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# DEVELOPMENT OF SEQUENTIAL INJECTION ENZYMATIC AND BEAD INJECTION ASSAYS IN A LAB-ON-VALVE FORMAT

Thesis submitted to the Universidade Católica Portuguesa to attain the degree of PhD in Biotechnology - with specialization in Chemistry

By

Susana Maria Socorro de Matos Peixoto Vidigal

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Susana Maria Socorro de Matos Peixoto Vidigal

Under the supervision of António O. S. S. Rangel, Professor.

Under the co-supervision of Ildikó V. Tóth, Ph.D.

June 2011

To Mário, love of my life.

Rodrigo, Diogo and Tomás, for giving a new meaning to my life.

# ABSTRACT

## ABSTRACT

The development of bioengineering and biotechnology has promoted the interest for faster and more reliable methods for monitoring biochemical processes. Flow-based systems stand out as the most effective in the automation and miniaturization of these methods. A significantly reduction on the analysis time and dispensing skilled labour is achieved, thus becoming an important tool for implementation and enforcement of these assays.

The main objective was the development of new methodologies for the miniaturization of biochemical analysis system using a sequential injection lab-on-valve (SI-LOV) system. In this context, analytical methodologies were developed based on enzymatic assays for the detection of enzyme substrates, for measurements of enzyme activity, or for enzymatic inhibition studies.

Under this program, some enzymatic methodologies were developed: determination of ethanol in alcoholic beverages; determination of hydrogen peroxide in cleaning solutions for contact lenses, with and without dilution in line; and determination of the activity of peroxidase in vegetable extracts.

The developed method for the enzymatic assay of ethanol in beverages (Chap.3) was based on the conversion of ethanol to acetaldehyde by alcohol dehydrogenase (ADH), using spectrophotometric detection. The quantification of hydrogen peroxide ($H_2O_2$) (Chap.4) and peroxidase activity (Chap.5) was based on the reaction

of oxidation of 2,2'-Azino-bis(3-Ethilbenzothiazoline 6-sulfonic acid) (ABTS) with hydrogen peroxide in the presence of peroxidase (HRP).

Another major goal was to explore the potential of the SI-LOV systems to execute solid phase spectrophotometry, demonstrating its suitability for handling complex samples. This process, in the form of bead injection, was implemented inside the flow system. For this purpose, we used the resin "NitriloaceticAcid (NTA) Superflow", capable of retaining metal ions, which can be derivatized and measured by spectrophotometry (solid-phase spectrophotometry). These methods were used for the quantification of iron and protein content in wine samples.

The developed method for the quantification of iron in wine samples (Chap. 6) was based on the reaction of $Fe^{3+}$ with $SCN^-$, where the $Fe^{3+}$ ions were retained at the surface of the NTA beads. The total iron content was achieved by performing the in-line oxidation of the $Fe^{2+}$ to $Fe^{3+}$. The methodology for total protein content in white wine samples (Chap.7) was based on the Lowry method. The NTA beads were initially charged with $Cu^{2+}$ to complex with the proteins present in the sample. The quantification was achieved by the colour reaction of the proteins with the Folin Ciocalteau reagent.

As for detection system, UV/Vis spectrophotometry was used in all of the developed methodologies.

# RESUMO

# RESUMO

O desenvolvimento da bioengenharia e da biotecnologia impulsionou o interesse por métodos mais rápidos e fiáveis para a monitorização de processos bioquímicos. Os sistemas de fluxo salientam-se como os mais eficazes na automatização e na miniaturização destes métodos. Diminuindo significativamente o tempo de análise, dispensando mão-de-obra qualificada, convertendo-se numa ferramenta fundamental para a execução e aplicação destes ensaios.

O presente trabalho de doutoramento teve como principal objectivo o desenvolvimento de novas metodologias para a miniaturização de ensaios bioquímicos usando um sistema de análise de injecção sequencial "Lab-on-valve" (SI-LOV). Neste contexto foram desenvolvidas metodologias analíticas com base em ensaios enzimáticos, para a detecção de substratos da enzima, para medições de actividade enzimática e para ensaios de inibição.

No âmbito deste programa foram desenvolvidas as seguintes metodologias enzimáticas: determinação de etanol em bebidas alcoólicas; determinação de peróxido de hidrogénio em soluções de limpeza de lentes de contacto, com e sem diluição em linha; e determinação da actividade da peroxidase em extractos vegetais. O método desenvolvido para o ensaio enzimático de etanol em bebidas alcoólicas (Cap. 3) foi baseado na conversão do etanol em acetaldeído na presença da enzima álcool desidrogenase. A quantificação de peróxido de hidrogénio (Cap.4)

e da actividade da peroxidase (Cap.5) teve por base a reacção de oxidação do 2,2'-Azino-bis(3-Ethilbenzothiazoline 6-sulfonic acid) (ABTS) com o peróxido de hidrogénio na presença da enzima peroxidase (HRP).

Outro objectivo principal foi o de explorar as potencialidades destes sistemas de fluxo em espectrofotometria em fase sólida, demonstrando a sua adequação a amostras com matrizes normalmente complexas. Este processo, na forma de "bead injection", foi implementado no sistema de fluxo. Neste trabalho utilizou-se a resina "NitriloaceticAcid (NTA) Superflow" que retém catiões metálicos, e que pode ser derivatizada e efectuada a medição por espectrofotometria. Assim, foram desenvolvidas metodologias empregando esta técnica de separação/concentração para a quantificação de ferro e de teor proteico em amostras de vinho. O método desenvolvido para o doseamento de ferro em amostras de vinhos (Cap. 6) teve por base a reacção de $Fe^{3+}$ com $SCN^{-}$, em que os iões de $Fe^{3+}$ são retidos na superfície das esferas de NTA. A quantificação de ferro total é conseguida efectuando a oxidação de $Fe^{2+}$ a $Fe^{3+}$em linha. A metodologia desenvolvida para a quantificação de proteínas totais em amostras de vinho branco (Cap.7) teve por base o método de "Lowry". Inicialmente as esferas foram derivatisadas com $Cu^{2+}$ de modo a complexarem as proteínas presentes na amostra. A quantificação foi efectuada pela reacção de coloração entre as proteínas retidas e o reagente de "Folin Ciocalteau".

No âmbito deste trabalho a espectrofotometria UV/Vis foi o sistema de detecção usado em todas as metodologias desenvolvidas.

# ACKNOWLEDGMENTS

## ACKNOWLEDGMENTS

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To my husband, Mário, particularly for his patience throughout these long, long years. Without you none of this would have come through. Thank you for loving me.

To my three little boys Rodrigo, Diogo and Tomás, who fulfilled my life.

# LIST OF ABBREVIATIONS

## LIST OF ABBREVIATIONS USED THROUGHOUT THE THESIS

µSPE – Micro solid phase extraction

AAS – Atomic Absorption Spectroscoty

ABTS – 2,2'-Azino-bis(3-Ethilbenzothiazoline 6-sulfonic acid)

ADH – alcohol dehydrogenase

BI – Bead injection

BIS – Bead injection analysis

BSA – Bovine serum albumin

CRM – Certified reference material

CV-AAS – Cold vapour- atomic absorption spectroscopy

CV-AFS – Cold vapour- atomic fluorescence

ETAAS – electrothermal atomic absorption spectrometry

FC – Flow cell

FCr – Folin Ciocalteau's reagent

FIA – Flow injection analysis

GFAAS – Graphite furnace atomic absorption spectrometry

HC – Holding coil

HRP – Horseradish peroxidase

IgG –Immunoglobulin G

KR – Knotted reactor

LOD – Limit of detection

LOQ – Limit of quantification

LOV – lab-on-valve

NTA – Nitrilotriacetic acid

pI – isoelectric point

PPO – Polyphenoloxidase

PTFE – Politetrafluoretileno

RSD – Relative standard deviation

SIA – Sequential injection analysis

SI-LOV – Sequential injection lab-on-valve

SP – Syringe pump

SPE – Solid phase extraction

SPS - Solid phase spectrometry

TLP – Thaumatin-like protein

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# CHAPTER 1

## GENERAL INTRODUCTION

## 1.1. OBJECTIVES

The main objective of this PhD project was to provide a contribution, based on the sequential injection lab-on-valve concept, to the tendency of automation and miniaturization in bioanalytical chemistry. The option for the SIA-LOV technique was mainly based on its potential to handle very low volumes of sample and reagents, and the possibility to integrate both physico-chemical treatments and instrumental measurements in a sole device.

In this context, different analytical methodologies were selected so that different aspects of bioanalytical measurements could be assessed in this format: (i) enzymatic bio-assays for the detection of substrates; (ii) evaluation of enzymatic activity; and (iii) inhibition studies of enzymatic activity.

As the SIA-LOV also presents a large potential to implement solid phase spectrophotometry, an option was made to use this technique, to carry out some in-line sample treatments in a complex matrix like wine samples.

Another objective of this work was to develop methods that would $(i)$ minimize the sample off-line treatment using direct introduction of the samples when possible and to $(ii)$ minimize the reagent consumption and waste production.

## 1.2. FLOW-BASED METHODS

Flow injection analysis (FIA), belongs to the first generation of flow-based methods, and is a well-established technology. From the time when Růžička and Hansen (1975) developed FIA, almost 20000 papers have been published in international scientific periodicals (Hansen, 2011).

In this method, a well-defined volume of sample is injected by means of a rotary valve into a carrier solution stream in a reproducible way. The reagents can be added downstream and the formed reaction product is measured in a suitable flow through detector.

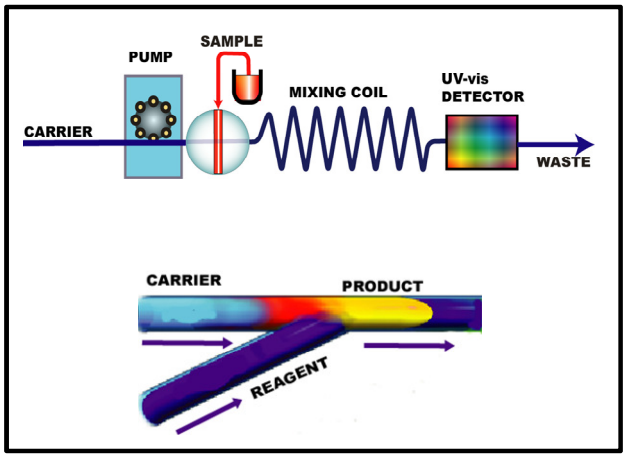


**Fig. 1.1.** Schematic representation of a generic manifold of a flow injection analysis system with spectrophotometric detection and a detailed description of the merging zones at the confluence point (Růžička, 2009).

Flow-based analytical methods rely on the combination of three principles: $(i)$ reproducible sample injection, $(ii)$ controlled dispersion of the sample zone and $(iii)$ reproducible timing of its movement from the injection point to the detection (Růžička, 2008). In contrast to all other methods of instrumental analysis, the chemical reactions are taking place while the sample material is dispersing within the reagent. This is why, in flow-based methods, the dispersion has to be controlled in time and space. The reproducible timing is also vital since neither physical nor chemical equilibrium is attained; therefore what happens to one injected sample happens in the exactly same way to all other subsequently injected samples (Růžička and Hansen, 1988).

With the use of the programmable flow approach, Růžička and Marshall (1990) developed a new system termed sequential injection analysis (SIA), the so-called second generation of flow-based methods. This important methodological innovation is based on the same principles as FIA, but offers more versatility for the systems and makes it possible to develop multipurpose flow manifolds with a significant decrease in reagent consumption and waste production.

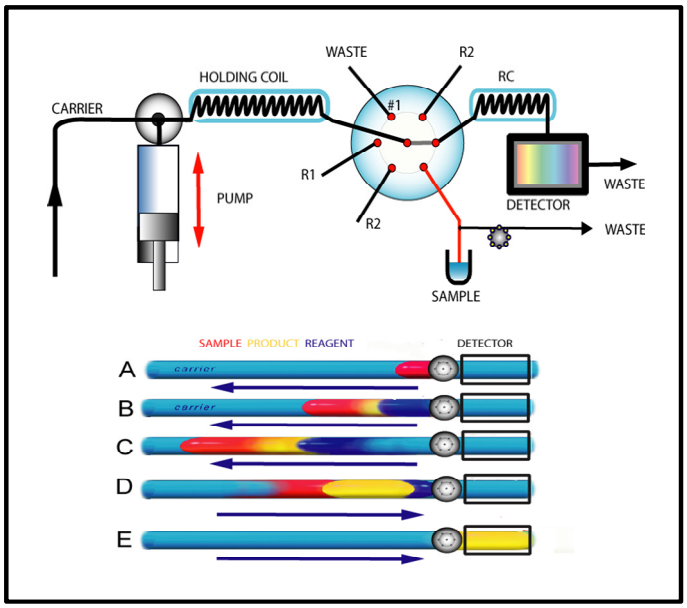


**Fig. 1.2.** Schematic representation of a generic manifold of a sequential injection analysis system with detailed description of the merging zones. A) aspiration of sample to HC; B) aspiration of reagent; C) the product is formed; D) flow reversal; and E) detection of the formed product (Růžička, 2009).

The main difference between FIA and SIA is the way by which the solutions are manipulated. In FIA, there is only one direction of the flow system and SIA is a two direction flow system. By means of a multi-position valve, sample (Fig. 1.2.A) and reagents (Fig. 1.2.B) are sequentially aspirated to a holding coil (HC), the product starts to form in the interface of the stacked zones (Fig.1.2.C) and then propelled by reversed flow (Fig. 1.2.D) towards detection (Fig. 1.2.E). Sample and reagents are mixed by reversing the flow. The dispersion is controlled by means of flow programming. This flow programming is also used to potentially reduce the solutions consumption because there is no need for continuous pumping.

Since the introduction of FIA and SIA, several progresses have been made in the development of new flow methodologies/equipment. Other emerging flow techniques have been developed, namely multicommutation flow injection analysis (MCFIA) (Reis et al, 1994) where the injection valve is replaced for solenoid valves. The introduction of sample and reagents can be performed by aspiration through a single channel pump placed after the detection system (Fig. 1.3.) (by selecting the positions of the solenoid valves) or by placing the pumping device before the commutation valves (Segundo and Rangel, 2002a).

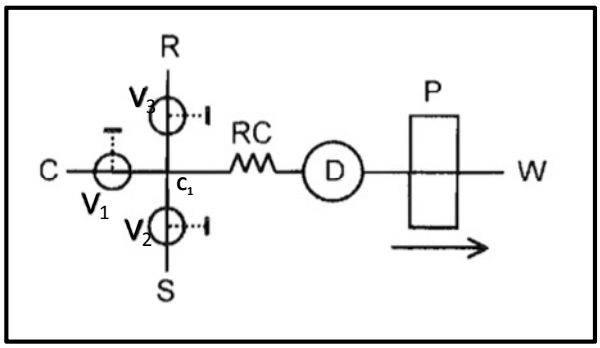


**Fig. 1.3.** Schematic representation of a generic manifold of a multicommutaion flow injection analysis (adapted from Segundo and Rangel, 2002a). C, carrier; P, pump; $V_i$, solenoid valve; $C_1$, confluence; D, detector; RC, reaction coil; S, sample; R, reagent; W, waste.

Later, Cerdà and co-workers (1999) presented multisyringe flow injection analysis (MSFIA). This multisyringe equipment comprises four syringes, each with a solenoid valve on the top, driven by a single motor of a usual automatic burette and controlled by computer software. This technique employs the multisyringe as

propulsion device and requires confluences and/or solenoid valves as well as a detection system (Fig. 1.4.).

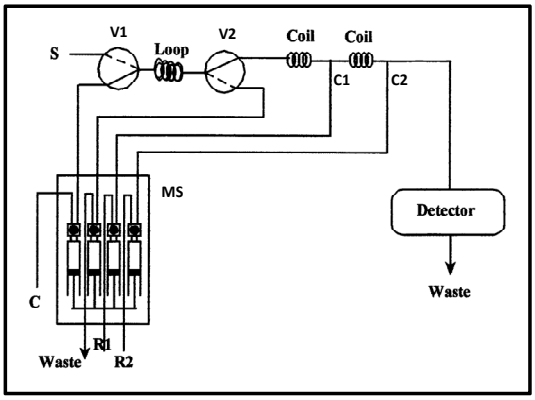


**Fig 1.4.** Schematic representation of a generic manifold of a multisyringe flow injection analysis (adapted from Cerdà et al., 1999). S, sample; C, carrier; $R_i$, reagents; MS, multisyringe; $V_i$, extra solenoid valves; $C_i$, confluences.

More recently, the multi-pumping flow system was proposed (Lapa et al.,2002) as a novel strategy, exploiting the action of individual micropumps for sample/reagent introduction and as component commutation (Fig. 1.5.). This technique ensures an effective and precise control of the sample volume and of its transportation towards the detector.

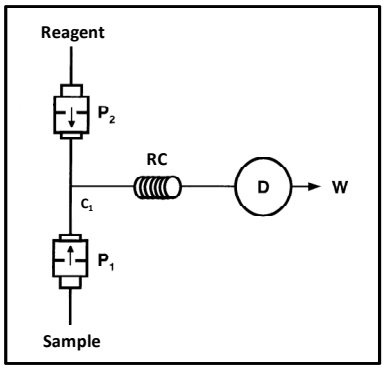


**Fig 1.5.** Schematic representation of a generic manifold of a multi-pumping flow system (adapted from Lapa et al., 2002). P1 and P2, solenoid micro-pumps; RC, reaction coil; D, detector; W, waste; $C_1$, confluence point.

Several other devices have been adapted for the development of methods improving the potentials of these flow-based systems. The flow-based systems coupled with these devices have been the main tools for replacing labour-intensive manual operations (Wang, 2003b). Particularly in the development of new bioassays, where the cost of reagents is an important limiting factor, further miniaturization must still be achieved.

## 1.3. SEQUENTIAL INJECTION LAB-ON-VALVE (SI-LOV)

Sequential injection lab-on-valve was developed by Růžička (2000). This system, several times referred as the third generation of flow-injection systems (Wang and Hansen, 2003b), was designed to operate at the micro-litre level with a further

downscaling option to the nano-litre range, while maintaining relatively large bore conduits that minimize surface contamination and clogging (Růžička, 2000).

The system was designed to integrate all necessary operations for a variety of analytical schemes; it is made to include connecting ports, working channels and a flow-through cell. The main components of the LOV system (Fig. 1.6.) consist of a propulsion device, a multiposition valve, a flow-through port and a central sample processing unit (CSPU).

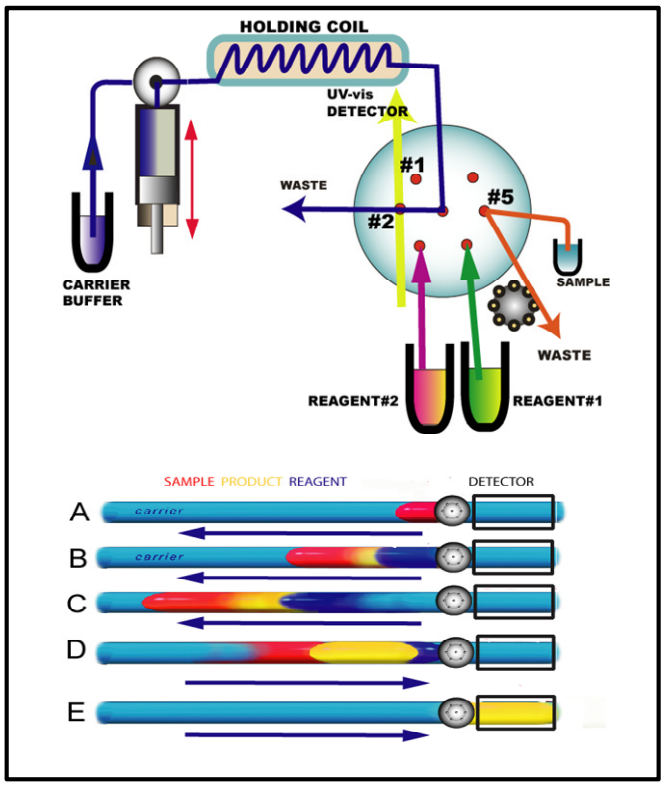


**Fig. 1.6.** Schematic representation of a generic manifold of a sequential injection lab-on-valve system with detailed description of the merging zones. A) aspiration of sample to HC; B) aspiration of reagent; C) the product is formed; D) flow reversal; and E) detection of the formed product (Růžička, 2009).

As a propulsion device, generally a syringe pump (SP) is used to propel the solutions in the manifold. The multiposition valve is responsible for the connection of the several ports to a HC. The flow-through port connects two channels of port #5; one channel serves as sample solution inlet, while the other serves as the sample outlet. The auxiliary peristaltic pump allows the sampling conduit to be thoroughly washed between samples of standards independently from the syringe pump functioning. The CSPU integrates the “heart” of the LOV manifold. Initially made on Perspex and more recently on PEEK and ULTEM for improved chemical resistance (Miró and Hansen, 2007), it is a single monolithic structure mounted atop of the multiposition valve where the sample path from injector to flow cell must be minimised. This manifold was developed to accommodate sample metering, dilution, reagent addition, mixing, incubation, separation and detection, ideally in any desired sequence (Růžička, 2000). This is not possible to achieve with a continuous unidirectional flow, but is quite feasible in a sequential injection (SI) mode that employs flow programming. Therefore, SI was the ideal platform for miniaturization. One of the main features of this system is its robustness, offered by conventional sized pumps, valves and miniaturized spectrometers. As additional benefit of this choice is the proximity of the main components, with simple manipulation, assuring robustness and repeatability (Vidigal et al., 2008; Růžička, 2000).

