. To do so, requires the establishment of a calibration model made up of standards in which the value of the property to be determined is known. One of the chemometric tools that enable researchers to work with data matrices are the so-called resolution methods. In our group, these methods have been applied to chromatography data [3] and to near infrared data [4].

Another way to generate second-order data (evolving systems) is sequential injection analysis (SIA) [5] with a multivariate detector, e.g. diode-array spectrophotometric detector. SIA was introduced by Jaromir Ruzicka in 1990 in the laboratories of the Centre for Process Analytical Chemistry (CPAC) at the University of Washington (Seattle). Our group has experience in generating and treating multivariate data using sequential injection analysis [6].

One interesting field of application for these methods is the analysis of pharmaceuticals—for example, to control the quality of the pharmaceutical itself and, in clinical analysis, to study the effects and evolution of the pharmaceutical in the human body. Within this field of application, the analysis of antibiotics is also important due to the wide-ranging application of these drugs.

1.2. OBJECTIVES OF THE THESIS

The objective of this thesis is to study and develop analytical methods to determine amoxicillin and clavulanic acid using sequential injection analysis (SIA) with a diode-array spectrophotometric detector to obtain second-order data. To treat these data, the chemometric tool used was multivariate curve resolution with alternating least squares (MCR-ALS). More specifically, we:

1. Develop and study the experimental conditions to obtain second-order data.

    a) We study all the instrumental and chemical reaction factors (flow, volume and concentration of sample and reagents) that can influence the analytical signal.

    b) We study multivariate curve resolution with alternating least squares (MCR-ALS) and the techniques involved in the resolution process e.g. principal analysis components (PCA), simple-to-use interactive self-modelling mixture analysis (SIMPLISMA) or evolving factor analysis (EFA), and alternating least squares (ALS) and its constraints.

2. Develop analytical methods to determine amoxicillin in the presence of interferents, and amoxicillin and clavulanic acid simultaneously in pharmaceuticals.

3. Apply methods based on concepts of experimental design to find the optimal analytical sequence by considering multiples responses.

1.3. STRUCTURE OF THE THESIS

This thesis is divided into five chapters.

- Chapter 1. *Introduction and objectives.* This chapter contains the introduction, objectives and structure of the thesis.

- Chapter 2. *Theoretical backgrounds*. This chapter contains a brief description of the theoretical backgrounds that have been used during this thesis. Section 2.1 discusses the characteristics and properties of amoxicillin and clavulanic acid. Section 2.2 describes the instrumental and the process that takes place when the reagents and sample are introduced to the system. Section 2.3 introduces the chemometric tools used in the treatment of the signal (PCA, EFA, SIMPLISMA, ALS, etc.). Section 2.4 introduces the experimental designs used in the thesis. These are the Plackett-Burman design, full and fractional factorial designs, central composite design and the simplex approach. Due to ours multiresponse system, desirability function was explained. Section 2.5 contains the references for this chapter.

Chapters 3 and 4 contain the bulk of the work carried out for this thesis and incorporate papers published in journals. These papers describe the methods used, the results, the discussion and the conclusions. These papers have been edited to provide a uniform format.

- Chapter 3. *Sequential injection analysis and multivariate curve resolution with alternating least squares. Determination of amoxicillin and clavulanic acid.* Section 3.1 justifies the experiments carried out for this thesis and presents the papers resulting from these experiments. Section 3.2 is a paper in which we explored the possible interferents present with amoxicillin in several pharmaceuticals. Section 3.3 analyses a series of parameters in order to optimise the second-order calibration step. Section 3.4 describes the simultaneous determination of clavulanic acid and amoxicillin. Finally, section 3.5 discusses the potential of using sequential injection analysis (SIA) for generating an evolving system.

• Chapter 4. *Optimisation of analytical sequence using experimental designs. Response surface and simplex approach.* This chapter comprises two papers. Section 4.1 contains a brief introduction and justification of the experimental work. In section 4.2, the factors and responses of interest for the determination of amoxicillin are defined and the response surface method is then applied in order to find the optimum analytical sequence. In section 4.3, after a screening step, we apply the simplex approach to optimise the analytical sequence for determining amoxicillin and clavulanic acid simultaneously.

• Chapter 5. This chapter contains the *conclusions* of the thesis. The advantages and limitations of the methods described are discussed and suggestions for further research are outlined.

• The A*ppendix* contains the list of the abbreviations used in the thesis and the list of papers and meeting presentations given by the author during the period of development of this thesis.

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2. Theoretical backgrounds

2.1. ANTIBIOTICS: AMOXICILLIN AND CLAVULANIC ACID

Antibiotics are drugs used to kill or harm organisms such as bacteria, viruses, funguses and protozoon in living organisms. Since their discovery in the 1930s, antibiotics have made it possible to cure diseases caused by bacteria such as pneumonia, tuberculosis and meningitis— saving the lives of millions of people around the world. Some antibiotics are produced from live organisms such as bacteria and funguses. Other antibiotics are totally or partially produced synthetically.

Antibiotics act *via* two mechanisms: they kill the microorganisms (bactericide action) and prevent them from reproducing (bacteriostatic action).

Table 1 shows antibiotics classified by their mechanism. In the bactericides group, we find the beta-lactams such as penicillins. Though penicillins are the oldest antibiotics, they are still the antibiotics of choice for most infections.

Table 1.- Classification of antibiotics by their mechanism.

Bactericides	Bacteriostatic
● Beta-lactams (penicillins and cephalosporin)	● Macrolids (erythromycin group)
● Glycopeptides (vancomycin, teicoplanin)	● Tetracycline
● Aminoglycoside (streptomycin group)	● Cloranfenicol
● Quinolone (norfloxacin group)	● Clindamycin, Lincomycin
● Polimixinas	● Sulfamide

One of the most important penicillins is amoxicillin (α-amino-4-hidroxibenzilpenicilin), whose chemical structure is shown in Figure 1. Amoxicillin is a white, or almost white, crystalline powder that is slightly soluble in water and in alcohol, and can be present in various acids forms depending on the pH. Its pKa are 2.4, 7.4, 9.01, and 10.29 [1, 2].

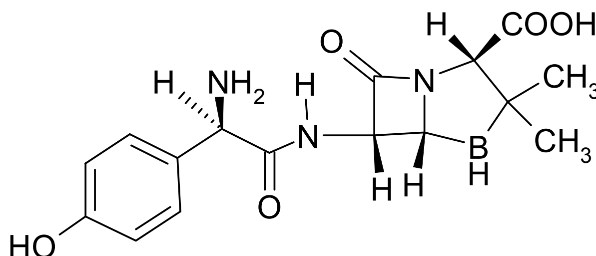


Figure 1.- The chemical structure of amoxicillin.

Amoxicillin is absorbent in the UV-VIS zone, which the spectra change depending on the pH media. The amoxicillin spectra has two maximum of absorbance at λ=230 and 280 nm when pH is lower than 10, and its spectra in a pH higher than 10 has a maximum of absorbance at λ=250 and 300 nm.

Clavulanic acid, (Z)-(2R,5R)-3-(2-hydroxyethylidene)-7-oxo-4-oxa-1-azabicyclo [3.2.0]-heptane-2-carboxylate, whose structure is shown in Figure 2, contains a beta-lactams ring linked to an oxazolidine ring instead of the thiazolidine ring found in all penicillins. Clavulanic acid is freely soluble in water but not very stable in aqueous solution. Its stability is optimal at a pH of 6.0 to 6.3 and it is also soluble in methanol. Clavulanic is a white powder produced from *streptomyces clavuligerus*. It possesses several functional groups (proton donor). Since it has acid-base characteristics, it has three values of pKa 2.7, 12.17 [2].

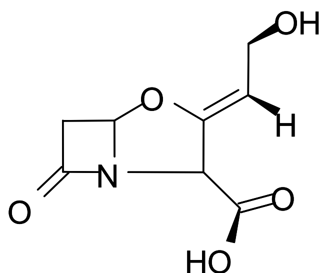


Figure 2.- The chemical structure of clavulanic acid.

In a UV-VIS detector, the spectra of clavulanic acid changes depending on the pH buffer. The spectra for clavulanic acid in a pH lower than 12 has a maximum of absorbance at λ=290 and is much less sensitive than the basic species of clavulanic acid (pH higher than 12). The basic species of clavulanic acid has a maximum of absorbance at λ=260.

2.1.1. Bibliographic review

We have reviewed the literature on amoxicillin and the various methods for determining it. Amoxicillin can be determined in two fields: in living organisms (humans or animals), where its presence is due to the effects of medical treatments [3-6] and in pharmaceuticals [7-10].

- In the first field, all methods include a pre-concentration step followed, mainly, by the use of the HPLC technique (high performance liquid chromatography), which helps to determine it in the presence of other components. Several methods use a UV-VIS detector, which measures the absorbance to a wavelength of 230 nm [11-15]. The quantification limit for this type of analysis is around 0.5μg/ml in plasma [11-14]. Chulavatnatol and Charles [15] reported a method for measuring amoxicillin in urine. Other techniques use derivatization pre-columns [16] or reaction post-columns [17-21] to improve sensitivity.

- In the second field (pharmaceuticals), the methods are more varied. These methods do not contain a pre-concentration step because the amoxicillin content in pharmaceuticals is higher. The most common technique is UV-VIS spectroscopy with univariate calibration. To perform this technique, either the permissible concentrations of substances that can accompany amoxicillin in several pharmaceuticals are studied so that they do not interfere [22], or, if interferents are present, a previous chemical reaction is provoked in order to obtain selectivity [23]. The first and second derivate was used to determine amoxicillin when it was found in combination with other penicillins and when it was found with cephalosporin [24]. Other methods have been

published that incorporate other techniques such as polarography [24], fluorescence spectroscopy [25] and HPLC [26-28].

Clavulanic acid has been determined by derivatization using chemiluminescence and UV-VIS detection respectively [29, 30]. In most cases it has been determined together with amoxicillin by chromatography [31-38] but another method is to use chemometric tools [39-41] and flow systems [42, 43].

2.2. SEQUENTIAL INJECTION ANALYSIS (SIA)

The automation of analytical processes has been increasing since the 1970s. One way of achieving automation is using a flow system such as flow injection analysis (FIA) [44, 45], which was introduced in 1975 by Ruzicka and Hansen [46]. Since then many papers and reviews on automated analytical processes have been published and their field of application has been extended to food analysis [47], clinics analysis [48], pharmaceuticals analysis [49], etc.

The advantages of this type of system are:

- It can be easily automated and miniaturised.
- The cycles can be executed repeatedly.
- It is highly versatile because it can be adapted to most analytical instruments.
- The frequency of analysis can be high.
- The consumption of samples and reagents is low.

In 1990, Professor Ruzicka developed the second generation of flow systems known as sequential injection analysis (SIA) [50]. The classic configuration of a sequential injection analyzer is shown in Figure 3. The principle components of the sequential injection analyzer are: an automatic pump or syringe (1), which is used to

aspirate the reagent and sample *via* a valve and push them towards the detector; a selection valve (2), which is used to select the reagents and introduce them into the system; the detector (3), which in our case is a diode-array spectrophotometer; the holding coil (4); the reactor coil (5); and finally a computer that controls the functions of each component.

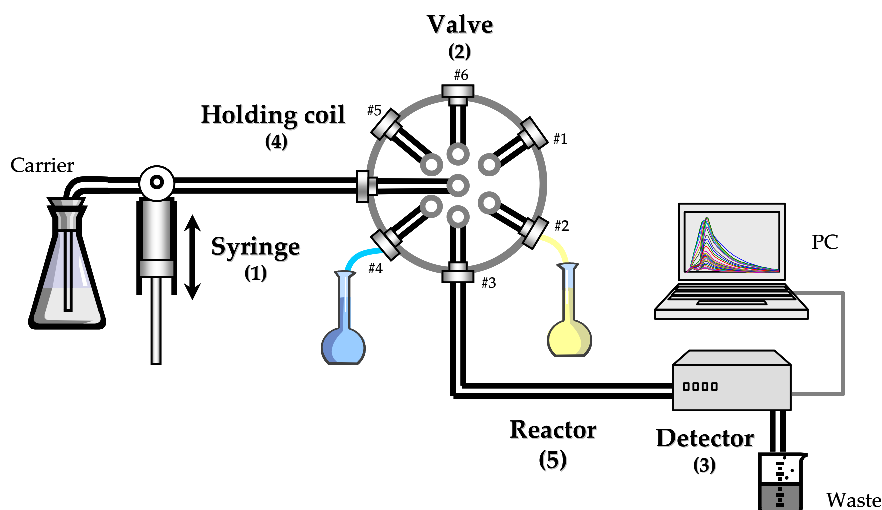


Figure 3.- Scheme of the sequential injection analyzer.

The process involves the following steps. First, the valve (#1, #2, #4, #5 and #6) selects the order of aspiration of the reagents. The syringe then aspirates the carrier, the sample and the reagents. Next, the syringe pushes the reagents towards the detector. When the reagents are aspirated and pushed towards the detector, a change in the flow direction takes places. Finally, the detector is activated and provided with information about the number of measurements and the range of wavelength, etc. Once the sample has passed through the detector, it is expelled as waste.

During a process of sequential injection, the reagents and samples are mixed *via* a phenomenon of dispersion. A scheme of this process is shown in Figure 4. In the first two steps the reagent and sample are introduced into the system (yellow and blue respectively) and the dispersion between the reagent and sample are considered minimum. In the final steps the reagents are displaced towards the detector by a

syringe. This provokes dispersion between the reagents and generates a product (green).

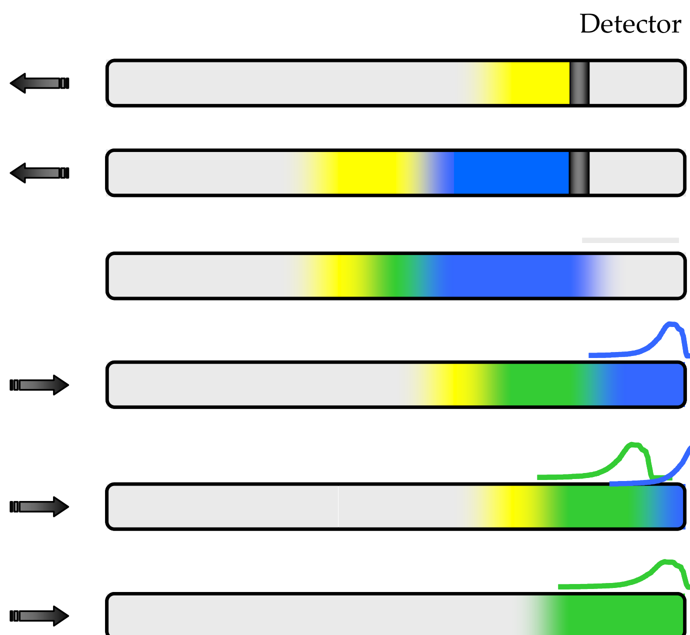


Figure 4.- Illustration of the dispersion phenomenon in SIA.

The type of data obtained from SIA (zero-order data, first-order data or second-order data) depends on the type of detector used and whether the reagents have interacted totally or partially before passing through the detector. If the reagent has interacted totally, the analyte (blue) will be converted into green colour, which is the reaction product. However, if the reagent has interacted partially, the following areas will be observed as it passes through the detector: the analyte that did not react (blue), a mixture of green and blue colour, and a reaction product (green). This characteristic, together with the versatility of the system in coupling to different types of detectors, enables data of different dimensions to be obtained. This means that various chemometric tools can be used to provide information about the system.

2.2.1. Bibliographic review

Our laboratory has been working with SIA in the fields of multivariate analysis (first-order data) [51-53]. Sequential injection analysis has numerous analytical applications. We should mention in particular the work carried by the research groups of Ruzicka, Cerdà and Van Staden.

The Ruzicka team developed the SIA method for determining a stop flow [54] and used it to automatize analytical methods—for example, for determining total nitrogen and ammonia in fermentation processes [55]; for determining hydrogen peroxide and glucose using luminescence detection [56], and for separating phases for the preparation of samples [57]. More recently, a micro-sequential injection system (μ-SI) has been produced to reduce waste and the consumption of reagents. This novel concept in micro-flow analysis has led to the development of a lab-on-valve system (LOV) [58]. These techniques have been explored for their usefulness in biomedical clinical diagnostics [59-61], biotechnology [62, 63], pharmaceutical research [64] and coupled with other techniques [65-67].

Cerdà et al. described the implementation of an SIA system using a syringe as the means of propulsion [68]. They studied the influence of several design factors that affect results [69] and the use of the sample as carrier [70]. More recent studies have focused on the application of multisyringe flow injection analysis (MSFIA) [71-73]. This technique has largely been applied to environmental samples in, for example, the analysis of stable and radioactive yttrium [74], the determination and speciation analysis of iron [75-77], the determination of sulphide [78], and the determination of orthophosphate in waters [79]. This technique was coupled with other techniques such as chemiluminescence detection [80], solid-phase reflectometry [81] and fluorescence detection [82].

Van Staden et al. have published papers describing the SIA method [83] in which they discuss the computer programs needed to control the various components and the variables that affect the results [84]. More recently studies have focused on

determining enantiomers using amperometric detection [85, 86], chemical speciation [87, 88] and the determination of metals such as: bromate [89], iodide [90] and Pb^{2+}, Cu^{2+}, Zn^{2+}, Co^{2+}, Cd^{2+}, Fe^{2+} and Hg^{3+} [91]. Other applications have been applied in the field of pharmaceuticals, where phenylephrine hydrochloride [92], magnesium [93] and Fe^{2+} [94] have been determined. Other interesting studies can be consulted in the references [95-107].

2.3. MULTIVARIATE CURVE RESOLUTION WITH ALTERNATING LEAST SQUARES (MCR-ALS)

The main aim of multivariate curve resolution with alternating least squares is to resolve a data matrix to obtain the concentration and spectra profiles of every important component that provides information to the system.

Applying MCR-ALS involves using various chemometric tools. From a data matrix, in a first step, we apply principal component analysis (PCA) to determine how many significant principal components or sources of variation are present in the system. In a second step, we find an initial estimation, of either the concentration profiles or the spectra profiles, from the number of significant principal components previously found. This initial estimation can be made using chemometric techniques based on principal components analysis e.g. evolving factor analysis (EFA) or by other techniques, such as simple-to-use interactive self-modelling mixture analysis (SIMPLISMA), based on finding pure variables.

From an initial estimation and the number of significant principal components selected in the principal component analysis, alternating least squares (ALS) is applied to obtain a matrix of concentration profiles and a matrix of spectra profiles in order to quantify the species (quantification analysis) or identify the species (qualification analysis). To afford the solution a chemical significance, information is introduced in

the form of constraints. Figure 5 is a scheme of the multivariate curve resolution with alternating least squares process and its sequence of application.

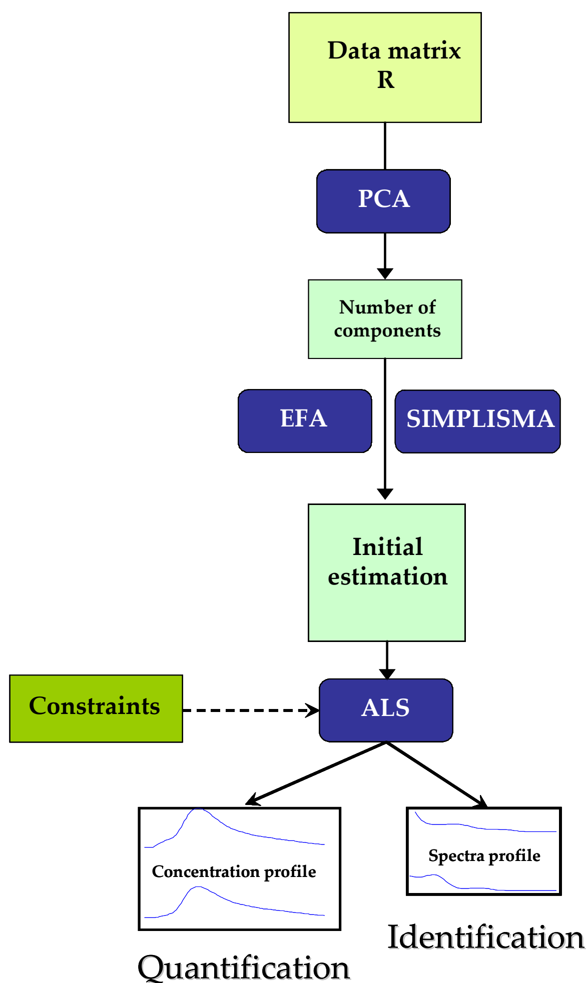


Figure 5.- Scheme of MCR-ALS.

2.3.1. Principal components analysis (PCA)

The main aim of principal components analysis [108, 109] is to express the important information contained in the raw matrix **R**(*m*x*n*) made up of *m* objects (samples measured at different times) and *n* variables (wavelengths) in a small number of new variables that are linear combinations of the original variables called principal components (PC).

The experimental matrix **R** can be expressed as a product of two matrices:

$$\mathbf{R}=\mathbf{Q}\cdot\mathbf{P}^{T} \qquad (1)$$

where $\mathbf{P}^{T}$ is a data matrix of eigenvectors known as loading, which provides information about the importance of the variables in each of the new variables generated; and **Q** is the projection of experimental data, known as scores, onto an orthonormal base.

Each eigenvector is assigned one eigenvalue, the size of which is a measure of its relative importance. Each eigenvector represents one principal component or source of variation in the response signal.

This decomposition process is used to extract the important information from the studied system. The number of eigenvalues obtained is the lower of the two values *m* and *n*. However, not all of these *n* eigenvectors contain useful information about the system. Most of the eigenvectors basically contain information about the noise associated with the experimental measurements, which are also a part of the experimental signal. The importance of the information from each eigenvector is indicated by the size of the eigenvalues. Noise is the component that predominates in the eigenvectors, whose eigenvalues will be low because noise contributes very little to the variation of the data. Excluding some eigenvectors does not make it difficult to reconstruct the information in the raw matrix; rather, it improves this matrix. A key

step in principal components analysis is to determine the number of significant principal components.

Principal component analysis can be performed using various algorithms. One of the most important of these is singular value decomposition, SVD [110, 111].

2.3.2. Evolving factor analysis (EFA)

The main aim of this algorithm is to determine how many species there are at any time during an evolving system. The process of evolving factor analysis [112, 113] can be summarised in the steps shown graphically in Figure 6.

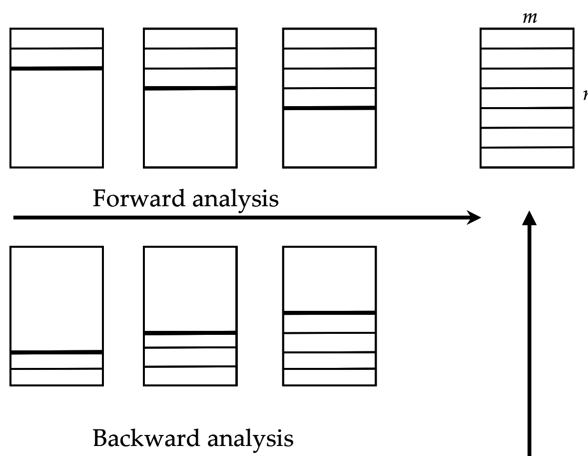


Figure 6.- Steps in EFA for a data matrix. The marked region indicates the sub-matrix to which PCA is applied.

The first step involves applying singular value decomposition to a sub-matrix that contains only the first two rows (spectra) of the raw matrix (**R**). We then add the third spectra of the data matrix to the initial sub-matrix and again apply singular value decomposition with this new data matrix. This process is repeated until singular value decomposition is applied to the whole matrix. EFA can be applied in both directions: forward analysis and backward analysis. Forward analysis provides information about

the appearance of the different principal components throughout the experiment. Backward analysis provides information about the disappearance of these principal components.

Once the number of principal components for the forward and backward analyses has been calculated, a graph such as that in Figure 7 can be obtained. In this graph, only the values of eigenvalues logarithm higher than a prefixed value were represented, this means that the values of eigenvalues logarithm less important were deleted.

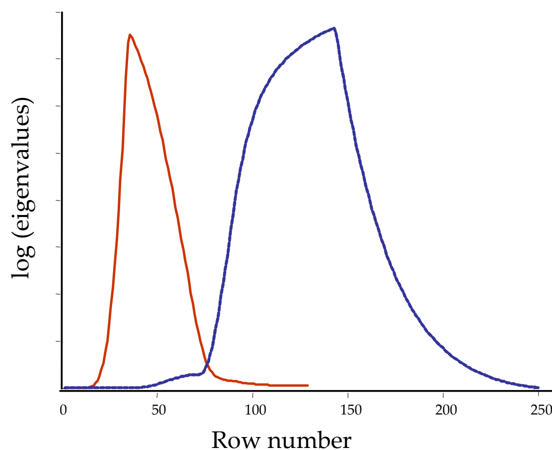


Figure 7.- Reconstructed concentration profiles from the combination of forward and backward analyses.

This graph shows the result of a data matrix with two significant principal components. We may conclude from this graph that, from row 20 to row approximately 50, there is just one significant principal component, that from row 100 to the end there is a just one principal component, and that in the intermediate range there are two principal components. This graph will be used like an initial estimation of concentration profile.

2.3.3. Simple-to-use interactive self-modelling mixture analysis (SIMPLISMA)

Unlike the previous methods, SIMPLISMA [114, 115] is not based on principal components analysis. Instead, it looks for "pure variables". This method is based on evaluating the relative standard deviation of the column n, p_n, defined from equation 2.

$$p_n = \frac{s_n}{\overline{x}_n + \delta} \tag{2}$$

where s_n is the standard deviation of the column n, $\overline{x}$ is the average of the column n and δ is a correction factor that is added in order to avoid columns with a low average value (generally associated with noise) being the purest variables. A large relative standard deviation (p_n) indicates a high purity of the column.