This system works in SIA mode, where precisely metered zones of sample (Fig. 1.6.A) and reagents (Fig. 1.6.B) are aspirated individually from the port of the

selection valve to a HC where mixing, dilution and incubation take place (Fig. 1.6.C). The mixture is propelled by reversed flow (Fig. 1.6.D) towards detection (Fig. 1.6.E). Once again the system functions in two way direction. The mixing of sample and reagents is mainly attained by reversing of the flow. In SIA systems, the mixing can be increased by coiling the flow path. In the LOV format, mixing is enhanced by the geometry of the platform. The geometry (Fig. 1.7.) is not straight; the sharp bends and narrow sections are important ways for promoting the mixing, by disrupting the laminar flow.

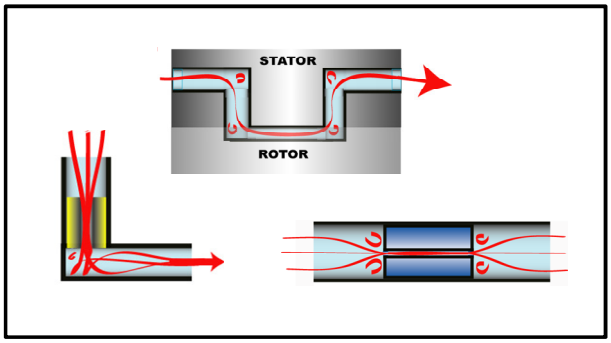


**Fig. 1.7.** Details of the path geometry of the SI-LOV system (Růžička, 2009).

As for detection, this system has a multipurpose flow cell that can integrate optical detection devices where communication is established by optical fibres (Fig. 1.8.). The fibres can be placed in different positions, configured for absorption or fluorescence detection. By combining configurations, absorbance and fluorescence measurements can be used in the same set-up (Lähdesmäki et al., 2009).

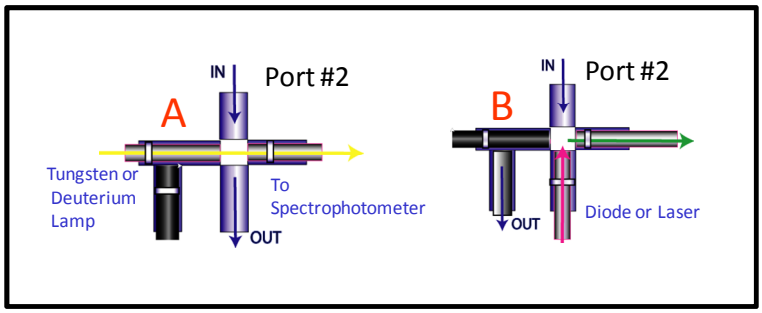


**Fig. 1.8.** Schematic representation of the multipurpose flow cell. Configuration for A) UV/VIS spectroscopy and B) fluorescence spectroscopy (Růžička, 2009).

Although the CSPU is one piece only, the length of the light path is not fixed; it can be adjusted by changing the position of the end of the optical fibre. It is possible to obtain from 2 mm to 10 mm, and with an extension (Garth's Cell) the light path can increase up to 50 cm (Fig. 1.9.).

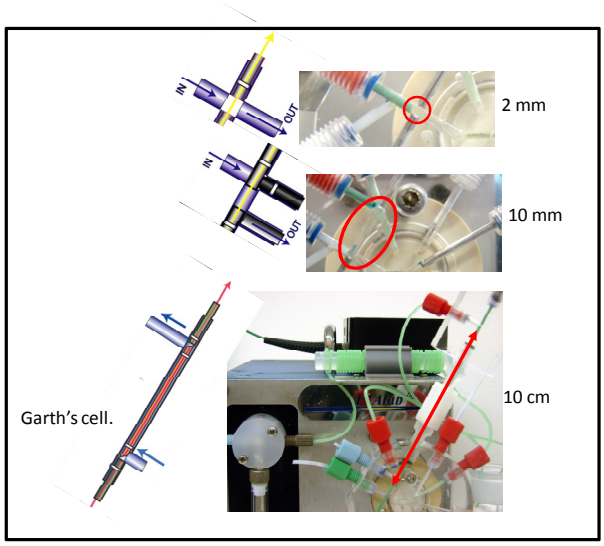


**Fig. 1.9.** Representation of the different light path dimensions that can be used in a LOV format (Růžička, 2009).

Considering all these features, the system has proved to be a valuable tool for solution handling, mixing of sample and reagent, with a multipurpose flow cell. This platform served not only for reagent-based assays methodologies but also for bead injection assays (BIA). BIA is based on the introduction and trapping of functionalized beads in the flow conduit (Růžička, 2000). The beads are the solid surface that extract the analyte or accommodate the chemical reaction. In the BIA system, a small amount of micro-spheres is injected into the flow channel, where the beads are trapped and perfused by analyte solutions and auxiliary reagents. The BIA protocol can be divided into five steps. First the microspheres are injected in the flow channel and trapped in the flow cell building a bead column (Fig. 1.10.A); then the sample is injected and transported towards it (Fig. 1.10.B). Afterwards, the target analyte is captured on the bead surface, while the matrix components are washed away. After that, a chromogenic reagent is injected (Fig. 1.10.C) and some species are detected by spectroscopy(Fig. 1.10.D). Finally, beads are discharged (Fig. 1.10.E) or regenerated.

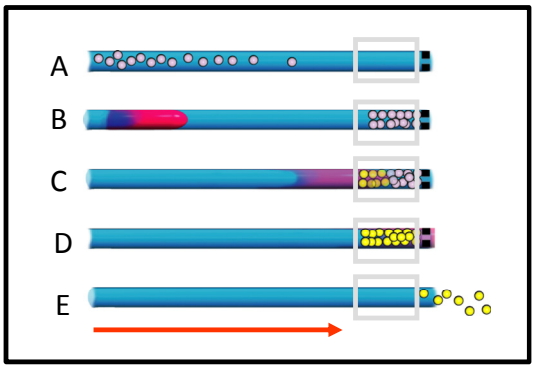


**Fig. 1.10.** Schematic representation of the BIA principles. A) Load of beads suspension; B) load of sample solution; C) injection of the chromogenic reagent; D) development of colour; and E) beads are discharged (Růžička, 2009).

The reaction can take place on the bead surface allowing real time monitoring, directly on the solid phase, solid phase spectrometry (SPS), or on the eluted liquid phase, solid phase extraction (SPE) (Růžička, 2000). In the traditional SPE concept, the detection will occur on the eluted phase; however, there is a partial loss of preconcentration capabilities gained in the sorption step (Miró and Frenzel, 2004). To enhance sensitivity, SPS has been developed based on the direct measurement of the light attenuation of adsorbent particles packed in an optical cell, in which the target analyte is concentrated and coloured (Matsuoka and Yoshimura, 2010). Considering that the measurement occurs without elution, SPS turns to be a more sensitive method than the conventional SPE. SPS can be performed by two different approaches: batch or flow method.

To perform SPS in a batch mode, besides being very time-consuming, above-average skills are required to pack the solid particles into the flow cell. In flow-

based methods, the filling of the particles into the cell is simplified. Moreover, the flow methods are more suitable since they can easily measure the light attenuation in the adsorbed species in the flow-through cell with considerable reduction of the volumes used. However, the implementation of SPE/SPS in flow systems can be tricky, as the solid phase can become too much packed and increase overpressure. Additionally, if it is not renewed, it might become saturated, either with the analyte or with interfering compounds. To overcome this problem, instead of reusing the particles, it would be interesting to renew the optosensor. This is quite feasible using a SI-LOV system (Růžička, 2000).

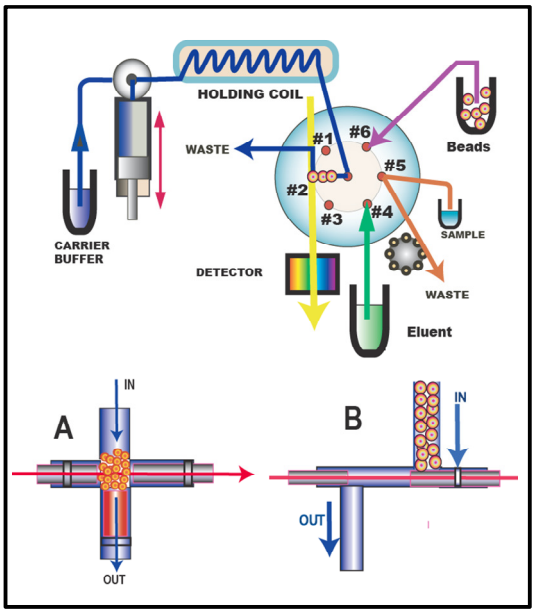


**Fig. 1.11.** Bead injection in Lab-on-valve format with details of the bead column for A) SPS measurement and B) SPE measurement (Růžička, 2009).

When the bead column is placed in the detection zone, it is labelled as flow-through sensor (Ruedas Rama et al., 2003). In some applications, after the measurement, the beads can be discharged, occurring physical regeneration, or in other cases the sensor surface can be renewed, being considered as chemical regeneration. The physical regeneration of the sensor is an advantage since there is no need for the elution step and its applicability is not limited by the lifetime of the sensor, at the same time any possible contamination or carryover is also eliminated. The major interest of using these sensors is the increase insensitivity and selectivity. The sensitivity is enhanced since the analyte is retained in the detection zone, and the selectivity also increases since interfering species are excluded from the bead column, therefore they don't develop any signal. In SPS assay, when the chemical

reaction takes place on the bead surface, the baseline must be established after the application of the sample mixture to the bead column. In this way the interference of the colour of the sample can be also reduced. The main difficulty of this approach is to obtain a solid phase that can be applied to the separation, concentration and determination of the chemical species of interest (Sartini et al., 2003).

In BIA applications the size, the shape and the material of the beads are the main characteristics to consider when selecting them. According to Růžička (2009) they should be spherical with a size range from 20 to 150 µm, and in terms of its nature material can be soft (polymer-made) or rigid (glass, silica).

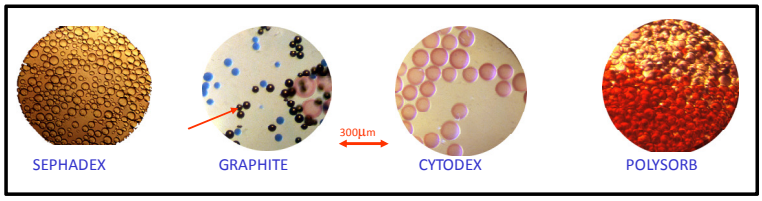


**Fig. 1.12.** Examples of beads usually used in BIA (Růžička, 2009).

Beads of different material nature are commercially available and have become useful as surface media for retaining reagents, analytes or reaction products. Sephadexis usually used for the linkage of reagents; sepharose 4B; agarose; and aminolink, which provide adequate bead support for bioligand immobilization; cytopore is the most usual for cell linkage (Luque de Castro et al., 2008).

## 1.4. Applications of SI-LOV

The trend for miniaturization is highly justified by the elevated costs of the reagents involved in enzymatic and immuno-assays, as well as by the often limited amount of the samples available. The possibility to perform the bioassays within small integrated manifolds through flow based automation of sample and reagent handling has received increased attention. Therefore, several methodologies were developed in a lab-on-valve format for diverse fields coupled to different detection systems. Several papers have been published describing different applications of lab-on-valve platform.

A literature search in SciVerse Scopus using the different terms of: lab-on-valve; lab-on-a-valve; “SIA-LOV”; “micro SI-LOV”; “SI-LOV”; “sequential injection-LOV” returned a total number of 112 papers on 2$^{nd}$of June 2011. This search was limited to the record fields of article title, abstract and keywords, for documents published from 1975 to present. Using the same date and field limits, the search for “bead injection” returned 75 results. Cross-search of “bead injection” AND NOT LOV was used to exclude from the list of papers those articles that are based on Bead Injection but not performed in the LOV format. Using the same terms, a similar search was made on ISI Web of Knowledge – Web of Science. As a result, 121 articles were listed since the first publication by Růžička in 2000 until 2$^{nd}$ of June of 2011.

The diagram on Fig. 1.13. represent the evolution of the scientific literature during the last decade on SIA-LOV.

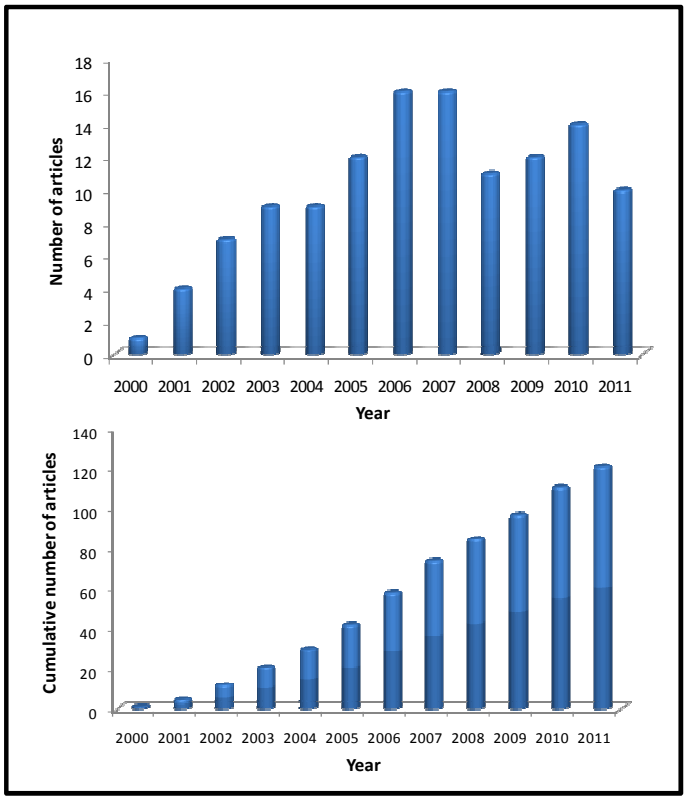


**Fig. 1.13.** Number of articles dealing with lab-on-valve published over the years.

The results obtained in this search can be divided into two different types of manuscript: reviews and applications. Within the applications, it was possible to establish a subdivision into: biologically active analytes, organic chemical analytes, and inorganic chemical analytes. Figure 1.14. presents this subdivision based on the subject of the articles.

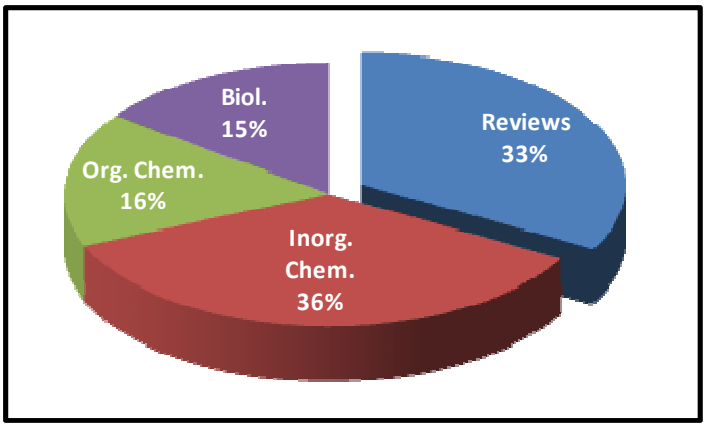


**Fig. 1.14.** Diagram on the subject of Lab-on-valve articles published.

As it can be noticed from Fig. 1.14, one third of the published articles are reviews. These articles describe the potentials of the flow-based methods in general (Anthemidis and Miró, 2009; Chen et al., 2007a; Chen et al., 2008a; Economou, 2010; Fajardo et al., 2010; Grudpan, 2004; Hansen and Wang, 2002; Hansen, 2004; Hansen and Wang, 2004; Hansen, 2005; Hansen and Miró, 2007; Hartwell et al., 2004; Irdis, 2010; Matsuoka, 2010; Miró and Frenzel, 2004; Miró et al., 2008; Růžička and Hansen, 2008; Sakai and Teshima, 2008; Sardans et al, 2010; Wang and Hansen, 2003a; Wang and Hansen, 2005; Wang et al., 2007; Zagatto et al, 2009). These reviews describe the potentials and applications of the three generations of flow, FIA, SIA and LOV. Other reviews are more focused on the potentials of the LOV system (Hansen and Miró, 2008; Wang and Hansen, 2003b; Wang, 2005) when applied to bioassays (Chen and Wang, 2007; Luque de Castro et al., 2008); or to environmental assays (Miró et al., 2006; Miró and Hansen, 2007) where sample pre-

treatment is an important issue (Hansen et al., 2006; Miró et al., 2011; Wang et al., 2003). There are other reviews centred in the potential of SIA (Santos and Masini, 2010; Ecomonou et al., 2007; Mesquita and Rangel, 2009; Miró and Hansen, 2006; Silvestre et al., 2011; Solic et al., 2003) and multicommutation (Leal et al., 2011) systems making critical comparisons with the other flow-based methods/techniques. Matsuoka and Yoshimura (2010) presented the advantages of the flow-based methods in the application of SPS and Taylor et al. (2009) discussed the recent developments in the interfacing with atomic absorption spectrometry (AAS).

**Table 1.1.** LOV systems applied to the determination of biologically active analytes.

| Analyte | Matrix | Determination Characteristics | Detection system | Dynamic range | LOD | RSD | Deter. Rate | Reference |
|---|---|---|---|---|---|---|---|---|
| AChE | No application | Enzyme kinetics and inhibition studies in micro reactor with acetylthiocholine as substrate | Spectrophotometry | -- | -- | -- | -- | Chen et al., 2006 |
| ACE | No application | Enzyme kinetics and inhibition studies in micro reactor by the hydrolysis of FAPGG | Spectrophotometry | -- | -- | -- | -- | Chen et al., 2006 |
| Biotin conjugate | Cultured human skin fibroblasts | Affinity capture, release kinetics and enzyme assay studies with Streptavidin agarose beads | UV/VIS and ESI-MS | -- | -- | -- | -- | Ogata et al., 2002 |
| DNA | Human whole blood | DNA purification via µSPE by silica beads | Laser induced fluorescence | up to 20 ng/µL | -- | 3.8% | 10 /h | Chen et al., 2007b |
| DNA | No application | Sepharose streptavidin beads retained the analyte | Spectrophotometry and fluorescence | 1 – 1000 pmol synthetic DNA | -- | 7.2% | 20 min | Edwards and Baeumner, 2006 |
| ssDNA | No application | Streptavidin-coated Sepharose 4B beads preconcentratesbiotinylated telomerase subtract | Fluorescence | 0 –993ng/mL | 111 pgss DNA | -- | 24 /h | Decuir et al., 2007 |

**Table 1.1.** LOV systems applied to the determination of biologically active analytes. (cont.)

| Analyte | Matrix | Determination Characteristics | Detection system | Dynamic range | LOD | RSD | Deter. Rate | Reference |
|---|---|---|---|---|---|---|---|---|
| DNA | No application | Discoloration reaction of crystal violet in a meso-fluidic analytical system | Spectrophotometry | 0.2 –6.0 µg/mL | 0.07 µg/mL | 1.2% | 30 /h | Chen et al., 2005a |
| dsDNA | no application | Fluorescence enhancement of etidium bromide in the presence of nucleic acids | Laser induced fluorescence | 0.03 – 3.0 µg/mL | 0.009 µg/mL | 1.9% | 60 /h | Chen et al., 2005b |
| Female hormones | Human urine | SPE by micro column with Chromabond $C_{18}$ or $C_{18}$-Hydra | Tandem mass spectrometry | -- | -- | 1.93 % 10.99% | -- | ÁlvarezSánchez et al., 2008 |
| GAD65 auto-antibody | Human serum samples | ELISA in a streptavidin sepharose 4B beads | Spectrophotometry | 250 – 300 ng/mL | 20 ng/mL | 2 to 5% | 2 /h | Carroll et al., 2003 |
| HRP | Vegetable extracts | Enzymatic reaction with HRP in the presence of ABTS and $H_2O_2$ | Spectrophotometry | Up to 2 mg/L | 0.3 mg/L | 3.5% | 1 /min | Vidigal et al., 2010 |
| IgG | No application | Sepharose CL-4B beads were used for µAC and µBIS | Spectrophotometry | 0.100 - 1.00 µg/µL 0.100 – 0.400 µg/µL | 5 ng/µL µAC 50 ng/µL µBIS | -- | -- | Gutzman et al., 2006 |

**Table 1.1.** LOV systems applied to the determination of biologically active analytes. (cont.)

| Analyte | Matrix | Determination Characteristics | Detection system | Dynamic range | LOD | RSD | Deter. rate | Reference |
|---|---|---|---|---|---|---|---|---|
| IgG | No application | Protein A sepharose and recombinant Protein G Sepharose 4B Conjugate | Spectrophotometry/ fluorescence spectroscopy | 0 – 0.4 µg/µL IgG 0 – 0.8 µg/µL BSA | 470 ng | -- | -- | Carroll et al., 2002 |
| IgG | No application | Sepharose B with recombinant protein G on their surface or bead injection | Fluorimetry | 10 – 100 µg/mL IgG | 10 µg/mL IgG | -- | -- | Růžička, 2000 |
| Protease | No application | Enzymatic degradation of a fluorescence substract | Fluorimetry | Up to 10 KNPU | -- | -- | -- | Růžička, 2000 |
| Proteins | Human serum, urine, milk, yogurt | Reaction with Congo red at pH 4.1 | Spectrophotometry | 12.5 - 200 µg/mL | 5.6 µg/mL | 1.9% | 60 /h | Chen et al., 2008b |
| Protein | No application | Immobilisation of the proteins in Aminolink monitored by BIS | Spectrophotometry | -- | -- | -- | -- | Růžička et al., 2006 |
| Proteins | No application | Sepharose Protein A beads in a micro reactor performing affinity chromatography | sSpectrophotometry | -- | 6 ng mouse IgG/µL | -- | Less than 2 min | Erxleben and Růžička, 2005b |

**Table 1.1.** LOV systems applied to the determination of biologically active analytes. (cont.)

| Analyte | Matrix | Determination Characteristics | Detection system | Dynamic range | LOD | RSD | Deter. rate | Reference |
|---|---|---|---|---|---|---|---|---|
| Proteins | Peptide mixture | CNBr-activated Sepharose B for bead injection | ESI-MS | $10^{-5}$ to $10^{-7}$ M for Kd value | -- | -- | 15/ min | Ogata et al., 2004 |
| Peptide C | Rat pancreatic islet excretions | In-situ derivatisation of the analyte | Capillary electrophoresis | 260 – 781 nM | -- | 2.8% | -- | Wu et al., 2003 |
| Sphingoid precursors | Human urine, serum | Spe by $C_{18}$ , Chromabond $C_{18}$ Hydra, BondElut $NH_2$, Chromabond HR-P, $C_8$ Strata | Laser induced fluorescence | -- | 4.2 - 10.20.56 - 1.36 ng/mL | -- | -- | ÁlvarezSánchez et al., 2011 |

µAC – micro affinity chromatography; ACE - angiotensin-converting enzyme; AChE - acetyl cholinesterase;µBIS – micro bead injection analysis; DNA - deoxyribonucleic acid; ds DNA – double strained deoxyribonucleic acid; ss DNA – single strained deoxyribonucleic acid; ELISA - Enzyme Linked Immunosorbent Assay; GAD65 - Glutamate decarboxylase; IgG – immunoglobulin G; RNA - Ribonucleic acid.