The process involves, in a first step, finding the column with the highest value of relative standard deviation and then normalising this column. The variable with the second highest purity, as well as having a high relative standard deviation, must have the least correlation with the first pure variable found. A weight factor, w_n, is therefore calculated as follows:

$$w_n = \det(\mathbf{Y_n}^T \mathbf{Y_n}) \tag{3}$$

where $\mathbf{Y}$ is a matrix made up of the pure variables found and each nth column of the data matrix that has not yet been selected. The value calculated by the determinant will be proportional to the independence between the pure variables found and the nth row, which has been used to build the matrix $\mathbf{Y_n}$. In this way, this determinant will have a high value when the variables are not correlated and a value close to zero when they are.

To calculate the new pure variable p_i, therefore, the weight factor will be applied. This changes equation 2 slightly,

$$p_i = w_n \left(\frac{s_n}{\bar{x}_n + \delta} \right) \quad (4)$$

The algorithm selects the maximum value of p_i, which corresponds to the variable of greatest purity, and so on until all the pure variables are found.

With the purest variables, the columns (purest spectra) are obtained. These will be used as the initial estimation. This technique is very useful when spectra overlap or shift [116, 117].

2.3.4. Alternating least squares (ALS)

The aim of alternating least squares [118] (ALS) is, from an initial estimate using EFA or SIMPLISMA, to obtain a result with chemical significance that corresponds satisfactorily to the experimental behaviour observed. ALS allows several constraints to be imposed [119], based on our knowledge of the inner structure of the data and the chemical characteristics of the experimental measurements, to improve the final result. This method imposes a linear model on experimental data and, when working with spectroscopic responses, it satisfies Lambert-Beer's law.

The alternating least squares method consists of two steps which are repeated iteratively:

a) From the estimation of concentration profiles, the spectra profiles are obtained from the least squares resolution according to equation 5 below:

$$\mathbf{S}^{T} = \mathbf{C}^{+} \mathbf{R}^{*} \quad (5)$$

where $\mathbf{C}^{+}$ is the pseudoinverse matrix ($\mathbf{C}^{+}=(\mathbf{C}^{T}\cdot\mathbf{C})^{-1}\cdot\mathbf{C}$) of $\mathbf{C}$ obtained from the initial estimation of EFA and $\mathbf{R}^{*}$ is the data matrix reconstructed from the number of

significant principal components selected in the principal component analysis with noise removed. $\mathbf{S}^T$ is the matrix of pure spectra calculated by least squares.

b) A new estimate of the concentration profile is then obtained, from the spectra profile of the previous step, by least squares from equation 6.

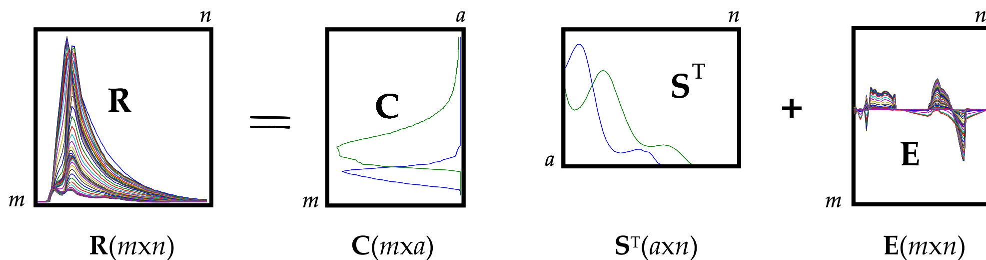
$$\mathbf{C} = \mathbf{R}^* (\mathbf{S}^T)^+ \quad (6)$$

where $(\mathbf{S}^T)^+$ is now the pseudoinverse matrix of $\mathbf{S}^T$.

Steps *a)* and *b)* are repeated until the difference between the residual of one iteration and the next is less than a prefixed value of the order of 0.1 (this number can be changed).

With this method, we use an initial estimate of the concentration profile obtained by evolving factor analysis. If, on the other hand, we use an initial estimate of the spectra profile obtained by SIMPLISMA, the process is reversed i.e. the pure variable is a row, we begin with equation 6 instead of with equation 5, and in step *b)* we use equation 5.

The final result of the process is schematized as follows:



where **R** is the raw matrix, **C** is the matrix of the resolved concentration profiles, $\mathbf{S}^T$ is the matrix of the resolved pure spectra of all the species, **E** is the residual matrix with

the data unexplained by $\mathbf{CS^T}$ and a is the number of chemical species that we considered significant and that contribute to the signal.

2.3.4.1. Augmented matrices analysis

An augmented data matrix is an arrangement of various matrices obtained from different experiments. Several types of augmented data matrices can be used: a column-wise augmented data matrix (a), a row-wise augmented data matrix (b), or a column- and row-wise augmented data matrix (c) (see Figure 8). The most common type of augmented matrix is the column-wise augmented data matrix (a).

a) R_1 R_2 = C_1 C_2 * S + E_1 E_2

b) R_1 R_2 = C * S_1 S_2 + E_1 E_2

c) R_1 R_2 R_3 R_4 = C_1 C_2 * S_1 S_2 + E_1 E_2 E_3 E_4

Figure 8.- Types and information obtained from augmented matrices for MCR-ALS analysis.

When there is no selectivity, the augmented matrix analysis can provide better results than the analysis of individual matrices because the individual experimental response of the common components is obliged to be the same for all data matrices. In this way, the number of possible solutions with augmented matrix analysis is considerably lower than with individual matrix analysis.

A problem when using MCR-ALS is rank deficiency. Rank deficiency occurs when the number of significant principal components is less than the number of chemical species present. The most usual case is when various chemical species have common profiles in one of the two orders of measurement. It is not possible to resolve the profiles of each component if we want to analyse only one data matrix.

2.3.4.2. Constraints

One advantage of using methods with multivariate curve resolution with alternating least squares is that they do not need any prior physical or chemical model postulation as in the case of hard-modelling. However, they have an intrinsic disadvantage in that there are multiple solutions to this equation ($\mathbf{R}=\mathbf{C}\cdot\mathbf{S}^{T}$). When there is no selectivity for any of the species, we cannot totally guarantee that the calculated solutions are the true ones because they could contain what is known as rotational ambiguity [120, 121]. It is known that rotational ambiguity does not exist for individual responses located in an area in which no other component presents a response or for concentration profiles in areas where only one component is present.

Herein lies the importance of the constraints in this kind of resolution. In order to restrict the possible number of solutions for $\mathbf{C}$ and $\boldsymbol{S}^{T}$ so that the solutions have chemical significance.

The constraints [122, 123] used are:

a) *Non-negativity.* The concentrations of the chemical species and/or the values of absorbance of the spectra must have positive values or be equal to zero. Although this constraint is very general, there are some spectroscopic data for which the spectra can be positive or negative (circular dichroism spectroscopy).

b) *Unimodality.* This constraint can only be used when there is one maximum peak per response, which is very common in concentration profiles (e.g. in chromatography systems or FIA peaks) but not in spectra profiles.

c) *Closure.* This constraint is used for systems for which the sum of the concentrations of all the species involved in the reaction or the sum of some of them is forced to be constant at each stage of the reaction. Closure may be a mass balance constraint.

d) *Selectivity.* This constraint is used when there are areas in which there is total selectivity for each chemical species. It is the same as restricting the concentrations or spectra signals of the other components to zero.

e) Constraint related to the *correspondence of species* (components). These are used when performing augmented matrix analysis of several data matrices. This constraint identifies the same species in matrices from different experiments.

f) *Trilinearity* (three-way structure). This involves imposing a unique response profile for the common components in all three measurement orders. In our case these orders were time, wavelength and concentration. Trilinearity involves decomposing a data cube into the product of three matrices so that for each sample (matrix) the only variations are the concentrations of its components and where two of the matrices, such as those of the concentration profiles and spectra profiles, are fixed.

2.3.5. Evaluation of the process quality

One of the critical steps in the resolution process is to choose the correct number of principal components. One way to determine whether the estimate of principal components has been corrected is to study the lack of fit (lof), expressed in equation 7, of the model with respect to the original:

$$lof = \sqrt{\frac{\sum_{i,j}\left(d_{ij} - \hat{d}_{ij}\right)^2}{\sum_{i,j} d_{ij}^2}} \qquad (7)$$

where d_{ij} may be each of the elements of the raw data matrix or each of the elements of the matrix reconstructed from the principal components selected in the principal components analysis (PCA), and $\hat{d}_{ij}$ are the corresponding values calculated after the optimisation process (ALS). Therefore, depending on the matrix used, either lof_{exp} or lof_{pca} is obtained.

If the value of lack of fit is close to zero, this indicates a good fit of the model and, therefore, a good estimate of the number of principal components. A high value of lack of fit means that the model is not well fit.

Another way to check whether the model is well fit is to observe the structure of the residuals matrix (**E**). If this matrix has a random data structure, we consider that the model explains the experimental data. If it does not, it may contain an important component that has not yet been considered.

When pure analyte is available, another way to check whether the model is well fit is to determine the correlation between the spectra obtained in the resolution process and the spectra of the pure species, as well as the quantification error measured by the relationships between the areas of the concentration profiles obtained from analyses of the pure analyte and the sample.

2.3.6. Bibliographic review

We have reviewed the literature on studies that have used MCR-ALS techniques and shown how they can be useful for resolving a wide range of analytical problems.

These techniques have been used to analyse transition conformations in nucleic acid [124], its thermodynamic [125] and its synthesis [126]. They have also been used to study the union of these polynucleotides to Cu [127], Cd [128], Pb [129], Zn

[130], Pt [131] and the union of metals to RNA (ribonucleic acid) [132]. They have also been used in biological systems [133].

Another field of application is chromatography. In the field of environmental pollution they have been used to determine pesticides [134, 135] and fulvic acid [136] and to analyse water [137, 138].

For flow systems, such as flow injection analysis (FIA) and sequential injection analysis (SIA), in which a pH gradient is generated [139], they can be used to determine the pK_a of the analytes [140]. They can also be used when they had an acid-base reaction [141, 142].

Our group has been working on second-order data applied to chromatographic data [143, 144], NIR data [145, 146] and sequential injection data [147].

2.4. EXPERIMENTAL DESIGN

Experimental design, also called design of experiments (DOE), involves planning experiments systematically in order to extract the maximum efficient information with the least number of experiments possible using statistical tools [148, 149]. Experimental design helps the experimenter to select an optimal experimental strategy to achieve the proposed objectives. This includes:

- The determination of the influence of the factors which, *a priori*, have been selected as significant in the chemistry process using screening design.

- Optimisation of a response by mean of modelling the response in an experimental domain to obtain a response surface or using sequential technique such as simplex.

The conclusions obtained after applying an experimental design can only be generalised in the domain of interest. These conclusions usually refer to a single response. However, when there are several responses of interest, it is better to transform the responses into a single response using the overall desirability function.

2.4.1. Screening designs

2.4.1.1. Full factorial design

A full factorial design is an experimental set-up consisting of all the possible combinations between the different factors and their levels. The set of all experiments with codified factor values is the design matrix. In general, a design in which k factors are studied at m levels contains m^k different experiments. Of all these experimental design, full factorial design at two levels is the one that is most used.

In a full factorial design, not only the effects of factors A, B and C (main factors) on the response can be estimated, but also the occurrence of the interactions of the factors. A two-factor interaction occurs when the effect of the first factor (A) on the response is different at both levels of the second factor (B), or vice versa. A three-factor means that a two-factor interaction effect is different at the two levels of the third factor. And so on for higher order interactions.

The number of p-factor interactions (p=2,3,....) in a 2^k design is calculated from equation 8:

$$\frac{k!}{p!\,(k-p)!} \qquad (8)$$

When for example, we have 3 factors (k=3), the number of experiments is 8 and 8 statistics can be calculated. These are the estimates of three main effects, three two-factor interactions and one three-factor interaction. An eighth statistic, the mean

response, can also be calculated. The columns of the design matrix are orthogonal, which means that they allow uncorrelated estimates of the effects of the different factors and interactions.

A disadvantage of full factorial designs is that the number of experiments to perform increases rapidly as the number of factors examined increases. For 7 factors, for example, $2^7 = 128$ experiments are necessary; for 8 factors, $2^8 = 256$.

2.4.1.2. Fractional factorial design

If the number of factors is high, and the full experimental cost associated with a full factorial design cannot be assumed, an alternative is to perform only a fraction of the experiences of a full factorial designs. This fraction should be selected so that the experimental space is mapped as well as possible and orthogonality (all factor effects uncorrelated) is preserved.

The practical set-up of a fractional factorial design 2^{k-q} is *via* a full factorial design for *k-q* factors, where *k* is the number of factors and *q* is the number that indicates the reduction (a half, a quarter, etc.). For example, for a 2^{4-1} fractional factorial design, a full factorial design for 4-1=3 factors (A, B and C) is first constructed. The fourth factor, e.g. factor D, is assigned to one of the interactions (i.e. ABC). The relationship D = ABC is called the generator. By multiplying both parts of the generator with each other, a defining relation or defining contrast (I) is produced:

I = D x ABC = ABCD

The alias of any factor or interaction is then obtained by multiplying it with the defining relation. An additional rule is that when a term appears an even number of times in the product, it disappears. The generator can determine how the factors were confounded. In the previous example, some of the confounded effects are shown:

A x ABCD = BCD

B x ABCD = ACD

……

AB x ABCD = CD

where factor A is confounded with interaction BCD, factor B is confounded with interaction ACD, factor AB is confounded with interaction CD, etc.

The main effects tend to be more important than two-factor interactions, which in turn tend to be more important than three-factor interactions and so on. However, one should be careful with this assumption, as two-factor or even three-factor interactions can be larger than non-significant main effects.

2.4.1.3. Plackett-Burman design

The Plackett-Burman design is a saturated design, where the number of experiments is very similar to the number of factors. This type of design consists of *(r x 4)* experiments (r = 1, 2, 3, ..) and is suitable for studying up to *(r x 4)* -1 factors.

The design matrix is obtained from a given first line describing the first experiment, which will define the low and high values for each factor. For example, when we have 8 factors, the first line will be:

+ + + - + - -

The next experiences are obtained by a cyclical permutation of this line: the sign for the last factor of the first row becomes the sign for the first factor of the second row. For the others factors, all the signs are shifted one place to the right. The last experiment will only contain minus signs i.e. low factor values.

If the number of factors to be examined is less than *(r x 4)* -1, dummy factors are attributed to the remaining columns. These are imaginary factors from which the change from one level to another has no physical meaning.

With this design, interactions are assumed to be smaller than the main effect. This may not always be true in optimisation procedures when a non-significant main effect is confounding an important interaction term.

2.4.1.4. Study of the factor influences

The influence of the factors A on the response in an experimental design can be calculated as:

$$b_A = \frac{\Sigma\, Y(+)}{n} - \frac{\Sigma\, Y(-)}{n} \quad (9)$$

where ΣY(+) and ΣY(-) are the sums of the responses where factor A is at its high (+1) and low (-1) level, respectively, and n is the number of times each factor is at the (+1) or (-1) level. Equation 9 can be applied for each factor or interaction.

There are several statistical tools for studying the significance of the estimated effects when working on screening designs [150]. These statistical tools can be classified by: (i) visual interpretation or (ii) ANOVA.

(i) A *Pareto chart* displays a frequency histogram where the length of each bar on the chart is proportional to the absolute value of its associated estimated effect or the standardized effect.

(ii) One of the most important statistical techniques for deciding which factors and which interactions are significant is ANOVA, which shows the results of partitioning the variability in the selected response into separate items for each of the effects. We then test the statistical significance of each effect by comparing the mean square with an estimate of the experimental error.

2.4.2. Optimisation of a response

2.4.2.1. Response surface methodology

Response surfaces are used to build a mathematical model for the experimental response as a function of the factors values. The type of experimental design could be related to the type of function we wish to model (linear, quadratic, etc.). Normally, the type of function that related the response to the factors is unknown, but we know it can be modelled by a polynomial function.

Typical polynomial functions are given below for two factors, x_1 and x_2, and the single response y:

$$y = b_o + b_1x_1 + b_2x_2 \quad \text{(a)}$$

$$y = b_o + b_1x_1 + b_2x_2 + b_{12}x_1x_2 \quad \text{(b)}$$

$$y = b_o + b_1x_1 + b_2x_2 + b_{12}x_1x_2 + b_{11}x_1^2 + b_{22}x_2^2 \quad \text{(c)}$$

y = polynomial function of higher order (d)

where b_o, b_1, b_2, b_{12}, b_{11} and b_{22} are the coefficients of the model. Such a model contains a constant term b_0, which estimates the response when the values of all the factors are set at zero (coded factors), linear coefficients (b_1 and b_2), which describe the sensitivity of the response to the variations in the corresponding factors (equivalent to the main effects discussed earlier), cross-product coefficients (b_{12}), which are measures of the interactions between factors, and quadratic terms (b_{11} and b_{22}), which describe curvature.

When we do not know which model is valid, the experimental procedure is to: (i) begin with the simplest model and obtain its coefficients; (ii) check the validity of the model and, if it is valid, end the process; (iii) if the model is not valid, conduct more experiments to obtain the coefficients of a higher-degree polynomial and then repeat step (ii) and (iii).

If the postulated model is type (a) or type (b), a full factorial design is used. If it is type (c), one possibility is to make a central composite design.

This design contains three parts: a full factorial design 2^k, where k is the number of factors, a number of centre experiments and a number of star experiments with a pre-determined axial distance. The centre points are often replicated, which gives an immediate idea of experimental precision. Figure 8 shows the geometric figure to represent this design for 2 factors.

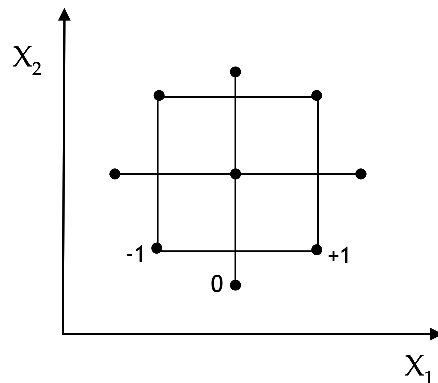


Figure 8.- Representation of a central composite design for 2 factors.

Usually only a limited number of factors are examined (two or three) since the number of experiments increases rapidly as the number of factors increases. Only continuous factors can be studied.

This response surface allows us to choose the best experimental conditions, either the optimum response or the best relationship between the response and values of the factors more suitable for experimenter.

2.4.2.2. Simplex approach

Simplex is a sequential method in which we first conduct a restricted number of experiments. Depending on the results of these experiments, the conditions for the next experiments are determined in the direction of the best response.

A simplex is a geometrical figure defined by a number of points equal to one more than the number of the factors considered in the optimisation. This means that for the optimisation of two factors, the simplex has three vertices and is therefore a triangle. Each vertex reflects a set of experimental conditions.

To build the first simplex, we should define a starting value for each factor and a step size. The step size will determine the dimensions of simplex. Table 2 shows the rules for generating the starting simplex.

Table 2.- Construction of the initial simplex.

Vertex	Factor x_1	Factor x_2	Factor x_3		Factor K
1	c_1	c_2	c_3	·	c_k
2	$c_1 + p_1$	$c_2 + q_2$	$c_3 + q_3$	·	$c_k + q_k$
3	$c_1 + q_1$	$c_2 + p_2$	$c_3 + q_3$	·	$c_k + q_k$
4	$c_1 + q_1$	$c_2 + q_2$	$c_3 + p_3$	·	$c_k + q_k$
.	.	.	.	.	.
.	.	.	.	.	.
k + 1	$c_1 + q_1$	$c_2 + q_2$	$c_3 + q_3$	·	$c_k + p_k$

where c_i correspond to the starting coordinates for factor *i* and s_i correspond to the step size for factor *i.* To calculate the values of the factors for the rest of the experiments, we used the following equations.

$$p_i = s_i \cdot \left(\frac{\sqrt{k+1} + k - 1}{k\sqrt{2}} \right) \tag{10}$$

$$q_i = s_i \cdot \left(\frac{\sqrt{k+1} - 1}{k\sqrt{2}} \right) \tag{11}$$

where *k* is the number of factors. Once the results of the experiments with the initial simplex are obtained, some rules are applied to establish the next experiment [151]. The search is terminated when reflections provide no further improvement, when a satisfactory result is obtained or when the responses of the vertices of the last simplex are similar.

The number of experiments for obtaining the optimum depends on:

- how close the coordinates of first simplex are to the optimum
- the step size, because if the step size is too small, a large number of experiments will have to be executed before the optimum is reached. On the other hand, when the step size is too large, the optimum can be exceeded in one movement. In this case, we could start a new simplex with a smaller step size around this optimum. Another way to avoid this problem is to use the modified simplex [152, 153].

Optimisation methods such as simplex are easy and can obtain good responses. However, they also have a number of disadvantages. For example, simplex can only be used to optimise continuous factors and not for discrete ones. Also, the overall optimum may be found but there may be several local optima: simplex will find one of these but we do not know if this is the best optimum.

2.4.3. Multi-response optimisation: desirability function

It is common today to determine the effects of the experimental factors in only one response. However, there is a need to make joint analyses of multiple responses

due to the ever-increasing capacity to measure more parameters in any process or product.

When several responses are evaluated by experimental design, it is unlikely that the coordinates of the optimum points obtained individually will always coincide. In this situation it is necessary to look for a compromise so that all the experimental responses will satisfy the specifications or restrictions imposed by the researcher in order to meet his or her objectives. The oldest procedure is to resolve the problem by superimposing the individual results. Obviously, this method is limited by the number of factors and responses. When there are multiple responses to evaluate, an overall desirability function is suitable [154].

The form of the desirability function was originally proposed by Harrington (1965) [155]. The basic idea of the desirability function approach is to transform a multiresponse problem into a single response problem by means of mathematical transformations. The procedure calls for introducing for each response Y_j = 1,2,...,m, a individual desirability function $d_j(Y_j)$ with a range of values between 0.0 and 1.0. Once this function is defined for each of the m responses of interest, an overall objective function (the total desirability) is defined as the geometric mean of the individual desirabilities:

$$D = \sqrt[n]{d_1^{p1} \; x \; d_2^{p2} \; x \cdots\cdots d_n^{pn}} \qquad (12)$$

where p_n is the weight of the responses, n is the number of responses and d is the individual desirability function of each experiment.

2.4.4. Bibliographic review

We have reviewed some of the literature on experimental design. Today more than 20,000 papers refer to the use of experimental design for various applications. Simplex has been applied to flow injection analysis (FIA), for example, for optimising

the concentration for the determination of salbutamol [156], copper in water [157], dodecylsulfate anion [158], heavy metal ions [159], sulfadiazine and sulfamethoxazole [160] and tartaric acid in wines [161].

Simplex also has been applied to sequential injection analysis (SIA) to determine salbutamol [162], antibacterial drug trimethoprim [163], sulphonamides [164], bromazepam anxiolytic drug [165]. Many recent publications refer to the desirability function [166-171], one of whose applications is to FIA [172] systems.

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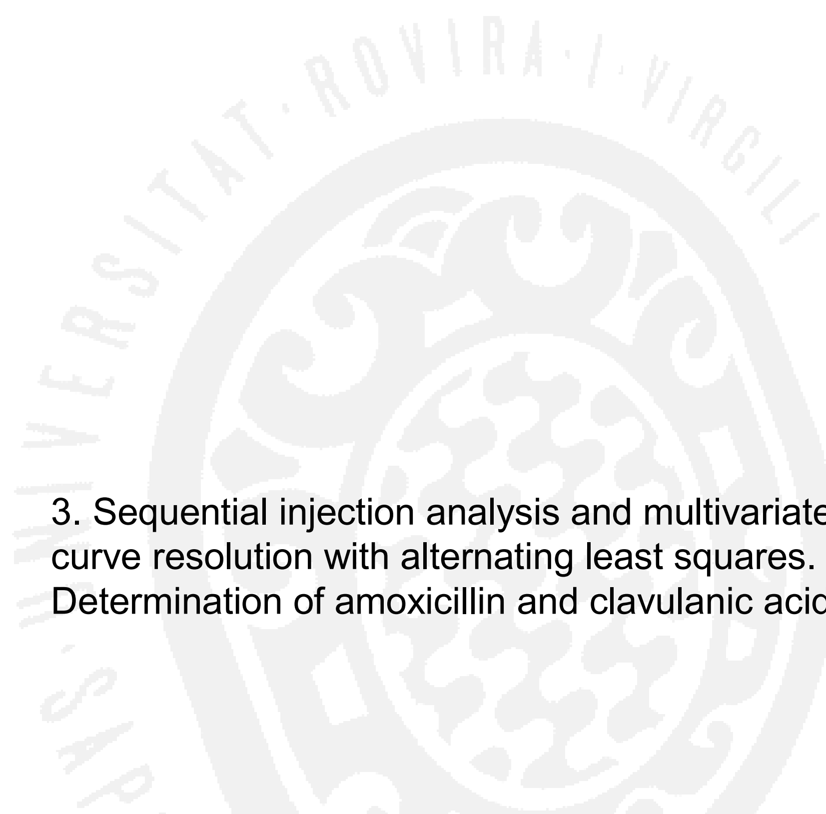

3. Sequential injection analysis and multivariate curve resolution with alternating least squares. Determination of amoxicillin and clavulanic acid

3.1. INTRODUCTION

This chapter includes, in four papers, the experimentation and methodology for determining amoxicillin and clavulanic acid in pharmaceuticals using sequential injection analysis (SIA) and multivariate curve resolution with alternating least squares (MCR-ALS). We also studied the different parameters related to the analytical sequence and the chemometrics tools applied. We have used the conclusions drawn from these papers to expand the objectives proposed and establish different analytical methods in function of the type of sample and the number of species we wish to determine in just one analysis.

In the first paper, entitled *"Determination of amoxicillin in pharmaceuticals using sequential injection analysis (SIA). Evaluation of the presence of interferents using multivariate curve resolution (MCR)"*, the pharmaceuticals were classified according to their selective zones. In a first step, the experimental work was conducted to find an analytical sequence that enabled the acid and basic species of amoxicillin to be obtained sequentially. The factors taken into account were: the volume of sodium hydroxide, flow, the concentration of sodium hydroxide and the volume of amoxicillin. We studied different conditions related to the MCR-ALS process. In this study, we established an analytical sequence to obtain a good resolution of the amoxicillin contained in the pharmaceuticals (Augmentine, Flubiotic, Eupen, Ardine, Mundogen and Clamoxyl).

If there were selective zones, i.e. zones in which either no appreciable response appears in the concentration profile or the spectra corresponding to the interferent do not give a signal in any wavelength maximum of the analyte spectra, we performed univariate calibration. The determination of amoxicillin was carried out in those pharmaceuticals that contained no interferents (Mundogen, Eupen and Ardine). This method is useful for observing the presence or otherwise of interferents.

In the second paper, entitled *"Determination of amoxicillin in pharmaceuticals using sequential injection analysis and multivariate curve resolution"*, the quantity of

amoxicillin in the pharmaceuticals with interferents or without selective zones was determined. This method involved studying second-order calibration. Because they can affect the results, in an earlier step in the calibration process we studied the following parameters associated with the optimisation process: (i) the effects of imposing trilinearity at the resolution stage; (i) how to choose the species that will be used for quantification (acid, basic or the sum of the two); (ii) what the most suitable concentration of the reference standard is. Once these parameters were established, the amoxicillin was determined.

In the third paper, *"Sequential injection analysis (SIA) for the simultaneous determination of clavulanic acid and amoxicillin in pharmaceuticals using second-order calibration"*, we propose the simultaneous determination of amoxicillin and clavulanic acid. Due to the similarity of the acid-base characteristics and spectra of these compounds, the existence of rank-deficiency was favoured and the determination of the two substances with only one reagent (sodium hydroxide) was impossible. To solve these problems, we worked with augmented matrices. So that we could view the species, we added a new reagent to extend the range of pH.

In the fourth paper, entitled *"Sequential injection analysis linked to multivariate curve resolution with alternating least squares"*, we describe the state of the art of sequential injection analysis (SIA) and multivariate curve resolution with alternating least squares (MCR-ALS) by reviewing the bibliography since 2004. We discuss the potential of SIA for generating second-order data and the necessary conditions for applying chemometric tools to treat this type of data.

3.2. Paper

"Determination of amoxicillin in pharmaceuticals using sequential injection analysis (SIA). Evaluation of the presence of interferents using multivariate curve resolution (MCR)."
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Analytica Chimica Acta 485 (2003) 195-204.

Determination of amoxicillin in pharmaceuticals using sequential injection analysis (SIA). Evaluation of the presence of interferents using multivariate curve resolution (MCR).

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Abstract

In this study, we report a method for determining amoxicillin in pharmaceuticals using sequential injection analysis (SIA) with a diode-array spectrophotometric detector. Before determining the amoxicillin, we use multivariate curve resolution with alternating least squares (MCR-ALS) to investigate whether any interferents are present. With a suitable analytical sequence, we can use SIA to generate a pH gradient and, for each sample, obtain a matrix of data that have been analysed by several chemometric techniques based on multivariate analysis: principal components analysis (PCA), simple-to-use interactive self-modelling mixture analysis (SIMPLISMA) and MCR-ALS. In this way we obtain the concentration profiles and spectra of the species in the sample.

We studied six pharmaceuticals containing amoxicillin. Two of these pharmaceuticals contained no interferents, one contained an interferent but amoxicillin had a selective spectral area with respect to it, and the other three contained interferents. For the first three samples, we set up a system of univariate calibration, which determines amoxicillin quickly (it can analyse 20 samples per hour) using inexpensive instrumentation and reagents.

Keywords: SIA, MCR, Amoxicillin.

1. Introduction

In this study, we propose a system of sequential injection analysis (SIA) with a diode-array spectrophotometric detector to determine amoxicillin in pharmaceuticals. The advantages of flow systems are a high frequency of analysis and a low consumption of reagents.

Amoxicillin, whose structure is shown in Figure 1, is a wide-spectrum bactericide that is present in many medicines in general use. It is a white or almost white crystalline powder. It is slightly soluble in both water and alcohol, and may be present in various acid forms depending on the pH. Its first two pK_a are 2.4 and 7.4 [1].

Figure 1.- Chemical structure of amoxicillin.

Amoxicillin is determined in two fields: living organisms (humans and animals) [2-5] and pharmaceuticals [6-9]. For the first field, all methods include a preconcentration step and HPLC, which allows the quantification of amoxicillin in the presence of other components. In the second field, the available methods are more varied and do not include a preconcentration step, since the concentration of amoxicillin in pharmaceuticals is higher. The most common technique is spectrophotometry with univariate calibrations. For this reason other authors have studied the level of concentration of the substances that are permitted to accompany amoxicillin in several pharmaceutical preparations so that interference does not occur [10], or, if it does, so that a reaction is provoked to produce selective areas [11].

First we explored the possible interferents of amoxicillin. Then, depending on the result of this exploration, we proposed a method for determing it. Multivariate

curve resolution methods are often used to resolve the components in unknown mixtures [12]. They can be used when second order data are obtained, i.e. one matrix for each sample. SIA, introduced by Ruzicka in 1990 [13], with a diode-array spectrophotometric detector is an easy way to generate second order data [14, 15].

With SIA techniques, carrier, reagents and sample are stacked sequentially into the coil using a selector valve. Amoxicillin is a polyprotic species, so if the reagent introduced is NaOH, a pH gradient is generated in the interdiffusion process that carries the reagents through the channel to the detector. In this way, when the sample reaches the detector, all the spectra are recorded at regular intervals during the SIA peak evolution, so spectra at different pH [15] are recorded. Figures 2a and 2b illustrate this process. At time zero (Figure 2a), we assume that the sample, reagents and carrier have not mixed. After a certain time (Figure 2b), when the sample reaches the detector, interdiffusion of the carrier with NaOH and of NaOH with amoxicillin takes place, in such a way that the most acid species appears first, followed by the mixture of this species with its conjugate base. And so on if there are more species with differentiated spectra. Interferents with acid-base characteristics would experience the same process. Interferents that do not have these characteristics would have just one profile of concentrations.

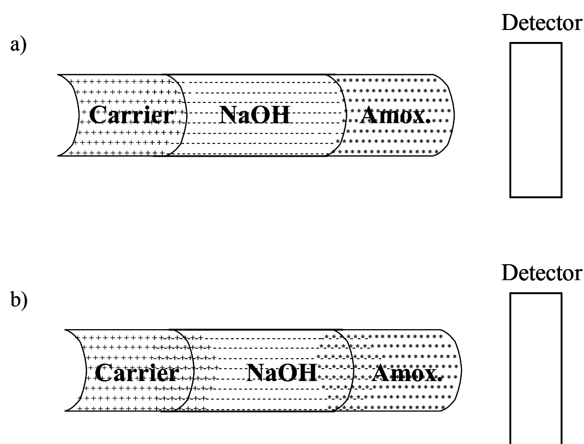

Figure 2.- Scheme of the process for mixing sample, reagent and carrier in the channel of an SIA system. A) Time zero: interdiffusion has not taken place B) Time t: some interdiffusion has taken place.

When multivariate curve resolution (MCR) is applied to ordered data matrices, a profile in each order (mode) of the data matrix is recovered for each component in the mixture. In this study, we recovered different profiles of concentration of the species over time and the spectra of each species. With these profiles of concentration and spectra, we determined whether there were any selective areas, i.e. areas where there was no appreciable response in the profile of concentrations, or where the interferent does not absorb in an area where the analyte does. In such cases the concentration of amoxicillin can be determined using a simple univariate calibration.

2. Experimental

2.1. Reagents and solutions

We prepared amoxicillin and sodium hydroxide stock standard solutions by weighing the required amount of the respective compounds (amoxicillin from Sigma and sodium hydroxide from Prolabo) and dissolved them in purified water (from a Milli-Q water system from Millipore).

Pharmaceuticals: Clamoxyl (500 mg of amoxicillin per packet) and Augmentine (500 mg of amoxicillin per packet) from SmithKline Beecham, S.A.; Eupen (1g of amoxicillin per packet) from J. Uriach, S.A; Ardine (1g of amoxicillin per packet) from Antibioticos Farma, S.A; Flubiotic (500 mg of amoxicillin per packet) from Pharmazam; Amoxicilina Mundogen (500 mg of amoxicillin per packet) from Mundogen Farma, S.A.

2.2. SIA manifold

The sequential injection analyser comprised: CAVRO XL 3000 stepper motor-driven syringe pump connected to the PC with an RS-232 interface; A 6-position Eurosas EPS 1306 BPB automatic valve connected to the computer through a PCL-711S PC-Lab-Card; Omnifit PTFE tubing reaction coil: 70cm x 0.8mm; Holding coil: 200cm x 0.8mm; An

HP8452A diode-array spectrophotometer controlled by an HP Vectra 5/75 computer equipped with an HP-IB IEEE 488 interface for communications; a Hellma 178.711QS flow-through cell. A scheme of the analyser is shown in Figure 3.

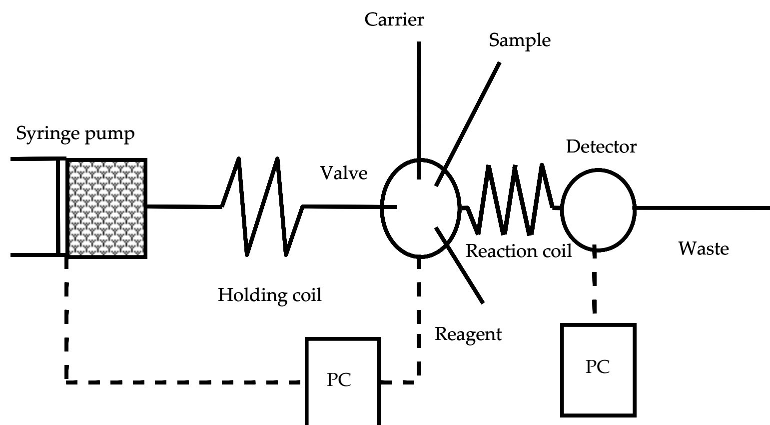

Figure 3.- Scheme of the sequential injection analyzer

2.3. Software

HP89531A software was used to record and store the spectra. Customized software was used to control the SIA. All calculations relating to multivariate curve resolution with alternating least squares (MCR-ALS) were performed with laboratory-written software under a MATLAB 5.3 computer environment [16]. This software is available from the authors [17].

3. Theory

3.1. MCR: Data treatment

For each sample analysed one matrix **R***(mxn)* made up of *m* rows and *n* columns is obtained. Each row *m* is made up of the spectrum at time *m*. Each column *n* is the absorbance at wavelength *n* for all times recorded. The **R** is mathematically decomposed into the product of two factor matrices:

$$\mathbf{R} = \mathbf{C}\mathbf{S}^{T}+\mathbf{E} \tag{1}$$

where matrix **C***(mxa)* is the concentration matrix describing the evolution of the *a* chemical components during the experiment; matrix $\mathbf{S}^{T}$*(axn)* is the spectroscopic matrix describing the pure individual spectra of these components; and **E**(*mxn*) is the error or residual data matrix that provides the data variation not explained by the proposed *a* contributions.

Equation 1 assumes that **R** is bilinear, i.e. that the spectroscopic signal can be decomposed into the sum of individual contributions, each described by a concentration profile in matrix **C** and by a pure spectra in matrix $\mathbf{S}^{T}$. The number of components or contributions, *a*, to be considered in the mathematical decomposition of equation 1 can be initially estimated by singular value decomposition (SVD) analysis [18].

Equation 1 is solved iteratively using an alternating least squares (ALS) procedure based on the two following matrix equations:

$$\mathbf{C} = \mathbf{R}\,(\mathbf{S}^{T})^{+} \tag{2}$$

and

$$\mathbf{S}^{T} = \mathbf{C}^{+}\mathbf{R} \tag{3}$$

where $(\mathbf{S}^{T})^{+}$ and $\mathbf{C}^{+}$ are the pseudo-inverse matrices of $\mathbf{S}^{T}$ and **C** [19]. Initial estimates, which are needed to start the ALS procedure described by these two equations, can be obtained by algorithms such as SIMPLISMA, which is described in detail elsewhere [20-25]. SIMPLISMA searches for the "pure variables", which are actually wave numbers where only one component in the system gives a spectral response or, more precisely, where the selectivity (defined as the intensity divided by the concentration) of a given component is maximised.

Once we have determined the pure variables that represent all the species in the system, we can use them to calculate the corresponding pure species spectra because the intensity changes in the pure variables are proportional to the concentration changes and, if they are aligned in separate columns, they form matrix **C**.

The resolution can be improved by treating it with what are known as augmented matrices [15]. These matrices result from adding the matrix generated by the sample to other matrices that contain some information about some of the species contained in the sample.

We can also improve the resolution by applying several constraints during optimisation. These depend on the nature and structure of the data. Some of the possible constraints are:

- *Non-negativity*. The elements in one or both profiles must be positive.
- *Unimodality*. Only one maximum per profile is allowed.

We can also introduce a small binary matrix containing the information about the correspondence of species (components) among the submatrices in the data set. This is applicable when working with augmented matrices.

When the optimisation process ends, the performance of the model can be evaluated from lack of fit (lof) data as:

$$lof = \sqrt{\frac{\sum_{i,j}\left(d_{ij} - \hat{d}_{ij}\right)^2}{\sum_{i,j} d_{ij}^2}} \qquad (4)$$

where d_{ij} are the experimental values and $\hat{d}_{ij}$ are the corresponding values calculated by PCA or ALS.

4. Results and Discussion

Figure 4 shows the spectra, recorded at between 210 and 410 nm, of a solution of 50 mg/l of amoxicillin in water (which produces a pH of approximately 4.6), in NaOH 0.1 M and in HCl 0.1 M. The spectra for amoxicillin in water and acid are the same, but they are different from the spectra in NaOH. From the values for the pK_a of amoxicillin, at pH below 2.4 there should predominantly be one specie, at pH between 2.4 and 7.4 there should be a second species, and at pH above 7.4 there should be a third species. Since the spectra at pH=1 and 4.6 are the same, we can deduce that the first two species have the same spectrum, and that the third species, which we call basic, appears when the pH reaches 7.4. Therefore, in the 1-13 pH range there are at least two absorbent species of amoxicillin. Absorbance was linear with concentrations of up to 800 mg/l, both in the acid zone and in the base zone. We selected a concentration of 50 mg/l as the working concentration for later studies. The water solution of amoxicillin remained stable for at least 21 days.

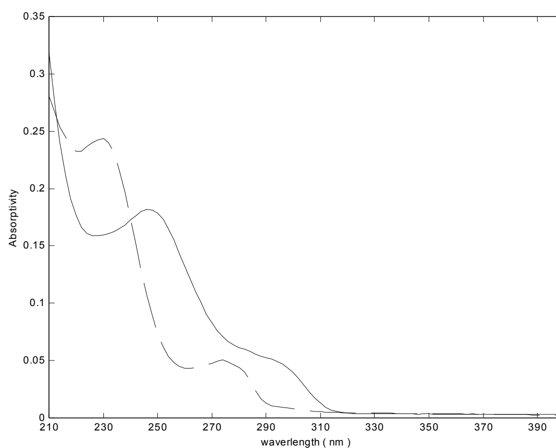

Figure 4.- Spectra of a solution of 50 mg/l of amoxicillin at pH = 1, pH = 4,6 (---------) and pH = 13 (— — — —).

The aim of this study is to obtain an evolving system. We will do this by working from the pH of the aqueous solution of the reactant up to basic pHs. To establish an analytical sequence so that the pH would change gradually in the channel containing the amoxicillin, we chose the order of aspiration of the reagents in Figure 2.

Table 1 shows the operational variables and their working intervals. The flow directly influences the time spent in the channel and, therefore, the interdiffusion of NaOH and amoxicillin. The volume of amoxicillin affects the number of rows in the response matrix. The concentration of NaOH affects the size of the change in pH. Finally, we studied the volume of NaOH. Although this was the least relevant variable, we wanted to make sure there was enough of it to ensure that the change in pH was always upwards. We chose the range of values in accordance with operational restrictions (the length of the tubes, the volume of the syringe, etc.) and previous knowledge.

Table 1.- Operational parameters for selecting the analytical sequence.

Flow	0,5 / 2 ml/min
Volume of NaOH	8 / 33 μl
Concentration of NaOH	0.01 / 0.5 M
Volume of amoxicillin	0,08 / 0,25 ml

The experiments we performed with amoxicillin, which correspond to a factorial design of 2^4, are shown in Table 2. We applied the curve resolution algorithms to the data matrix obtained from them. In experiments 4, 8 and 16 we only observed the base species. In experiments 9 and 10 we only observed the acid species. In the other experiments we observed both species, and these coexisted the longest in experiment 13. We selected the conditions of this experiment because they increased the pH in a suitable way.

First we used principal components analysis (PCA) to examine the data and concluded that the number of principal components was 2, 3 or 4, depending on the pharmaceutical.

Table 2.- Experiments performed to establish the analytical sequence.

num. exp.	Flow [a]	[NaOH] [b]	Volume NaOH [c]	Volume Amox. [d]	Result [e]
1	0.5	0.01	8	0.08	ac / bas
2	2	0.01	8	0.08	ac / bas
3	0.5	0.5	8	0.08	ac / bas
4	2	0.5	8	0.08	bas
5	0.5	0.01	33	0.08	ac / bas
6	2	0.01	33	0.08	ac / bas
7	0.5	0.5	33	0.08	ac / bas
8	2	0.5	33	0.08	bas
9	0.5	0.01	8	0.25	ac
10	2	0.01	8	0.25	ac
11	0.5	0.5	8	0.25	ac / bas
12	2	0.5	8	0.25	ac / bas
13	0.5	0.01	33	0.25	ac / bas
14	2	0.01	33	0.25	ac / bas
15	0.5	0.5	33	0.25	ac / bas
16	2	0.5	33	0.25	bas

The system we studied may be rank-deficient, due to the presence of species with similar characteristics to those of the analyte, such as clavulanic acid in augmentine. We therefore decided to use augmented matrices.

After making an approximate assumption of which components were relevant, we constructed the augmented matrices by columns by adding the matrix obtained from analysing the amoxicillin pattern to the matrices of each pharmaceutical. We used SIMPLISMA to estimate the pure spectra and applied the ALS optimisation program with the following restrictions: non-negativity for concentration profiles and spectra; unimodality for concentration profiles; and a small binary matrix containing the information about the correspondence of species (components) in the submatrices of the data set.

Our results indicate that there were two relevant components for Eupen and Ardine, three for Mundogen, Flubiotic and Clamoxyl and four for Augmentine. With regard to composition, all the indications of the formulations include, as well as excipients of unknown composition, saccharose and/or sodium saccharine, except

Augmentine, which contains clavulanic acid. In all cases, we checked the resolution by adding one more component to those already evaluated. The spectrum of this component was the same as that of an existing one was established, so the number of relevant components was established. We considered the fit of the models to the experimental data, evaluated by lack of fit to be satisfactory (in all cases lack of fit was between 3 and 4%). We also studied the residual matrix and checked that it had a noise structure.

Figures 5-7 show the concentration profiles and spectra for Ardine, Flubiotic and Augmentine, respectively. In the profile of concentrations, we can see first the profiles for the samples and then the profiles for the amoxicillin pattern. In the profiles of the spectra, we can see the profile of the acid species of amoxicillin (dashed line), the profile of the basic species of amoxicillin (continuous line) and, depending on the pharmaceutical, the spectra for the interferent or combination of the interferents.

In Figure 5, which shows the data for Ardine, we can only see the signal for amoxicillin (profiles of concentration and spectra). This indicates that, for this pharmaceutical, no interferent produced a signal in the area in which we were working. The situation with Eupen was the same.

In Figure 6, which corresponds to Flubiotic, there is a third component. The profile of concentrations of this component is located between the two species of amoxicillin. Its spectrum in the first wavelengths is superimposed on those of the first acid species and the base species of amoxicillin. In the case of Mundogen and Clamoxyl, there is also a third species. The spectra of all the third species are different. With Mundogen, the third species produces a spectral signal that corresponds to the first wavelengths that interfere with the spectrum of acid amoxicillin.

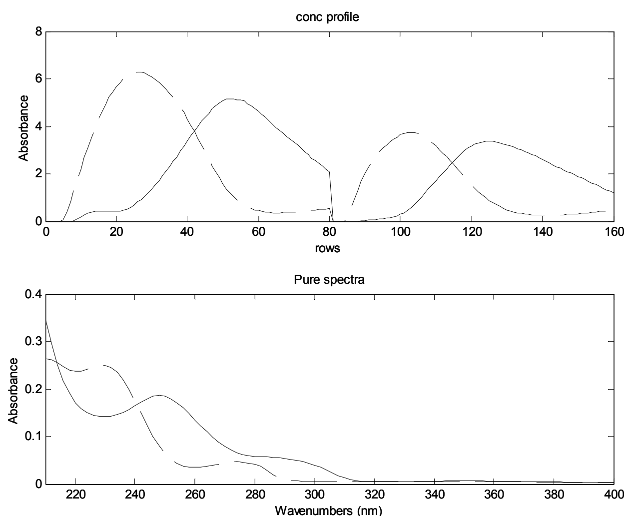

Figure 5.- Normalized concentration profiles (a) and pure spectra (b) recovered by multivariate curve resolution of the analysis of the Ardine-amoxicillin augmented data matrix. (———) Acid species of amoxicillin; (– – – -) basic species of amoxicillin.

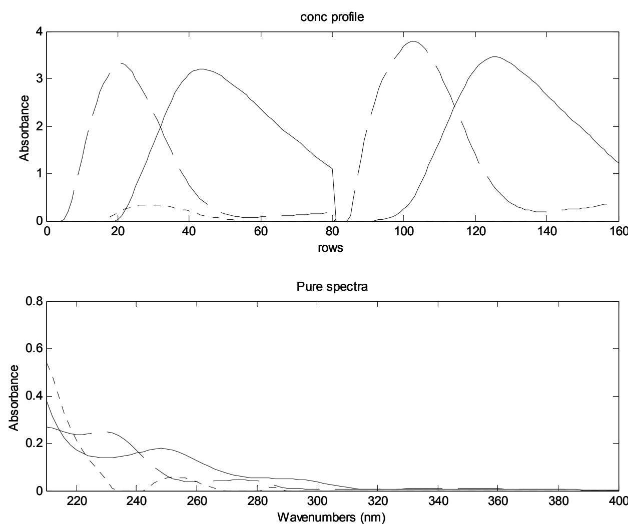

Figure 6.- Normalized concentration profiles (a) and pure spectra (b) recovered by multivariate curve resolution of the analysis of the Flubiotic-amoxicillin augmented data matrix. (– – – -) Acid species of amoxicillin; (———) basic species of amoxicillin; (- - - - - - -) interferents.

With Augmentine (Figure 7), four species appeared in the resolution. The structure of the two species that interfered in the profile of concentrations suggests that some interferent, probably clavulanic acid, behaves as an acid species.

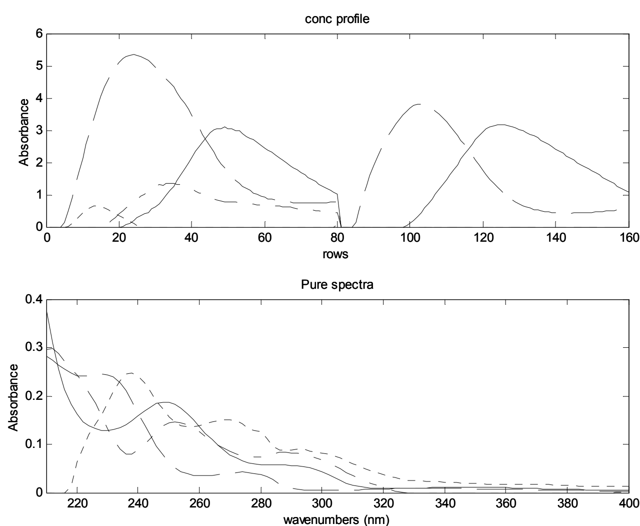

Figure 7.- Normalized concentration profiles (a) and pure spectra (b) recovered by multivariate curve resolution of the analysis of the Augmentine-amoxicillin augmented data matrix. (− − − -) Acid species of amoxicillin; (———) basic species of amoxicillin; (- - - - -) and (− · − · −) interferents.

After obtaining these results, we decided to use linear calibration to determine amoxicillin in the pharmaceuticals that had no interferents (Eupen, Ardine) and in Mundogen, which had no interferents in the maximum of the basic species of amoxicillin. With pharmaceuticals that have no interferents, it does not matter if the amoxicillin is determined in its acid form or in its base form. As Mundogen has to be determined in base form, we used the conditions of experiment 4 in Table 2 because a fast and total conversion to base form is required in a short analysis time. The absorbance measured is the one that corresponds to the maximum in the SIAgram and the working wavelength was 250 nm.

Table 3.- Parameters of the analytical methods developed.

Working λ	250 nm
Straight line equation	y= 7x[a] + 51,9
Correlation coefficient	0.9953
Linear range	10 to 120
num. standards	15
RMSEC	3.7

[a] x is expressed in mg/ml

Our results are shown in Tables 3 and 4. Table 3 shows the calibration characteristics and Table 4 shows the results from analyses of the pharmaceuticals. Due to the instability of amoxicillin, the amount of amoxicillin added to the pharmaceuticals is generally 10% higher than in the formulation. This is to ensure bacteriostatic activity. This may be the reason for the discrepancies in Table 4. The standard deviation was obtained by analysing 6 samples treated independently from the start. The variation coefficient ranges from 9 to 16%. Considering that the samples were analysed independently, we think the results are acceptable. We should point out that the concentrations indicated for these pharmaceuticals are subject to a certain degree of uncertainty, about which we have no data.