Considering the applications of LOV systems, there are some works reporting the development of new methodologies for the determination of biologically active analytes, in different types of samples. Among these analytes, DNA, immunoglobulins, proteins and enzymes have been subject of study (Table 1.1). Most of the developed methodologies for the quantification of DNA were based on the capture of the DNA on a bead column. The retained DNA was quantified *in-situ* or after an elution step. Using fluorescence as detection measurement, the DNA retained on the surface of the beads was also quantified after reaction with OligGreen (Decuir et al., 2007) or after reaction with fluorescein (Edwards and Baeumner, 2006). This quantification was also carried out on the eluted phase, where the product of the reaction of the DNA with etidium bromide was excited with laser beam and the fluorescence intensity recorded (Chen et al., 2007b). In the development of these methodologies, different types of beads were used to retain the DNA, from silica (Chen et al., 2007b) to streptavidin-coated sepharose (Edwards and Baeumner, 2006; Decuir et al., 2007).

However, DNA was not always quantified by BIA approach. Chen and co-workers developed two different methodologies for this quantification, one based on the reaction of DNA with crystal violet, resulting on the discoloration of this reagent, with posterior spectrophotometric quantification (Chen et al., 2005a), and another based on the reaction with etidium bromide for fluorescence quantification (Chen et al., 2005b).

Between these biologically active analytes, several papers can be encountered reporting methodologies for protein quantification and protein studies, in particular enzymatic studies. The methodologies for protein quantification use, in general, spectrophotometry as detection system; only one paper reports the use of an electrospray ionization ion-trap mass spectrometer (ESI-MS) as detection device. In this work, the proteins were retained in a CNBr-activated sepharose 4B beads and quantified after elution by ESI-MS (Ogata et al., 2004). Spectrophotometric protein quantification was carried out employing the reaction of protein with congo red (Chen et al., 2008a). Making use of the BIA approach, Sepharose protein A beads were used to, perform affinity chromatography (Erxleben and Růžička, 2005b) and in Aminolink beads that served as support for protein immobilisation (Růžička et al., 2006).

Sequential injection lab-on-valve was also used for enzyme kinetic and inhibition studies in different enzymes. Acetylcholinesterase (AChE) (Chen et al., 2006) activity was measured spectrophotometrically using a method employing acetylthiocholine (ASCh) as substract, forming thiocholine and acetate. The produced thiocoline reacted with a chromogen monitored by measuring the absorbance increase at 412 nm. Angiotensin-converting enzyme (ACE) (Chen et al., 2006) activity was measured based on the hydrolysis of FAPGG (N-[3-(2-furyl)acryloyl]-L-phenylalanylglycylglycine) that resulted in a decrease in absorbance at 340 nm. Horseradish peroxidase (HRP) was also aim of kinetic studies (Vidigal et al., 2010). This determination was based on the reaction of $H_2O_2$ (hydrogen peroxide) with

ABTS (2,2'-Azino-bis(3-Ethilbenzothiazoline 6-sulfonic acid)) catalysed by HRP. The oxidation of ABTS was followed by observing the increase in absorbance at 410 nm during 30 s. As for inhibition studies, these were performed by the addition of the inhibition factor of each enzyme to AChE and ACE (Chen et al., 2006) and by the increase of the temperature in the inhibition of the HRP (Vidigal et al., 2010).

Some methodologies were developed for the quantification of immunoglobulin G (IgG) with spectrophotometric and/or fluorescence detection. The methodologies used the BIA mode employing sepharose beads. Sepharose CL-4B beads were used for performing micro affinity chromatography (μAC) and micro bead injection spectrometry (μBIS) (Gutzman et al., 2006). Sepharose B with recombinant protein G covalently bound at the surface was used to retain IgG for spectrophotometric and fluorometric detection (Růžička, 2000), and were compared with Protein A sepharose for the same purpose using the same types of quantification (Carrol et al., 2002).

Due to the versatility of the sepharose beads, they were chosen to separate IgG (Gutzman et al., 2006; Carroll et al., 2002; Růžička, 2000), to retain proteins (Růžička et al., 2006; Erxleben and Růžička, 2005b; Ogata et al., 2004) and as solid support for ELISA (Carroll et al., 2003). To compare the performance of different types of beads, sphingoid precursors (Álvarez-Sánchez et al., 2011) and female hormones (Álvarez-Sánchez et al., 2008) were used as case study.

**Table 1.2.** LOV systems applied to the determination of organic chemical analytes.

| Analyte | Matrix | Determination Characteristics | Detection system | Dynamic range | LOD | RSD | Deter. rate | Reference |
|---|---|---|---|---|---|---|---|---|
| Acidic drugs | Urine, water | Oasis HLB was used for preconcentration, elution of the analyte towards Liquid Chromatography | UV-spectra 200 - 350 nm | 0.4 - 40 ng/mL | 0.02 - 0.67 ng/mL | < 8.6% | 20 min | Quintana et al., 2006 |
| Amantadine | Pharmaceutical formulations, urine | No sample pre-treatment | Potentiometry (cyclodextrin-based) | $2.0x10^{-4}$ - $1.0x10^{-2}$ $8.2x10^{-5}$ - $1.0x10^{-3}$mol/L | $5.4x10^{-5}$mol/L | 2.9% | -- | Amorim et al., 2007b |
| BMDBM | Bathing waters spiked with Uv-filters | Oasis HLB and Focus for bead injection, elution of the analyte towards Liquid Chromatography | Spectrophotometry 360 nm | 60 – 420 ng | 0.45 µg/L | 13 | 9 min | Oliveira et al., 2010a |
| BP3 | Bathing waters spiked with Uv-filters | Oasis HLB and Focus for bead injection, elution of the analyte towards Liquid Chromatography | Spectrophotometry 290 nm | 60 – 480 ng | 0.81 µg/L | 2.5 | 9 min | Oliveira et al., 2010a |
| Catechol | Beer | No sample treatment | Amperometry, irreversible electrochemical oxidation of catechol | $1.0x10^{-6}$– $5.0x10^{-4}$mol/L | $5.09x10^{-5}$mol/L | 2.39% | 35/h | Wang et al., 2010a |
| Diazepam | Pharmaceutical formulations | No sample treatment | Potentiometry (cyclodextrin-based ESI) | $1.2x10^{-1}$ – 1.7 g/L | $6.8 \times 10^{-2}$ g/L | -- | -- | Amorim et al., 2008 |

**Table 1.2.** LOV systems applied to the determination of organic chemical analytes. (cont.)

| Analyte | Matrix | Determination Characteristics | Detection system | Dynamic range | LOD | RSD | Deter. rate | Reference |
|---|---|---|---|---|---|---|---|---|
| EHMC | Bathing waters spiked with Uv-filters | oasis HLB and Focus for bead injection, elution of the analyte towards Liquid Chromatography | Spectrophotometry 305 nm | 60 – 420 ng | 0.90 µg/L | 12 | 9 min | Oliveira et al., 2010a |
| Epinephrine | Commercial pharmaceutical products | no sample treatment | Potentiometry (pertiodate selective electrode) | $2.0x10^{-4}$ - $2.5x10^{-3}$ mol/L | -- | 0.34 | -- | Amorim et al., 2007a |
| Ethanol | Red and white table wine, beer and spirits | Peak height and initial rate measurement of the enzymatic reaction with ADH in the presence of $NAD^+$ | Spectrophotometry | up to 0.04% (v/v) | 0.003% (v/v)<br>0.004% (v/v) | < 5.0% | 27/h<br>37/h | Vidigal et al., 2008 |
| Ethanol | No application | Enzymatic assay performed with ADH in the presence of $NAD^+$ with stop flow | Spectrophotometry | 50 - 250 ppm | -- | 3% | -- | Chen and Růžička, 2004 |
| Glucose | No application | Enzymatic assay with glucose oxidase and peroxidase with stop flow | Spectrophotometry | 100 - 1000 ppm | -- | 3% | -- | Chen and Růžička, 2004 |
| Glucose | Hepatocyte cell line | Enzymatic reaction with cytopore bead micro reactor for monitor cell rates | Spectrophotometry | 0.1 - 5.6 mM | -- | -- | < 2 min /det | Schulz and Růžička, 2002 |

**Table 1.2.** LOV systems applied to the determination of organic chemical analytes. (cont.)

| Analyte | Matrix | Determination Characteristics | Detection system | Dynamic range | LOD | RSD | Deter. rate | Reference |
|---|---|---|---|---|---|---|---|---|
| Glucose | Hepatocyte line cell | Enzymatic reaction with cytopore bead micro reactor for monitor cell rates | Spectrophotometry | 0.1 – 5.6 mM | -- | ± 4.5% | -- | Schulz et al., 2002 |
| Glucose | *E.coli* culture | Two step enzymatic assay with glycerol kinase, glycerol phosphate oxidase and peroxidase | Spectrophotometry | 20 – 120 ppm | -- | -- | -- | Wu et al., 2001 |
| Glycerol | *E.coli* culture | Enzymatic assay with hexokinase and glucose-6-phosphate hydrogenase | Spectrophotometry | 35 – 1000 ppm | -- | -- | -- | Wu et al., 2001 |
| Histamine | Food samples (fish) | No sample treatment | Potentiometry (histamine ion selective electrode) | $10x10^{-5}$ -$10x10^{-2}$ mol/L | $4.7x10^{-6}$ mol/L | 7% | -- | Amorim et al., 2010 |
| HMS | Bathing waters spiked with Uv-filters | Oasis HLB and Focus for bead injection, elution of the analyte towards Liquid Chromatography | Spectrophotometry 235 nm | 240 – 1920 ng | 3.2 µg/L | 2.1 | 9 min | Oliveira et al., 2010a |
| Insulin | Rat pancreatic islet excretions | In-situ derivatisation of the analyte | Capillary electrophoresis | 3.43 – 6.87 µM | -- | 2.8% | -- | Wu et al., 2003 |

**Table 1.2.** LOV systems applied to the determination of organic chemical analytes. (cont.)

| Analyte | Matrix | Determination Characteristics | Detection system | Dynamic range | LOD | RSD | Deter. rate | Reference |
|---|---|---|---|---|---|---|---|---|
| Lactate | Hepatocyte line cell | Enzymatic reaction with cytopore bead micro reactor for monitor cell rates | Spectrophotometry | 0.05 – 1.00 mM | -- | ± 4.5% | -- | Schulz et al., 2002 |
| LAS | Effluent, waste, surface and sea water | SPE by Chromabond $C_{18}$ Hydra | Spectrophotometry and capillary electrophoresis | LOQ – 10 mg/L | < 15 µg/L | <5.4% | 2/h | JimenezandLuque de Castro, 2008 |
| Midazolam | Pharmaceutical formulations | No sample treatment | Potentiometry (cyclodextrin-based ESI) | $2.3x10^{-3}$ – $4.4x10^{-1}$ g/L | $1.2x10^{-3}$ g/L | -- | -- | Amorim et al., 2008 |
| OPDA | Tap, industrial, waste wayter | No sample treatment | Biamperometry detection of Fe(III)/Fe(II) reduction couple | $5.0x10^{-7}$ – $1.0 x10^{-4}$mol/L | $1.1x10^{-7}$ mol/L | 2.8% | 40/h | Wang et al., 2011b |
| PCB's | Solid waste leachates | SPE by oasis HLB, LIchrolut EN, Bond ElutPlexa, modified silica Upti-Clean $C_{18}$ | Gas chromatography | -- | 0.5 -6.1 ng/L (LOQ) | 9% | -- | Quintana et al., 2009 |
| Proinsulin | Rat pancreatic islet excretions | In-situ derivatisation of the analyte | Capillary electrophoresis | 0.39 – 1.96 pM | -- | 2.8% | -- | Wu et al., 2003 |

**Table 1.2.** LOV systems applied to the determination of organic chemical analytes. (cont.)

| Analyte | Matrix | Determination Characteristics | Detection system | Dynamic range | LOD | RSD | Deter. rate | Reference |
|---|---|---|---|---|---|---|---|---|
| Propranolon | No application | SPE by oasis HLB, Lichrolut EN, SupelMIP β-receptores and discovery DSMCAX | High performance liquid chromatography | -- | -- | -- | -- | Oliveira et al., 2011 |
| Quercetin | Food supplements, pharm.product | Oxidation by MBTH | Spectrophotometry | 5 - 50($10^{-5}$)M | 1x$10^{-5}$ M | < 5% | 59/h | Matsyushina et al., 2010 |
| Riboflavin | Foodstuffs, CRM | Renewable molecularly imprinted solid-phase | Liquid chromatography | 0.450 – 5.00 mg/L | 0.05 mg/L | <4% | 16.6 min | Oliveira et al., 2010b |
| Rution | Food supplements, pharm.product | Oxidation by MBTH | Spectrophotometry | 6 – 50 ($10^{-5}$)M | 2x$10^{-5}$ M | < 5% | 59/h | Matsyushina et al., 2010 |
| Tetracycline | Milk | Oxidation micro column, with biamuthate immobilised in silica beads | Chemiluminescence by generation of bromide | 60 – 10000 µg/L | 2.0 µg/L | 2.2% | 120/h | Yang et al, 2006 |

BMDBM - butylmethoxydibenzoylmethane; BP3 - benzophenone-3; EHMC – ethylhexylmethoxycinnamate; HMS – homosalate; LAS - Linear alkilbenzenesulfonates ; MBTH – 3-methilbenzothiazolinon-2-hydrazone; OPDA - o-phenylenediamine; PCB's - polychlorinated biphenyls.

New methodologies were developed for application of organic chemical compounds (Table 1.2). Within this group of methods, several were applied to pharmaceutical compounds, hyphenated to different separation techniques like gas or liquid chromatography (Quintana et al., 2006; Oliveira et al., 2010a; Quintana et al, 2009; Oliveira et al., 2011), most of them were coupled to spectrophotometry (Wu et al., 2001a; Schulz and Růžička, 2002; Chen and Růžička, 2004; Schulz et al., 2002; Jimenez and Luque de Castro, 2008; Matsyushina et al., 2010). Some analytes were detected using electroanalytical techniques (Amorim et al., 2007b; Wang et al., 2010a; Amorim et al., 2007a; Amorim et al., 2010; Amorim et al., 2008; Wang et al., 2011b); Capillary electrophoresis (Wu et al., 2003) and chemiluminescence (Yang et al., 2006).

Curiously, BIA was not so used in the development of these methodologies as often as I expected when compared to biologically active assays. A more extensive use of the BIA approach was expected due to the specificity of the analytes to be determined; on the other hand the determinations were performed mostly in pharmaceutical formulations where the complexity of the matrix is usually not a problem. Probably this methodology will gain popularity when applied to environmental or to biological samples. Less than half of the reported works used bead injection; oasis HLB were used for the development of methodologies for acidic drugs (Quintana et al., 2006), organic UV-filters components (Oliveira et al., 2010a), solid waste leachates (Quintana et al., 2009), and propanolol (Oliveira et al., 2011). Cytopore was used for the determination of glucose (Schulz and Růžička,

2002) and lactate (Schulz et al., 2002); Shepharose B (Růžička, 2000), chromabond (Jimenez and Luque de Castro, 2008) and silica (Yang et al., 2006) beads were also used. As it can be noticed, the same type of bead, for example: oasis HLB can be used for the development of methodologies for different analytes.

**Table 1.3.** LOV systems applied to the determination of inorganic chemical analytes.

| Analyte | Matrix | Determination Characteristics | Detection system | Dynamic range | LOD | RSD | Deter. rate | Reference |
|---|---|---|---|---|---|---|---|---|
| Acidification rates (extracellular) | No application | pH indicator immobilised in Cytopore and sephadex G-50 beads | Spectrophotometry | -- | -- | -- | -- | Erxleben et al., 2004 |
| Acidity | Fruit juices | Monosegmented flow titration, sample and reagents are sandwiched between air segments | Spectrophotometry | 0.2 – 1.2% (w/v) | -- | 1.2% | 30 /h | Jakmunee et al., 2005 |
| Ammonia, | *E. coli* culture | Berthelot's method was used for ammonia quantification | Spectrophotometry | 3 – 1200ppm | -- | -- | -- | Wu et al., 2001 |
| Anions | No application | Sample was pre-treated in the lov manifold | Capillary electrophoresis | 0.5 – 3.0 mM | < 521 ppb | -- | -- | Wu et al., 2002 |
| As | Ancient papers | SPE by Strong acidic Dowex-50W cation and Strong basic Dowex-1W anion exchange | X-ray fluorescence spectrometry, after elution | 0.2 – 2 mg/L | 0.02 µg/g | 5.62% | -- | Alcalde-Molina et al., 2009 |
| As | CRM river water | Immobilised cells onto Sephadex G-50 beads were used to retain analyte | After elution the analyte was analysed by GFAAS | 0.15 – 2.5 µg/L | 0.05 µg/L | 1.7% | -- | Chen et al., 2009 |

**Table 1.3.** LOV systems applied to the determination of inorganic chemical analytes. (cont.)

| Analyte | Matrix | Determination Characteristics | Detection system | Dynamic range | LOD | RSD | Deter. rate | Reference |
|---|---|---|---|---|---|---|---|---|
| As | Tap, underground water, CRM | SPE performed by Q-Sepharose Fast Flow strong anion exchanger | Atomic fluorescence spectroscopy | 0.05 – 0.3 ng/mL | 0.02 ng/mL | 5.7% | 9 /h | Long et al., 2006a |
| As | Rice, human hair | Integrates hydride generation, gas-liquid separation | Atomic fluorescence spectroscopy | 0.10 – 5.00 µg/L | 0.03 µg/L | 2.8% | 80/h | Yu et al., 2008 |
| Bi | CRM 320, water, human urine | Sepahadex C-25 cation exchanger for on-line preconcentration | ICPMS | 0.04 – 3.2 µg/L | 4 ng/L | 1.7% | 12 /h | Wang and Hansen, 2001a |
| Bi | CRM 320, water, human urin | Sephadex C-25 beads used for on-line preconcentration | ETAAS after elution | 0.05 – 3.0 µg/L | 27 ng/L | 2.3% | 10 /h | Wang and Hansen, 2001b |
| $Ca^{2+}$ | No application | No sample treatment | Potentiometry with all-solid state electrode | $10^{-1}$ – $10^{-4}$ M | -- | 2 % | 10 to 20 /h | Kikas and Ivaska, 2007 |
| Cd | CRM, urine sample | SPE was evaluated for PTFE and Copolymeric $C_{18}$ beads | ETAAS after elution of Cd | 0.05 – 1.00 µg/L<br>0.2 – 1.5 µg/L | 15 ng/L<br>126 ng/L | <3.4% | 12 /h | Miró et al., 2003 |

**Table 1.3.** LOV systems applied to the determination of inorganic chemical analytes. (cont.)

| Analyte | Matrix | Determination Characteristics | Detection system | Dynamic range | LOD | RSD | Deter. rate | Reference |
|---|---|---|---|---|---|---|---|---|
| Cd | CRM | SPE was evaluated for Granular PTFE and Aldrich PTFE beads | ETAAS after elution of Cd | 0.05 – 0.25 µg/L<br>0.05 – 1.00 µg/L | 5.5 µg/L<br>5.0 µg/L | 1.3%<br>3.0% | 12 /h | Long et al., 2004 |
| Cd | River sediment, sea lettuce, frozen cattle blood | Target ions were sorbed by chelation in Sepharose Fast Flow beads | ETAAS after elution | 0.005 – 0.050 µg/L | 0.001 µg/L | 3.6% | 12 /h | Long et al., 2005c |
| Cd | River sediment, sea lettuce, frozen cattle blood | $C_{18}$ silica, Copolymeric $C_{18}$ and silica microbeads for bead injection | ETAAS after elution | 0.01 – 0.2 µg/L | 1.7 ng/L | 2.1% | 13 /h | Wang et al., 2005 |
| Cd | River sediment, sea lettuce, frozen cattle blood | Copolymeric $C_{18}$ beads for bead injection | HG - AFS | -- | 3.5 ng/L | 1.6% | 11 /h | Wang et al., 2006 |
| Cd | River sediment, sea lettuce, frozen cattle blood | Performance of $C_{18}$ versus PTFE KR beads | ETAAS after elution | 0.01 – 0.2 µg/L<br>0.02 – 0.3 µg/L | 1.7 ng/L<br>3 ng/L | 2.1%<br>4.7% | 13 /h<br>20/h | Yang and Jianhua, 2006 |
| Cd | Environmental water | Out-line sample treatment | Stripping voltametry, nafion coated bismuth electrode | 2.0 – 100.0µg/L | 0.88 µg/L | 3.65% | 22 /h | Wang et al., 2010b |