Table 4.- Determination of amoxicillin in pharmaceutical preparations.

Sample	Present (mg)[a]	Found (mg) ± s (n=6)
Mundogen	500	459 ± 73
Eupen	1000	1109 ± 100
Ardine	1000	1229 ±107

[a] indicated in the pharmaceutical

5. Conclusions

SIA with a diode-array spectrophotometric detection is an attractive technique for checking the presence of interferents in unknown samples. A suitable of design,

suitable for analytical sequences obtains a data matrix for each sample. Chemometric methods can then be applied to deduce the number of absorbent species and their spectral characteristics.

If no interferents are observed, or if the analyte has selective areas, the determination can be carried out using univariate calibration. In such cases the advantages of using this system is that the determination is fairly automatic, the frequency of analysis is high and the consumption of reagents is low. We used this system to successfully determine amoxicillin in pharmaceuticals.

For samples that contain interferents, the same system can carry out more complex calibrations. This will be the object of later studies.

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3.3. Paper

"Determination of amoxicillin in pharmaceuticals using sequential injection analysis and multivariate curve resolution."
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Analytica Chimica Acta 515 (2004) 159-165.

Determination of amoxicillin in pharmaceuticals using sequential injection analysis and multivariate curve resolution.

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Abstract

In this study, we report a method for quantifying amoxicillin in pharmaceuticals in the presence of interferents using sequential injection analysis (SIA) with a diode-array spectrophotometric detector and multivariate curve resolution with alternating least squares (MCR-ALS). With a suitable analytical sequence, we can use SIA to generate a pH gradient and, for each sample, obtain a data matrix. We used augmented matrices to resolve the system and obtain the spectra and concentration profiles of the components in the sample.

We studied what are the effects of imposing trilinearity at the resolution stage, how to choose the species that will be used for quantification (acid, basic or the sum of the two), and which is the most suitable concentration of the reference standard. Once the optimum conditions were established, we performed the quantification in three amoxicillin-containing pharmaceuticals (Flubiotic, Augmentine and Clamoxyl). With this method determination is quick, the reactants and instrumentation are inexpensive, and pre-treatment of the sample is unnecessary.

Keywords: SIA, MCR, Amoxicillin, Three-ways.

1. Introduction

In this study, we present a method for determining amoxicillin in pharmaceutical products in the presence of interferents using sequential injection analysis (SIA) with a diode-array spectrophotometric detector and using chemometric techniques for second-order data.

In accordance with Sanchez and Kowalski [1], the analytical data generated by instrumental techniques can be classified as follows: zero-order data (e.g. a univariate or single data per sample), first-order data (e.g. a data vector per sample), or second-order data (e.g. a data matrix per sample). Correspondingly, zero-, first- and second-order calibration methods have been developed for extracting chemical information from each type of data.

Univariate calibration can only be performed if there are no interferents. If there are interferents, these must be removed by physical or chemical methods or by first- or second-order calibration methods. For first-order multivariate calibration (e.g. using principal component regression and partial least square regression), the nature and chemical matrix of the standard samples have to be similar to those of the unknown samples. The standards are really samples analysed previously by an independent method. In second-order calibration methods, pure analyte standards are frequently used to quantify unknown samples, even in the presence of unknown and uncalibrated interferences. This is a clear advantage of second-order calibration over first-order calibration [1, 2].

In calibration methods, the level of mathematical complexity increases as the order increases. At the same time, the number of instrumental configurations able to provide one type of data or another decreases as the complexity of the data increases.

By using a suitable analytical sequence and acquiring spectra at regular intervals, SIA provides second-order data [3, 4]. This technique also has some very

interesting characteristics, such as a high frequency of analysis and a low consumption of reactants.

The most common techniques for determining amoxicillin in pharmaceuticals are UV-visible spectroscopy and univariate calibration. Before these techniques are used, we must either check that there are no interferents or pretreat the signal in some way to remove its effects [5-8]. In a previous study [3], we set up an SIA system for acquiring second-order data to analyze amoxicillin-containing pharmaceuticals. After using multivariate curve resolution with alternating least squares (MCR-ALS) [9, 10] to chemometrically treat these data, we resolved the system and determined whether interferents were present in a number of medicines.

In this study, we have designed a second-order calibration method to determine amoxicillin in pharmaceuticals that contain interferents. To set up this calibration, we studied several operational aspects that can influence the quality of the results e.g. the effects of various mathematical restrictions at the resolution stage, the signal provided by the acid or basic species of amoxicillin (or both) to establish the calibration, and the concentration of the reference standard. Finally, once the process was optimised, we quantified amoxicillin in three pharmaceuticals that contain interferents.

2. Experimental

2.1. Reagents and solutions

We prepared amoxicillin and sodium hydroxide stock standard solutions by weighing the required amount of the respective compounds (amoxicillin from Sigma and sodium hydroxide from Prolabo) and dissolved them in purified water (from a Milli-Q water system from Millipore). The pure amoxicillin standards were as follows: P1, 10 mg/l; P2, 20 mg/l; P3, 30 mg/l; P4, 50 mg/l; P5, 60 mg/l. Every standard was prepared twice.

Pharmaceuticals: Clamoxyl (500 mg of amoxicillin per packet) and Augmentine (500 mg of amoxicillin per packet) from SmithKline Beecham, S.A.; Flubiotic (500 mg of amoxicillin per packet) from Pharmazam. Carrier was purified water from a Milli-Q water system. Problem samples were aqueous solutions of these pharmaceuticals that, according to their specifications, contained 50 mg/l of amoxicillin.

2.2. SIA manifold

The sequential injection analyzer comprised: CAVRO XL 3000 stepper motor-driven syringe pump connected to the PC with an RS-232 interface; A 6-position Eurosas EPS 1306 BPB automatic valve connected to the computer through a PCL-711S PC-Lab-Card; Omnifit PTFE tubing reaction coil: 70cm x 0.8mm; Holding coil: 200cm x 0.8mm; An HP8452A diode-array spectrophotometer controlled by an HP Vectra 5/75 computer equipped with an HP-IB IEEE 488 interface for communications; a Hellma 178.711QS flow-through cell. A scheme of the analyzer is shown in Figure 1.

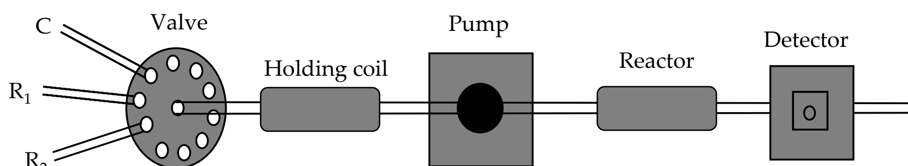


Figure 1.- Scheme of the sequential injection analyzer.

2.3. Process and conditions of the analytical sequence

The analytical process involved sequential aspiration of the carrier (water), reagent (sodium hydroxide) and sample (standards of amoxicillin or pharmaceuticals). The interdiffusion process of the sample and reactants lead to a gradual fall in pH through the channel to the detector. The amoxicillin is a polyprotic species, so when the sample reaches the detector, the most acid species appears first, followed by the mixture of this species with its conjugate base.

The working conditions for obtaining a suitable response (sequential appearance of the acid and basic species of amoxicillin) were determined using several amoxicillin standards [3].

The sequence of the analysis was as follows: (1) carrier aspiration was 4.74 ml; (2) sample aspiration was 0.25 ml; (3) NaOH aspiration was 0.01 ml (4) NaOH concentration was 0.025 mol/l; (5) the flow rate was 1 ml/min for all steps. The spectra were recorded every 2 nm in the 210 to 340 nm range, with an integration time of 0.1s. every 0.7s. The sampling frequency achieved was 25 h^{-1}.

2.4. Software

HP89531A software was used to record and store the spectra. Customized software was used to control the SIA.

All calculations relating to MCR-ALS were performed with laboratory-written software under a MATLAB 5.3 computer environment [9]. This software is available from the authors [10].

3. Data treatment

3.1. MCR method

The multivariate curve resolution (MCR) method is based on principal component analysis (PCA) [11]. After applying evolving factor analysis (EFA) or simple-to-use interactive self-modelling mixture analysis (SIMPLISMA) [12] and constrained alternating least squares (ALS) [13], MCR is implemented through MATLAB [9]. Details of the data treatment are described elsewhere [13, 14].

With MCR-ALS, we can simultaneously study several matrices, if these are correlated. Figure 2 shows how augmented matrices are obtained from individual

matrices. The individual matrices $\mathbf{D}_1$ and $\mathbf{D}_2$ are obtained from experiments 1 and 2, which have some component in common, and from these a matrix **D** is obtained that is augmented by columns (Figure 2a) or rows (Figure 2b). The simultaneous analysis of several matrices is done as with the individual matrices. The only difference is that more restrictions are applied. The advantage of working with augmented matrices is that rotational ambiguity is minimised, i.e. the number of different results obtained from an augmented matrix is more restricted, and so the resolution is better.

One of the advantages of MCR over other second-order techniques such as GRAM [15] is that it allows us to work with data in which the trilinearity is not so strict. It also allows us to impose several restrictions on the data in accordance with our knowledge of the system. Restrictions of non-trilinearity or trilinearity can be imposed. If restrictions of trilinearity are imposed, we can also impose conditions on the synchronization and shape of some or all of the species.

When the optimisation process ends, the performance of the model can be evaluated from lack of fit (lof) data as;

$$lof = \sqrt{\frac{\sum_{i,j}\left(d_{ij} - \hat{d}_{ij}\right)^2}{\sum_{i,j} d_{ij}^2}} \qquad (1)$$

where d_{ij} may be each of the elements of the raw data matrix or each of the elements of the matrix reconstructed from the principal components selected in the principal components analysis (PCA), and $\hat{d}_{ij}$ are the corresponding values calculated after the optimisation process (ALS). Therefore, depending on the matrix used, either lof_{exp} or lof_{pca} are obtained.

3.2. Building the calibration model

To establish a calibration model, we will use a series of calibration standards whose concentrations are known and found within the lineal range (called calibration standard (st)) and a standard solution whose composition is constant throughout the calibration process (called reference standard (rst)).

The simultaneous analysis of data matrices was performed using column-wise augmented data matrices (Figure 2a) by putting one matrix on top of another and keeping common wavelengths in the same column.

Matrix $\mathbf{D}_1$ corresponds either to different calibration standards of amoxicillin or to the problem sample, which in this case is the pharmaceutical (parameters related to the sample have the subscript (s)). Matrix $\mathbf{D}_2$ is always the matrix that corresponds to reference standard (rst), which is used throughout the calibration process.

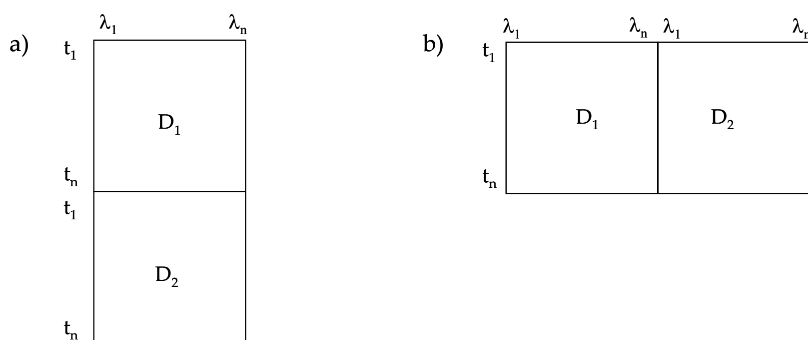


Figure 2.- Arrangements for spectra generated in the different experiments. a) Augmented data matrix in the wavelength direction. b) Augmented data matrix in the time direction.

The area of the acid or basic species, or the sum of these areas, recovered by MCR-ALS, were chosen as the analyte responses in order to relate it with the concentration of the reference standards.

Relative values were calculated as follows:

$$r_i = a_i / a_{rst} \quad (2)$$

where r_i is the relative area and a_i and a_{rst} are the areas of acid or basic species, or the sum of the two, in the sample *i* and in a reference standard, respectively. From the values of r_i obtained from the standards, the following univariate linear relation is established:

$$r_i = b_1 \cdot c_i + b_0 \quad (3)$$

where c_i is c_{st}/c_{rst}, in which c_{st} is the concentration of the standards and c_{rst} is the concentration of the reference standard, and b_1, b_0 are the parameters of the regression line. The value of the concentration of the analyte in the sample is obtained from its corresponding r_i value and the calibration parameters in accordance with the following expression:

$$Cs = \frac{r_i - bo}{b1} * Crst \quad (4)$$

where c_s is the concentration of the sample and c_{rst} is the concentration of the reference standard.

4. Results and Discussion

Figure 3 shows the spectra of acid and basic species of amoxicillin obtained in the HCl 0.1M and NaOH 0.1M media. These spectra will serve as references for evaluating the quality of the resolution.

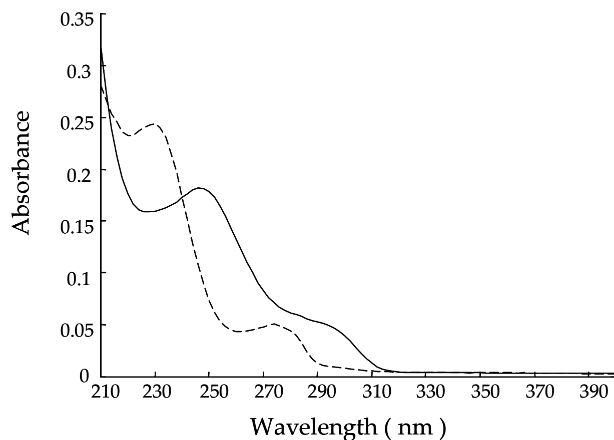
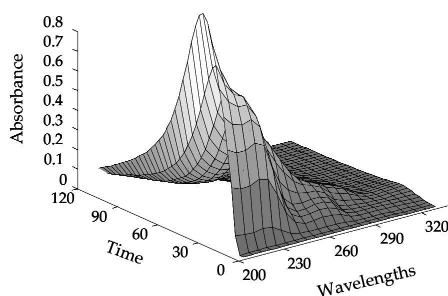
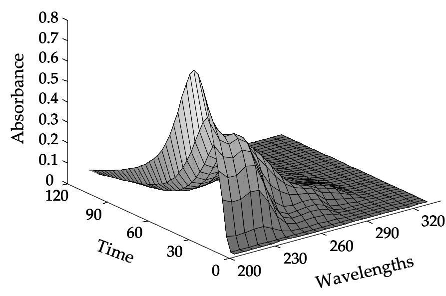


Figure 3.- Spectra of a solution of 50 mg/l of amoxicillin, (a) pH=13: basic species (———). (b) a pH=1: acidic species (– – – -).

Figure 4 shows the spectra, obtained from the analytical sequence, of a sample of augmentine (Figure 4a) and a calibration standard of amoxicillin (Figure 4b). We can see that these two figures are very similar. This indicates that, although there are differences in the first wavelengths, probably due to the effects of the interferents, the main contribution to the signal in the sample is made by amoxicillin.



Sample of augmentine (4a)



Standard of amoxicillin (4b)

Figure 4.- Spectrum obtained throughout the analytical sequence: a) Sample of augmentine. b) Standard of amoxicillin.

From the data matrices of the calibration standards and a reference standard, all of which were of amoxicillin, we built the augmented matrices and studied the effects of applying the following conditions, which are related to the possible trilinearity of the data: (a) the data matrix is not trilinear; (b) the data matrix is trilinear and its shape and synchronization are similar for the acid and basic species; (c) the data matrix is trilinear but the acid and basic species are not synchronized; and (d) the data matrix is trilinear and synchronized for acid species. In all cases, the constraints applied at this stage were: (i) correspondence between common species in different matrices; and (ii) non-negativity for concentration profiles and spectra and (iii) unimodality for concentration profiles.

We had two criteria to determine which of the four conditions was the most suitable for our data. The first one was the quality of the resolution, i.e. the degree of similarity between the spectra obtained in the resolution process and the spectra of the pure species (Figure 3). The second was the lack of fit, which was obtained as a measurement of the performance of the model.

Figure 5 shows the spectra and concentration profiles we obtained after applying each of the above conditions to an augmented matrix made up of a standard of 60 mg/l and one reference standard of 20 mg/l. The MCR process was performed using column-wise augmented data matrix, the graph of concentration profiles shows the profiles of the acid species and basic species corresponded to standard of 60 mg/l, and after these profiles we can see the profiles of the acid species and basic species corresponded to reference standard of 20 mg/l).

Table 1 shows the parameters for quality, indicated by lack of fit (lof) and the percentage of the explained variance (r^2) of the resolution with each condition. In all cases, we selected two principal components, the first of which explained 82% of the variance and the second of which explained 14% of the variance.

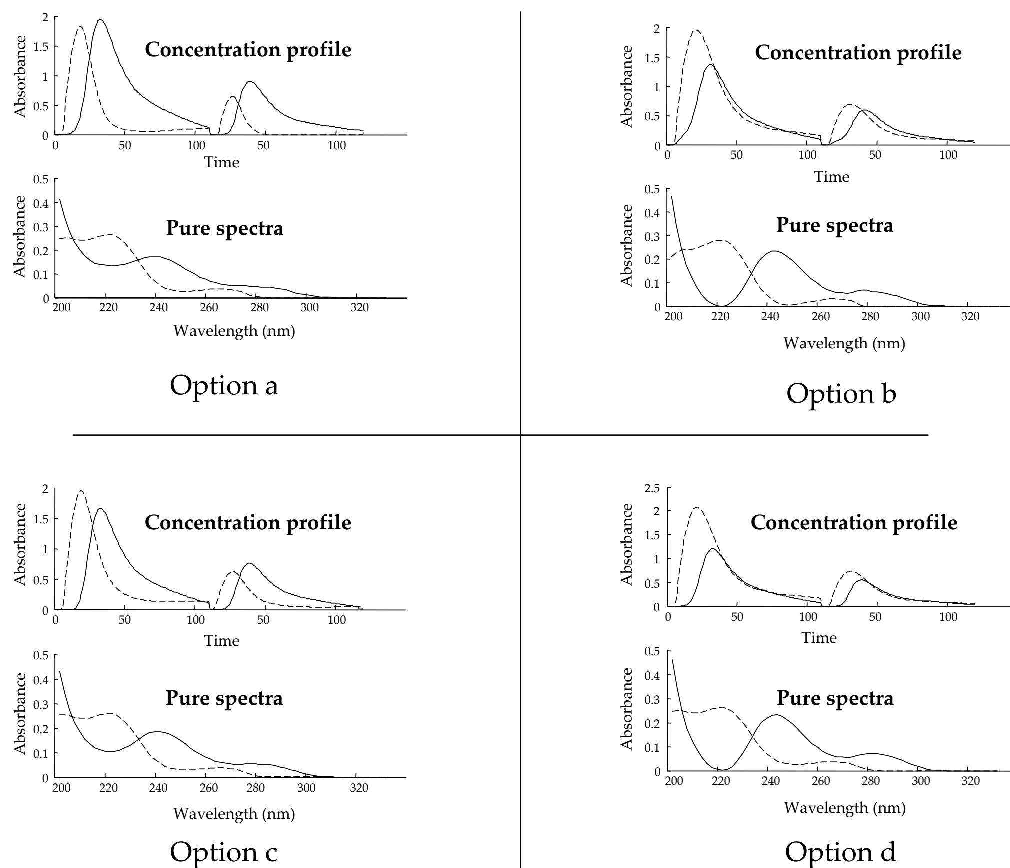


Figure 5.- Normalized concentration and pure spectra recovered by multivariate curve resolution of the analysis of the augmented data matrix for amoxicillin. (a) Non-trilinear data matrix. (b) Trilinear data matrix with equal shape and synchronization for acid and basic species. (c) Trilinear data matrix without synchronization for acid and basic species. (d) Trilinear data matrix with synchronization for acid species.

From the data in Table 1, we can see the best values for option (a) and the worst values for option (b). With regard to the other two options, the lack of fit and the percentage of explained variance are better with option (d). However, Figure 5 shows that the spectra fit the real spectra better with option (c). This is due to the calculation of the r^2 values from mathematical decomposition of raw matrix, however, the best mathematical decomposition is not always the best approach to our chemical system.

Table 1.- Parameters for the quality of the resolution.

	Option a	Option b	Option c	Option d
Lof_{pca}	0,77	3,9	2,63	1,67
Lof_{exp}	3,56	5,232	4,3672	3,86
r^2	99,88	99,72	99,81	99,85

We are interested in obtaining the best model which has to be similar to our chemical system. Also, unlike what we expected, the concentration profile presented no selective area for the basic species (option c). The profiles of the other two options were correct but the values indicating the fitness of the model were better with option (a). These results suggest that, because the system is not completely trilinear, the resolution improves when conditions of trilinearity are not imposed on the system. The system may not be trilinear for several reasons. Firstly, the elution of the analyte may, due to random variations in the analytical process (flow, volumes, etc.), be slightly different from one analysis to another. Secondly, the differences in the concentrations of the standard analyte and the reference standard may, for two reasons, lead to differences in the profiles. One reason is that the velocity of diffusion increases as the concentration increases and produces wider peaks (concentration profiles) in the more concentrated solutions. Another is that the time at which the basic species appears is influenced by the concentration of the analyte. In all subsequent treatments, condition (a), in which the trilinear data are not considered, will be imposed.

We then studied whether quantification could be done on the basis of the area of the acid or basic species, or the sum of the two species (total response of the analyte). Every standard was analysed twice. The results are shown in Table 2. We can see that when there are difference in the repetitions (relative concentrations of 0.33, 0.83 and 1), the trends are opposite, i.e. when the area of the acid species increases, the area of the basic species decreases. Therefore, reproducibility is better when the total response (the sum of the areas) is considered.

Table 2.- Response according to whether the species considered is acid, basic or the sum of the two.

Rel. conc.[a]	Rel. ar. acid[b]	Rel. ar. basic[c]	Rel. Ar. total[d]
0,17	0,14	0,24	0,2
0,17	0,14	0,25	0,2
0,33	0,29	0,45	0,38
0,33	0,34	0,4	0,37
0,5	0,56	0,55	0,55
0,5	0,57	0,54	0,55
0,83	0,87	0,91	0,9
0,83	0,71	0,96	0,88
1	1	1	1
1	0,67	1,15	0,98

[a] relative concentrations
[b] relative area of the acid species
[c] relative area of the basic species
[d] relative area of the sum of the species

We confirmed this by studying the results of six repeated measurements of a pharmaceutical, obtaining relative standard deviation of 6.7, 2.3 and 1.3% for the acid species, basic species and combination, respectively.

To determine the calibration lines in accordance with equation (3), we had to resolve the augmented matrices made up of each of the standard and one reference standard (any one of the standards), which is constant in all cases. We studied the effects of using one concentration or other of the reference standard from the corresponding calibration lines.

Ideally, the regression line should have a slope of one and an ordinate at the origin of zero because when the ratio of concentration is 1 (i.e. when the same data matrix is used for the standard and for the reference standard), the ratio of the areas is one and this proportionality must be maintained for all cases. To check whether the regressions lines satisfy this condition, we compared the slope and ordinates at origin of each line with those of the ideal line.

Our results are given in Table 3. Our response and concentration work intervals were different for each line because we were working with a relative measurement. The fit of the regressions lines, evaluated by the correlation coefficient, was similar in all cases, i.e. the fit was not affected by the reference standard used. As the concentration of the reference standard increased, the slope of the regression line tended to increase and the ordinate at the origin tended to decrease. However, as we can see in the final column, all the lines except those constructed with the reference standards with the lowest concentrations passed the joint gradient and ordinate test, but the line that best fit the theoretical values is the one used as reference standard for 60 mg/l. This standard will be used as the reference standard and in the quantification of the pharmaceuticals. For this regression line the limit of detection was 0.06 mg/l (α = 0,05 y k = 2), expressed in absolute terms.

Table 3.- Parameters of the calibrations in function of the concentration of the reference standard.

Conc. Ref [a]	A_{st} /A_{rst} [b]	C_{st}/C_{rst} [c]	b_1	b_0	r^2	Test T[d]
10	1.00 - 5.03	1.00 - 6.00	0,824	0,227	0,998	NO
20	0.53 - 2.63	0.50 - 3.00	0,863	0,127	0,998	NO
30	0.36 - 1.83	0.33 - 2.00	0,889	0,091	0,998	SI
50	0.22 - 1.12	0.20 - 1.20	0,906	0,060	0,998	SI
60	0.20 - 0.99	0.16 - 1.00	0,972	0,050	0,997	SI

[a] reference standard concentration (mg/l)
[b] range of area of standard divided by area of reference standard
[c] range of standard concentration divided by concentration of reference standard
[d] test T (α=0.05)

Table 4 shows the results from the analyses of the pharmaceuticals. The standard deviation was obtained by analyzing 6 samples treated independently from the beginning of the experiment. These results are similar to those of a previous study in which we determined amoxicillin in pharmaceuticals without interferents using zero-order calibration.

Table 4.- Determination of amoxicillin in pharmaceuticals preparations.

Sample	Present (mg)[a]	Found (mg) ± s (n=6)
Flubiotic	500	548 ± 36
Clamoxyl	500	550 ± 80
Augmentine	500	634 ± 35

[a] indicated in the pharmaceutical

The discrepancy between the value indicated in the pharmaceutical and the observed value may be due to the fact that, because amoxicillin is unstable, the amount of amoxicillin added to the pharmaceuticals is generally 10% higher than in the formulation in order to ensure bacteriostatic activity.

5. Conclusions

SIA with diode-array spectrophotometric detection is an interesting technique for quantifying amoxicillin in the presence of interferents in pharmaceuticals. With a suitable analytical sequence we can obtain a data matrix for each sample. Chemometric methods can then be applied to obtain the response of the amoxicillin in the form of a chromatographic profile and determine the concentration of the acid and basic species of the analyte. We can quantify the analyte of interest from the relationship between the response and a reference standard of known concentration.

As well as a suitable design for the analytical sequence, the optimisation of this method requires suitable constraints at the resolution stage for the problem under consideration and the selection of signals (area of acid or basic species or the combination of the two) that improve results. We also found that we had to choose the higher concentrations of reference standard.

The advantages of using this system are that the determination is fairly automatic, the frequency of analysis is high, and the consumption of reactants is low.

Also, we can quantify in the presence of interferents without having to do any sample pre-treatment.

Acknowledgements

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3.4. Paper

"Sequential injection analysis (SIA) for the simultaneous determination of clavulanic acid and amoxicillin in pharmaceuticals using second-order calibration."

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Analytical Science submitted, 9 September 2005

Sequential injection analysis (SIA) for the simultaneous determination of clavulanic acid and amoxicillin in pharmaceuticals using second-order calibration

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Abstract

In this study, we report a method for quantifying clavulanic acid and amoxicillin simultaneously using sequential injection analysis (SIA) with a diode-array spectrophotometric detector and multivariate curve resolution with alternating least squares (MCR-ALS). Clavulanic acid and amoxicillin are the main agents of several pharmaceuticals. Their acid-base properties and spectral responses in the UV-visible are similar. We used an analytical sequence in which NaOH, the sample and HCl were aspirated to generate a suitable pH gradient for resolving all the species studied.

We optimised the experimental parameters so that the analytical sequence could distinguish the concentrations and spectrum profiles of the species of interest with optimum resolution quality. The quality of this resolution has been evaluated by the degree of similarity between the spectra obtained in the resolution process and the spectra of the pure species, and by the lack of fit (lof) of the predicted model with respect to the raw matrix. To evaluate any quantification error, we also considered the relationship between the areas obtained in analyses of two standards (amoxicillin and clavulanic acid) and a synthetic mixture, of known concentration, of both standards.

After establishing the optimum conditions, we quantified clavulanic acid and amoxicillin in four pharmaceuticals. In most cases our results were slightly higher than those in the prospectus of the pharmaceutical. Relative standard deviations were

below 5% for amoxicillin and below 7% for clavulanic acid. These results are acceptable because, to prevent degradation due to bacteriostatic activity, the concentration of the main reactant is usually higher.

Keywords: Multivariate curve resolution, Sequential injection analysis, Amoxicillin, Clavulanic acid, Experimental design.

1. Introduction

A derivative of penicillin, amoxicillin belongs to a group of β-lactam antibiotics. It is often dispensed together with clavulanic acid, which has weak antibacterial properties but produces a reduction of bacterial resistance. As both are components of widely used preparations such as “Augmentine“ and “Clavucid”, it would be very interesting to have an analysis method to simultaneously determine them in only one step. Several methods have been reported for analysing these two compounds simultaneously. Some authors, for example, have proposed determination by ultraviolet spectrometry by derivate spectrophotometry [1, 2]. Although these methods present good results, they do not allow to work with the maximum signal. Consequently, sensitivity is low and uncertainty is high. One way of getting high sensitivity is shown in [3], where the authors propose the simultaneous determination of amoxicillin and clavulanic acid by multivariate calibration using fluorimetric detection— the disadvantage of these methods is that involve analysing many samples in a calibration step. Other papers discuss HPLC with amperometric detection [4, 5], which allows work with interferents but extends analysis time.

In previous studies, we proposed applying multivariate curve resolution with alternating least squares to determine amoxicillin. If no interferents were observed in the sample, the determination was carried out using univariate calibration [6] and if interferents were observed, the determination was carried out using second-order calibration [7]. In these papers, the analytical sequence focused on determining only amoxicillin and we did not study the composition or identification of the interferents.

In the present study, we propose the simultaneous determination of clavulanic acid and amoxicillin in pharmaceuticals using second-order calibration. Three aspects of this determination are problematic;

- Firstly, as the acid-base properties of the two species are similar, this may lead to rank-deficient chemical data [8]. The first two pKa of amoxicillin are 2.4 and 7.4 [9] but its structure (Figure 1a) also has two proton donor groups. The pKa of clavulanic acid is 2.7 [10] but, like amoxicillin, it has other donor groups (see Figure 1b). We found no references to the pKa values of the least acidic protons, so we used software [11] to predict them. We obtained values of 9.01 and 10.29 for amoxicillin and 12.17 for clavulanic acid.

- Secondly, because of the acid-base and spectral characteristics of both compounds, we need to establish a new analytical sequence to widen the pH range in the reactor coil. This involves studying and optimising the analytical sequence.

- Thirdly, as amoxicillin is the majority species and as the sensitivity of the response is similar, it can be difficult to find good conditions for the simultaneous determination.

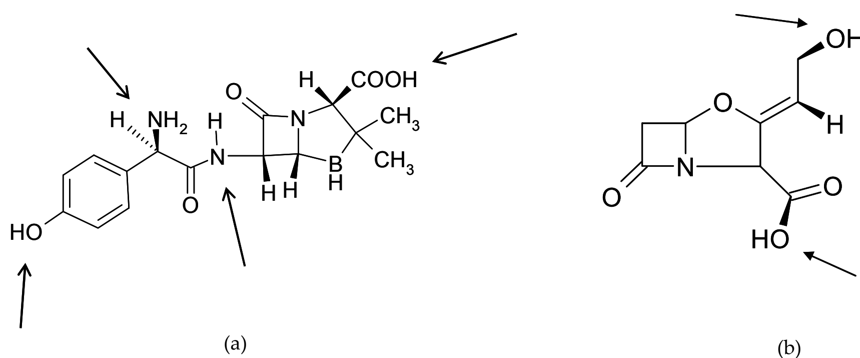


Figure 1.- (a) Chemical structure of amoxicillin (b) chemical structure of clavulanic acid.

To obtain an analytical sequence with which to visualise all the species, we used an experimental design. We selected the optimal analytical sequence on the basis of several criteria: the concentration profiles and spectra of all the species of interest; the quality of the resolution, measured by the degree of similarity between the spectra

obtained in the resolution process and the spectra of the pure species; the fit of the model to the experimental data; and the quantification error, measured by the relationships between the areas obtained from analyses of two standards and a synthetic mixture.

2. Experimental

2.1. Reagents and solutions

We prepared amoxicillin, clavulanic acid, sodium hydroxide and hydrochloric acid stock standard solutions by weighing the required amount of the respective compounds (amoxicillin from Sigma and sodium hydroxide and hydrochloric acid 37% from Prolabo) and dissolved them in purified water (from a Milli-Q water system from Millipore). Clavulanic acid was supplied by Laboratorio Reig Jofré S.A. The pure amoxicillin standards were 120, 150, 210, 250, 270 and 300 mg/l. The pure clavulanic acid standards were 20, 25, 35, 40, 45 and 50 mg/l. Each standard was prepared twice.

Pharmaceuticals: Augmentine (500 mg of amoxicillin and 125 mg of clavulanic acid per packet) and Augmentine suspension (100 mg of amoxicillin and 12.5 mg of clavulanic per 1 mL per packet) from ClaxoSmithKline S.A.; Clavucid (875 mg of amoxicillin and 125 mg of clavulanic acid per packet) from Recordati; generic medicine (500 mg of amoxicillin and 125 mg of clavulanic acid per packet) from Normon S.A. Carrier was purified water from a Milli-Q water system.

2.2. SIA manifold and measurement conditions

The sequential injection analyzer comprised: CAVRO XL 3000 stepper motor-driven syringe pump connected to the PC with an RS-232 interface; A 6-position Eurosas EPS 1306 BPB automatic valve connected to the computer through a PCL-711S PC-Lab-Card; Omnifit PTFE tubing reaction coil: 70cm x 0.8mm; Holding coil: 200cm x 0.8mm; An

HP8452A diode-array spectrophotometer controlled by an HP Vectra 5/75 computer equipped with an HP-IB IEEE 488 interface for communications; a Hellma 178.711QS flow-through cell.

The spectra were recorded every 2 nm in the 220 to 340 nm range, with an integration time of 0.1s. every 0.7s. The sampling frequency achieved was 25 h^{-1}.

The analytical process, shown in Figure 2, involved sequential aspiration of the carrier (water), first reagent, sample (standard of amoxicillin, standard of clavulanic acid or pharmaceuticals) and second reagent. The interdiffusion process of the sample and reagents led to a gradual fall in pH through the channel to the detector.

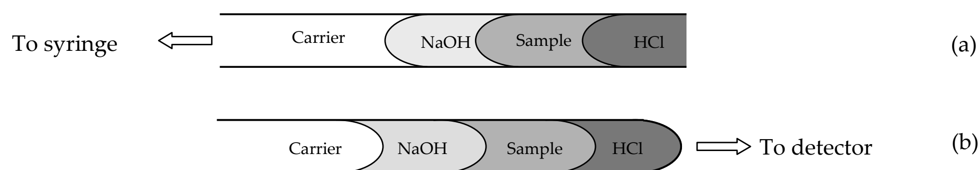


Figure 2.- Scheme of the process for mixing sample, reagent and carrier in the channel of an SIA system: (a) Reagents go to syringe; (b) reagents go to detector, interdiffusion has taken place.

2.3. Software

HP89531A software was used to record and store the spectra. Customized software was used to control the SIA.

All MCR-ALS calculations were performed with laboratory-written software under a MATLAB 5.3 computer environment [12]. This software is available from the authors [13].

3. Data treatment

3.1. MCR method

Multivariate curve resolution (MCR) is based on decomposition of the experimental matrix into two matrices: the concentration matrix and the spectra matrix. In a first step, we apply principal component analysis (PCA) [14] to determine how many significant principal components are present in the reaction, after evolving factor analysis (EFA) [15] or simple-to-use interactive self-modelling mixture analysis (SIMPLISMA) [16] to obtain an initial estimation of the spectra profiles. The last step is an optimisation process with alternating least squares (ALS) [17].

In chemical systems with very similar acid-base properties that change as the pH changes, rank-deficient chemical data are possible [8]. The chemical rank is defined as the number of chemical species distinguishable from noise. The rank of the matrix was analysed by means of singular value decomposition (SVD) [18]. The number of chemical components was estimated by inspecting the size of the singular values. It was assumed that the singular values associated with the chemical components are much larger than other possible contributions such as noise. Chemical data is rank-deficient when the number of significant principal components is less than the number of chemical species present.

This can be avoided by working with augmented matrices by columns where the augmented matrix ($\mathbf{D}_{aug}$) is built from the experimental matrices corresponding to sample (**S**), amoxicillin (**A**) and clavulanic acid (**C**). Whether or not the chemical data is rank deficient, working with augmented matrices improves the resolution.

The iterative optimisation started with the evaluation of the unknown species spectra using an initial estimation of the **S** data matrix:

$$\mathbf{C}^{T} = (\mathbf{S})^{+} \cdot \mathbf{D}^{*} \qquad (1)$$

where $\mathbf{D}^*$ is the reproduced data matrix for the number of components considered, performed by PCA, and $(\mathbf{S})^+$ is the pseudoinverse of **S**.

In a second stage, the matrix **S** was updated using equation 2:

$$\mathbf{S} = \mathbf{D}^* (\mathbf{C}^T)^+ \tag{2}$$

These steps were repeated until convergence was achieved.

All the matrices (**D**, **C** and **S**) can be individual (only one matrix) or augmented matrices. Various constraints depending on the nature and structure of the data can be applied during ALS optimisation. The constraints applied at this stage were: (i) correspondence between common species in different matrices, (ii) non-negativity for concentration profiles and spectra, (iii) unimodality for concentration profiles, and (iv) non-trilinear data matrix.

Any unmodelled data variance in the residuals was evaluated by lack of fit, using the following equation:

$$lof = \sqrt{\frac{\sum_{i,j}\left(d_{ij} - \hat{d}_{ij}\right)^2}{\sum_{i,j} d_{ij}^2}} \tag{3}$$

where d_{ij} are the corresponding values of the raw data matrix and $\hat{d}_{ij}$ are the corresponding values calculated after the ALS optimisation process. High values of lof show that the established model does not fit the experimental data.

3.2. Calibration and quantification steps

The calibration was performed using column-wise augmented data matrices. In all cases, the top matrix corresponds to calibration standards whose concentrations vary

and the lower matrix corresponds to the reference standard, whose concentration is constant. The concentration of the reference standard will always be the calibration standard with the highest concentration [7].

After applying the ALS algorithm, we obtain a_i and a_{rst} in which, a_i are the areas of each analyte in the sample *i* or the calibration standard and a_{rst} is the area of the reference standard.

From these values, the following univariate linear relation is established for each analyte.

$$r_i = b_1 \cdot c_i + b_0 \qquad (4)$$

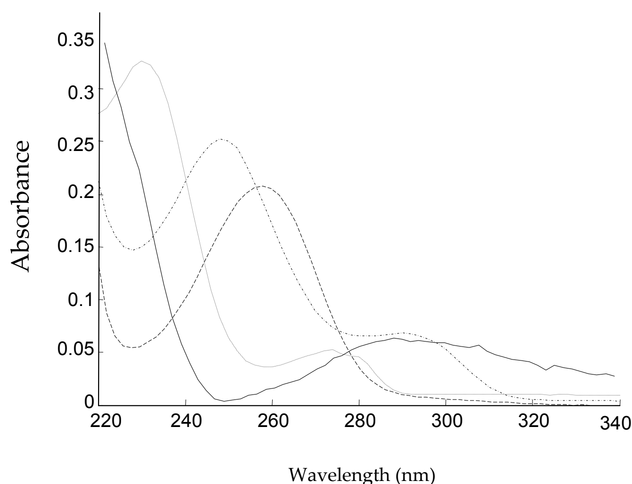
where r_i is a_i/a_{rst} and c_i is c_{st}/c_{rst}, in which c_{st} is the concentration of the calibration standards and c_{rst} is the concentration of the reference standard, and b_1, b_0 are the parameters of the regression line.

The value of the concentration of the analyte in the sample is obtained from its corresponding r_i value and the calibration parameters.

4. Results and discussion

The first step was the spectroscopic characterisation of the clavulanic acid species with different pHs with their pKas taken into account. Figure 3 shows the spectra of clavulanic acid. In hydrochloric acid (pH=1) and in water (pH=7.7), we obtained the spectrum indicated by a continuous line, and in sodium hydroxide (pH=13) we obtained the spectrum indicated by a discontinuous line. These are superimposed onto the two spectra of amoxicillin [6, 7]. We found that the acid species of clavulanic acid has a maximum level of absorbance at λ=290nm and is much less sensitive than both species of amoxicillin and the basic species of clavulanic acid. We

checked that the absorbance was linear with concentrations of 120 to 300 mg/l for amoxicillin and concentrations of 20 to 50 mg/l for clavulanic acid.



Wavelength (nm)

Figure 3.- Spectra of acid species of amoxicillin (·················). Spectra of basic species of amoxicillin (-·-·-·). Spectra of basic species of clavulanic acid (— — ·). Spectra of acid species of clavulanic acid (——).

To establish an analytical sequence for sequentially distinguishing all the species of amoxicillin and clavulanic acid, we carried out studies with sodium hydroxide as the only reagent, modifying its concentration (0,05 - 1 mol/l) and volume (10-60 μl). In these conditions, we checked the chemical rank of the system. Table 1 (a) shows the singular values obtained by SVD analysis of the sample matrix and augmented matrices when sodium hydroxide was the only reagent. We found that the sample had only two principal components, which could indicate the presence of rank deficiency. However, when we applied SVD to the augmented matrix, we also observed two principal components. We therefore concluded that we could not see all the species not because of rank deficiency but because we did not have suitable experimental conditions. In these studies (depending on concentration of NaOH used); (a) we obtained the basic species of both analytes and in consequence we did not have an evolving system, or (b) we observed acid and basic species of amoxicillin but not the basic species of clavulanic acid (like previous papers [6, 7]).

Table 1.- Rank analysis of individual matrices (**S**,**A**,**C**) and the column-wise augmented matrices M (the first six eigenvalues are shown).

a) Sodium hydroxide as the only reagent

Number of factors	S	M = [S;A;C]
1	**13.3013**	**16.0089**
2	**2.8872**	**4.1413**
3	0.2911	0.1837
4	0.1345	0.1495
5	0.0681	0.0869
6	0.0403	0.0613

b) Sodium hydroxide and hydrochloric acid as reagents

Number of factors	S	M = [S;A;C]
1	**42.2306**	**63.7817**
2	**11.9252**	**18.7969**
3	**1.5966**	**5.1361**
4	0.5853	**2.8794**
5	0.395	1.2804
6	0.2088	1.074

To widen the range of pHs, we introduced a second reagent (hydrochloric acid). An analytical sequence was therefore established in which first sodium hydroxide was aspirated, then the sample and finally hydrochloric acid. These reached the detector in reverse order, as we can see in Figure 2. In a first step, we decomposed the principal components to observe the number of species in the system. In Table 1 (b), we can see that the number of principal components for the sample was three and we knew that the number of chemical species was four. However, when we applied SVD to the augmented matrix the number of principal components was four. Rank deficiency is therefore solved by working with augmented matrices.

After resolving the rank-deficient problem, we experimentally designed the system variables to find the optimal analytical sequence. The experiments were

performed with a synthetic mixture of amoxicillin and clavulanic acid. The design of the experiment was a factorial design of 2^4, as shown in the first columns of Table 2. The choice of the domain of the quantitative factors was evaluated from previous experiments and instrumental limitations. For each experiment, the sample and the standard of amoxicillin and clavulanic acid were analysed. This produced three matrices: matrix (**A**), obtained from analysing an amoxicillin concentration of 300mg/l; matrix (**C**), obtained from analysing a clavulanic acid concentration of 50 mg/l; and matrix (**S**), obtained from analysing a synthetic mixture of 300 mg/l amoxicillin and 50 mg/l of clavulanic acid.

The criteria for selecting the optimal analytical sequence were: (a) the appearance of the concentration profiles and spectra of the all species of interest; (b) the degree of similarity between the spectra obtained in the resolution process and the spectra of the pure species by means of the correlation of the two vectors; (c) lack of fit (lof), obtained as a measurement of the performance of the model; and (d) a ratio of one for the areas, obtained by analysing two standards of clavulanic acid and amoxicillin and a synthetic mixture with the same concentration as the two standards.

The last eight columns of Table 2 show the results of all the experiments. The first of these columns indicates whether all the species were resolved: (+) indicates that they were and (-) indicates that they were not. In the negative cases, as the analytical sequence is considered not to be suitable, the other results are not shown. The next four columns show the correlation between the spectra obtained in the resolution process and the spectra of the pure species. The next column shows the lack of fit (lof) and the last two columns show the ratio of the areas.

Only experiments 3, 4, 7, 13 and 15 gave the four species of interest after applying the resolution process. Of all these experiments we considered experiments 7 and 15 because the relationship between the areas of both analytes was close to one, which, because the synthetic mixture and the reference standard are at the same concentration, is the expected value. In all cases, the acid species of clavulanic acid provided the worst correlation because of the type of signal obtained at low

wavelength (see Figure 3). As in our opinion experiment 15 was generally the one with the best characteristics, we used it for the quantification of the pharmaceuticals.

Table 2.- Experiments performed to establish the analytical sequence and the responses of each analytical sequence.

						Spectrum[d]					Ratio of areas	
nº exp	Flow[a]	Volume[b]	[NaOH][c]	[HCl][c]	Profile	Amox. A	Amox. B	Clavu. A	Clavu. B	lof	Amox	Clavu
1	0,5	17	0,25	0,25	-	-	-	-	-	-	-	-
2	1	17	0,25	0,25	-	-	-	-	-	-	-	-
3	0,5	50	0,25	0,25	+	0,9972	0,9943	0,8141	0,9775	8,32	0,89	1,87
4	1	50	0,25	0,25	+	0,9979	0,9961	0,8938	0,9191	8,77	1,06	1,8
5	0,5	17	0,5	0,25	-	-	-	-	-	-	-	-
6	1	17	0,5	0,25	-	-	-	-	-	-	-	-
7	0,5	50	0,5	0,25	+	0,9988	0,9993	0,9315	0,9963	7,2	0,93	1,17
8	1	50	0,5	0,25	-	-	-	-	-	-	-	-
9	0,5	17	0,25	0,5	-	-	-	-	-	-	-	-
10	1	17	0,25	0,5	-	-	-	-	-	-	-	-
11	0,5	50	0,25	0,5	-	-	-	-	-	-	-	-
12	1	50	0,25	0,5	-	-	-	-	-	-	-	-
13	0,5	17	0,5	0,5	+	0,9977	0,9949	0,9315	0,9825	7,95	0,98	1,68
14	1	17	0,5	0,5	-	-	-	-	-	-	-	-
15	0,5	50	0,5	0,5	+	0,9984	0,9987	0,8993	0,9974	9,9	1,02	1,03
16	1	50	0,5	0,5	-	-	-	-	-	-	-	-

[a] flow injection ml/min
[b] volume µl for HCl and NaOH
[c] concentration mol/l
[d] amox. A correspond to correlation between the spectrum of the acid species of amoxicillin
amox. B correspond to correlation between the spectrum of the basic species of amoxicillin
clavu. A correspond to correlation between the spectrum of the acid species of clavulanic acid
clavu. B correspond to correlation between the spectrum of the basic species of clavulanic acid

For the calibration step, we prepared 10 standards and repeated each standard twice. The parameters used to establish the calibration model were the sum of the areas of the acid and basic species of amoxicillin and the area of the basic species of clavulanic acid. Our results would be less reproducible if we considered the acid specie of clavulanic acid.

The results of the quantification are shown in Table 3. The standard deviation was obtained by analyzing six samples treated independently from the beginning. As in previous studies [6, 7], there was a slight discrepancy in the results —generally they were 15% higher than is indicated by the pharmaceutical itself. This discrepancy may arise because both amoxicillin and clavulanic acid have bacteriostatic activity, so a greater quantity than is indicated in the pharmaceutical is added. The relative standard

deviation was less than 5% for the determination of amoxicillin and less than 7% for the determination of clavulanic acid.

Table 3.- Determination of amoxicillin and clavulanic acid in pharmaceutical preparations.

	Amoxicillin		Clavulanic acid	
Sample	Present (mg)[a]	Found (mg) ± s (n=6)	Present (mg)[a]	Found (mg) ± s (n=6)
Augmentine	500	570 ± 24	125	134 ± 3
Normon	500	574 ± 28	125	144 ± 9
Augmentine Susp.	100	115 ± 3	12.5	11.8 ± 0.8
Clavucid	875	997 ± 13	125	117 ± 4

[a] indicated in the pharmaceutical

5. Conclusions

By generating a pH gradient using sequential injection analysis (SIA) with a diode-array spectrophotometric detector and later applying MCR-ALS techniques, we can resolve two species, amoxicillin and clavulanic acid, in pharmaceuticals.

These two substances have very similar acid-base properties and spectral responses. Resolving them therefore requires working with a wide interval of pHs. We therefore needed to use two reactants (HCl and NaOH) and make a detailed study of the experimental conditions that produce the optimal analytical sequence. The optimal analytical sequence is that which not only resolves the four species but also provides a suitable quality of resolution, measured by the degree of similarity between the spectra obtained in the resolution process and the spectra of the pure species. It is also measured by the lack of fit (lof) and the quantification error, which is measured by the relation of areas obtained by analysing two standards and a synthetic mixture and must be minimal.

Working under the optimal conditions, and after applying multivariate curve resolution to the augmented matrix made up of the sample and the reference

standards, we simultaneously quantified amoxicillin and clavulanic acid from the corresponding areas obtained from the concentration profiles.

This method has several advantages: in one single analysis the two analytes of interest are determined, the sample does not need pre-treatment, the reagents and instrumental system are easily acquired, the frequency of analysis is high (a sample per 3 minutes) and experimental cost in both the calibration and the quantification steps is low (1 ml per analysis.).

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3.5. Paper

"Sequential injection analysis linked to multivariate curve resolution with alternating least squares."

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Sequential injection analysis linked to multivariate curve resolution with alternating least squares

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Abstract

This paper discusses the potential of using sequential injection analysis (SIA) for generating second-order data. To treat these data, we used multivariate curve resolution with alternating least squares (MCR-ALS) as the chemometric tool. This combination can be used for both qualitative and quantitative analyses, since it provides concentration and spectra profiles for the various species. By combining these techniques (SIA-MCR-ALS), several analytes can be determined simultaneously in the presence of interferents without the need to pretreat the sample.

We describe the state of the art of both techniques by reviewing the bibliography since 2004 and the necessary conditions for applying chemometric tools to treat this type of data. We also discuss the advantages and disadvantages of this combined technique and examine their future prospects.

Keywords: Alternating least squares, MCR-ALS, Multivariate curve resolution, Second-order data, Sequential injection analysis, SIA.

1. Introduction

Since Ruzicka and Hansen [1] introduced the flow systems in 1975, many papers and reviews have used this technique, known as flow injection analysis (FIA) [2-8]. In 1990, Prof. Ruzicka [9] developed the second generation of flow systems, known as sequential injection analysis (SIA), in which the samples and reagents are introduced sequentially and with a double flow direction. Table 1 lists the number of reviews and tutorials that indicate the popularity of this technique and its scope of application.

Table 1.- Published reviews and tutorials about sequential injection analysis (SIA).

Focus	R/T[a]	Num.ref.[b]	Year	ref
Food analysis (Wine)	R	21	2004	[10]
Food analysis	R	105	2005	[11]
Monolithic columns	T	75	2003	[12]
Multicommutation based on solenoid valves	R	51	2002	[13]
Operational parameters of SIA	T	112	1998	[14]
Operational parameters of SIA	R	301	2002	[15]
Pharmaceutical	R	54	2003	[16]
Process analytical chemistry	R	25	1999	[17]

[a] review or tutorial
[b] number of references

One characteristic of the sequential flow system is that the reagents mix with the sample *via* an interdiffusion process. Depending on the experimental conditions, after passing through the detector, the reagent will have interacted totally or partially with the analyte. If the reagent has interacted totally, the analyte (A) will be converted into the reaction product (P). However, if the reagent has interacted partially, the following areas will be observed as it passes through the detector:

- the analyte that did not react (A);
- a mixture of P and A; and,
- a reaction product (P).