**Table 1.3.** LOV systems applied to the determination of inorganic chemical analytes. (cont.)

| Analyte | Matrix | Determination Characteristics | Detection system | Dynamic range | LOD | RSD | Deter. rate | Reference |
|---|---|---|---|---|---|---|---|---|
| Cd | Water | No sample treatment | Stripping voltametry, morin modified electrode | 1 – 125 µg/L | 0.41 µg/L | < 3.1% | 27 /h | Wang et al., 2011a |
| Chloride | No application | Reagent and sample were aspirated by a spacer | Spectrophotometry | 0 – 1000 ppb<br>0 – 100 ppm | 45 ppb | -- | -- | Nishihama et al., 2002 |
| Co | Environmental, aqueous biological samples | MNPs- PAA for nano absorbent for on-line trace analysis | ICPMS | 0.5 – 50 µg/L | 0.04 – 0.6 µg/L | < 4% | 5 min | Lee et al., 2009 |
| Cr(IV) | Natural, tap and sea water | DPC loaded beads are used as reagent carrier | ETAAS after elution | 0.12 - 1.5 µg/L | 0.03 µg/L | 3.8% | 15 /h | Long et al., 2005a |
| Cr(III)<br>Cr(IV) | Tap, river, sea water and CRM | SPE performed by Q-Sepharose Fast Flow strong anion exchanger | ETAAS after elution | 0.02 – 0.28 µg/L<br>0.035- 0.4 µg/L | 0.010 µg/L<br>0.020 µg/L | 2.4%<br>2.2% | 12 /h<br>8 /h | Long et al., 2005b |
| Cr(IV) | CRM agricultural soil | Q-Sepharose Fast Flow beads used for preconcentration | ETAAS after elution | 0.02 – 0.6 ng | -- | < 6.8% | -- | Long et al., 2006c |

**Table 1.3.** LOV systems applied to the determination of inorganic chemical analytes. (cont.)

| Analyte | Matrix | Determination Characteristics | Detection system | Dynamic range | LOD | RSD | Deter. rate | Reference |
|---|---|---|---|---|---|---|---|---|
| Cr(IV)<br>Cr(III)<br>total Cr | Water | Sodium bismuthate immobilised in silica beads for oxidation in micro column | Spectrophotometry | 24 – 2000 µg/L<br>32 – 2000 µg/L<br>32 – 2000 µg/L | 5.6 µg/L<br>8.2 µg/L<br>6.8 µg/L | 2.0%<br>3.2%<br>2.8% | 60 /h | Yang et al., 2007 |
| Cu | Environmental, aqueous biological samples | MNPs- PAA for nano absorbent for on-line trace analysis | ICPMS | 0.5 – 50 µg/L | 0.04 – 0.6 µg/L | < 4% | 5 min | Lee et al., 2009 |
| Cu(II) | Wastewater | Reaction with zincon in air segmented system | Spectrophotometry | 0.1 – 2.0 mg/L | 0.05 mg/L | 2.0% | 120 /h | Leelasattara-thkul et al.,2006 |
| Cu(II) | Wastewater | 5-Br-PSAA as chromogenic reagent | Spectrophotometry | 0.1 – 2 mg/L | 50 µg/L | < 2% | 18 /h | Ohno et al., 2006 |
| Fe(II) | Wastewater | 5-Br-PSAA as chromogenic reagent | Spectrophotometry | 0.1 – 5 mg/L | 25 µg/L | 1.8% | 18 /h | Ohno et al., 2006 |
| Fe(III) | Wine | SPE performed by NTA superflow resin | Spectrophotometry | 0.09 – 5.0 mg/L | 0.02 mg/L | -- | 20 /h | Vidigal et al., 2011 |

**Table 1.3.** LOV systems applied to the determination of inorganic chemical analytes. (cont.)

| Analyte | Matrix | Determination Characteristics | Detection system | Dynamic range | LOD | RSD | Deter. rate | Reference |
|---|---|---|---|---|---|---|---|---|
| Fe | *E. coli* culture | Reaction of iron with ferrozine | Spectrophotometry | 80 –400ppm | -- | -- | -- | Wu et al., 2001 |
| $H_2O_2$ | Lens care solutions | In-line dilution by means of dialysis; enzymatic reaction with HRP and ABTS | Spectrophotometry | Up to 4.28 mg/L<br>Up to 342 mg/L | 0.20 mg/L<br>16.1 mg/L | < 3% | 45 /h | Vidigal et al., 2009 |
| $H_2O$ | Live cells immobilised | Cytodex 2 micro carrier beads used for immobilisation | Voltametry | 10 – 1000 µM | 9 µM | -- | -- | Lähdesmäki et al., 2007 |
| $H_2O_2$ | No application | Cytodex micro carrier beads for immobilisation | Fluorimetry and spectroscopy | Up to 100 µM | -- | -- | -- | Lähdesmäki et al., 2009 |
| Hg | Ancient papers | SPE by Strong acidic Dowex-50W cation and Strong basic Dowex-1W anion exchange | X-ray fluorescence spectrometry, after elution | 0.2 – 2 mg/L | 0.06 µg/g | 6.37% | -- | Alcalde-Molina et al., 2009 |
| Hg | No application | Integrates a gas expansion separator | CV-AFS | 0 -300 µg/L | 9 µg/L | ± 5% | 43 /h | Erxleben and Růžička, 2005a |

**Table 1.3.** LOV systems applied to the determination of inorganic chemical analytes. (cont.)

| Analyte | Matrix | Determination Characteristics | Detection system | Dynamic range | LOD | RSD | Deter. rate | Reference |
|---|---|---|---|---|---|---|---|---|
| Hg | CRM, water, waste incineration ash | Thin layer of $NaBH_4$ | Atomic fluorescence spectroscopy | 0.2 – 10.0 µg/L | 0.06 µg/L | 2.9% | 60 /h | Yu et al., 2007a |
| Hg | CRM, water, waste incineration ash | Integrates a vapour generator, gas-liquid separation | Atomic fluorescence spectroscopy | 0.06 – 10.0 µg/L | 0.1 µg/L | 4.4% | 90 /h | Yu et al., 2007b |
| Hg | Spiked environmental samples | SPE by oasis HLB beads | CV- AFS | 0.1 – 60 µg/L | 0.04 – 0.2 µg/L | < 3.8% | -- | Anthemidis et al., 2010 |
| Mn | Environmental, aqueous biological samples | MNPs- PAA for nano absorbent for on-line trace analysis | ICPMS | 0.5 – 50 µg/L | 0.04 – 0.6 µg/L | < 4% | 5 min | Lee et al., 2009 |
| Ni | River sediment, sea lettuce, frozen cattle blood | Target ions were sorbed by chelation in Sepharose Fast Flow beads | ETAAS after elution | 0.05 – 1.00 µg/L | 0.02 µg/L | 3.2% | 12 /h | Long et al., 2005c |
| Ni | Saline matrices, tap, sea water | Oasis HLB and $C_{18}$ PS/DVB for bead injection | ETAAS after elution | 0.2 – 2 µg/L | 0.05 µg/L | 4.5% | 10 /h | Long et al., 2006b |

**Table 1.3.** LOV systems applied to the determination of inorganic chemical analytes. (cont.)

| Analyte | Matrix | Determination Characteristics | Detection system | Dynamic range | LOD | RSD | Deter. rate | Reference |
|---|---|---|---|---|---|---|---|---|
| Ni | CRM 320, water, human urine | Sepahadex C-25 cation exchanger for on-line preconcentration | ICPMS | 0.05 – 2.4 µg/L | 15 ng/L | 2.9% | 12 /h | Wang and Hansen, 2001a |
| $O_2$ | Live cells immobilised | Cytodex 2 micro carrier beads used for immobilisation | Voltametry | 160 – 616 µM | 13 µM | -- | -- | Lähdesmäki et al., 2007 |
| Pb | No application | SPE by sephadex impregnated with dithizone | ETAAS | 1 – 4 ng/L | 0.3 ng/L | 1.9% | 12 /h | Ampan et al., 2003 |
| Pb | River sediment, sea lettuce, frozen cattle blood | Target ions were sorbed by chelation in Sepharose Fast Flow beads | ETAAS after elution | 0.10 – 2.00 µg/L | 0.07 µg/L | 5.1% | 12 /h | Long et al., 2005c |
| Pb | Water | stripping processes took place between the renewable mercury film carbon electrode | Anodic stripping voltametry | 1.0 – 100.0 µg/L | 0.3 µg/L | 3.14% | 45 /h | Wang et al., 2009b |
| Se(IV) | Human hair | Hg film coated glassy carbon electrode was used | Anodic stripping voltametry | 1 – 600 µg/L | 0.11 µg/L | 3.81% | 20 /h | Wang et al., 2009a |

**Table 1.3.** LOV systems applied to the determination of inorganic chemical analytes. (cont.)

| Analyte | Matrix | Determination Characteristics | Detection system | Dynamic range | LOD | RSD | Deter. rate | Reference |
|---|---|---|---|---|---|---|---|---|
| Th (IV) | Water | SPE performed by UVETA resin; arsenazo-III as chromogenic reagent | Spectrophotometry (LWCC) | 0 – 2.000 µg/L | 6.0 ng/L | 1.6% | 6 /h | Avivar et al., 2011 |
| U (VI) | Water, phosphate fertiliser | SPE performed by UVETA resin; arsenazo-III as chromogenic reagent | Spectrophotometry (LWCC) | 0 – 0.3 µg/L | 10.3 ng/L | 1.6% | 5.5 /h | Avivar et al., 2010 |
| U (VI) | Water | SPE performed by UVETA resin; arsenazo-III as chromogenic reagent | Spectrophotometry (LWCC) | 0 – 1.200 µg/L | 5.9 ng/L | 1.6% | 6 /h | Avivar et al., 2011 |

Most of the developed methodologies in SI-LOV format were used for determinations of inorganic chemical analytes. Among them, cadmium(II) is the most reported one (Fig. 1.15.).

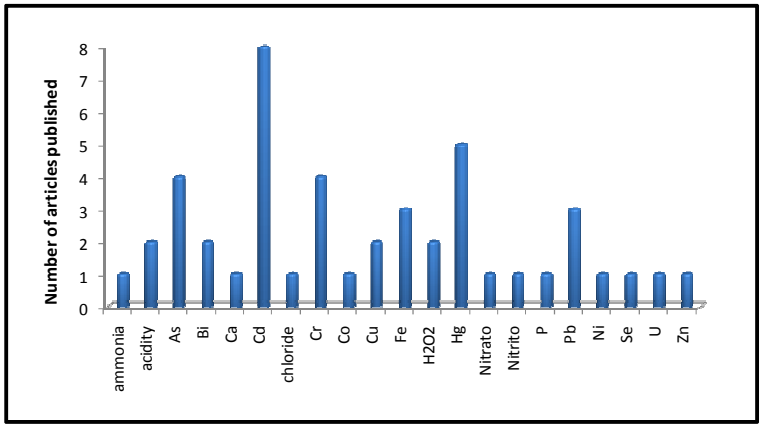


**Fig. 1.15.** Number of articles published reporting development of methods applied to inorganic chemical analytes.

The determinations in total Cd were accomplished by bead injection, using poly(tetrafluoroethylene) (PTFE) (Miró et al., 2003), granular PTFE (Long et al., 2004) or Aldrich PTFE (Long et al., 2004), Copolymeric $C_{18}$ (Miró et al., 2003; Wang et al., 2006; Wang et al., 2005c; Yang and Jianhua, 2006b; Long et al., 2005c), and silica (Wang et al., 2005c) beads. As for detection system, in most of the cases, the detection is performed by electrothermal atomic absorption spectrometry (ETAAS) (Long et al., 2004; Miró et al., 2003; Wang et al., 2005c; Yang and Jianhua, 2006b; Long et al., 2005c) and by hydride generation atomic fluorescence spectrometry

(HG-AFS) (Wang et al., 2006). Stripping voltammetry was also used for this determination in water samples without the bead injection approach (Wang et al., 2010b; Wang et al., 2011a).

The determination of Hg(II) was also reported by several authors; in the majority of the studies atomic fluorescence spectrometry (AFS) was chosen as detection system. The system integrates vapor generation, gas-liquid separation, cold vapor excitation and fluorescence detection (CV-AFS) onto the SI-LOV apparatus (Anthemidis et al., 2010; Yu et al., 2007a; Yu et al., 2007b; Erxleben and Růžička, 2005a). Among the methodologies reported for this determination, only two of them employed bead injection, using oasis HLB (Anthemidis et al., 2010) and Downex, ion-exchange (Alcalde-Molina et al., 2009) beads.

Bead injection coupled to ETAAS was used for the determination of Cr(III) (Long et al., 2005b) and Cr(VI) (Long et al., 2006c; Long et al., 2005a; Long et al., 2005b). A methodology for the determination of total Cr was also developed (Yang et al., 2007) however without the use of BI nor ETAAS; in this case the determination was quantified spectrophotometrically after the reaction with 1,5-diphenylcarbazide (DPC).

Atomic fluorescence spectrometry was used for the determination of As(II) integrating hydride generation (Yu et al., 2008) or with SPE performed by a sepharose exchanger for total As (Long et al., 2006a). These types of beads were even used to retain this analyte that was quantified by X-ray fluorescence

spectrometry after elution (Alcalde-Molina et al., 2009). Total As was also retained by immobilized cell on sephadex beads and quantified by GFAAS after elution (Chen et al., 2009).

Lead(II) was quantified in two different methodologies: one with detection by anodic stripping voltammetry (Wang et al., 2009b) the other with SPE by magnetic nanoparticles with polyacrylic acid (MNPs-PAA) and detection by inductively coupled plasma mass spectrometers (ICPMS) (Lee et al., 2009). The Pb(II) ions were also chelated on sepharose beads and quantified by ETAAS after elution (Long, 2005c). A methodology for the determination of total Pb was developed combining SPE with shepharose G-25 beads were directly propelled into a graphite tube where they were pyrolyzed and lead ions were subsequently atomized by ETAAS (Ampan et al., 2003).

Total iron content was quantified spectrophotometrically after reaction with ferrozine (Wu et al., 2001) and Fe(II) after reaction with 5-Br-PSAA (2-(5-bromo-2-pyridylazo)-5-[*N*-*n*-propyl-*N*-(3-sulfopropyl)amino]aniline) (Ohno et al., 2006). A BIA methodology was developed for the quantification of Fe(III) (Vidigal et al., 2011), the ions were retained by NTA sepharose beads and the quantification was attained by spectrophotometry after reaction with $SCN^-$.

A methodology was developed for the enzymatic determination of $H_2O_2$ with in-line dilution by means of a dialysis with spectrophotometry detection (Vidigal et al., 2009). BIA mode with cytodex microcarrier beads was used to retain this analyte

that was later quantified by spectrophotometry and fluorimetry (Lähdesmäki et al., 2009) or by voltametry (Lähdesmäki et al., 2007); in this latter application the determination of $O_2$ was also carried out.

Different types of beads were used for the development of methods for Ni(II) quantification. Chelating Sepharose TM Fast Flow (Long et al., 2005c) and oasis HLB (Long et al., 2006b) beads were used to retain the analyte quantified by ETAAS after elution. Sephadex C-25 cation exchanger was also used for on-line preconcentration of Ni(II) and Bi(II) with quantification by ICPMS (Wang et al., 2001a).

Nitrite, nitrate and phosphate were also aim of study. Nitrate reduction was accomplished by a Cd/Cu column and the resulting nitrite was quantified by spectrophotometry after reaction with sulfanilamide. The phosphate was quantified after reaction with molibdate (Wu and Růžička, 2001).

The examples presented here have undeniably demonstrated that SI-LOV is increasingly attracting interest as a programmable, flow-based, mesofluidic platform for the quantification of a vast number of different analytes. One added value of the system is the possibility of coupling different devices simplifying the sample pretreatment and/or the quantification.

The ability to deliver beads precisely in a flow-based system offers versatile manipulation for in-line sample pretreatment with a solid-phase reactor. With

increasing interest in automation and miniaturization, SPS shows promise for matrix removal and appropriate pre-concentration of targets or even chromatographic separation of multi-components by selecting the proper solid phase and suitable eluents. In addition, it also offers real-time information about binding chemistry via BIS.

# CHAPTER 2

## GENERAL MATERIAL AND METHODS

## 2.1. INTRODUCTION

The sequential injection lab-on-valve system was used in every developed methodology presented in this thesis. Therefore a general description of the components involved as well as the computer software programme, is detailed in this chapter. The optimisation procedures used in the development of the flow methods is also described here, as well as the statistical treatment used to assess the quality of the results, i.e. in terms of repeatability and accuracy.

## 2.2. REAGENTS AND SOLUTIONS

All chemicals were of analytical grade and no further purification was applied. Milli-Q water with a resistivity of 18 mΩ $cm^{-1}$ was used in the first methodology presented in this thesis (Chap. 3). In the following works (Chap. 4-7), deionised water with specific conductance less than 0.1 μS $cm^{-1}$ was used.

Standard stock solutions were obtained by weighing the respective reagent in a Sartorius analytical balance (model SB 2105), followed by dissolution in appropriate solutions. Otherwise, the solutions were prepared by rigorous dilution from a concentrated solution.

When necessary, the concentration of the stock solution was determined by titration with a standard solution (Chap. 4 and 5). The working standards were

obtained by rigorous dilution of the stock solution using glass pipettes and volumetric flasks (class A) of different volumes.

In the enzymatic assays (Chap. 3 and 5), Gilson micropipettes (models P100 and P1000, with corresponding maximum capacities of 100 and 1000 µL) were also used. The micropipettes were regularly calibrated with deionised water.

When required, pH of solutions was measured using a combined glass pH electrode (Mettler U402-S7/120) and a Crison (model 2002) potentiometer.

## 2.3. COMPONENTS OF THE FLOW SYSTEM

The SI-LOV system used to develop the methodologies presented in this thesis was a FIAlab-3500 from FIAlab Instruments, Medina, WA, USA. The principal components are described below.

The SI-LOV system consisted of a bi-directional syringe pump (2500 µL of volume), a holding coil, a bi-directional variable speed peristaltic pump, and a lab-on-valve manifold mounted on the top of a six-port multi-position valve.

## 2.3.1. Propulsion devices

The FIAlab 3500 system has a high resolution bi-directional micro syringe pump (Fig. 2.1.A) and a 4 channel peristaltic pump (Fig. 2.1.B) as propulsion devices. The micro syringe pump with a glass syringe (Cavro) of 2500 µL of volume was the main component used to aspirate or propel the solutions from the multi-position valve to or from the holding coil, respectively. The peristaltic pump was employed as auxiliary propulsion device to circulate the sample solution (Chap. 3, 4 and 5) or the bead injection suspension (Chap. 6 and 7).

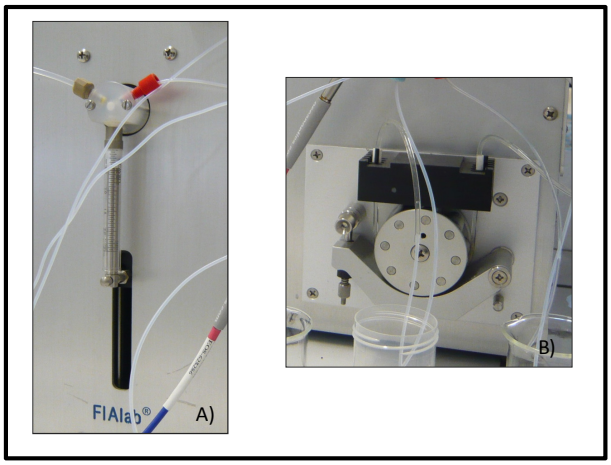


**Fig. 2.1**. Photographs of the propulsion devices: **A)** Syringe pump; **B)** Peristaltic pump.

## 2.3.2. Valves

A two way commutation valve was placed on the top of the syringe of the syringe pump.

A six-port selection valve multi-position from Vici Cheminert® was used. The valve body is placed inside the FIAlab 3500 box and the lab-on-valve CSPU is mounted on the place of its stator (Fig. 2.2).

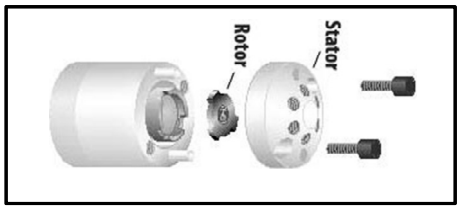


**Fig. 2.2**. Exploded view of the ViciCheminert® selection valve (www.vici.com).

A schematic representation of the valve is given in Fig. 2.3. This type of valve acts as a stream selector, connecting just one side port to the central one, and this central port is connected to the holding coil.