This characteristic, together with the versatility of the system in coupling to different types of detectors, enables data of different dimensions to be obtained. This means that various chemometric tools can be used to provide information about the system.

Figure 1 shows a scheme of the various possibilities. Figures 1 (a)-(c) represent a situation in which the chemical reaction has been completed before the sample passes through the detector. Figures 1 (d)-(f) represent a situation in which the chemical reaction is still taking place when the sample passes through the detector. There are three types of detectors, depending on their ability to provide data independently. Some detectors provide single data (e.g., the photometer), some provide vector data (e.g., the diode-array spectrophotometer (DAD)) and others provide matrix data (e.g., the excitation–emission spectrofluorimeter). The signal obtained is that provided by the detector over time. If the sample contains the same species, the only thing that changes over time is the concentration of the species due to the phenomenon of dispersion; the information obtained at two different times is the same. If, over time, different species pass through the detector, there is an effective change in the signal, which increases the dimension supplied by the detector by one. For this reason, Figures 1 (d)-(f) have one more dimension than Figures 1 (a)-(c).

The study of chemical systems using data obtained by flow techniques and later subjected to chemometric treatment has highlighted the usefulness of such techniques [18]. Worth mentioning in this respect are those by R. Tauler, S. Hernández-Cassou and J. Saurina [19-22] with FIA and MCR-ALS. The aim of this article - i.e., to focus on one flow system (SIA) and one type of data treatment (MCR-ALS) - is to show the possibilities of SIA for generating this type of data, the conditions necessary for applying this chemometric tool and the advantages and disadvantages of this type of

combination. We also discuss the state of the art of SIA and MCR-ALS by reviewing the bibliography since 2004 and examine their future prospects.

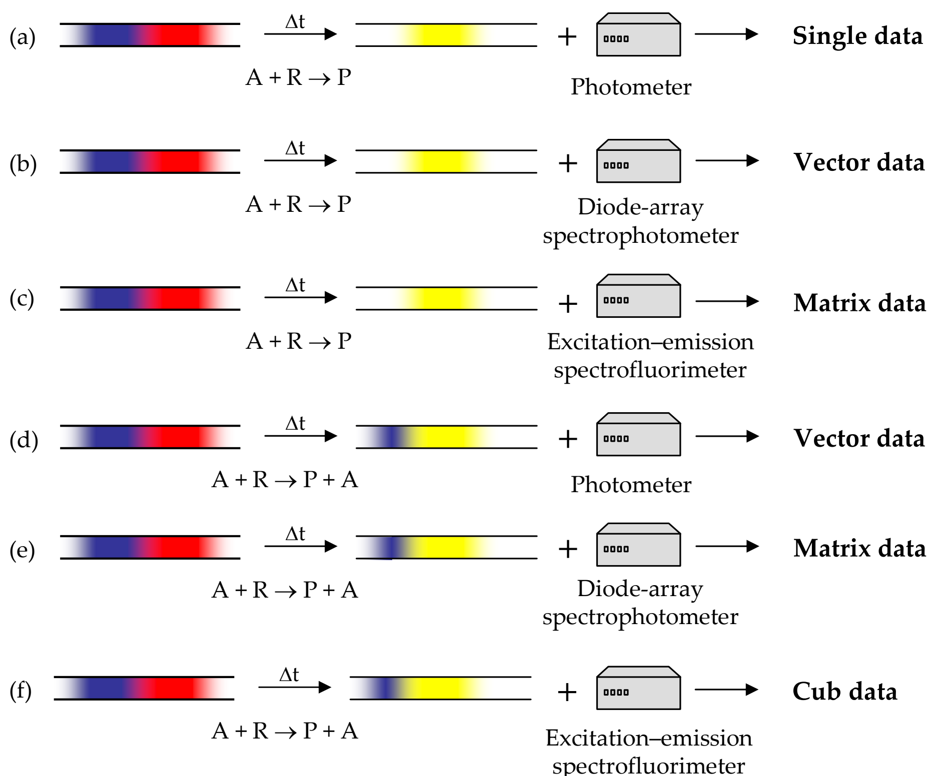

Figure 1.- Scheme of the different types of data that can be obtained by a sequential flow system and different types of detector. A, R y P stand for analyte, reagent and product, respectively.

2. Sequential injection analysis (SIA)

The classical configuration of SIA is shown in Figure 2. In a first step, a syringe pump aspirates the sample and the reagents *via* a valve that enables the desired reagent to be aspirated. In a second step, the syringe pump pushes the sample towards the detector through channels (in which the sample and reagents mix by dispersion) that join together the valve, the syringe and the detector. A computer controls the functions of every component.

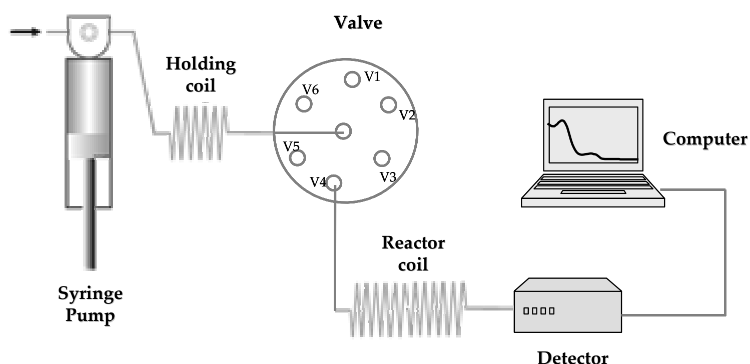

Figure 2.- Scheme of a sequential injection analysis system.

The advantages of SIA are that it can be easily automated and miniaturised, the cycles can be executed repeatedly, it is highly versatile because it can be adapted to suit most analytical instruments, the frequency of analysis can be high and the consumption of samples and reagents is low. Thanks to these advantages, SIA has been used in a wide range of applications.

To illustrate the state-of-the-art of this technique, Table 2 shows some studies published since 2004. The most widely studied applications have been in the pharmaceutical, environmental, food and bioprocessing fields. The chemical species studied were very diverse and the detector most often used was UV-VIS. In practically all cases, the data were of the type shown in Figure 1a - i.e., the chemical reaction was completed before it passed through the detector and a single datum was obtained per sample. In most cases, the sample was pre-treated.

Table 2.- Overview of recent applications of sequential injection analysis.

Focus	Analyte/sample	Detection system	Description	ref.
Alloys	Copper, zinc and lead in brass	UV-VIS	Method based on the catalytic effects of Cu^{2+}, Pb^{2+} and Zn^{2+} by $K_3Fe(CN)_6$	[23]
Bioprocess	γ-aminobutyric acid in human fluids	Fluorescence	On-line coupling of sequential injection with liquid chromatography	[24]
Bioprocess	Anti-oxidation/radical-scavenging activity	UV-VIS	Method based on the reaction of DPPH with antioxidants in organic or aqueous-organic medium	[25]
Bioprocess	Phosphate in urine	UV-VIS	Use of the molybdenum blue method	[26]
Bioprocess	Lead for Chinese citizens in blood	Fluorescence	Lead hydride was generated from acid solution, with potassium ferricyanide by reaction with alkaline tetrahydroborate	[27]
Pharmaceuticals	Differents thyroxine (L-T4, D-T4 and L-T3)	Amperometry	Simultaneous determination. L-T4 and L-T3 was detected by immunosensors and biosensor for the assay of D-T4.	[28]
Environmental	Formaldehyde in air	UV-VIS	Method based on the inhibitory effect of formaldehyde on the ethyl green (EG)-sulfite	[29]
Environmental	Calcium, magnesium and alkalinity in water	UV-VIS	The determination of both metals is based on cresolphtalein complexone and alkalinity is based on Bromcresol Green	[30]
Environmental	Simultaneous analysis of Np-237 and Pu	Mass spectrometry	Use of ICP with an automated sequential injection	[31]
Environmental	Aluminum in drinking water	Fluorescence	Solid phase extraction of aluminum on a chelating resin and detection of a complex formed between HQS and Al^{3+}	[32]
Environmental	Orthophosphate in wastewaters and urine	UV-VIS	Use of the molybdenum blue method	[33]
Environmental	Caffeine in coffee and beverages	UV-VIS	Method based on the SIA-solid-phase extraction on a molecularly imprinted polymer coupled on-line to HPLC	[34]
Environmental	Chloride and iodide in water	Potentiometry	Both ion concentrations is accomplished by titration with silver ions using the Gran's plot	[35]
Environmental	pH, chloride and nickel in electroplating baths	Potentiometry	Use of two ion-selective electrodes in a tubular configuration. Nickel concentrations were assessed using colorimetric detection	[36]
Environmental	Ca, Fe, Mn, Cu, Zn in a soil (CRM)	FAAS	Sequential injection system furnished with an extraction microcolumn	[37]
Environmental	Chloride and nitrate in waters	Potentiometry	Simultaneous determination of these two chemical species	[38]
Environmental	Lead in water	ETAAS	The concentration was assessed from its catalytic effect on the reduction reaction of resazurine by sulfide	[39]
Food	Urea in milk	UV-VIS and conductometry	The system is based on the coupling of gas diffusion separation and SIA	[40]

Table 2.- Continued.

Focus	Analyte/sample	Detection system	Description	ref.
Food	Polyphenol index in wines	UV-VIS	The determination is based on the Folin-Ciocalteu reaction	[41]
Food	Chloride in milk	Potentiometry	Milk was sandwiched between two silver nitrate plugs (titrant)	[42]
Food	Glycerol in wine	UV-VIS	This method is based on multicommutation and enzymatic reaction	[43]
Food	Nitrite and nitrate in cured meat	UV-VIS	Based on the Shinn reaction. Nitrate is previously in-line reduced to nitrite	[44]
Food	Vitellogenin (Vg) of carp	Chemiluminescence	Sandwich immunoreaction of anti-Vg monoclonal antibody on the magnetic beads, Vg, and the anti-Vg antibody are labeled with horseradish peroxidase (HRP)	[45]
Pharmaceuticals	Amoxicillin	UV-VIS	Determination was based on second-order calibration	[46]
Pharmaceuticals	Phenylephrine hydrochloride	UV-VIS	Based on the condensation reaction of the analyte with 4-aminoantipyrine in presence of potassium ferricyanide	[47]
Pharmaceuticals	Iron as Fe^{2+} in multi-vitamin preparations	UV-VIS	Use of the 1,10-phenanthroline as complexing agent	[48]
Pharmaceuticals	Piroxicam	Fluorescence	Method based on the europium reaction	[49]
Pharmaceuticals	Chloride	UV-VIS and Potentiometry	Chloride-selective membrane electrodes and optodes based on an indium^{3+} porphyrin	[50]
Pharmaceuticals	Fenoterol hydrobromide	UV-VIS	Method based on the reaction of fenoterol hydrobromide with 4-aminoantipyrine and potassium hexacyanoferrate	[51]
Pharmaceuticals	Lisinopril	Fluorescence	Method based on reaction of LSP with o-phthalaldehyde in the presence of 2-mercaptoethanol	[52]
Pharmaceuticals	Paracetamol	UV-VIS	Based on the on-line nitrosation with sodium nitrite in an acidic medium	[53]
Pharmaceuticals	Prazosin hydrochloride	Fluorescence	Develop of a fully automated flow system based on the coupling of the SIA technique	[54]
Pharmaceuticals	Bismuth	UV-VIS	Methylthymol blue was used as a color forming reagent	[55]
Pharmaceuticals	Magnesium	UV-VIS	Method is based on the reaction between o-cresolphthalein complexone and Mg^{2+}	[56]
Pharmaceuticals	Ascorbic acid in Vitamin C tablets	Chemiluminescence	Comparison of soluble manganese^{4+} and acidic potassium permanganate for determining ascorbic acid	[57]
Pharmaceuticals	Penicillin-G	Potentiometry	Different polymeric membranes (PVC, EVA) using Mn^{3+}TPP-Cl as electroactive material	[58]
Pharmaceuticals	S-enalapril, ramipril and pentopril	Amperometry	Use of immobilization of L-amino acid oxidase in carbon paste	[59]
Pharmaceuticals	Boron in water and pharmaceuticals	Fluorescence	Method based on the enhancement of chromotropic acid complexed with boric acid.	[60]
Pharmaceuticals	Aspartame in sweetener tablets	UV-VIS	Enzymatic conversion of aspartame by the a-chymotrypsin–alcohol oxidase	[61]

3. Second-order data

The aim of second-order data-treatment techniques is to decompose the raw matrix into the product of two matrices. One matrix provides information about concentration profiles and the other provides information about the spectra profiles of all the components that provide information of the system. If the data obtained from the configuration in Figure 1 (e) are considered, a matrix is obtained whose columns represent the wavelengths and whose rows represent the times at which the measurements were taken.

Figure 3 illustrates this decomposition graphically. In this Figure, **R** is the raw matrix, **C** is the matrix of the concentration profiles, which indicates the presence of the various species over time, $\mathbf{S}^T$ is the matrix of the spectra of the components, **E** is the matrix of the residuals, which provides information about the fit of the model, m and n are the number of spectra recorded over time and the number of wavelengths, respectively, and a is the number of species in the system that provide a signal.

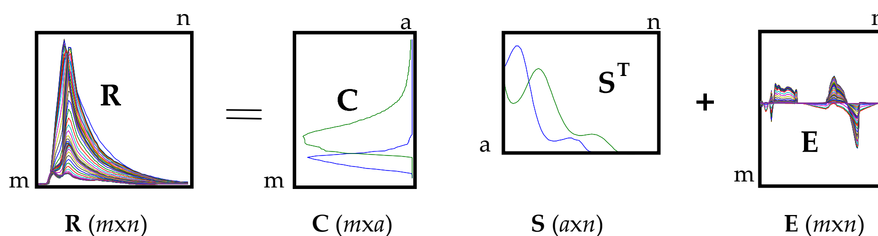

Figure 3.- Decomposition of the original matrix into the product of matrices.

Several second-order chemometric techniques can perform this decomposition. These are parallel factor analysis (PARAFAC [62]), multivariate curve resolution with alternating least squares (MCR-ALS [63]) and Tucker3 [64]. In this study, we focus on MCR-ALS. Unlike other techniques, MCR-ALS is highly flexible when defining the type of data because it enables us to work with trilinear data when two modes are in common (spectra and time) and with non-trilinear data when only one mode (the spectra) is in common.

Table 3.- Overview of recent applications of multivariate curve resolution with alternating least squares since 2004.

Focus	Objective	Detection system	Descripcion	ref.
Bioprocess	Study protein dynamics	FTIR	Comparation of resultats from the analysis of individual and augmented matrices.	[65]
Bioprocess	Study of the photocycle of bacteriorhodopsin	FTIR	Resolution of intermediate with time constant from the microsecond to the millisecond.	[66]
Environmental	Water in oil emulsions	Raman	Use of the varimax extended rotation to resolve image data. MCR-ALS was one step	[67]
Environmental	Analysis of 9,10-anthraquinone reduction in dimethylformamide	Electrochemical	Factor analysis revealed that three chemical components coexist, which can be attributed to diferents species of anthraquinone	[68]
Environmental	Study of the conversion of a bismaleimide during curing	FTIR	Identification of the curing temperature and the optimum experimental conditions	[69]
Environmental	Determination of pesticides and phenolic compounds in river and wastewater	DAD	Comparition of three second-order calibration algorithms GRAM, PARAFAC and MCR-ALS	[70]
Environmental	Modeling of contamination sources in surface waters	DAD	PCA, MCR-ALS, PARAFAC and Tucker3 was compared.	[71]
Environmental	Simultaneous determination of carbaryl and chlorpyrifos	DAD	Strategies such as PARAFAC and MCR-ALS have been employed.	[72]
Environmental	Analysis of complex biocide	DAD	Determination by LC under lack of chromatographic resolution and spectral selectivity	[73]
Food	Determination of benzoic and sorbic acids in juices	DAD	This method is based on a pH gradient within flow injection technique	[74]
Kinetics reaction	Study of the reaction between cisplatin and amino acid-nucleotide	NMR	The number and structures of the different species have been examined using MCR-ALS	[75]
Kinetics reaction	Characterize the stoichiometry of solvent molecules in the liquid phase	IR	Resolution of eight unique spectra: four mobile phase components, and four unique spectra of acetaldehyde in different environments.	[76]
Pharmaceuticals	Determination of amoxicillin	DAD	Study of optimum conditions for the determination	[77]
Polimers	Reaction of curing epoxy resins	NIR	Rank deficiency in reaction was solved by assembling an augmented column-wise matrix	[78]
Polimers	Reaction of curing epoxy resins	NIR	The reaction between phenyl glicidyl either and aniline (2:1) was studied at 95 ºC	[79]
Syntetic sample	Determination of 1-methyl-6,7-dimethoxy-3,4-dihydroisoquinoline	FTIR	This study is presented as an alternative method to HPLC.	[80]
Syntetic sample	Determination of stability constants of Cu2+-L-histidine	DAD	This multivariate analysis data treatment simultaneously reveals the species Cu, CuL, CuLH, CuL2, CuL2H, and CuLOH	[81]
Syntetic sample	Determination of aspirin/polyethylene	TOF-SIMS and Raman	Raman image of an aspirin/polyethylene, and a Raman image of polymer fibers in an epoxy.	[82]

Table 3 shows a review of the bibliography since 2004 in which MCR-ALS has been used. We can see that both the analytes and the type of signals used are very diverse and that the main fields of application are bioprocessing and the environment. The most often used detectors were the diode-array and the infrared spectrophotometer.

MCR-ALS provides the following information [83, 84]:

- *Qualitative.* This enables us to visualize the concentration and spectra profiles of the various species that are sensitive to the detector and contained in the sample. Sometimes this information allows us to solve the problem at hand (e.g., kinetics studies [19, 20, 78]). However, if our main objective is to quantify, it allows us to observe the presence of selective areas and so choose the type of calibration— univariate if there are no interferents or if there are selective areas —, or second-order [46].

- *Quantitative.* From the information obtained from concentration profiles (see Figure 3), MCR-ALS enables us to rebuild the response of the analyte in the presence of other substances, so an analyte can be quantified in the presence of interferents, or to make a simultaneous determination of several analytes. The analytes of known concentration are used as standards and the interferents do not need to be considered in the calibration step, which is a clear advantage over multivariate calibration [21, 22, 77].

Multivariate curve resolution with alternating least squares consists of two steps [85] (see Figure 4):

(i) *First estimations.* In a first step, we estimate the number of species of the sample that give a signal (*a*). The chemometric tool used for this process is principal component analysis (PCA) [86]. We then estimate the concentration ($\mathbf{C}'$) or spectral profiles ($\mathbf{S}^{T\prime}$) of these species. The most common techniques are evolving factor

analysis (EFA) [87] and simple-to-use interactive self-modelling mixture analysis SIMPLISMA [88].

(ii) *The optimisation process.* The next step, and the nucleus of the whole process, is to apply the ALS algorithm, which, from the initial estimation (**C′** or **S**$^{T\prime}$), searches for the optimal solution *via* iterations (**C** and **S**^{T}). In order for this solution is as close as possible to the one true solution, restrictions related to the studied system can be placed on this optimisation process. An example of these restrictions is non-negativity, which means that the mathematical solution cannot contain negative concentrations or negative absorbance [89].

Figure 4.- Scheme of the MCR-ALS optimisation process.

Correct application of MCR-ALS requires several conditions to be met [90]:

- The sample must be evolving when it passes through the detector (i.e., a data matrix must be available). In mathematical terms, the rows must not be linear combinations of each other. One way to check whether we have a data matrix or a vector is to calculate the determinant of the matrix, which must not be zero.

- Another important factor is the bilinearity of the system. Sometimes the data distance themselves from bilinearity, so the pure matrix of each analyte cannot be decomposed into the product of two matrices—the matrix of concentration profiles and the matrix of spectral profile — as in the following model:

$$\mathbf{R} = \Sigma\, c_i \cdot s_i^T \quad (1)$$

where **R** is the raw matrix, c_i are the vectors that define the concentration profile of the *i* species and s^T_i are the vectors corresponding to the signals of these species.

4. Limitations of SIA-MCR-ALS

Below we describe several limitations of SIA, MCR-ALS and the combined technique:

- To obtain the data in Figure 1 (c) — i.e., in evolution when passing through the detector — many variables are involved: flow, the length and dimension of the reactor tubes, and the volume and concentration of the sample and reagent. A previous experiment to establish the optimum conditions is therefore needed.

- The lack of bilinearity, which could be caused by the drift in the signal or the noise [90]. This problem is related to the type of detector used.

- The difficulty in correctly determining the number of analytes in the sample. This problem is intrinsic to all PCA decompositions—sometimes it is not clear how many principal components there are. This number is extremely important and strongly influences the result, so if there are doubts about the number of components, the process will continue by testing more than one value.

- Rank deficiency, which occurs when the number of important species considered is lower than the number of species in the sample. In most cases this is due to the fact that the concentrations and spectra profiles of the species are similar. To eliminate the effect of rank deficiency, we use augmented matrices, which involves adding one or more matrices with new information [91].

- The possible high number of species to resolve. The more species there are, the less likely it is that the algorithm will obtain the correct solution. So, the fewer variables there are in the system of equations, the easier it will be to obtain the correct solution.

- Rotational ambiguity, which causes MCR-ALS to find more than one solution. To make it easier to find just one solution, all the known information must be introduced into the system (spectra, selective zone and augmented matrices [92]).

The problems mentioned in relation to MCR-ALS have been discussed in several papers [90, 92].

5. Trends in SIA-MCR

5.1. From the instrumental point of view.

Since sequential injection systems were first introduced, several new techniques [93] based on SIA, such as micro-SIA-LOV [94], bead injection [95],

sequential injection chromatography (SIC) [13] and the multisyringe system [96] have appeared. Such techniques do not exclude the use of advanced chemometric methods.

5.2. From the second-order SIA point of view.

Combining SIA and MCR-ALS can be used to determine analytes in the presence of interferents. Many of the applications in Table 3 could then be adapted. This would avoid the need for pre-treatments or highly selective reagents.

5.2.1. Reaction kinetics studies.

The high degree of automation enables the SIA system to rapidly obtain reaction kinetics graphs and observe how variables such as temperature, concentration of reagents and stoichiometry affect the reaction kinetics.

5.3. From the data point of view.

Chemometric techniques that use date cubes per sample are in their early stages of development. Although techniques such as PARAFAC [62] are currently using cube data, which are obtained from combinations of matrices, we can expect a promising future for the vast amount of information contained in the cube data per sample.

6. Conclusions

SIA and MCR-ALS can be used independently to resolve various analytical problems in fields such as bioprocessing, the environment and pharmaceuticals.

Combining a chemometric tool such as MCR-ALS and SIA is an attractive technique for evaluating the composition of a chemical system and determining an analyte in the presence of interferents or for determining several analytes

simultaneously. The advantages of such a technique are that there is a high frequency of analysis, the system is completely automated and the consumption of the reagents is low. Another advantage, associated with the use of a chemometric tool, is that there is no need to use physicochemical methods to eliminate interferents or other analytes.

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4. Optimisation of analytical sequence using experimental designs. Response surface and simplex approach

4.1. INTRODUCTION

In previous chapter, we found that the most critical step in the development of analytical methods based on SIA and MCR-ALS was to obtain an analytical sequence that provides an evolving system. This is so that the species of interest will show a selective zone when the sample and reagent pass through the detector and the species studied can then be visualised. To do so, the values of the parameter associated with SIA and the chemical reaction must be studied in order to obtain satisfactory responses or direct the experiments forward the optimal response. In this chapter, we developed the method of experimental design to obtain the optimal analytical sequence. To do so, we wrote two papers.

In the first paper, entitled *"Optimisation by means of responses surface of an analytical sequence using a sequential injection system"*, we studied all the factors and analysed how they affect to the analytical sequence. We also proposed responses to quantitatively represent a good resolution. Once these factors and responses were proposed, we used a Plackett-Burman design to conduct factor screening and then modelled a response surface. In the maximum of response surface, the optimum conditions for the analytical sequence could be visualised. To transform several responses into a single response, we used the overall desirability function.

In the second paper, entitled *"Fractional factorial design and simplex algorithm for optimizing sequential injection analysis (SIA) and second-order calibration"*, we applied an alternative optimisation method knows as the simplex approach. We aimed to determine amoxicillin and clavulanic acid simultaneously when the number of factors and responses was higher than in the previous paper. For the factor screening, we used a fractional factorial design to study the interactions and direct the factors towards the optimal conditions in order to later apply the simplex approach.

4.2. Paper

"Optimisation by means of responses surface of an analytical sequence using a sequential injection system."

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Talanta, available online 12 September 2005

Optimisation by means of responses surface of an analytical sequence using a sequential injection system

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Abstract

An experimental design method was applied to determine the optimum working conditions for sequential injection analysis (SIA) to obtain second-order data that will be treated using multivariate curve resolution with alternating least squares (MCR-ALS).

The critical step is to design an analytical sequence that provides relevant information. This sequence depends on parameters related to the system, the chemical reaction and the chemometric treatment of the data. Also, from the multiple responses that quantify the quality of this analytical sequence, a single response is determined from the desirability function.

This method involves a factor screening step, in which both the global desirability function and the individual responses are considered and a response surface modelling step, in which the most relevant factors are considered.

Keywords: Experimental design, Desirability function, MCR-ALS, SIA, Amoxicillin, Responses surface.

1. Introduction

Currently available analytical systems generate various sizes of data: zero-order data, when the signal obtained for each sample is a single datum; first-order data, when a vector is generated; and second-order data, when a matrix is generated. These second-order data are very interesting because, using suitable chemometric treatment and from one analysis, we can obtain qualitative information about the sample or/and quantitative information about the analytes in the presence of interferents [1].