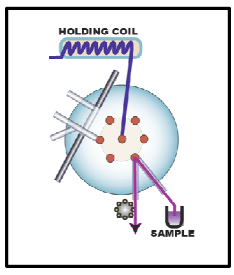


**Fig. 2.3.** Schematic representation of the selection valve, illustrating the six ports, the sample load port and the connection to the holding coil (Růžička, 2009).

### 2.3.3. Flow tubes, connectors and other devices

All the tubing connecting the different components of the system was made of Teflon from Upchurch Scientifics, as well as the flangeless nuts and ferrules used in the end-fittings.

In Chap. 4, a laboratory made diffusion unit was incorporated in the manifold. It consisted of two acrylic blocks with matching cavities of 72 $mm^2$ of surface area and 0.5 mm of channel depth in a linear path (Fig. 2.4.). The two blocks were pressed against each other by four screws. The dialysis membrane was placed between these two blocks making two symmetrical flow channels. The in-line dilution was performed by means of a pre-mounted cellulose acetate dialysis membrane.

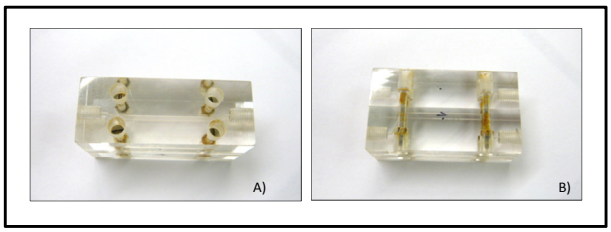


**Fig. 2.4.** Photograph of the dialysis unit. **A)** Top view of the unit and **B)** side view of the dialysis unit.

### 2.3.4. Lab-on-valve manifold

The flow cell integrated in the lab-on-valve module has four outlets capable of accommodating optical fibres that were mounted axially to perform a

spectrophotometric detection. The optical path length within this module can be varied from 2 to 10 mm. Two different lengths of light paths were used, 10 mm (Chap. 3, 4 and 5) and 2 mm (Chap. 6 and 7).

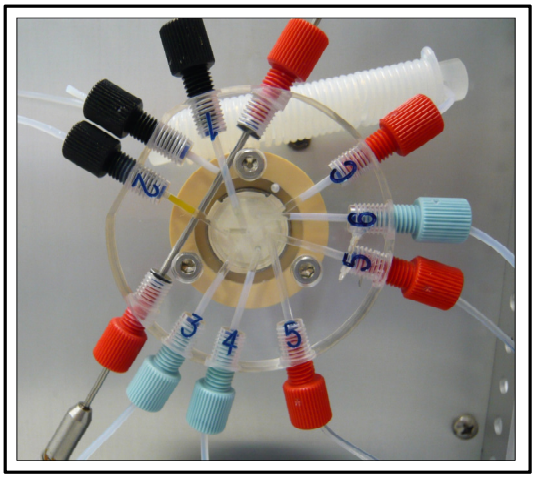


**Fig. 2.5.** Photograph of the CSPU lab-on-valve manifold; the flow cell is positioned as used in Chap. 6 and 7.

## 2.3.5. Detection system

The detection system comprised a USB 2000 Ocean Optics a charge coupled device (CCD) spectrophotometer, a DH-2000-BAL Mikropack, UV/VIS/NIR light source and fibre optics cables. For the methods described in chap. 3, 4 and 5 the optical fibres were P200 with 200 µm of i.d. For the other methods (Chap. 6 and 7), the optical fibres were P400-SR with an i.d. of 400 µm.

## 2.4. COMPUTER SOFTWARE PROGRAM

The FIAlab for Windows 5.0 (Fig. 2.6) program on a personal computer was used. This software can be used for data collection from the FIAlab-3500 system and for control of their family of peripheral devices. The family of external devices controllable by FIAlab for Windows include: a multi-port valve, an Alitea peristaltic pump, a syringe pump, an USB 2000 Ocean Optics spectrometer, and data acquisition devices.

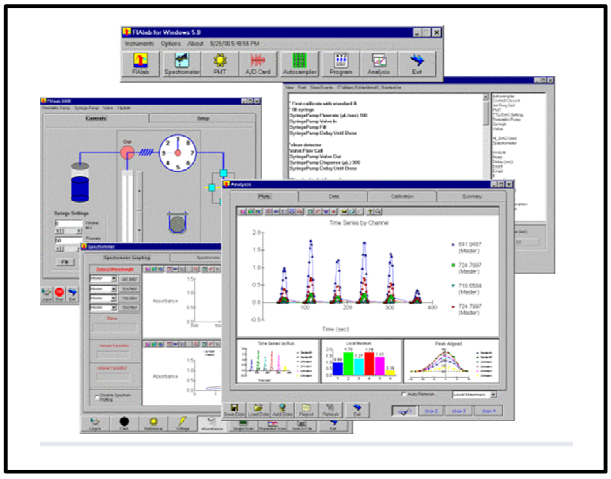

**Fig. 2.6** Layout of the FIAlab for Windows 5.0 program user interface (www.flowinjection.com).

## 2.5. OPTIMISATION OF THE FLOW SYSTEM

When the sequential injection lab-on-valve method was initially established, the parameters were roughly optimised on a trial and error basis to allow each

determination to be performed between the expected concentration limits. Afterwards, the parameters were optimised in order to attain maximum sensitivity and sampling rate with the lowest reagent consumption. During the optimisation studies the univariate method was used; each parameter to be optimised was varied within a certain interval, keeping the other parameters fixed. Exception was made in the optimisation of the methodology for the determination of total protein content (Chap. 7); in this work, one part of the optimisation was based on the experimental design that is further detailed in the corresponding chapter.

Afterwards, the optimised methods were characterised in terms of reagent consumption, application range, limits of detection and quantification, determination rates and repeatability.

The application range was determined by injecting a series of working standards solutions with different concentrations, and by establishing the range where the analytical signal was related with the concentration in a linear or a second order polynomial function.

The limit of detection (LOD) was calculated (Chap. 4, 5 and 6) according to IUPAC recommendations (IUPAC, 2007). When expressed in concentration units, it derived from the smallest measure, $\chi_{LOD}$ , that can be detected with reasonable certainly for the developed analytical signal. The value of $\chi_{LOD}$ is given by the equation:

$$\chi_{LOD} = \overline{\chi}_b + 3S_b$$

Where $\overline{\chi_b}$ is the mean of the blank measurements (10 consecutive measurements), $S_b$ is the standard deviation of those blank measurements, and 3 is a numerical factor determined assuming a 95% confidence level. In Chapter 3 and 7, the LOD was calculated using the unweighted least-squares method (Miller and Miller, 1993) where each point of a plot of the calibration curve has a normally distributed variation with a standard deviation estimated by $S_{y/x}$. This measure can be used in the place of $S_b$ for the estimation of the limit of detection and the value of limit of detection can be calculated based on the calibration curve:

$$\chi_{LOD} = b + 3S_{y/x}$$

The quantification limit (LOQ) was calculated using a similar procedure as for the LOD and was also expressed in concentration units (IUPAC, 1995). The calculated value derived from the smallest measure, $\chi_{LOQ}$, that can be quantified with reasonable certainty for the developed analytical procedure. The value of $\chi_{LOQ}$ is given by the equation:

$$\chi_{LOQ} = \overline{\chi}_b + 10S_b$$

where $\overline{\chi_b}$ is the mean of the blank measurements (10 consecutive measurements), $S_b$ is the standard deviation of those blank measurements, and 10 is a default value by IUPAC. In Chapter 3 and 7, the LOQ was calculated as the LOD using the

unweighted least-squares method (Miller and Miller, 1993). The value of limit of detection can be calculated based on the calibration curve:

$$\chi_{LOQ} = b + 10S_{y/x}$$

The determination rate was calculated as the sum of the time necessary for each operation step, that is to say the time required for a complete analytical cycle. This rate was expressed as number of determinations per hour.

Repeatability of the methods developed was evaluated in terms of relative standard deviation (RSD), expressed in percentage, corresponding to consecutive determinations of the same sample. Samples with different analyte concentrations were injected to provide a reliable estimate of the repeatability of the method. The repeatability was also assessed by performing calibration curves under identical physical and chemical conditions between and within different working days. The results obtained were analysed in a 95% confidence level.

## 2.6. ANALYSIS

Calibration curves were established by injecting working standard solutions into the flow system and registering their corresponding analytical signal (absorbance).The relationship between signal and the corresponding concentration was linear for all of the methodologies developed with the exception of the determination of ethanol

in beverages (Chap. 3) that followed a polynomial (second order) relationship. The concentration of the samples was calculated by interpolation in the previously established calibration curves.

For the initial rate measurements, the calibration curve was established by recording the variation of absorbance during a period of time and plotting that variation over time against the corresponding standard concentration.

The accuracy of the developed methods was evaluated by comparing the results obtained by the proposed method ($C_{SI\text{-}LOV}$) with those obtained by the corresponding reference procedure ($C_{REF}$). A linear regression was established with the results obtained for each variable. The parameters of the regression line, intercept ($b$) and slope ($m$), were calculated. In a perfect agreement of the methods, $b$ should be equal to 0 and $m$ equal to 1. Thus the methods are statistically comparable if $b$ and $m$ do not differ significantly from 0 and 1, respectively. This was verified by estimation the errors in the slope and intercept values through calculation of their confidence limits at 95% significant level (Miller and Miller, 1993).

The relative deviation (RD) values, expressed as percentage, were calculated based on the expression:

$$\%RD = [(C_{SI\text{-}LOV}-C_{REF})/C_{REF}]x\ 100$$

In Chap. 3, the accuracy of the developed method was also evaluated by the application to a certified reference sample (CRM). The comparison to the certified

value was based on a significant test (Miller and Miller, 1993). Using the following expression:

$$t = (\bar{\chi} - \mu)\sqrt{n}/s$$

where $\bar{\chi}$ is the mean of the results obtained, $n$ is the number of samples, $s$ is the standard deviation of those results and $\mu$ is the value to be compared, in this case is the reference value. The value of $t$ calculated is compared with the critical value listed in $t$-distribution tables. If the value calculated, $|t|$, is below the $t_{critical}$ it means that the results are not statistically different; if the value exceeds the $t_{critical}$ it means that there is a systematic error in the results.

In the methodology developed for the determination of total protein content in white wines (Chap. 7) a significant test was also used to evaluate the results obtained in the interferences studies. These studies were carried out by adding the specie at the expected level present in the sample to a standard. In this case, $\bar{\chi}$ is the mean of the results obtained in the spiked standard and $\mu$ is the expected value, that is to say the result obtained for the standard without addition.

In the same chapter, recovery studies were carried out. The samples were analysed before and after addition of the analyte and the recovery ($R_A$) was calculated as recommended by IUPAC (IUPAC, 2002), using the following expression:

$$R_A = [Q_A(O+S) - Q_A(O)] / Q_A(S)$$

Were $Q_A(S)$ is the quantity if analyte A added and $Q_A(O+S)$ the quantity of A recovered from the spiked sample and $Q_A(O)$ the quantity of the original sample.

The recovery value was presented in percentage and a significant test was used to evaluate if the percentages were statistically different from 100%. Here, $\overline{\chi}$ is the mean of the percentages obtained for each sample and $\mu$ is the expected value, i.e., 100%.

In chapter 5, significance two-tailed $F$ -test (Miller and Miller, 1993) was performed to evaluate the variance of the results obtained for the sample analysis. The $F$ value is calculated considering the squares of the standard deviations, and is given by:

$$F = s_1^2 / s_2^2$$

where $s_1^2$ and $s_2^2$ were the squares of the standards deviations of the results obtained. The calculated value is then compared to the critical value (obtained from tables), if the $F_{value}$ is lower than the $F_{critical}$, there is no significant difference between the two variances at the 5% level. On the contrary, if e $F_{value}$ is higher than the $F_{critical}$, there is evidence of a systematic error.

# CHAPTER 3

## ENZYMATIC DETERMINATION OF ETHANOL BY PEAK HEIGHT AND KINETIC MEASUREMENT MODES: APPLICATION TO ALCOHOLIC BEVERAGES

## 3.1. INTRODUCTION

Flow analysis systems have been gaining an increased importance in wet analytical chemistry. This fact can be mainly explained by the possibility of automating analytical chemical procedures with a simultaneous dramatic decrease in reagents consumption. The trend for automation and miniaturization is particularly important for biochemical methods of analysis, due to the elevated costs of the reagents involved in enzymatic and immuno-assays, as well as by the often-limited amount of the samples available. Therefore, the possibility to perform the biochemical assays within small-integrated manifolds through flow-based automation of sample and reagent handling has received increased attention.

In this scenario, flow methods became widely popular among the scientific community, due to the possibility of automatic sample handing resorting to mostly simple and low cost apparatus. Therefore the objective of this work was to study the potential of the SI-LOV format for the miniaturization of enzymatic assays. As a case study, we selected the enzymatic determination of ethanol in alcoholic beverages, an important parameter to define the quality and the stability of the product. The reference methods proposed by Office International de la Vigne et du Vin (OIV) are complex, laborious and most of them require distillation as sample pre-treatment (OIV, 2005; Analytica EBC, 2005; NP 2143, 1987). To overcome these disadvantages enzymatic flow systems were developed for this determination (Worsfold et al., 1981; Matos et al., 1995; Künnecke and Mohns, 1995; Rangel and

Tóth, 2000; Segundo and Rangel, 2002b; Mori et al., 2003; Páscoa et al., 2006; Lázaro et al., 1987; Rangel and Tóth, 1999; Hedenfalk and Mattiason, 1996; Lapa et al., 2003). Most of these methods exploit the reaction between the immobilized alcohol dehydrogenase with ethanol in the presence of $NAD^+$ to produce acetaldehyde and NADH. The detection is generally spectrophotometric of the produced NADH at 340 nm. The methods are fast, simple and do not require any other sample pre-treatment than dilution, however the process of enzyme immobilization frequently involves the manipulation of toxic reagents (glutaraldehyde), and the support material used for the immobilization process (controlled pore glass) can add to the cost of analysis.

In this scenario, our study comprised two main aspects: (i) the use of the SI-LOV format to further miniaturize the flow system and prevent the need for the enzyme immobilization; this study implied the assessment of the efficiency of the zones overlapping in LOV (ii) comparison of two quantification methodologies, peak-height based versus initial-rate measurement; the latter one to cope with the possible interference of the sample intrinsic absorption (OIV, 2005; Chen and Růžička, 2004).

## 3.2. EXPERIMENTAL

### 3.2.1 Reagents and solutions

A 0.05 M phosphate buffer (106591, Merck) solution was prepared weekly and the pH of this solution was adjusted with phosphoric acid 8% v/v to 9.5. The 0.01M buffer, pH 7.5, for the enzyme suspension was prepared from $Na_2HPO_4.2H_2O$ (106580, Merck) in 100.0 mL of water.

To daily prepare the working enzyme solution, 1 mg of lyophilized ADH (alcohol dehydrogenase, EC 1.1.1.1, from baker's yeast, A7011, Sigma) was re-suspended in 1.00 mL of enzyme buffer pH 7.5, and this suspension was further diluted to 1.00 mL in the same buffer in a way to achieve the concentration of 24 U/mL in the working reagent solution. The cofactor solution was prepared daily by dissolving 0.0664 g of $NAD^+$ ($NAD^+$, free acid grade II, ~98%, 10621650001, Roche) in 5.00 mL of water.

The working standard solutions of ethanol were prepared daily from the stock standard solution (ethanol absolute pro analysis, 1210861212, UN1170, Panreac Quimica, SA) in a range between 0.010 and 0.040% (v/v).

For the zone overlapping study, a 24 mg/L bromothymol blue solution was prepared as described by Růžička and Hansen (1988). The carrier solution used for this study was borax 0.01 M.

### 3.2.2. Samples

Samples were purchased in a local supermarket. A total of ten samples were analyzed, using the content of the same bottle for the reference and for the developed flow methods. For the table wine and spirit samples no other treatment than dilution was applied before sample analysis. Table wines were 350 times diluted, and the spirit samples were diluted 2500 times. The beer samples were filtered, degassed and 400 times diluted before analysis.

A certified reference sample of low alcohol level wine was also analyzed (CRM 653, wine, nominal 0.5% vol). This sample was 20 times diluted before introduction into the SI-LOV system.

### 3.2.3. Manifold and flow procedures

The manifold for sequential injection lab-on-valve system for the enzymatic determination of ethanol in alcoholic beverages is depicted in Fig. 3.1.

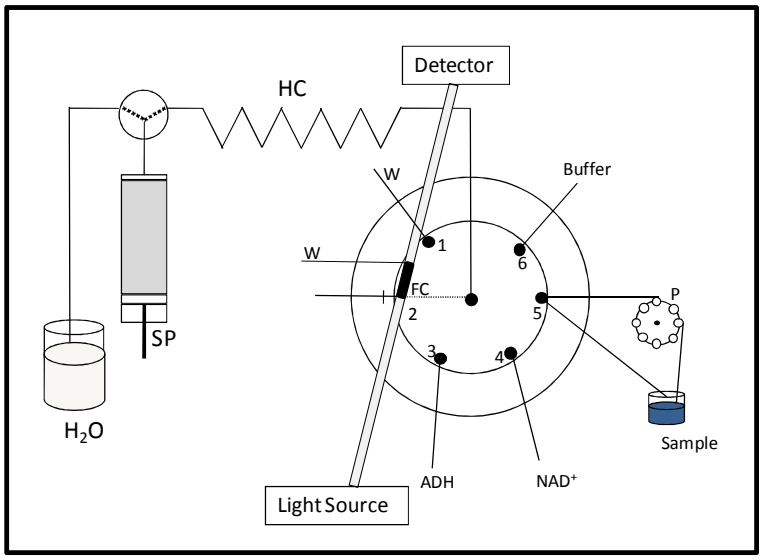


**Fig. 3.1** Configuration of SI-LOV system for the determination of ethanol; ADH, alcohol dehydrogenase 24 U/mL; $NAD^+$, cofactor 20 mM; Buffer, phosphate buffer pH 9.5; W, waste; SP, syringe pump (2.5 mL); HC, holding coil; FC, flow cell; P, peristaltic pump; Detector, CCD spectrophotometer.

The flow protocol was similar for the peak height and for the initial rate measurement, given in Table 3.1. The initial steps (A-F) consisted in the aspiration of carrier, buffer, sample, and reagents to the holding coil: 1000 µL of carrier, then 50 µL of buffer, 15 µL of sample solution, 5 µL of ADH (enzyme), 5 µL of $NAD^+$ (cofactor), and finally 100 µL of buffer. In the case of the peak height measurement, the following steps (G-J) consisted in reversing the flow and propelling the mixture, after 30 seconds of stop time in the holding coil, to the flow cell where the absorbance was measured.

To perform the initial rate measurement, the stacked zones (steps K-M) were directed to the flow cell and the flow was stopped and the change in absorbance was monitored during 15 seconds. Afterwards, the reaction zone was dispensed.

**Table 3.1** Flow Protocol for the enzymatic determination of ethanol in alcoholic beverages.

| Step | Description | Volume (mL) | Flow rate (mL/s) | Selection valve position |
|---|---|---|---|---|
| **A** | Aspirate carrier to HC | 1000 | 100 | - |
| **B** | Aspirate buffer to HC | 50 | 80 | 6 |
| **C** | Aspirate sample to HC | 15 | 25 | 5 |
| **D** | Aspirate enzyme to HC | 5 | 25 | 3 |
| **E** | Aspirate cofactor to HC | 5 | 25 | 4 |
| **F** | Aspirate buffer to HC | 100 | 25 | 6 |
| | Peak height measurement | | | |
| **G** | Reverse flow, reference scan | 10 | 15 | 2 |
| **H** | Stop period (30s) | - | - | - |
| **I** | Dispense HC content, data acquisition | 450 | 15 | 2 |
| **J** | System washing, SP empty | - | 100 | 2 |
| | Initial rate measurement | | | |
| **K** | Dispense selection of stacked zones | 100 | 15 | 2 |
| **L** | Stop period (15 s), data acquisition | - | - | - |
| **M** | System washing, SP empty | - | 100 | 2 |

## 3.2.4. Reference procedure

The reference method used for beer and wine samples consisted in the distillation of the sample (OIV, 2005; Analytica EBC, 2005), and Anton Paar DMA 5000 Density

meter was used for the measurement of the volumetric alcohol content of the distillates (OIV, 2005). The spirit samples were diluted (NP 2143, 1987) before Density meter reading (OIV, 2005).

## 3.3. RESULTS AND DISCUSSION

### 3.3.1. Study of the overlapping of reagent zones

Enzymatic assays usually comprise the mixing of various solutions like sample, enzyme, buffer, and frequently cofactor solutions. Therefore, when these assays are carried out in SIA mode, the number of plugs and the aspiration order are determined by the involved enzymatic reaction. In this work, the enzymatic assay is based on the reaction between ethanol and alcohol dehydrogenase in the presence of $NAD^+$ (cofactor), producing acetaldehyde and NADH. The formation of the reduced cofactor is measured spectrophotometrically at 340 nm. Additionally, the reaction occurs under controlled pH. Based on these conditions, the overlapping and mixing of the reagent zones is of great importance. Firstly the aspiration sequence was defined as buffer-sample-enzyme-cofactor-buffer; buffer solutions in the front and in the rear part of the sequence were used to sandwich the other reagents and assure the adjustment of the reaction pH. Within the sandwiched zone the sequence of the other reagents was selected to promote the penetration of the sample zone through the enzyme and cofactor sections (Růžička and Gübeli,

1991; Gübeli et al., 1991). In preliminary experiments, the aspiration flow rates were studied with the objective of using low sampling volumes with good repeatability. These preliminary studies were carried out using a bromothymol blue model solution (24 mg/L): when a combination of aspiration flow rate and volume uptaken fulfilled the criteria of RSD< 5% (n=10) for the absorbance values, it was applied in the enzymatic method. To assess the effect of the dimensions of the introduced reagent zones on the efficiency of the mixing, a study similar to Gübeli et al (1991) was carried out using also the model solution of bromothymol blue (24 mg/L). Using a borax solution (0.01 M) as carrier, the bromothymol blue solution was introduced sequentially in each port involved and the resulted peak profiles were recorded. The distance across the dispersed zone ($W_t$), the zone of total overlapping between the reagents ($W_o$), and the peak height absorbance (H) were measured. These parameters and the volumes studied are resumed in Table 3.2.