Several instrumental configurations provide second-order data e.g. chromatographic or flow techniques with amperometric detectors, UV-VIS with a diode-array spectrophotometer detector and fluorimetric spectrophotometric detector [2-5].

If we use sequential injection analysis (SIA) [6] with a diode-array spectrophotometric detector (DAD), the sample and reagent zones are sequentially aspirated into a channel using a selection valve to subsequently reverse the flow and transport the stacked zones into the detector. During the course of these operations, the zones undergo some mutual dispersion and the analyte interacts with the reagents, evolving into another species to obtain a matrix data.

In previous papers [7, 8], we observed that the critical step to determine amoxicillin in pharmaceuticals is to design an analytical sequence that not only provides second-order data (to generate an evolving system) but also, in the final results of the process, we have to visualize all the analyte and the quantification error must be optimum. Finding this analytical sequence depends on factors related to the system, the chemical reaction and the chemometric treatment of the data.

In this study, we will apply experimental design methods to find the optimum response for determining amoxicillin in pharmaceuticals. First, we screened the factors and with the selected factors, we made a central composite design to obtain a response

surface. To define the quality of the analytical sequence, we need to have several responses. To reduce the number of responses to only one, we use the desirability function. We use like a chemometric tool multivariate curve resolution with alternating least squares (MCR-ALS) [9]. Unlike other chemometric treatments, such as classical least squares (CLS), MCR-ALS does not need to know the composition of the interferents. This characteristic is known as the advantage of second-order data [10].

2. Experimental method

2.1. Reagents

We prepared amoxicillin and sodium hydroxide stock standard solutions by weighing the required amount of the respective compounds (amoxicillin from Sigma and sodium hydroxide from Prolabo) and dissolved them in purified water (from a Milli-Q water system from Millipore). The Pharmaceuticals drug was Augmentine (500 mg of amoxicillin per packet) from SmithKline Beecham, S.A.

2.2. Apparatus

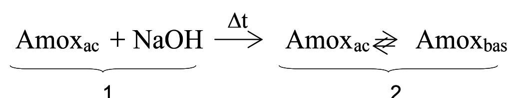
The sequential injection analyser comprised: CAVRO XL 3000 stepper motor-driven syringe pump connected to the PC with an RS-232 interface; A 6-position Eurosas EPS 1306 BPB automatic valve connected to the computer through a PCL-711S PC-Lab-Card; Omnifit PTFE tubing reaction coil: 70cm x 0.8mm; Holding coil: 200cm x 0.8mm; An HP8452A diode-array spectrophotometer controlled by an HP Vectra 5/75 computer equipped with an HP-IB IEEE 488 interface for communications; a Hellma 178.711QS flow-through cell. The spectra were recorded every 2 nm in the 220 to 340 nm range, with an integration time of 0.1s. The spectra measurements were taken every 0.7s.

2.3. Software

HP89531A software was used to record and store the spectra. Customized software was used to control the SIA. All calculations relating to MCR-ALS were performed with laboratory-written software under a MATLAB 5.3 computer environment [11]. This software is available from the authors [12]. The adjustment and optimisation of the response surfaces for the desirability function were done with NemrodW [13].

2.4. Chemical reaction [7, 8]

The acid-base properties of amoxicillin (pKa: 2.4, 7.4, 9.01, 10.29) and its spectral characteristics enable us to generate an evolving system that leads to a pH gradient between the pH of the aqueous solution of amoxicillin (pH=4.5) and basic pH. The spectra of amoxicillin in its acid ($amox_{ac}$) and basic ($amox_{bas}$) forms are shown in Figure 1b. The spectrum indicated by a dashed line (called the acid species of amoxicillin ($amox_{ac}$)) was obtained by measuring the amoxicillin in hydrochloric acid (pH=1), in water (pH=4.5) and in a buffer of NaH_2PO_4/Na_2HPO_4 (pH=7.5). The spectrum indicated by a continuous line (called the basic species of amoxicillin ($amox_{bas}$)) was obtained by measuring the amoxicillin in a buffer of NH_3/NH_4Cl (pH=10.1) and in sodium hydroxide (pH=13). The proposed chemical system is:



$$\underbrace{Amox_{ac} + NaOH}_{1} \xrightarrow{\Delta t} \underbrace{Amox_{ac} \rightleftarrows Amox_{bas}}_{2}$$

This system includes a dynamic part (step 1), which corresponds to the interdiffusion between NaOH and $amox_{ac}$, which involves a pH gradient into the reactor coil of the SIA, and an equilibrium part (step 2) between $amox_{bas}$ and $amox_{ac}$, which is established according to the pH in the various zones of the reactor coil.

3. Data Treatments

3.1. Application of MCR-ALS

The aim goal of MCR-ALS is to decompose the raw matrix, whose columns represent the wavelengths and whose rows represent the times at which the measurements were taken, into the product of two matrices; one matrix will give information about concentration profiles and the other matrix will give information about the spectra profiles of every component. The final results of applying MCR-ALS are shown in Figure 1. The chemometric tool used for this process is: principal component analysis (PCA) [14] to fix the number of species and simple-to-use interactive self-modelling mixture analysis (SIMPLISMA) [15] to make the initial estimation. We can also start ALS using the pure spectrum, but an initial estimation of the spectra of the interferents is needed.

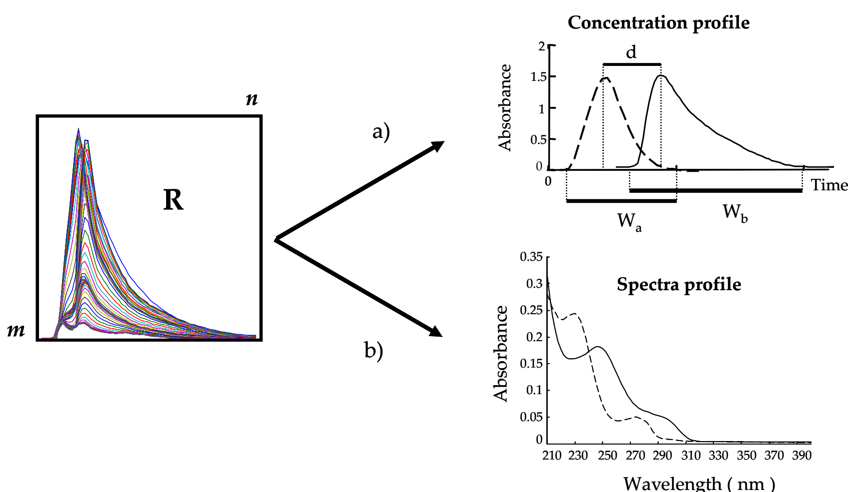


Figure 1.- Result obtained by MCR-ALS. **R** corresponds to raw matrix, where *m* is the number of spectra recorded over time and *n* is the number of wavelengths. a) Correspond to the matrix of concentration profiles and b) correspond to spectra profiles.

Depending on the nature and structure of the data, different constraints can be applied during the ALS optimisation. Another way to improve the resolution is to use

augmented matrices by columns, which involves adding one or more matrices that have one or two orders in common with new information [16].

3.2. Evaluation of the responses

To find an optimal analytical sequence, we must select a response, or responses, that reflect the quality of the results. In our study, we aimed to achieve a good resolution between species, but this qualitative response had to be transformed into quantifiable responses. The quantifiable responses we considered were:

1) The resolution of the concentration profile (*Rs*), which, because of its similarity to the chromatographic process, we evaluated using the following expression:

$$R_s = \frac{2 \cdot d}{W_a + W_b} \quad (1)$$

where d is the distance between the maxima of the peaks of the concentration profiles (Figure 1a) of the acid and basic species, and W_a and W_b are the widths of the two peaks.

2) The correlation between the spectra obtained in the resolution process and the spectra of the pure species.

3) The lack of fit of the model from experimental data was evaluated from the following equation.

$$lof = \sqrt{\frac{\sum_{i,j}\left(d_{ij} - \hat{d}_{ij}\right)^2}{\sum_{i,j} d_{ij}^2}} \quad (2)$$

where d_{ij} are the corresponding values of the raw data matrix and $\hat{d}_{ij}$ are the corresponding values calculated after the optimisation process (ALS).

4) The quantification error was evaluated from the following equation:

$$Error = \frac{\left| r_{exp} - r_{theo} \right|}{r_{theo}} * 100 \tag{3}$$

From the areas obtained in the resolution process with augmented matrices (samples + reference standard), we get the relative area r_{exp}:

$$r_{exp} = a_s / a_{rst} \tag{4}$$

where a_s is the area of the sample of amoxicillin and a_{rst} is the area of the reference standard. Ideally, r_{exp} should be equal to the relation between the concentration of the analyte in the sample (c_s) and the concentration of the analyte in the reference standard (c_{rst}). We thus define r_{theo}:

$$r_{theo} = c_s / c_{rst} \tag{5}$$

3.2.1 Desirability function [17, 18]:

When there are multiple responses to evaluate, an overall desirability function is suitable. The overall desirability function, D, is defined as the geometric mean, weighted or otherwise, of the individual desirability functions. The weight of the individual functions reflects the importance of each response. The expression that defines the overall desirability function is:

$$D = \sqrt[i]{d_1^{p1} \, x \, d_2^{p2} \, x \cdots\cdots d_i^{pi}} \tag{6}$$

where p_i is the weight of the response, i is the number of responses and d_i is the individual desirability function of each response obtained from the transformation of the individual response of each experiment. At this stage, the value d_i=1 is assigned when all the previous specifications are fully met and the value d_i=0 is assigned when

they are not. Values between 0 and 1 are obtained using a continuous function of the measured response.

3.3. Evaluation of the factors

The factors associated with the system and the chemical reaction were: (a) the flow, which directly influences the time spent in the channel and the interdiffusion of sodium hydroxide and amoxicillin, (b) the volume of sodium hydroxide and amoxicillin, which must be enough to produce the reaction and generate the pH gradient correctly, (c) the concentration of sodium hydroxide, since it affects the pH interval that can be obtained in the reactor. Also, the interval achieved must ensure the presence of the two species of amoxicillin, (d) the concentrations of amoxicillin in the sample.

The factors associated with MCR-ALS were: (i) the concentration of the reference standard, and (ii) whether to impose conditions of trilinearity when treating the data [18].

The choice of the domain of the quantitative factors can be evaluated from previous experiments. The experimental domain of these factors is shown in Table 1.

3.4. Experimental design [19].

3.4.1. Screening design

With a limited number of experiments, screening designs evaluate how a large number of factors affect the response. The most common screening designs are two-level fractional saturated designs and Plackett-Burman design. The effects of the factors can be evaluated using a Pareto chart, which shows important factors in the response in the form of a graph.

Table 1.- Factors and experimental domain.

Factors	Domain	
	Low	High
V. Amox [a]	0.13	0.22
V. NaOH [b]	8	42
[amox] [c]	50	300
[NaOH] [d]	0.01	0.5
Flow [e]	0.5	2.5
Type of data	Trilinearity	No-trilinearity
Ref. Stand [f]	50	300

[a] volume of amoxicillin ml
[b] volume of sodium hydroxide ml
[c] concentration of amoxicillin μg/l
[d] concentration of sodium hydroxide mol/l
[e] flow ml/min
[f] concentration of amoxicillin in reference standard μg/l

3.4.2. Central composite design

The second-order polynomial model is usually suitable for estimating the experimental response and finding the optimal point. One of the designs that can be used to optimise the second-order response surface is the central composite design. This type of experimental design includes a full factorial 2^k (where k is the number of factors), a series of replications in the centre and points centred on the faces with a pre-determined axial distance.

4. Results and discussion

Table 2 shows the responses of our experiments when we applied Plackett-Burman design. It indicates the maximum and minimum values got of each response that fixes the range of the factors in the responses. For responses such as lack of fit or quantification error, the maximum value of response is assigned the value of d_i=0 and the minimum value of response is assigned the value of d_i=1. For responses such as the resolution of the concentration profile, the correlation between the spectra obtained in

the resolution process and the spectra of the pure species for acid and basic species, the maximum value of response is assigned the value of d_i=1 and the minimum value of response is assigned the value of d_i=0. The individual desirability values for experimental values between these limits are linear between 0 and 1. Overall desirability function was obtained from equation (6). We considered that all the responses were equally important so, to obtain overall desirability, they were not weighted.

From the overall and individual desirability functions, we obtained the Pareto chart, which shows the most important factors. Figure 2 shows the Pareto chart with the overall desirability function. We can see that the only important factor was the volume of the sample with a p-value of 10%. The next two factors (volume of NaOH and flow) had p-values of 30%. However, when we studied the experimental responses individually, we found that the p-values of these two factors were around 10%. For the volume of NaOH, the responses were the lack of fit and the correlation between the basic spectra. For the flow, the responses were the lack of fit and the correlation between the acid spectra and the resolution.

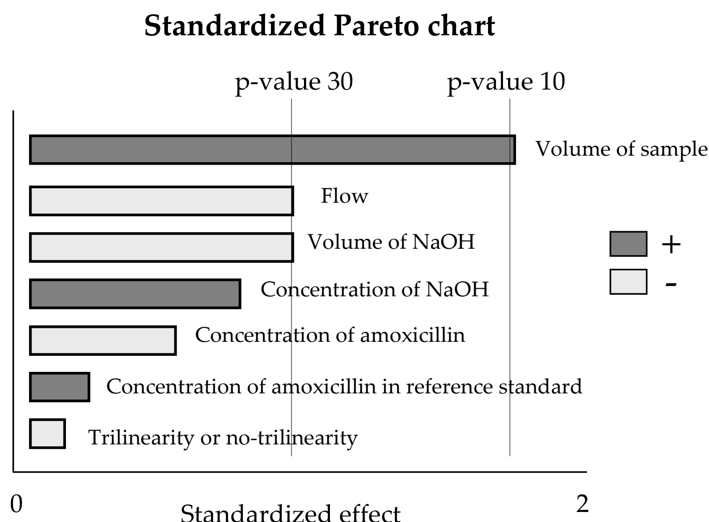


Figure 2.-. Pareto chart of the overall desirability function from the responses in the Plackett-Burman experiments.

Table 2.- Responses of Plackett-Burman design.

Exp. Num.	Lof [a]	Quan. E. [b]	Rs [c]	Cor. Acid [d]	Cor. Basic [e]
1	**2.4**	0.6	0.13	**0.998**	**0.991**
2	3.7	**0.3**	0.15	0.967	0.988
3	5.6	31.3	0.13	0.700	0.979
4	2.9	31.6	0.07	0.996	0.849
5	8.2	3.2	0.20	0.975	0.991
6	4.6	36.8	**0.04**	0.942	**0.441**
7	**16.2**	15.0	0.28	0.983	0.946
8	13.7	24.0	0.26	0.984	0.931
9	9.2	1.0	**0.29**	0.919	0.989
10	3.7	0.4	0.26	0.991	0.975
11	7.5	**40.0**	0.08	**0.656**	0.966
12	4.4	1.9	0.23	0.991	0.831

[a] lack of fit of model
[b] quantification error
[c] resolution of the peak
[d] correlation between the spectra of acid specie obtained in the resolution process and the spectra of the pure acid species
[e] correlation between the spectra of basic species obtained in the resolution process and the spectra of the pure basic species

As an example, Figure 3 shows the spectra and concentration profile we obtained after applying MCR-ALS. Figure 3a shows the result of a bad resolution and Figure 3b shows the result of a good resolution. At the top of each figure, we can see the concentration profile (augmented matrix made up of the augmentine sample and a standard of 60 mg/l). At the bottom of each figure, we can see the pure spectra.

To evaluate the response surface, we considered the first three factors in the Pareto Chart. For the other factors, we fixed those that provided the best values for the overall desirability function (the concentration of sodium hydroxide was 0.5 mol/l, the concentration of amoxicillin was 50 µg/l and the concentration of reference standard was 300 µg/l) and imposed the no trilinearity condition on the data.

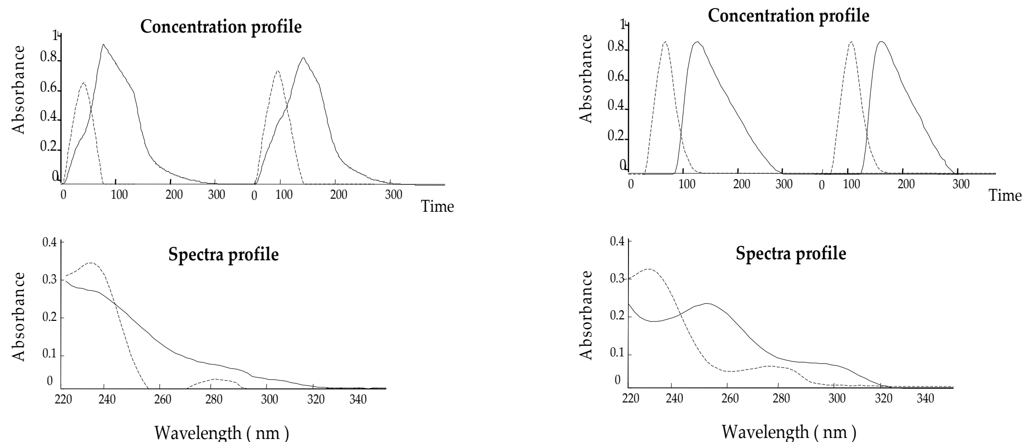


Figure 3.- Results obtained after applying MCR-ALS (a) a bad resolution and (b) a good resolution.

With the chosen factors we carried out an experiment corresponding to a central composite design 2^3, with four replications in the central point and with the points concentrated on the faces with an axial distance of one. See Table 3, where the first column is the number of experiments, the next three columns corresponds to the previously selected factors (flow, volume of sodium hydroxide and volume of amoxicillin, respectively) and the last five columns correspond to the responses. Experiments 15-18 were performed in the central point, which enabled us to estimate the experimental error.

After obtaining the results, we transformed the responses into individual desirability. The conditions for obtaining these values are given in Table 4. The first column indicates whether we wished to maximise or to minimise each of the responses. The next two columns show the experimental maximum or minimum values of the responses. The next two columns show the upper and lower limits we chose to apply the desirability function. These limits are more restrictive than the experimental ones because the aim is not to show the influence of the factors but to establish a response surface in a range where the global desirability function will be optimum.

Table 3.- Experiments and responses of the central composite design.

Exp. num.	Flow	V. NaOH	V. amox	Lof	Quan. E.	Cor. Basic	Cor. Acid	Rs
1	0,5	8	0,13	14,2	1,53	0,98	0,27	0,07
2	2,5	8	0,13	10,7	1,82	0,95	0,34	0,23
3	0,5	42	0,13	16,6	0,40	0,98	0,97	0,26
4	2,5	42	0,13	5,9	1,23	0,98	0,98	0,32
5	0,5	8	0,22	19,3	1,70	0,97	0,77	0,32
6	2,5	8	0,22	11,3	2,00	0,98	0,81	0,29
7	0,5	42	0,22	14,3	2,43	0,98	0,96	0,30
8	2,5	42	0,22	5,2	1,01	0,98	0,97	0,22
9	1,5	25	0,22	8,5	1,97	0,98	0,92	0,17
10	1,5	25	0,13	10,3	13,17	0,99	0,95	0,26
11	1,5	42	0,18	7,9	7,70	0,99	0,97	0,23
12	0,5	25	0,18	18,3	1,87	0,98	0,94	0,34
13	1,5	8	0,18	18,1	8,98	0,99	0,75	0,32
14	2,5	25	0,18	7,7	1,41	0,98	0,95	0,25
15	1,5	25	0,18	9,1	0,60	0,99	0,94	0,22
16	1,5	25	0,18	8,9	0,61	0,98	0,93	0,25
17	1,5	25	0,18	9,1	0,61	0,98	0,93	0,22
18	1,5	25	0,18	9,2	0,62	0,98	0,93	0,21

Table 4.- Limits of experimental values for applying desirability function.

		Response range [a]		Transformation range [b]	
	Goal	Low	High	Low	High
Cor. Acid	Max.	0.950	0.992	0.970	0.990
Cor. Basic	Max.	0.270	0.980	0.970	0.990
Lof	Min.	5.200	19.280	6.000	14.000
E. quant	Min.	0.018	13.170	4.000	10.000
Rs	Max.	0.070	0.340	0.240	0.290

[a] experimental responses range
[b] transformation of experimental responses range

After setting these limits, we calculated the overall desirability function for each experiment. We adjusted these desirability functions to a response surface that provides an equation depending on three factors and where the response will be the overall desirability function. The mathematical equation that represents this response surface is:

$$y = 0.458 + 0.072x_1 + 0.556x_2 + 0.295x_3 - 0.014x_1x_2 - 0.021x_1x_3 - 0.421x_2x_3 - 0.314x_1^2 - 0.036x_2^2 - 0.05x_3^2$$

where y is the global desirability function, x_1 is the flow, x_2 is the volume of NaOH and x_3 is the volume of the sample. To graphically represent this equation, we have to fix a factor, e.g. the volume of amoxicillin, and represent a response surface (see Figure 4) for the flow and volume of sodium hydroxide factors.

As the maximum of this response surface represents the highest value of the overall desirability function, we have the optimum conditions. These high values of overall desirability function are achieved for high values of flow and volume of sodium hydroxide. If the value of the overall desirability function is zero, and the values of flow and volume of sodium hydroxide are low, at least one of the responses is outside the interval permitted.

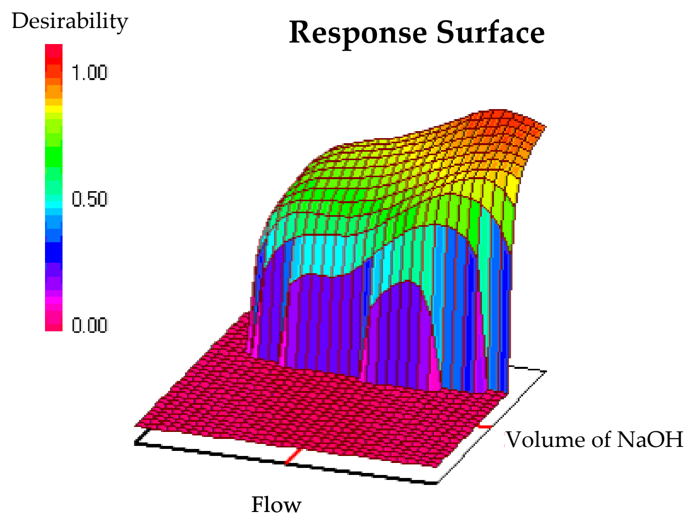


Figure 4.- Response surface (desirability) for volume of NaOH and flow. The third factor, the volume of amoxicillin, is 0.175 ml.

5. Conclusions

For a system made up of SIA and MCR-ALS, an attractive way to find an optimal analytical sequence that can be used to carry out a quantitative or qualitative analysis of the sample is to make a response surface. To obtain this response surface, we need to use a correct experimental design and a desirability function.

Two important steps are: (i) to choose the correct responses that reflect the quality of the results and transform these responses into quantifiable responses, and (ii) to define the experimental domain and correctly set the values of desirability.

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4.3. Paper

"Fractional factorial design and simplex algorithm for optimizing sequential injection analysis (SIA) and second-order calibration."
A. Pasamontes, M. P. Callao
Chemometrics and Intelligent Laboratory Systems submitted,
22 July 2005

Fractional factorial design and simplex algorithm for optimizing sequential injection analysis (SIA) and second-order calibration

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Abstract

In this study, we report a method for finding an analytical sequence that allows to simultaneously determine two analytes with very similar physical and chemical characteristics, such as amoxicillin and clavulanic acid, using sequential injection analysis (SIA) with a diode-array spectrophotometric detector and multivariate curve resolution with alternating least squares (MCR-ALS).

This method comprises two stages. In the first stage, a fractional factorial design (2^{6-2}) is carried out in order to address the responses, reduce later experimentation and study how the factors, though confounded, interact with each other. In the second stage, optimisation is carried out using the simplex algorithm.

This method has advantages over response surface modelling, for example, when the number of significant factors is high. If simplex is begun in a suitable zone, the number of experiments may be considerably reduced.

Keywords: Fractional factorial design, Simplex algorithm, MCR-ALS, SIA, Amoxicillin, Clavulanic acid.

1. Introduction

To research the optimal conditions in chemical experimentation, normally we just consider one response and one can use the "one factor-at-a-time strategy". With this strategy, one variable is changed while the others are held constant. When the best response is found, this value is held constant and another variable is studied, and so on. This strategy is time consuming and depends on the starting conditions and the number of factors to optimise.

Another type of experimentation is to use experimental design [1]. Experimental design for optimisation can involve (i) designing an experiment to model the response in an experimental domain to obtain a response surface [2] or (ii) finding the optimum conditions without having to model the experimental domain using a sequential technique such as simplex [3]. Previously, if the number of factors is high, a screening design [4] is recommended and if the number of responses is high, a desirability function is recommended because the information contained by each response can be transformed into one response.

If factor screening were carried out, twice as much information would be obtained. First, all the significant factors would be evaluated in order to conduct experiments with them alone and minimise subsequent experimentation. Second, the direction in which these factors obtain the best responses would be determined. With this information, choosing a simplex optimisation would enable us to begin in a good area, obtain the optimal responses with few experiments, and minimise the chance that simplex will be in a local optimum.

Sequential injection analysis with a diode-array spectrophotometric detector is an analytical system for obtaining second-order data that can be chemometrically treated to obtain information about the concentration and spectra profiles of all species [5-7]. Our research group has been developing analytical methods for determining amoxicillin [8, 9] and clavulanic acid [10] in pharmaceuticals using sequential injection analysis (SIA) and multivariate curve resolution with alternating least squares (MCR-

ALS). This analytical system incorporates multiple factors associated with both chemometric and instrumental tools and the number of responses to consider is high. If response surface is carried out and the number of important factors is greater than four, the number of experiences increases considerably.