**Table 3.2** Study of the overlapping of reagent zones using a model solution of bromothymol blue (24 mg/L); A to F correspond to different configuration and volumes tested.

| Aspiration sequence | Volume (μL) | | | | | |
|---|---|---|---|---|---|---|
| | **A** | **B** | **C** | **D** | **E** | **F** |
| Plug1: Buffer | 50 | 100 | 50 | 50 | 50 | 50 |
| Plug2: Sample | 50 | 50 | 50 | 25 | 15 | 15 |
| Plug3: Enzyme | 25 | 25 | 25 | 10 | 5 | 5 |
| Plug4: Cofactor | 25 | 25 | 25 | 10 | 5 | 5 |
| Plug5: Buffer | 50 | 50 | 100 | 100 | 100 | 50 |
| $W_t$[a], s | 25 | 32 | 34 | 28 | 20 | 15 |
| $W_o$[b], s | 13 | 9 | 16 | 17 | 16 | 8 |
| H[c] | 0.331 | 0.337 | 0.270 | 0.138 | 0.096 | 0.127 |

[a] total baseline width of the dispersed sample-enzyme-cofactor reagent zones; [b] baseline width of the sample-enzyme-cofactor reagent zone with total overlapping; [c] peak height absorbance of the enzyme zone.

In configurations A to C, the relative volumes of the buffer zones were studied maintaining the total volume of the other zones at 100 µL. In configuration C; although $W_t$ was the largest, the zone of total overlapping between the reagents had also the largest extension, therefore this combination of the buffer solutions was maintained. To further improve the mixing conditions, the total volume of the sample-enzyme-cofactor sequence was reduced (configurations C to E); as expected, the dispersion of the enzyme zone increased (H decreased) with decreasing the total volume. Total overlapping between the sample, the enzyme and the cofactor zone was achieved in configuration E. With the objective of reducing the dispersion of the critical enzyme zone, while maintaining the low injection volumes, the last buffer zone was reduced to half (configuration F), the degree of overlapping of the zones deteriorated. Moreover, there is a need for a larger zone of the buffer solution to reach the flow cell before the reaction zone to perform a reference scan before the measurement. Therefore the configuration of E (Fig. 3.2) was maintained during the further studies. The repeatability of this aspiration sequence was also evaluated using the bromothymol blue solution. Good repeatability was found (n=10) for all the volumes used in the sequence; 50 µL of buffer (RSD< 0.7%), 15 µL sample (RSD< 1.0%); 5 µL enzyme solution (RSD< 2.9%); 5 µL cofactor solution (RSD< 3.0%), and 100 µL of buffer (RSD< 0.4%).

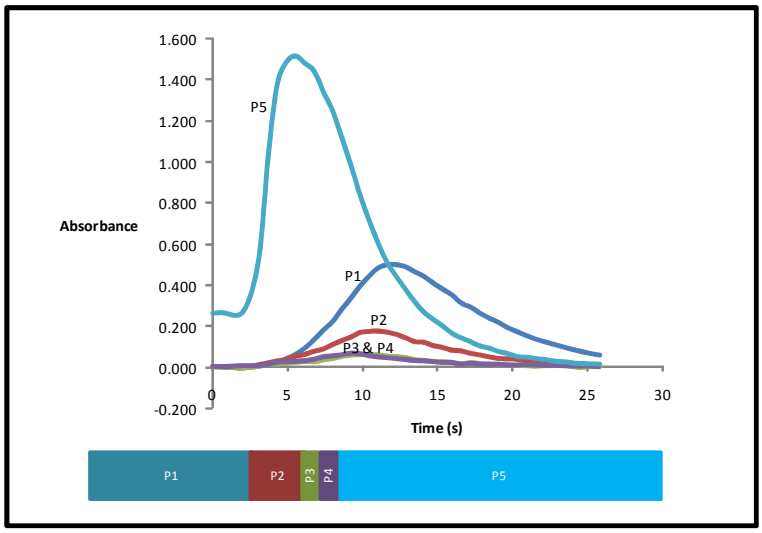


**Fig. 3.2** Recorded peak profiles ($P_i$) obtained by the injection of 24 mg/L of bromothymol blue and corresponding aspiration sequence: P1 corresponds to 50 µL of buffer;P2 15 µL of sample;P3 5 µL of enzyme (ADH);P4 5 µL of cofactor ($NAD^+$); and P5 100 µL of buffer.

It can be also concluded from Fig. 3.2. that in this aspiration sequence the dispersion of the cofactor and the enzyme zone is equal; therefore the order of aspiration of these two zones would not affect the overlapping of the reagent zones.

### 3.3.2. Study of the enzymatic reaction for peak height measurement mode

After establishing the volume of the reagent zones, a study of the influence of the chemical parameters (pH, $NAD^+$ and ADH concentration) on the sensitivity of the enzymatic reaction was studied. Univariate procedure was applied and the

sensitivity achieved in the range of 0.00 to 0.040% (v/v) of ethanol was monitored using the peak height measurement mode.

Initial conditions were established as diphosphate buffer pH 9.5 and 20 mM and 24 U/mL as $NAD^+$ and ADH concentrations, respectively, based on the previously published work (Páscoa et al., 2006) for the same determination using a SIA manifold.

The $NAD^+$ concentration was studied in a range between 10 to 30 mM. The sensitivity increased about 56% with the increase of the concentration from 10 to 20 mM. For higher concentrations the sensitivity decreased by 10%; therefore the concentration was set to 20 mM.

The effect of the enzyme concentration was studied for 14, 24 and 56 U/mL. The sensitivity increased 85% when the concentration was raised from 14 to 24 U/mL. By further increasing the concentration, the sensitivity did not show any further improvement, for that reason the concentration used was 24 U/mL.

The working pH is essential to assure the activity of the enzyme. This parameter was studied in a range between 8.5 and 10. The pH selected was 9.5 since it presented a higher sensitivity and was close to the reported optimum pH for this enzyme (Sigma, 2006).

One way to enhance the sensitivity of enzymatic reactions is to increase the reaction time before detection. In a LOV system this approach is quite easily

accomplished by introducing a stop period in the program sequence. A stop time (time elapsed between flow reversal of the stacked zones and propulsion to the detector) between 10 and 40 seconds was studied. The sensitivity increased with the stop time in the range of 10 to 30 seconds, but decreased about 57% when the stop time was 40 seconds. Thus the stop time selected was 30 seconds.

### 3.3.3. Study of the system for initial rate measurement mode

When samples exhibit either an intrinsic absorption or potential interferents with reaction kinetics different from the analyte, the initial reaction rate measurement could be the most efficient way to overcome these difficulties. The LOV format is particularly suitable for kinetic based measurements due to the easy manipulation of time sequences and the low volumes involved. In this case the stacked zones in the holding coil can be sent to the flow cell and the rate of product formation can be monitored during a pre-set time period. In this measurement mode, not only the length of the stop period is important for achieving adequate sensitivity and linearity, but also the volume used to propel the reagent zones to the flow cell. This volume will define what portion of the dispersed reagents/sample zone will be monitored during the initial rate measurement (Chen and Růžička, 2004; Růžička and Gübeli, 1991; Segundo and Rangel, 2003). This volume will also define the relative concentrations of the reagents and the sample inside the flow cell. These flow reversal volumes were studied between 90 and 120 μL, with 5 μL increments.

A higher initial rate ($\Delta A/\Delta t$) was achieved using a 100 µL volume; therefore this volume was set.

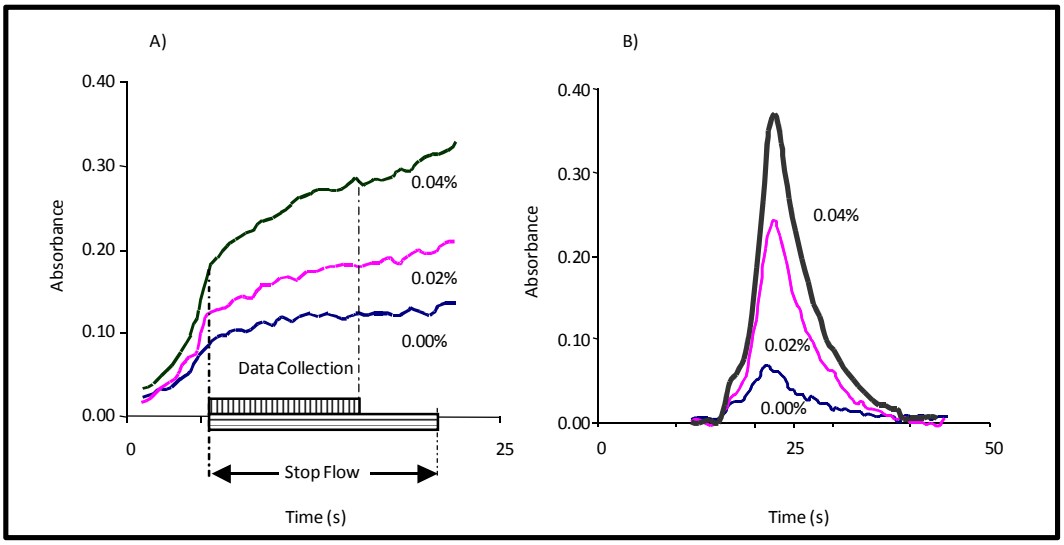


**Fig. 3.3**. Variation of the absorbance with the increase of the concentration of ethanol by (**A**) initial rate measurements and (**B**) peak height measurement.

The stop time in the flow cell was studied in the range 10 – 40s. When this stop time was higher than 15 seconds the total number of recorded data (spectrophotometer specifications allow a reading frequency of 2 Hz) did not produce a linear relationship. Therefore, the results of the kinetic method were obtained from initial reaction rate using the data collected during the first 10 seconds of the stop period. Figure 3.3 illustrates the increase of the initial rate with the increase of the ethanol concentration. It can be concluded that, besides the previously mentioned advantages concerning spectral and chemical interferences, the initial rate approach also presents a higher determination rate.

### 3.3.4. Figures of merit of the flow system

The performance of the proposed methods was evaluated in terms of reagent consumption, application range, determination rate, repeatability, and accuracy (Table 3.3). Both peak height measurement and initial rate measurement have the same reagent consumption, and they were linear up to 0.040% v/v. The sampling rate was higher for the initial rate measurement. RSD's were below 5.0% in a range between 5.2 to 40.3% (v/v). The accepted precision for a reference method is established as ± 0.1% (v/v) ethanol, referring to the measurement of different physical properties of the distilled samples. When looking at the results in Table 3.3 and 3.4, the developed methods show worse repeatability, but it must be kept in mind that these assays were performed on the whole sample without distillation, and that the obtained precision is adequate for the control of the fermentation process (Luque de Castro et al., 2005). The limit of detection and the limit of quantification were calculated as recommended by Miller and Miller (1993).

**Table 3.3**. Figures of merit of the developed enzymatic determination of ethanol in alcoholic beverages.

| Parameter | Peak height measurement | Initial rate measurement |
|---|---|---|
| Reagent consumption per assay | | |
| ADH | 0.12 U | 0.12 U |
| $NAD^+$ | 0.066 mg | 0.066 mg |
| Sample solution | 15 µL | 15 µL |
| Buffer | 150 µL | 150 µL |
| Waste production per assay | 1.2 mL | 1.2 mL |
| Application range | Up to 0.04% (v/v) | Up to 0.04% (v/v) |
| Determination rate | 27 det./h | 37 det./h |
| LOD | 0.003% (v/v) | 0.004% (v/v) |
| LOQ | 0.009% (v/v) | 0.01% (v/v) |
| Repeatability | 1.0% (9.1% v/v) (n=4) | 5.0% (9.4% v/v) (n=6) |
| (RSD) | 0.7% (11.1% v/v) (n=4) | 4.0% (11.0% v/v) (n=8) |
| | 1.0% (10.4% v/v) ) (n=4) | 4.0% (5.2% v/v) (n=7) |
| | | 2.5% (38.3% v/v) (n=10) |
| | | 4.6% (40.3% v/v) (n=10) |

The reproducibility of the initial rate measurement was evaluated by performing the calibration procedure under identical operation conditions during a working day. Applying a single factor ANOVA (Miller and Miller, 1993) treatment between and within day for the calibration curves revealed no significant differences for a 95% confidence level.

## 3.3.5. Application to samples

To evaluate the accuracy of the proposed methods, a total of ten beverage samples were analyzed. The reference procedure was also carried out using the same content of the bottle. The results obtained in the analysis are presented in Table 3.4.

**Table 3.4**. Comparison of the results obtained for the analysis of ethanol of different beverages according to the reference and the developed procedures.

| Sample | Ref. Met.[a] | % ethanol (v/v) | |
|---|---|---|---|
| | % ethanol (v/v) | Peak height meas. [b] | Initial rate meas. [b] |
| Red table wine 1 | 9.3 (± 0.1) | 9.1 (± 0.1) | 9.7 (± 0.6) |
| Red table wine 2 | 12.4 (± 0.1) | 12.3 (± 0.9) | 12.3 (± 0.2) |
| Red table wine 3 | 11.1 (± 0.1) | 11.1 (± 0.1) | 10.4 (± 2.9) |
| White table wine | 10.2 (± 0.1) | 10.4 (± 0.1) | 10.4 (± 0.1) |
| Beer 1 | 5.2 (± 0.1) | - | 5.1 (± 1.2) |
| Beer 2 | 4.6 (± 0.1) | - | 4.8 (± 0.5) |
| Beer 3 | 4.7 (± 0.1) | - | 4.7 (± 0.2) |
| Spirit 1 | 36.7 (± 0.1) | - | 36.7 (± 3.7) |
| Spirit 2 | 36.6 (± 0.1) | - | 36.3 (± 2.9) |
| Spirit 3 | 39.2 (± 0.1) | - | 39.8 (± 2.0) |

[a] mean and accepted precision for n=3; [b] mean and standard deviation for n=3.

The linear relationships ($C_{peak\ height\ meas.} = b + mC_{ref.meth.}$) and ($C_{initial\ rate\ meas.} = b + mC_{ref.meth.}$) were established (n= 4 and n=10, respectively) and are described by the equations $C_{peak\ height} = -0.056\ (\pm 4.245) + 1.003\ (\pm 0.393) \times C_{ref.\ meth.}$, and $C_{initial\ rate\ meas.}$

= -0.037 (± 0.446) + 1.003 (± 0.021) x $C_{ref.\ meth.}$ (Miller and Miller, 1993). Values presented in parentheses represent the limits of the 95% confidence intervals for the equation parameters. These values demonstrate that the obtained results were not statistically different.

A certified wine sample, CRM 653, with a low level alcohol (0.5% (v/v) nominal value) was also analyzed. In this case the sample was 20 times diluted before introduction into the system, resulting in a much higher matrix to analyte ratio than in the case of the table wines. The results obtained for the peak height and the initial rate measurement presented in Table 3.5 show good agreement with the certified value (Miller and Miller, 1993).

**Table 3.5.** Results obtained in the analysis of ethanol of the certified reference wine sample, CRM 653.

| **Certified value** | **Peak heightmeas. % ethanol (v/v)** | **t (α = 0.05) [c]** | **Initial rate meas. % ethanol (v/v)** | **t (α = 0.05) [c]** |
|---|---|---|---|---|
| 0.539 ± 0.0095 [a] | 0.537 ± 0.025 [b] | 0.03 | 0.548 ± 0.026 [b] | 0.15 |

[a] laboratory mean and standard deviation of laboratory means; [b] mean and standard deviation for n=7; [c] t critical: 2.45.

## 3.4. CONCLUSIONS

The results obtained for the enzymatic determination of ethanol in beverages were comparable to those obtained by the reference method with good repeatability, minimum sample treatment and low reagent consumption. The low reagent consumption is an advantage when compared with some flow methodologies for the same determination (Table 3.6). The application range and the corresponding detection limits are comparable to those obtained by the other flow methodologies. The limit of detection for the proposed method is lower than the ones obtained with other flow methodologies using enzymes in solution.

**Table 3.6**. Comparison of some analytical figures of enzymatic (ADH) flow methodologies for ethanol determination in beverages.

| | **Present work** | | **Worsfold et al., 1981** | **[a]Rangel and Tóth, 2000** | **[b]Mori et al, 2003** | **[b]Lázaro et al. , 1999** | **Rangel and Tóth, 1999** | **[a]Segundo and Rangel, 2002b** | **Páscoa et al., 2006** | **Hedenfalk and Mattiason, 1996** | **Chen and Růžička, 2004** |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Sample matrix | table wine, beer and spirits | | Beverages | wine | wine, saké | wine, beer | wine | wine | Wine | Ferment. broth | no application |
| Measurement | Peak height | Initial rate | Peak height | Peak height | Initial rate | Peak height | Peak height | Peak height | Peak height | peak height | initial rate |
| Enzyme | Solution | | Solution | immobilized | Immobilized | immobilized | immobilized | Immobilized | Solution | immobilized | solution |
| Reagent consumption | | | | | | | | | | | |
| ADH (U/assay) | 0.12 | | 225 | not given | 16.7 | 0.5 | ≈ 0.4 | not given | 1.1 | not given | 48 |
| $NAD^+$ (µmol/assay) | 0.1 | | 2.4 | 14 | 0.52 | 3.2 | 0.6 | 0.3 | 1 | 0.5 | 0.7 |
| Sample (µL/assay | 15 | | 30 | 3000 | 50 | 30 | 25 | 45 | 100 | 150 | 30 |
| Waste production (mL/assay) | 1.2 | | 1 | 8.1 | 5.2 | 1.8 | 11 | 1.8 | 1.8 | not given | 1 |
| Range of applicability (% v/v) | Up to 0.04 | | Up to 0.4 | 0.05 to 0.5 | 0.04 to 100 mM | Up to $50 \times 10^{-3}$ | 5 to 25 | 0.10 to 0.50 | Up to 0.024 | 0.25 to 100 mM | 50 to 250 mg/L |
| LOD (% v/v) | 0.003 | 0.004 | not given | $2 \times 10^{-3}$ | 0.02 mM | $2.5 \times 10^{-3}$ | 0.4 | 0.005 | 0.008 | not given | not given |
| Determination rate ($h^{-1}$) | 27 | 37 | 120 | 20 | 10 | 40 | 30 | 45 | 25 | 26 | 120 |
| RSD (%) | <1 | <5 | not given | <2.1 | 2 | ± 0.3 | <2.2 | <3.4 | <2.3 | <6 | <3 |

[a] with simultaneous determination of glycerol; [b] with simultaneous determination of acetaldehyde.

The determination rate can be increased when the initial rate measurement is performed as only a 10 second period is used for data acquisition while in the peak height measurement the maximum absorbance must be achieved.

The easy manipulation of time sequences and the low volumes used in the LOV format system makes it proper for the kinetic based assays. The initial rate measurement is the most efficient way when sample shows evidence of intrinsic absorption or contains potential interferents with a reaction rate different of the analyte. Additionally, the analytical response is not influenced by the schlieren effect in this type of measurement.

The use of a Lab-on-valve format with reduced injection volumes, and strategic selected aspiration sequence proved to be an efficient way to overcome the deficient overlapping of sample and reagent zones frequently attributed to conventional SIA assays.

# CHAPTER 4

## ENZYMATIC DETERMINATION OF HYDROGEN PEROXIDE: APPLICATION TO THE ON-LINE MONITORING OF THE DISINFECTION OF LENS CARE SOLUTIONS

## 4.1. INTRODUCTION

Hydrogen peroxide is widespread used in everyday life and in industrial applications due to its oxidative properties. Being a strong oxidative agent, it is commonly applied for industrial and household bleaching and it is an ecological friendly option when compared to the chlorine based alternatives. Hydrogen peroxide is also used as powerful antimicrobial agent, an effective sporicide (Pappas et al., 2002), applied in water treatment processes (Ikehata and Gamal El-Din, 2006), medical instrument sterilization (Guillaume et al., 2004) and it can be incorporated into pharmaceutical products (Vieira and Falibello-Filho, 1998) for example in the case of disinfections solutions for contact lenses (Kilvington, 2004; Hiti et al., 2005)

Within the contact lens disinfection processes (thermal and chemical), the hydrogen peroxide based methods show some advantages: it can be applied for repeated disinfection of the lenses, it is more effective against a larger range of microorganisms than the chemical disinfection, and its effervescence also provides mechanical cleaning. However, hydrogen peroxide is toxic to the ocular epithelium and to the cornea; therefore must be neutralized before lens wear (Kilvington, 2004). Some of the commercial products perform the neutralization of the hydrogen peroxide right after the disinfection of the lenses, also called one-step system; others, two-step systems, require a neutralization step after the disinfection. In one-step systems, the neutralization is achieved in the storage case using a platinum-coated disc (catalytic neutralization) or a soluble catalase tablet

(enzymatic neutralization) which catalyses the decomposition of hydrogen peroxide to water and oxygen (Kilvington, 2004). The study of the hydrogen peroxide concentration along the disinfection neutralization process is an important way to assess the efficiency and the safety of the procedure. The monitoring can be carried out in batch fashion using titrimetric, spectrophotometric or fluorimetric assays (Mattos et al., 2003), or more efficiently (with lower reagent consumption and higher sample throughput) in flow-based methods. Flow-based spectrophotometric methods combined with the advantages of highly specific enzyme-based assays could meet the requirements for fast and accurate process monitoring. Most of the spectrophotometric flow methods for the quantification of hydrogen peroxide (Table 4.1) are based on the use of the peroxidase (EC 1.11.1.7.) enzyme.

**Table 4.1.** Some analytical characteristics of spectrophotometric flow methods for the determination of hydrogen peroxide in different sample matrices.

| Flow System | Matrix | λ | Application range | LOD | References |
|---|---|---|---|---|---|
| Enzymatic FIA | Honey | 505 nm | 1 to 100 µmol/L | 0.7 µmol/L | Franchini et al.,2007 |
| Enzymatic FIA | Rainwater | 505 nm | 1 to 100 µmol/L | 0.7 µmol/L | Matos et al., 2006 |
| Enzymatic FIA | N/A | 420 nm | 55 to 2730 nmol/L | N/A | Fernandes et al.,2005 |
| Enzymatic FIA | N/A | 490nm | Up to 8.8 mmol/L | 0.3 mmol/L | Vojinović et al., 2004 |
| FIA, LWCC | Wash solution from cleaning vats in breweries | 590 nm | 20 to 700 nmol/L | 4 nmol/L | Pappas et al., 2002 |
| FIA | Disinfectants | 575 nm | $4x10^{-6}$ to $1x10^{-3}$mol/L | $1x10^{-6}$mol/L | Harms et al., 1999 |
| Enzymatic FIA | Pharmaceut.; Swimming pool waters | 470 nm | $1.6x10^{-5}$ to $6.6x10^{-4}$ mol/L | $2.1x10^{-6}$ mol/L | Vieira and Fatibello-Filho, 1998 |
| Enzymatic FIA | Olive oils and margarines | 458 nm | $2.5x10^{-5}$ to $2x10^{-4}$mol/L | 0.9 µmol/L | Avila et al., 1997 |
| FIA | N/A | 410 nm | $4x10^{-6}$ to $6x10^{-5}$mol/L[a]<br>$4x10^{-5}$ to $4x10^{-4}$ mol/L | $1x10^{-6}$ mol/L<br>$2x10^{-5}$ mol/L | Almuaibed and Townshend, 1994 |
| FIA | Rain water | 450 nm | $1x10^{-8}$ to $1x10^{-5}$mo/L | $5x10^{-6}$mol/L | Matsubara et al., 1993 |
| FIA | Milk | 450 nm | 10 to 150 mg/L | N/A | Cerdán et al., 1992 |
| FIA | Rain water | 508 nm | 1.36 to 1360 ppb | N/A | Matsubara et al.,1991 |
| Enzymatic FIA | N/A | 414 nm<br>514 nm<br>600 nm | 0.04 to 200 µmol/L<br>0.1 to 500 µmol/L<br>0.8 to 1000 µmol/L | 0.1 µmol/L | Olsson, 1985 |
| Enzymatic FIA | Rain water | 560 nm | $1.5x10^{-7}$ - $4x10^{-5}$ mol/L | $1.4x10^{-7}$mol/L | Madsen and Kromis, 1984 |

N/A - Not available; [a] determination with preconcentration.

In the analytical methodologies that make use of enzymatic reactions, the reduction in the cost of the determination is generally achieved by reducing the reagent consumption. This reduction can be maximised with the miniaturization of the system. Therefore, instead of using the previously mentioned flow injection methodologies (Table 4.1), the SI-LOV system was selected for this work. An enzymatic SI-LOV assay for the determination of hydrogen peroxide in bleaches and in pharmaceutical products was developed. At the same time the system was applied to the on-line monitorization of the disinfection-neutralization process of contact lenses. The quantification of hydrogen peroxide is based on the horseradish peroxidase (HRP) catalysed reaction between $H_2O_2$ (Hydrogen peroxide) and ABTS (2,2'-Azino-bis(3-Ethilbenzothiazoline 6-sulfonic acid)) (Durand and Monsan, 1992). The produced oxidized ABTS is measured at 410 nm. Due to the high concentration of hydrogen peroxide present in the lens care solution, a high dilution step is required. In the present work, this was achieved by incorporating a dialysis unit in which a reduced quantity of the analyte diffuses over the membrane resulting in a high sample dilution.

## 4.2. EXPERIMENTAL

### 4.2.1. Reagents and solutions

A 100 mM potassium phosphate buffer (104873, Merck) with pH 6.0 solution and an 40 mM enzyme diluents pH 6.8, containing potassium phosphate buffer, 0.25% (w/v) of bovine serum albumin (BSA, fraction V, 05484, Fluka) and 0.5% (v/v) of Triton X-100 (T9284, Sigma), were prepared. Potassium hydroxide (105033, Merck) 1.0 M was used to adjust the pH of these buffer solutions.

An enzyme stock solution was prepared by re-suspending 1 mg of lyophilized HRP (Peroxidase from horseradish, EC 1.11.1.7, type VI, P8375, Sigma) in 1.00 mL of enzyme diluent. The working enzyme solution was prepared daily by diluting 90 µL of the stock solution in 2.60 mL of enzyme buffer.

The ABTS (2,2'-Azino-bis(3-Ethilbenzothiazoline 6-sulfonic acid), A 9941,Sigma) stock solution was prepared by dissolving 10 mg (1 tablet) of ABTS in 25.00 mL of deionised water. The daily working solution was prepared by further dilution in 100 mM potassium phosphate buffer.

The working standard solutions of hydrogen peroxide were prepared from the stock solution (Perhydrol, 30% $H_2O_2$, 107210, Merck) in a range between 68.4 to 342 mg/L and 0.54 to 4.28 mg/L.

Solutions of 0.1 M of $Na_2S_2O_3$ and 0.01 M of $K_2Cr_2O_7$, concentrated $H_2SO_4$, concentrated $CH_3COOH$; 0.5% (m/v) starch indicator and solid KI were used (all from Merck) to carry out the reference titrimetric method.

### 4.2.2. Samples

The samples analysed were bleaches and disinfecting solutions for contact lenses containing hydrogen peroxide as an active component. Two brands of bleaches and two kinds of one-step disinfecting solutions for contact lenses (one with enzymatic and the other with catalytic neutralization) were purchased in a local market. The samples had no other treatment than dilution, when necessary.

### 4.2.3. Manifold and flow procedure

The schematic representation of the sequential injection lab-on-valve system for the enzymatic determination of hydrogen peroxide is depicted in Fig. 4.1.

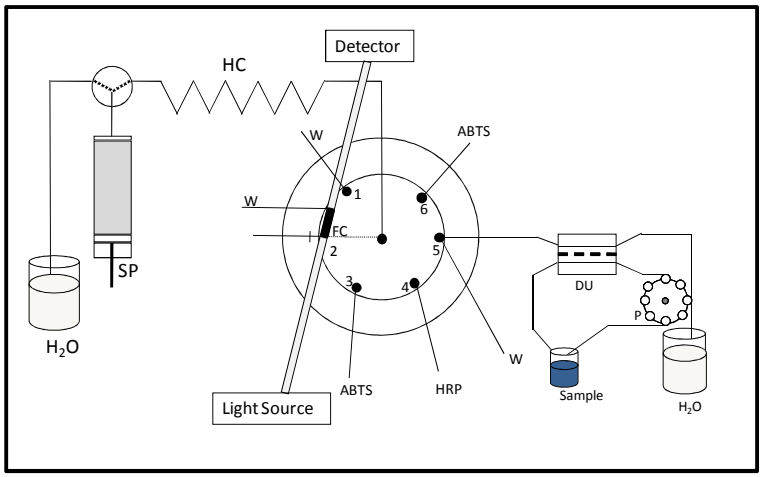


**Fig. 4.1**. Configuration of the SI-LOV manifold for the enzymatic determination of hydrogen peroxide. SP, syringe pump; HC, holding coil; FC, flow cell; ABTS, 0.06g/L ; HRP, horseradish peroxidase, 34.6 mg/L; DU, dialysis unit; P, peristaltic pump; W, waste.

The initial steps of B – C, and E – G, consisted in the aspiration of carrier, sample, and reagents to the holding coil; (in the order of: 1000 µL of carrier, 50 µL of ABTS, 15 µL of sample, 15 µL of enzyme and 100 µL of ABTS). The following steps (H - J) consisted in reversing the flow and propelling the mixture towards detection for absorbance scanning. The in-line dilution was carried out by means of a dialysis unit incorporated in the system. To execute this in-line dilution it is necessary to include steps A and D to drive the sample towards the dialysis unit and renew the solution at the sampling port while carrier and ABTS are aspirated to the holding coil.

The flow procedure for the determination of hydrogen peroxide using horseradish peroxidase and ABTS is summarised in Table 4.2.

**Table 4.2**. Flow protocol sequence for enzymatic determination of hydrogen peroxide.

| Step | Description | Volume (µL) | Flow rate (µL/s) | Selection valve position |
|---|---|---|---|---|
| **A** | Peristaltic pump On | - | 64 | - |
| **B** | Aspirate carrier to HC | 1000 | 150 | - |
| **C** | Aspirate ABTS to HC | 50 | 80 | 3 |
| **D** | Peristaltic pump Off | - | - | - |
| **E** | Aspirate sample to HC | 15 | 25 | 5 |
| **F** | Aspirate enzyme to HC | 15 | 25 | 4 |
| **G** | Aspirate ABTS to HC | 100 | 80 | 6 |
| **H** | Reverse flow, reference scan | 10 | 15 | 2 |
| **I** | Dispense HC content, data acquisition | 400 | 30 | 2 |
| **J** | System washing, SP empty | - | 150 | 2 |

## 4.2.4. Reference procedure

The reference method carried out for validation of the results consisted in the iodometric titration of the samples with standardised sodium thiosulphate solution in the presence of potassium iodide and acetic acid (APHA-AWWA-WPCF, 1998).

## 4.3. RESULTS AND DISCUSSION

### 4.3.1. Study of the flow system

The physical parameters studied were the volumes of the sample and reagent solutions and the aspiration sequence. Initial conditions were established as 0.108 mM and 23 mg/L as ABTS and HRP concentrations, respectively, with an aspiration order of: 75 µL of ABTS, 15 µL of enzyme, followed by 15 µL of hydrogen peroxide and 100 µL of ABTS. The ABTS solution was prepared in buffer (pH 6.0); therefore the two plugs of ABTS assured that the reaction occurred at a controlled pH. This optimization study was carried out by performing calibration curves with hydrogen peroxide standards up to 4.28 mg/L. The sample volume used in the developed method was studied in a range of 15 to 25 µL. As it was shown in the previous chapter, lower volumes would deteriorate the repeatability of the method. The highest sensitivity was obtained with the volume of 15 µL, as it was possible to achieve a better overlapping of the reagent zones involved in the reaction (Vidigal et al., 2008). For that reason this was the volume used for the developed method as a good compromise between sensitivity and repeatability.

The enzyme solution volume was studied in a range from 5 to 15 µL. A 15% increase in the sensitivity was achieved by the increase of enzyme solution volume from 5 to 15 µL; therefore, the chosen volume for the developed method was 15 µL. The volume of ABTS used in the rear part of the aspiration sequence was also studied (50, 75 and 100 µL). When the assay was performed with 50 µL, higher sensitivity

was obtained and with the increase to 75 µL of ABTS, a decrease in sensitivity of about 8% was found; therefore the volume of 50 µL was chosen. Additionally, the aspiration sequence was studied by altering the order of sample and enzyme aspiration. Calibration curves were established using standards of hydrogen peroxide in a range of 1.19 to 4.76 mg/L. When the sample was aspirated after the enzyme solution, the results showed higher analytical signal, but lower repeatability. As there were no significant differences in the sensitivity between the cases studied, the selected sequence of aspiration for the developed assay was: ABTS – sample – enzyme – ABTS.

The flow rate used to propel the product of the reaction towards detection was studied with the purpose of improving the sample throughput of the assay. For this study, a standard of 137 mg/L of hydrogen peroxide was analysed and flow rates in the range from 15 to 60 µL/s were tested. The results were evaluated in terms of repeatability and in terms of the analytical signal obtained. The value of the analytical signal obtained for all of the cases revealed no significant differences but the repeatability deteriorated for the flow rate of 60 µL/s. An intermediate flow rate (30 µL/s) was chosen for further work as good repeatability (RSD < 3%; n=9) was achieved under these conditions.

After establishing the physical conditions of the system, the effect of ABTS and enzyme concentration on the analytical signal was also studied to ensure that their concentrations were not the limiting one in this application range. Calibrations curves up to 4.28 mg/L of hydrogen peroxide were performed for the study. The

concentration of ABTS was studied in the range of 0.047 to 0.20 g/L. For concentrations of ABTS higher than 0.06 g/L, no significant increase on the sensitivity was achieved, therefore this concentration was chosen. The enzyme concentration was studied in a range between 23 to 46 mg/L. At concentrations higher than 34.6 mg/L there was no significant increase in the sensitivity (1.1%), thus this concentration was chosen for further work.

The developed method presented a sample consumption of 15 µL per assay and a consumption of enzyme and ABTS of 34.6 mg/L and 0.06 g/L, respectively. The determination rate was 45 per hour, and was possible to achieve a determination range up to 4.28 mg/L. The limits of quantification and determination (Miller and Miller, 1993) obtained were 0.45 and 0.20 mg/L, respectively.

Due to the elevated concentration of hydrogen peroxide present in the cleaning solutions, a high dilution of the samples is required. To carry out the in-line dilution, a mass transfer unit was introduced in the system, where the analyte diffuses across the membrane from the donor channel to the acceptor stream, resulting in the dilution of the sample. Three mass transfer units with different configurations (Table 4.3) were evaluated.

**Table 4.3**. Configuration of the mass transfer units studied for the in-line dilution of the enzymatic determination of hydrogen peroxide.

| Mass transfer unit | Configuration | Surface area ($mm^2$) | Channel depth (mm) |
|---|---|---|---|
| **A** | Linear | 72 | 0.5 |
| **B** | Linear | 140 | 0.5 |
| **C** | Zig-zag | 146 | 0.3 |

To evaluate the performance of the mass transfer units, a hydrophobic DURAPORE membrane filter (HVPV, 0.45 µm) was used and calibration curves were carried out with hydrogen peroxide standards in a range up to 51.3 g/L, as presented in Fig. 4.2.

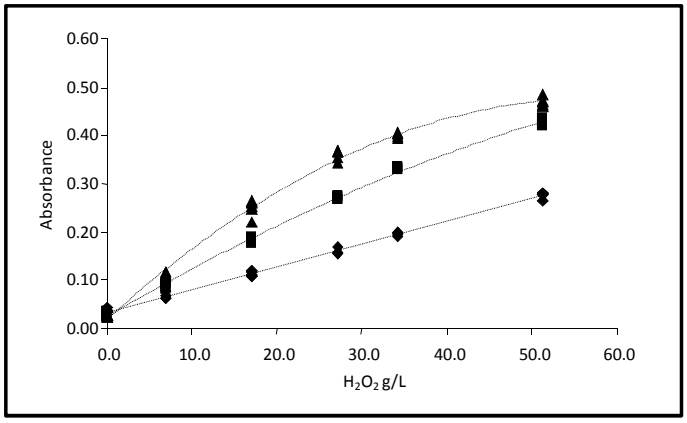


**Fig. 4.2.** Calibration curves obtained using different mass transfer unit configurations in the enzymatic determination of hydrogen peroxide: ♦ Unit A; ■ Unit B; ▲ Unit C. Other conditions: HVPV DURAPORE membrane filter; 0.06 g/L of ABTS; 34.6 mg/L of enzyme; hydrogen peroxide standards up to 51.3 g/L.

As expected, the sensitivity increased with the increase of the surface area of the units; therefore the lowest sensitivity was found using unit A. The chosen unit for the work was unit A for presenting a wider linear range with higher sample dilution.

Hydrophobic and hydrophilic membranes were tested to perform the in-line dilution. The hydrophobic membranes were not compatible with the samples. After contact with the sample matrix, the analytical signal obtained was constantly increasing, that can be explained by the presence of surface active substances in the sample that affect the hydrophobicity of the membrane. When hydrophilic dialysis membranes were used, this behaviour was not noticed.

Two different types of hydrophilic membranes were tested, cellophane and pre-mounted cellulose acetate dialysis membrane. With the use of the latter membrane the sensitivity increased about 65%, (while the linear response range was maintained in the same extent); therefore the pre-mounted membrane was chosen for performing the in-line dilution of the developed method. With the use of the selected membrane it was possible to achieve an in-line dilution of approximately 80 times.

### 4.3.2. Figures of merit of the flow system

The developed method with in line dilution was re-evaluated in terms of reagent consumption; determination rate and application range and was compared with the

system without in-line dilution (Table 4.4). The reagent consumption was maintained as in the previous system; however it was possible to achieve a determination range up to 342 mg/L with a limit of quantification of 44.3 mg/L, and a limit of detection of 16.1 mg/L.

**Table 4.4**. Figures of merit of the developed method for the enzymatic determination of hydrogen peroxide.

| **Parameter** | **Value** |
|---|---|
| Reagent consumption per assay | |
| ABTS | 9.0 µg |
| HRP | 0.52 µg |
| Sample | 15 µL |
| Waste production per assay | 1.2 mL |
| Application range | Up to 4.28 mg/L[a]<br>Up to 342 mg/L[b] |
| Determination rate | 45 det./h |
| LOD | 0.20 mg/L[a]<br>16.1 mg/L[b] |
| LOQ | 0.45 mg/L[a]<br>44.3 mg/L[b] |

[a] Measurement of hydrogen peroxide with off-line dilution;

[b] Measurement of hydrogen peroxide with in-line dilution.

## 4.3.3. Application to samples

Two different types of samples (two brands of bleaches and lens care solutions) were used to evaluate the accuracy of the developed methods. The values obtained for all samples were in agreement with those obtained by the reference method, as it can be concluded from the RD values achieved, as reported in Table 4.5.

**Table 4.5**. Results of the analysis of hydrogen peroxide in different samples.

| Sample | Reference method (g/L) [a] | Developed method (g/L) [b] | R.D.[c] % |
|---|---|---|---|
| Bleach 1 | 71.3 ± 0.3 | 70.2 [d] ± 2.5 | -1.3 |
| Bleach 2 | 84.4 ± 0.5 | 91.8 [d] ± 4.2 | 8.9 |
| Lens care solution 1, catalytic neutralization | 36.4 ± 0.3 | 36.6 [e] ± 0.8 | 0.5 |
| Lens care solution 2, enzymatic neutralization | 37.0 ±0.4 | 36.3 [e] ±¨1.8 | -1.9 |

[a] mean and standard deviation for n=3;
[b] mean and standard deviation for n=5;
[c] Relative deviation;
[d] with off-line dilution;
[e] with in-line dilution.

### 4.3.3.1. On-line monitoring of the neutralisation of the contact lenses disinfection/neutralization process

In this work, the neutralization process of two different one-step systems, catalytic and enzymatic (Fig. 4.3), were evaluated. The analytical signal resultant of the

presence of hydrogen peroxide during the disinfection-neutralization process was recorded. The initial concentration of hydrogen peroxide in the disinfecting solution obtained was situated over the upper limit of the linear response range of the method. Therefore, an option was taken to present the results as a percentage of degradation of the hydrogen peroxide, relative to the recorded initial absorbance.

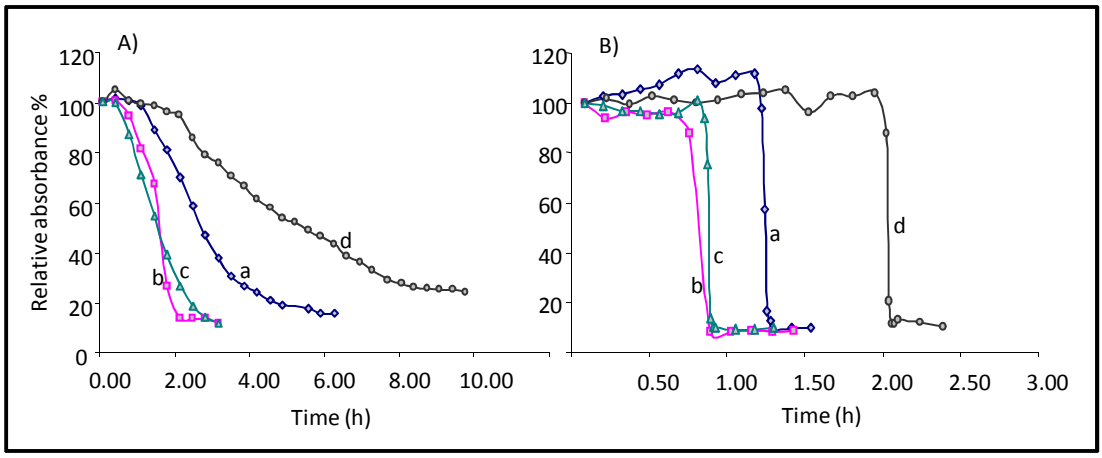


**Fig. 4.3.** Relative absorbance of the hydrogen peroxide disinfection-neutralization process for sample **A)** (catalytic neutralization) and for sample **B)** (enzymatic neutralization) in different conditions: a recommended; b with mechanical stirring; c at 30º C; d at 2º C. Other conditions: 0.06 g/L of ABTS; 34.6 mg/L of enzyme.