The aim of this study is to apply an optimisation method for simultaneously determining clavulanic acid and amoxicillin in pharmaceuticals. These species have similar chemical properties and spectra. Our method involves factor screening. To design the screening, we used fraction factorial designs because these provide information about the main effects with few experiments as well as information about interactions. With a suitable selection of the generators of the design, all the main effects are confounded with higher order interactions. We then optimised the analytical sequence using simplex. The response used will be the global desirability function.

2. Experimental

2.1. Reagents

We prepared amoxicillin, clavulanic acid, sodium hydroxide and hydrochloric acid stock standard solutions by weighing the required amount of the respective compounds (amoxicillin from Sigma and sodium hydroxide and hydrochloric acid 37% from Prolabo) and dissolved them in purified water (from a Milli-Q water system from Millipore). Clavulanic acid was supplied by Laboratorio Reig Jofré S.A. Clavucid (875 mg of amoxicillin and 125 mg of clavulanic acid per packet) was supplied by Recordati. Carrier was purified water from a Milli-Q water system.

2.2. Apparatus and analytical process

The sequential injection analyzer (see Figure 1) comprised: CAVRO XL 3000 stepper motor-driven syringe pump connected to the PC with an RS-232 interface; a 6-position Eurosas EPS 1306 BPB automatic valve connected to the computer through a PCL-711S PC-Lab-Card; Omnifit PTFE tubing reaction coil: 70cm x 0.8mm; holding coil: 200cm x 0.8mm; an HP8452A diode-array spectrophotometer controlled by an HP Vectra 5/75 computer equipped with an HP-IB IEEE 488 interface for communications; and a Hellma 178.711QS flow-through cell.

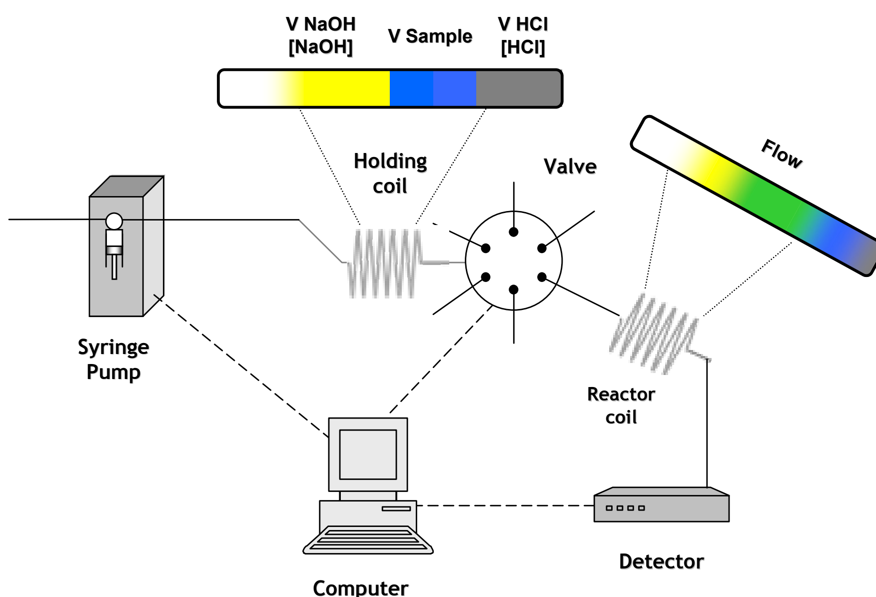

Figure 1.- Sequential injection analyzer and the analytical process. White corresponds to the carrier (water), blue corresponds to the pharmaceutical, yellow corresponds to sodium hydroxide, grey corresponds to hydrochloric acid and green corresponds to the product.

The analytical process is amplified in the same figure. In the first step, sodium hydroxide, sample and hydrochloric acid are aspirated towards the syringe. The factors that influence the registered signal are the volumes and concentrations of the various reagents and the volume of the sample. The reagent and sample are then pushed towards the detector through the reactor coil. During these operations, the zones undergo some mutual dispersion and the sample interacts with the reagents.

One factor that may influence the signal in this step is the flow. When the sample reaches the detector, all the spectra are recorded every 2 nm in the 220 to 340 nm range, with an integration time of 0.1s every 0.7s. As response we obtain a data matrix whose columns are the SIA peaks at a specific wavelength and whose rows are spectra recorded at a specific time.

2.3. Chemometric tools and software

We applied multivariate curve resolution with alternating least squares (MCR-ALS). The chemometric tools included in MCR-ALS are: principal component analysis (PCA) [11] and simple-to-use interactive self-modelling mixture analysis (SIMPLISMA) [12]. The final step is an optimisation process with alternating least squares (ALS) [13] in which different constraints can be applied during the ALS optimisation [14]. To delete rank-deficiency, we worked with augmented matrices [10].

All MCR-ALS calculations were performed with laboratory-written software under a MATLAB 5.3 computer environment [15]. The data were analysed with NEMRODW [16]. MCR was implemented through MATLAB [17].

2.4. Responses and desirability function

Figure 2 shows the decomposition of the experimental data matrix after MCR-ALS was applied. We obtained one matrix of concentration profiles, one matrix of spectra profiles and a residual matrix containing the signals not contemplated by the model. For the concentration matrix, the response considered for optimizing the analytical sequence was the distance between the peaks of two species, since greater selectivity (greater distance) leads to better results in the resolution process. This distance was calculated from the difference between the times at which the peaks of the two species were maximal. The quantification error was measured from the relationship between the areas obtained when two standards and a sample were analysed. For the spectra matrix, the response considered was the correlation between

the spectra obtained in the resolution process and the spectra of the pure species. Finally, for the matrix of residuals it was the lack of fit of the model.

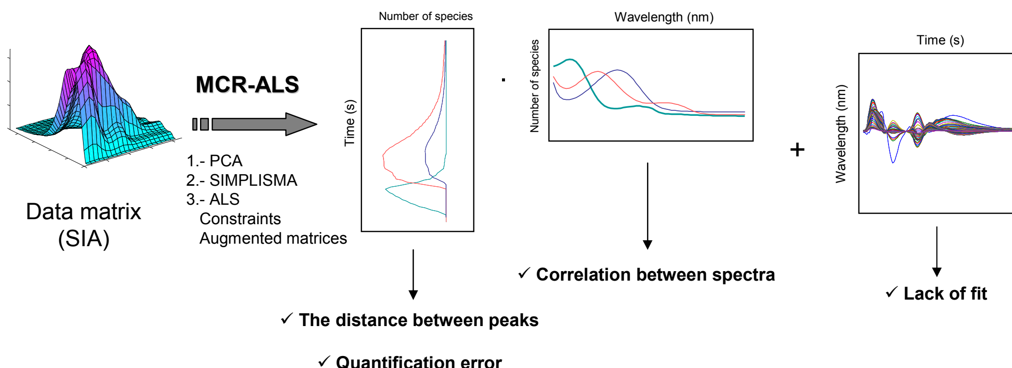

Figure 2.- Decomposition of the experimental matrix after MCR-ALS is applied.

Each experimental response was transformed into desirability values, in accordance with the scheme in Figure 3. Figure 3a illustrates the situation in which the optimal responses have a high experimental response and Figure 3b illustrates the situation in which the optimal responses have a low experimental response. For each experimental response, we must determine which values correspond to $d_i=0$ and $d_i=1$ in the individual desirability function. Values between 0 and 1 are obtained using a continuous function of the measured response. Once the individual desirability function has been established, the next step is to calculate the overall desirability function, D. This is defined as the geometric mean, weighted or otherwise, of the individual desirability functions:

$$D = \sqrt[i]{d_1^{p1} \, x \, d_2^{p2} \, x \cdots\cdots d_i^{pi}} \qquad (1)$$

where p_i is the weight of the response, i is the number of responses and d_i is the individual desirability function of each response.

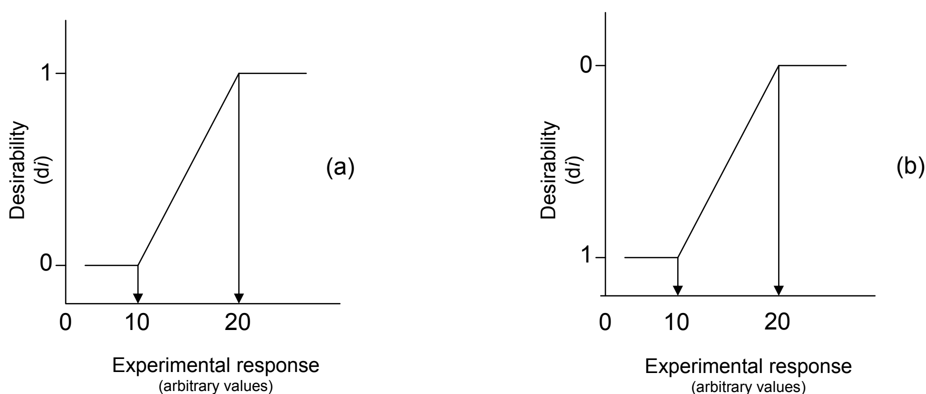

Figure 3.- Individual desirability functions constructed (a) when wishing to maximise the response and (b) when wishing to minimise the response.

3. Results and discussion

In the factor-screening stage, we decided to work with a 2^{6-2} design. Table 1 shows the factors, their codified name and their corresponding domains.

Table 1.- Factors and experimental domain.

		Domain	
Factors	Code	Low	High
V. sample [a]	A	0,05	0,2
V. NaOH [b]	F	0,03	0,08
V. HCl [c]	B	0,03	0,08
[NaOH] [d]	E	0,1	1
Flow [e]	C	0,5	4
[HCl] [f]	D	0,1	0,5

[a] volume of sample (ml)
[b] volume of sodium hydroxide (ml)
[c] volume of hydrochloric acid (ml)
[d] concentration of sodium hydroxide (mol/l)
[e] flow (ml/min)
[f] concentration of hydrochloric acid (mol/l)

Table 2 shows how the factors were confounded. We considered that three- (or more) factor interactions are neither likely nor important, so we were able to determine the main effects without these being confounded. As two-factor interactions are always confounded which each other, it is difficult to determine their influence independently.

Table 2.- Model matrix corresponding to a 2^{6-2} design.

Aliases
A = BCE = DEF = BCDF
B = ACE = CDF = ABDEF
C = ABE = BDF = ACDEF
D = AEF = BCF = ABCDE
E = ABC = ADF = BCDEF
F = ADE = BCD = ABCEF
AB = **CE** = ACDF = BDEF
AC = **BE** = ABDF = CDEF
AD = **EF** = BCDE = ABCF
AE = **BC** = DF = ABCDEF
AF = **DE** = BCEF = ABCD
BD = **CF** = ACDE = ABEF
BF = **CD** = ACEF = ABDE
ABD = ACF = BEF = CDE
ABF = ACD = BDE = CEF

To study the most significant factors, we used the standardized Pareto Chart of each response. In general, for a 95% confidence interval the significant factors in each individual response depended on the response considered. Table 3 is a summary of these results. The first column shows the responses affected by some factor. The second column shows the main effects (or two-factor interactions) on the responses. The third column shows the sign of the factors.

The only factor that did not affect any of the responses within the experimental domain considered was the volume of hydrochloric acid. The significant factors were the volume of the sample, the volume of sodium hydroxide, the flow and the

concentration of sodium hydroxide. The concentration of hydrochloric acid in some interactions was also important, though this was confounded with two-factor interaction. We studied whether some two-factor interactions could be considered *a priori* not to have chemical significance but we were not able to confirm this hypothesis. We therefore considered whether the concentration of hydrochloric acid could have an influence due to its interaction with the volume of the sample or to the concentration of sodium hydroxide.

Table 3.- Summary of the significant factors and their signs.

Responses	Importants factors	Sign [e]
Lof [a]	V. sample	+
d(aa-ab) [b]	Flow	-
Cor. A bas [c]	V. NaOH	-
	V. sample	+
	V. sample·[HCl] / V. NaOH·[NaOH]	-
	V. Sample·V. NaOH / [NaOH]·[HCl]	+
Cor. Clavu [d]	[NaOH]	+
	V. sample·[HCl] / V. NaOH·[NaOH]	-

[a] lack of fit of the model
[b] distance between the maximum of the acid species and the maximum of the acid species of amoxicillin
[c] correlation between the spectra of the basic species of amoxicillin obtained in the resolution process and the spectra of the pure acid species
[d] correlation between the spectra of the basic species of clavulanic acid obtained in the resolution process and the spectra of the pure acid species
[e] the sign of each factor

From these results, we selected the simplex starting zone. The first vertex and the step size are shown in Table 4. In accordance with the sign of the factor, the experimental values of the flow and volume of sodium hydroxide were set at low values of the domain. However, the flow was adjusted to 1 ml/min since the instrumental limit of the flow was 0.5, which would limit the movement of simplex to lower values. The volume of the sample and the concentration of sodium hydroxide were set at high values of the domain.

Table 4.- Values of the first vertex and step size for each factor.

Factors	First vertex	Step size
[HCl]	0,1	0,3
Flow	1	0,4
[NaOH]	1	0,3
V sample	0,2	0,016
V NaOH	0,03	0,016

The effect of the concentration of hydrochloric acid has not been unambiguously determined. However, we decided to begin with a low concentration because if this effect were important, this would be the most suitable concentration. The volume of hydrochloride acid was set at the average value for the domain (0.058 ml). The step size for each factor was selected so that the changes in the experimental conditions from one vertex to another would be effective i.e. so that they would not be comparable to the experimental random error. The analytical sequence used for the reference standards was the same as for the sample.

The transformations of the experimental responses into individual desirability functions are shown in Table 5. Depending on the kind of responses, the main aim will be to minimise or maximise them and the range of experimental responses will be different. To calculate the overall desirability function, we assigned the same weight to all the responses.

Table 6 shows the evolution of simplex. Note that, as expected, in the initial simplex all vertices except number 4 provided high overall desirability values. The value for vertex number 4 is explained from the definition of desirability function itself, since just one individual desirability value below the limits, however slightly, leads to values of zero in the overall desirability function. Simplex stops when one value (in our case, experiment 6) is maintained k+1 times in the geometric figure after it has been checked that this value is not an outlier. To check that this core was an outlier, we repeated the experiment three times and checked that the results were repetitive.

Table 5.- Experimental values and limits for applying desirability function.

Responses	d_i[d]	Exp. value[e]	d_i[d]	Exp. value[e]
Spectra correlation[a]	0	0,950	1	0,990
Distance peak[b]	0	2,000	1	5,000
Lof	1	5,000	0	12,000
Quant. Error[c]	1	4,000	0	20,000

[a] correlation between the spectra obtained in the resolution process and the spectra of the pure acid species
[b] distance between the maximums (λ)
[c] quantification error (%)
[d] value of individual desirability
[e] experimental values

The best results were for experiments 6 and 8. Both conditions provided the same overall desirability value but we can select one of these by studying the individual desirability values. Table 7 shows these values. We can see that experiment 6 provided good results for the quantification responses and that experiment 8 provided good results for the fit of the model. As our aim was to find an analytical sequence for simultaneously quantifying clavulanic acid and amoxicillin, it was more important to obtain good quantification results than to obtain a good fit, so we selected experiment 6. The other responses gave individual desirability values of 1.

Table 6.- All simplex algorithm experiments.

Exp. Num.	[HCl]	Flow	[NaOH]	V NaOH	V sample	D_{global}
1	0,10	1,0	1,00	0,030	0,200	**0,936**
2	0,38	1,1	1,07	0,036	0,203	**0,945**
3	0,17	1,4	1,07	0,036	0,203	**0,935**
4	0,17	1,1	1,28	0,036	0,203	**0,000**
5	0,17	1,1	1,07	0,048	0,203	**0,835**
6	0,17	1,1	1,07	0,036	0,215	**0,979**
7	0,23	1,2	0,83	0,040	0,206	**0,938**
8	0,25	1,2	0,95	0,025	0,208	**0,979**
9	0,28	0,8	0,90	0,032	0,210	**0,945**
10	0,42	1,2	0,93	0,035	0,216	**0,832**
11	0,37	1,0	1,13	0,026	0,215	**0,865**

Table 7.- Values of individual desirability for experiments 6 and 8.

	Exp. 6	Exp. 8
d_i (Lof)	0,827	1,000
d_i (err. A)	1,000	0,826
d_i (err. C)	1,000	1,000

4. Conclusions

Using a fractional factorial design and a simplex algorithm for sequential injection analysis linked to multivariate curve resolution with alternating least squares is an attractive chemometric tool for finding an optimal analytical sequence with which to simultaneously quantify two species with very similar physical and chemical proprieties because a high number (over 4) of significant factors leads to an optimum with few experiments.

Moreover, by using a fractional factorial design in the factor-screening stage we can address the factors to reduce the number of experiments in the simplex. Also, unlike with a Plackett-Burman design, we can obtain information about the interactions even if they are confounded.

Using the desirability function we were able to transform several responses into a single response and therefore to use simplex.

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5. Conclusions

5.1 CONCLUSIONS

In general, we conclude that a combined sequential injection analysis with a multivariate detector (i.e. diode array spectrophotometer) and multivariate curve resolution with alternating least squares can be used for both qualitative and quantitative analyses since, it provides concentration and spectra profiles for the different species of the sample. To make this possible, an evolving system must be generated in the SIA reactor so that, when absorbance is measured at different wavelengths and for the different times in which the sample passes through the detector, a data matrix is obtained. Also, the species present in the system must have a certain degree of selectivity with respect to the response of the detector.

In relation to the objectives, we outlined in chapter 1, our conclusions are as follows:

1. Develop and study the experimental conditions to obtain second-order data.

a) We study all the instrumental and chemical reaction factors (flow, volume and concentration of sample and reagents) that can influence the analytical signal.

We have studied both the chemical variables (reagents, volume of sample and volume of reagents) and the instrumental variables (flow, range of wavelengths) of the system in order to establish an analytical sequence with which to obtain second-order data. Given the acid-base nature of the analytes, this evolving system is achieved by generating a pH gradient. We conclude that when we wanted to determine amoxicillin with only one reagent (sodium hydroxide), we can achieve a suitable analytical sequence. To determine amoxicillin and clavulanic acid simultaneously, however, we have to work with a wide range of pH and use two reagents (hydrochloric acid and sodium hydroxide) in order to produce optimal results.

b) We study multivariate curve resolution with alternating least squares (MCR-ALS) and the techniques involved in the resolution process e.g. principal analysis components (PCA), simple-to-use interactive self-modelling mixture analysis SIMPLISMA or evolving factor analysis (EFA), and alternating least squares (ALS) and its constraints.

We have studied all the chemometric tools involved in MCR-ALS. We conclude that, for the problem discussed, there were no significant differences between making a first estimation using EFA or using SIMPLISMA and that the constraints to apply are: (i) correspondence between common species in different matrices; (ii) non-negativity for concentration profiles and spectra; and (iii) unimodality for concentration profiles. Also, imposing non-trilinearity at the resolution stage led to better values of lack of fit (lof).

2. Develop analytical methods to determine amoxicillin in the presence of interferents, and amoxicillin and clavulanic acid simultaneously in pharmaceuticals.

In section 3.2, we discussed the methodology involved in making a qualitative study of the pharmaceuticals that contained amoxicillin and observing the presence or absence of interferents. When interferents were not observed, the determination was carried out using univariate calibration because the risk of a systematic error being introduced due to interferents did not exist. In such cases, the advantages of this system are that the determination is fairly automatic, the frequency of analysis is high and the consumption of the reagents is low.

In section 3.3, amoxicillin was determined using second-order calibration for the cases in which interferents were observed. To do so correctly, in the calibration step we worked with augmented matrices made up of the matrix of the sample and the matrix of a reference standard. The most suitable concentration of the reference standard was the highest one within the working range since the regression line fits the ideal regression line better (a slope of 1 and an ordinate at the origin of 0). We also concluded that the species used for quantification must be the sum of the areas of the acid and basic species of amoxicillin because the variability of the responses was lower.

In section 3.4, amoxicillin and clavulanic acid were determined simultaneously. To do so, we re-designed the analytical sequence in function of the physical-chemical characteristics of the analytes (acid-base properties and spectral responses) and concluded that the various species could be analysed simultaneously. To determine clavulanic acid, we only took into account the area of the basic species since our results would be less reproducible if we also took into account the acid species.

We concluded that the critical step in these methodologies was to design an analytical sequence with which to obtain the species in the reactor coil sequentially.

3. Apply methods based on concepts of experimental design to find the optimal analytical sequence by considering multiples responses.

In sections 4.2 and 4.3, we studied several strategies based on experimental design in order to find the optimal analytical sequence. We concluded that, when the responses that measure the quality of the experiments are multiple, a good strategy is to transform these responses into individual desirability functions in order to weight them and obtain a single response called the overall desirability function.

In a first experimental step, when the number of theoretical factors is high, using a screening design often enables us not only to reduce later experimentation but also to obtain information about the direction of the best response for the important factors.

Also, when the number of important factors is low, a quick and easy way to obtain an optimal analytical sequence is to use response surface methodology. This provides not only an optimum but also an equation for the response in function of the factors, thus enabling us to decide which conditions are suitable even if they are not optimal.

If the number of important factors is high, a good strategy could be to use experimentation based on the simplex approach because, if our starting zone is good (obtained from the screening design), we can reach the optimum in fewer experiments.

5.2 SUGGESTIONS FOR FUTURE RESEARCH

This thesis focuses on the application of MCR-ALS to sequential injection data for determining amoxicillin and clavulanic acid in pharmaceuticals. The work presented here points to several areas that still need to be studied in greater detail. One area is the application of this method to another kind of sample or reaction kinetics studies. Another relates to improvements in the current system. More specifically,

1. Improvements in the current system and future work.

a) How the results are maintained over time should be checked by applying process control techniques and time series analysis.

b) The methodology should be applied to more complex samples such as to determine amoxicillin or clavulanic acid in blood or urine.

2. Application to other kinds of sequential injection systems.

This methodology should be checked with more advanced flow systems such as third-generation ones e.g. micro-sequential injection analysis using lab-on-valve (μSIA-LOV), bead injection (BI), sequential injection chromatography (SIC) and multisyringe flow injection analysis (MSFIA).

3. Application to reaction kinetics studies.

This methodology should be used to study antigen-antibody reaction and observe how variables such as temperature, the concentration of the reagents and stoichiometry affect the reaction kinetics.

Appendix

ABBREVIATIONS

Abbreviations used in the thesis

ALS	Alternating Least Squares
BI	Bead Injection
DAD	Diode Array Detector
DOE	Design of Experiments
EFA	Evolving Factor Analysis
FIA	Flow Injection Analysis
LOF	Lack of Fit
MCR	Multivariate Curve Resolution
PCA	Principal Component Analysis
SIA	Sequential Injection Analysis
SIC	Sequential Injection Chromatography
SIMPLISMA	SIMPLe-to-use Interactive Self-modeling Mixture Analysis
SVD	Singular Value Decomposition
UV-Vis	Ultraviolet Visible
μSIA-LOV	micro-Sequential Injection Analysis using Lab-on-valve

LIST OF PAPERS AND MEETING CONTRIBUTIONS

List of papers by the author presented in this thesis.

1.- A. Pasamontes, M. P. Callao; *Determination of amoxicillin in pharmaceuticals using sequential injection analysis (SIA). Evaluation of the presence of interferents using multivariate curve resolution;* Analytica Chimica Acta 485 (2003) 195 - 204.

2.- A. Pasamontes, M. P. Callao; *Determination of amoxicillin in pharmaceuticals using sequential injection analysis (SIA) and multivariate curve resolution*; Analytica Chimica Acta 515 (2004) 159 - 165.

3.- A. Pasamontes, M. P. Callao; *Sequential injection analysis linked to multivariate curve resolution with alternating least squares*; Trends in Analytical Chemistry, available online 11 August 2005.

4.- A. Pasamontes, M. P. Callao; *Optimisation by means of responses surface of an analytical sequence using a sequential injection system;* Talanta, available online 12 September 2005.

5.- A. Pasamontes, M. P. Callao; *Sequential injection analysis (SIA) for the simultaneous determination of clavulanic acid and amoxicillin in pharmaceuticals using second-order calibration;* Analytical Science submitted, 9 September 2005.

6.- A. Pasamontes, M. P. Callao; *Fractional factorial design and simplex algorithm for optimizing sequential injection analysis (SIA) and second-order calibration;* Chemometrics and Intelligent Laboratory Systems submitted, 22 July 2005.

The following papers have been omitted since their content is not related to the scope of the thesis. However, working on different projects has been very useful to broaden my knowledge of second-order data and experimental design.

7.- V. Gómez, A. Pasamontes, M. P. Callao; *On-line oxidation in a SIA-system for determining chromium using second order calibration. Use of factorial design for optimisation*; Analytical and Bioanalytical Chemistry submitted, 26 October 2005.

8.- A. Pasamontes, H. Erxleben, B. Marquardt, D. Veltkamp, J. Ruzicka; *Automated reaction optimization inside a micro-reactor with the sequential injection technique using the Lab-on-valve;* Paper in preparation.

Contributions to international meetings, directly related with the thesis:

1.- A. Pasamontes, M. P. Callao.
Determination of amoxicillin in pharmaceuticals using sequential flow injection analysis (SIA) and multivariate curve resolution.
V Colloquium Chemometricum Mediterraneum - Ustica (Italia) (2003).
Poster communication.

2.- A. Pasamontes, M. P. Callao.
Optimisation of operational and chemical variables in a sequential injection. Analysis (SIA) system and a multivariate curve resolution (MCR) system.
9th Chemometrics in Analytical Chemistry – Lisboa (Portugal) (2004).
Poster communication.

Other meeting contributions not directly related with the thesis:

3.- V. Gómez, A. Pasamontes, M. P. Callao.
A study to analyse chromiun in tannery samples using sequential injection analysis (SIA) and multivariate curve resolution (MCR).
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4.- V. Gómez, A. Pasamontes, M. P. Callao.
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