Performing the disinfection-neutralization process with catalytic neutralization (Fig. 4.3A) as recommended by the manufacture and described in the label of the product (Fig. 4.3A, curve a) it can be concluded that the total neutralization is achieved in almost six hours. When the process is performed with mechanical stirring (Fig. 4.3Ab), the neutralization is achieved in around two hours; this is also

the time necessary to attain the neutralization at 30º C (Fig. 4.3Ac). When the process occurs at 2 ºC (Fig. 4.3Ad) it is necessary to wait almost eight hours to obtain the neutralization of the hydrogen peroxide.

When the process occurs with enzymatic neutralization (catalase, Fig. 4.3B), the total neutralization of hydrogen peroxide is achieved faster than the same procedure with catalytic neutralization. When the process is performed as recommended by the manufacturer (Fig. 4.3Ba), it is not necessary wait more than one and half hour to achieve the neutralization (Hiti et al., 2005). After the disinfection time, about one hour, the catalase is released from the nucleus of the tablet and the neutralization process starts promptly. When the assay is performed with mechanical stirring (Fig. 4.3Bb) or at 30º C (Fig. 4.3Bc) the total neutralization is achieved in less than one hour; and in a little more than two hours the neutralization is complete if the process is performed at 2º C (Fig. 4.3Bd). The neutralization profile obtained for this process is very different from the one obtained for the process with catalytic neutralization.

## 4.4. CONCLUSIONS

The developed method proved to be a useful tool for the monitoring of the disinfection/neutralization process for the cleaning of contact lenses; the applied configuration is compact and miniaturized, and makes use of commercially available reagents. With the introduction of a dialysis unit in the system, it was possible to achieve a high in-line dilution factor of the sample, making the monitoring of this dynamic process possible.

The advantages of flow dialysis processes for analyte separation and/or sample dilution have been widely exploited since the first time applied in flow injection analysis by Růžička and Hansen (1976) and in sequential injection by van Staden(1997). However, to the best of our knowledge, this is the first time that the dialysis process was used to perform the in-line dilution of the sample in the sequential injection lab-on-valve format.

# CHAPTER 5

## DETERMINATION OF THE ACTIVITY OF PEROXIDASE IN VEGETABLES EXTRACTS

## 5.1. INTRODUCTION

Peroxidase (E.C. 1.11.1.7.) is an enzyme commonly found in vegetables that can catalyse a large number of reactions (Cruz et al.,2006). These enzymes are widely distributed in the plant kingdom however the most studied is the one obtained from horseradish roots (HRP), which is also the most readily available commercially (Cardinali et al., 2007). The action of these enzymes can have a negative effect on the colour and on the flavour of raw or processed food. They are involved in the enzymatic browning of the vegetables, either separately or together with polyphenoloxidase (PPO). Peroxidase appears to be the most heat stable enzyme in plants, and since it is very resistant to the thermal inactivation, it is widely used to evaluate the effectiveness of fruit and vegetable thermal blanching. The blanching process is a thermal procedure designed to inactivate the enzymes responsible for generation of off-flavours and off-odours. This procedure can be carried out by different methods, but water blanching is the most widely used technique for this purpose (Baçeci et al., 2005); and the on-line monitoring of the blanching process is important to control the time of exposure and to achieve an efficient process. It is generally accepted that if the peroxidase originally present in the food is destroyed, it is quite unlikely that other enzyme systems have survived (Burnette, 1977; Icier et al., 2006). To minimize the deterioration of the quality of the products during frozen storage, it is necessary to reduce the activity of peroxidase (Barrett and Theerakulkait, 1995). Nevertheless, the inactivation of peroxidase should not be

complete since there is evidence that the quality of the blanched and frozen products is better if there is some peroxidase activity left at the end of the blanching process. The complete absence of peroxidase activity is an indicator of over blanching (Garrote et al., 2004).

The determination of the peroxidase activity has been described based on colorimetric, chemiluminescence, electrochemical or fluorimetric detection of the product formed from the peroxidase reducing substrate. The possible substrates include guaiacol, phenol, pyrogallol, *o*-tolidine, *o*-phenylendiaminecathecol, resorcinol or *o*-dianisidine. One of the most frequently used substrate is guaiacol, but its reaction gives an undefined mixture of oxidation products and the absolute molar absorptivity cannot be determined (Vojinović et al., 2004; Vojinović et al., 2007). The high toxicity of these substrates is also a disadvantage. This limitation can be minimized by using flow-based methods, which comprise lower reagent consumption. Furthermore, they permit the reduction of analysis costs, which is an important parameter in the development of new methodologies for bioassays (Tóth et al., 2008).

The aim of this work was to develop a SI-LOV system for the determination of the peroxidase activity in vegetables. The determination was based on the reaction of $H_2O_2$ (hydrogen peroxide) with ABTS (2,2'-Azino-bis(3-Ethilbenzothiazoline 6-sulfonic acid)) catalysed by HRP (Durand and Monsan, 1992), the same as described in the previous chapter. The oxidation of ABTS was followed by observing the increase in absorbance at 410 nm during 30 s. Taking advantage of the flow system

versatility, the on-line monitoring of the thermal blanching process of an HRP enzymatic solution was also aim of study.

## 5.2. Experimental

### 5.2.1. Reagents and solutions

A 100 mM potassium phosphate buffer (104873, Merck) with pH 6.0 solution and an 40 mM enzyme diluents pH 6.8, containing potassium phosphate buffer, 0.25% (w/v) of bovine serum albumin (BSA, fraction V, 05484, Fluka) and 0.5% (v/v) of Triton X-100 (T9284, Sigma), were prepared. Potassium hydroxide (105033, Merck) 1.0 M was used to adjust the pH of these buffer solutions. For the preparation of the sample extracts, 100 mM potassium phosphate (pH 6.5) was prepared from monopotassium phosphate (Merck) and dipotassium phosphate (Merck). This buffer solution was kept cooled at 4 ºC until used.

The daily working solutions of hydrogen peroxide were prepared by dilution from the stock solution (Perhydrol, 30% $H_2O_2$, d = 1.11, 107210, Merck). The ABTS (2,2'-Azino-bis(3-Ethilbenzothiazoline 6-sulfonic acid), A 9941, Sigma) stock solution was prepared by dissolving 10 mg (1 tablet) of ABTS in 25 mL of deionised water and the daily working solution was prepared by additional dilution in potassium phosphate buffer (100 mM, pH 6.0).

An enzyme stock solution was prepared by re-suspending 1.0 mg of lyophilized HRP (Peroxidase from horseradish, EC 1.11.1.7, type VI, Sigma) in 1 mL of enzyme diluent. This stock solution was stable for at least one week when refrigerated. The working standards solutions were prepared daily by further dilution in the same buffer, in a concentration range between 0.5 and 2.0 mg/L.

### 5.2.2. Samples

Spinach (*Spinacia oleracea)*, green beans (*Phaseolus vulgaris, L.*) and watercress (*Nasturtium officinale*) were purchased in a local market and stored at 4 ºC until the preparation of the extracts. No vegetables were held longer than 72 h prior to processing. The extracts prepared were analysed within one hour without other previous treatment than dilution.

#### 5.2.2.1. Sample extract

A preliminary study was performed to verify the occurrence of peroxidase and to establish the ratio between sample weight and the buffer solution volume for optimal reproducibility and linearity between enzyme concentrations and observed activity. This study was carried out using various green vegetables, like green peppers (*Capsicum annuum***)**, collard greens (*Brassica oleracea*), rapini (*Brassica*

*rapa*), watercress, spinach, and green beans. Initially, a portion of 5 g of sample was homogenised in a blender with 200 mL of buffer solution. When no initial activity was detected, the sample weight was increased to augment the ratio between sample weight and buffer solution volume. For the first three vegetables studied, the ratio was increased but no peroxidase activity was possible to detect. For the other vegetable samples, the ones used on this work, it was possible to extract peroxidase and the extraction parameters were established for each vegetable. For the extracts, 3 g of watercress, 25 g of spinach and 30 g of green beans were used in 100 mL of buffer solution.

For the preparation of the sample extract, the vegetables were homogenized in 100 mL of buffer solution pH 6.5 at 4 ºC, with a laboratory blender for 1 min. The slurry was filtered through four layers of cotton gauze and was centrifuged (Universal 320R, Hettich Zentrifugen, Germany) at 6080 g during 20 min, to eliminate turbidity of the homogenates. The supernatant was filtered through filter paper and kept on ice until analysis.

### 5.2.3. Manifold and flow procedure

The schematic representation of the SI-LOV system for the determination of the activity of peroxidase in vegetables extracts is depicted in Fig. 5.1.

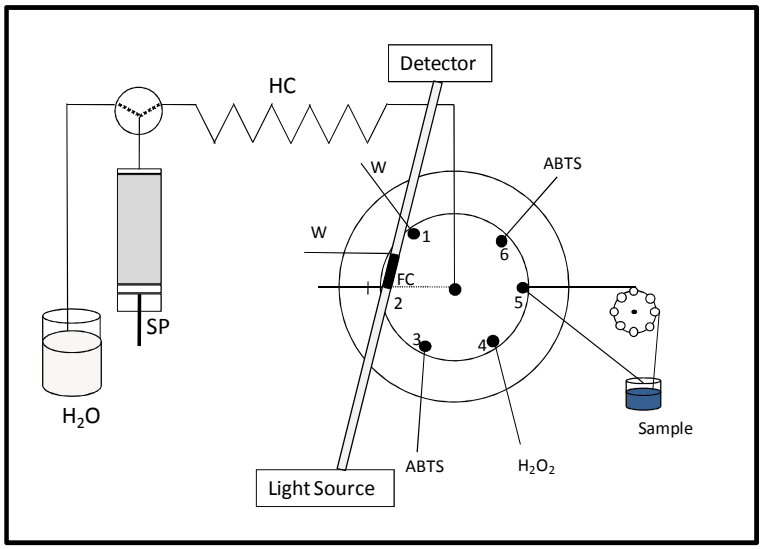


**Fig.5.1.** SI-LOV manifold for the determination of the peroxidase activity in vegetables extracts. SP, syringe pump; HC, holding coil; FC, flow cell; PP, peristaltic pump; ABTS, 160 mg/L; $H_2O_2$, hydrogen peroxide, 0.8 g/L; W, waste.

The SI-LOV flow procedure is summarised in Table 5.1. The initial steps (A to E), consisted in the aspiration of carrier, reagents, and sample to the holding coil (in the order of: 1000 µL of carrier, 50 µL of ABTS, 15 µL of $H_2O_2$, 15 µL of standard and 100 µL of ABTS). The following steps (F to I) consisted in reversing the flow and propelling the mixture towards detection for absorbance scanning (t=30 s) followed by a final washing of the system.

**Table 5.1** Flow protocol sequence of the determination of the activity of peroxidase.

| Step | Description | Volume (µL) | Flow rate (µL/s) | Selection valve position |
|---|---|---|---|---|
| **A** | Aspirate carrier to SP | 1000 | 150 | - |
| **B** | Aspirate ABTS to HC | 50 | 80 | 3 |
| **C** | Aspirate $H_2O_2$ to HC | 15 | 25 | 4 |
| **D** | Aspirate sample to HC | 15 | 25 | 5 |
| **E** | Aspirate ABTS to HC | 100 | 80 | 6 |
| **F** | Reverse flow, reference scan | 10 | 15 | 2 |
| **G** | Dispense HC content | 110 | 15 | 2 |
| **H** | Stop flow, data acquisition | - | - | - |
| **I** | System washing | - | 150 | 2 |

The activity of peroxidase in the vegetable samples was calculated by interpolation on the calibration curves plotting ΔA/Δt as a function of concentration of HRP. The calibration curve obtained under the optimised experimental conditions can be written as: ΔA/Δt = 0.0041 (± 0.0002) x [HRP] (mg/L) + 0.0016 (± 0.0006).

## 5.2.4. Comparison method

The accuracy of the results obtained in the analysis of the samples in the developed method was assessed by comparison with the method proposed by Sigma (2008): 2.90 mL of 200 mg/L of ABTS was transferred into suitable cuvettes, where 50 µL of sample solution was added. Subsequently, 100 µL of 3.3 g/L of $H_2O_2$ solution was mixed and the increase in absorbance was registered in a Thermo-Spectronic

(Cambridge, U.K.) Helios γ UV-Vis spectrophotometer at 405 nm for 2 minutes. The HRP activity was calculated by interpolation on the calibration curve obtained as the slope of the absorbance increase in function of the enzyme concentration (mg/L). This calibration curve was established using the same standards solutions as for the developed flow procedure.

## 5.3. RESULTS AND DISCUSSION

### 5.3.1 Study of the flow system

The physical parameters studied (like the volumes of sample and reagents, the aspiration sequence and the flow rates applied in this flow system) were detailed in the previous chapter. The aspiration sequence consisted in 50 µL of ABTS, 15 µL of sample, 15 µL of enzyme and 100 µL of ABTS (Vidigal, 2009). The flow rate of 30 µL/s was used to propel the product of the reaction towards detection and the flow was stopped after a pre-set time period for absorbance scanning.

With the physical conditions defined, the effect of the reagents concentration ($H_2O_2$, ABTS) on the analytical signal was studied with the aim to guarantee that they were not the limiting reagents in the reaction, and that the activity detected was only dependent on the quantity of the enzyme present. These conditions are necessary for an initial rate based assessment of enzyme activity. Calibration curves up to 5 mg/L of HRP were performed. The concentration of $H_2O_2$ was studied in a

range of 0.7 to 1.2 g/L. When the concentration was increased from 0.7 to 0.8 g/L, the sensitivity improved about 12%. With a further increase of $H_2O_2$ concentration, it was possible to observe a slight decrease of the sensitivity, for this reason the concentration chosen was 0.8 g/L.

The concentration of ABTS was studied between 100 and 200 mg/L. The sensitivity of the method increased with increasing concentrations of ABTS. However, for concentrations higher than 160 mg/L, no linearity was attained therefore this concentration was chosen for further work.

Based on the comparison method, the stop time for absorbance scanning was initially established as 2 minutes. With the decrease of this time down to 30 s, no significant difference was obtained in the quality of the results. Therefore, to increase the determination rate, 30 s stop period was used in the work.

### 5.3.2. Figures of merit of the flow system

The performance of the developed method was evaluated in terms of reagent and sample consumption, determination rate and application range. The method was also compared to the one chosen for its validation (Sigma, 2008). The method presented a sample consumption of 15 µL per assay and consumption of ABTS and $H_2O_2$ of 24 µg and 12 µg respectively, per assay. It was possible to achieve a linear range up to 2 mg/L with a throughput of 1 determination per minute, which

corresponds to an increase on the determination rate of 50% comparatively to the comparison method. The limits of determination and quantification (Miller and Miller, 1993) obtained were 0.3 and 0.9 mg/L, respectively.

The operational stability of the developed system was calculated by performing the calibration procedure under identical physical and chemical conditions between and within different working days. A calibration curve: $\Delta A/\Delta t = 0.0041 (\pm 0.0002) \times [HRP]$ (mg/L) + 0.0016 (± 0.0006) with a RSD < 5% for the slope was obtained; the values in parentheses are the standard deviation values of the calibration curve parameters (5 standard solutions injected 5 times each) which were assessed during a period of two months (n=7). The within day repeatability was evaluated at three different working days and was found to be better than 3.5% (assessed as the percentage of the standard error of the slope).

## 5.3.3 Application to samples

### 5.3.3.1 Determination of the activity of peroxidase in vegetables extracts

A total of twelve extracts for each sample were analysed with and without dilution. This study was conducted to obtain an insight on the possible effect of the vegetable extract matrix on the spectrophotometric activity assay. In the case that no matrix effect can be identified, the activity values obtained should be independent of the dilution. For this reason, and to evaluate the accuracy of the

developed method, the comparison method was also performed using the same sample extract. The results obtained in the sample analysis are summarized in Table 5.2 and present a linear relationship: $Act_{developed\ meth.} = m\ Act_{comp.meth.} + b$ described by the equations listed in Table 5.3. It can be demonstrated that the results obtained for the developed method are in good agreement with those obtained for the comparison method.

**Table 5.2.** Means and standard deviations of the results obtained of HRP activity for the samples analysed by the SI-LOV (n=5) and the comparison (n=3) method, and corresponding relative deviations (RD).

| Sample ID | | Watercress | | | Spinach | | | Green Beans | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Comp. Meth. (mg/L) | SI-LOV Meth. (mg/L) | RD (%) | Comp. Meth. (mg/L) | SI-LOV Meth. (mg/L) | RD (%) | Comp. Meth. (mg/L) | SI-LOV Meth. (mg/L) | RD (%) |
| A | 1 | 1.01 ± 0.13 | 0.93 ± 0.08 | -8 | 6.93 ± 0.16 | 6.65 ± 0.27 | -4 | 1.8 ± 0.09 | 1.84 ± 0.14 | 2.1 |
| | 2 | 0.95 ± 0.08 | 0.86 ± 0.10 | -9.4 | 5.56 ± 0.25 | 4.56 ± 0.21 | -18 | 1.96 ± 0.02 | 2.24 ± 0.00 | 14.4 |
| | 3 | 1.05 ± 0.10 | 1.39 ± 0.16 | 32.9 | 2.89 ± 0.11 | 3.57 ± 0.10 | 23.5 | 3.5 ± 0.52 | 3.96 ± 0.51 | 13 |
| | 4 | 1.08 ± 0.19 | 0.93 ± 0.08 | -13.8 | 5.57 ± 0.62 | 6.25 ± 0.58 | 12.2 | 3.1 ± 0.38 | 2.99 ± 0.25 | -3.5 |
| | 5 | 1.24 ± 0.08 | 1.46 ± 0.09 | 17.5 | 6.45 ± 0.70 | 4.31 ± 0.34 | -33.2 | 2.06 ± 0.19 | 2.13 ± 0.26 | 3.7 |
| | 6 | 1.3 ± 0.21 | 1.8 ± 0.10 | 38.2 | 6.26 ± 1.55 | 5.63 ± 0.83 | -10.1 | 1.08 ± 0.06 | 1.32 ± 0.11 | 21.8 |
| | 7 | 1.55 ± 0.18 | 2.32 ± 0.00 | 49.7 | 4.87 ± 0.59 | 5.63 ± 0.83 | 15.6 | 1.04 ± 0.13 | 1.36 ± 0.09 | 30.2 |
| | 8 | 1.84 ± 0.02 | 2.38 ± 0.23 | 29.6 | 4.13 ± 0.49 | 1.99 ± 0.07 | -51.8 | 1.24 ± 0.09 | 1.47 ± 0.12 | 18.3 |
| | 9 | 1.66 ± 0.19 | 2.27 ± 0.12 | 36.5 | 4.28 ± 0.07 | 1.92 ± 0.08 | -55.1 | 1.72 ± 0.44 | 1.8 ± 0.21 | 4.7 |
| | 10 | 1.83 ± 0.10 | 2.11 ± 0.35 | 15.1 | 3.95 ± 0.82 | 1.9 ± 0.10 | -52 | 1.8 ± 0.02 | 2.3 ± 0.11 | 28.1 |
| | 11 | 1.41 ± 0.11 | 1.57 ± 0.12 | 11.5 | 5.11 ± 0.27 | 4.25 ± 0.29 | -16.9 | 1.28 ± 0.21 | 1.5 ± 0.00 | 17.1 |
| | 12 | 2.33 ± 0.08 | 1.99 ± 0.14 | -14.7 | 5.73 ±1.20 | 2.11 ± 0.33 | -63.1 | 1.41 ± 0.04 | 1.95 ± 0.11 | 38.6 |
| B | 1 | 0.51 ± 0.03 | 0.54 ± 0.10 | 5.7 | 3.31 ± 0.14 | 4.82 ± 0.13 | 45.6 | 1.19 ± 0.11 | 1.22 ± 0.00 | 2.8 |
| | 2 | 0.52 ± 0.04 | 0.39 ± 0.08 | -24.2 | 3.4 ± 0.58 | 3.27 ± 0.27 | -3.8 | 1.17 ± 0.04 | 1.51 ± 0.11 | 29.2 |
| | 3 | 0.73 ± 0.05 | 0.43 ± 0.13 | -41.5 | 2.12 ± 0.37 | 1.79 ± 0.16 | -15.6 | 1.62 ± 0.05 | 2.16 ± 0.18 | 33.6 |
| | 4 | 0.62 ± 0.08 | 0.43 ± 0.00 | -31.1 | 5.28 ± 0.68 | 4.82 ± 0.20 | -8.7 | 1.31 ± 0.30 | 2.08 ± 0.12 | 59.1 |
| | 5 | 0.65 ± 0.04 | 0.5 ± 0.10 | -22.8 | 5.13 ± 0.72 | 4.91 ± 0.00 | -4.3 | 1.19 ± 0.05 | 1.54 ± 0.13 | 29 |
| | 6 | 0.9 ± 0.11 | 0.64 ± 0.12 | -28.1 | 4.8 ± 0.87 | 3.85 ± 0.36 | -19.8 | 0.82 ± 0.05 | 1 ± 0.00 | 22.6 |
| | 7 | 1.11 ± 0.09 | 1.24 ± 0.00 | 12.2 | 2.76 ± 1.40 | 3.85 ± 0.95 | 39.5 | 0.65 ± 0.02 | 0.84 ± 0.09 | 30 |
| | 8 | 1.22 ± 0.08 | 1.51 ± 0.00 | 23.7 | 2.59 ± 0.21 | 1.22 ± 0.07 | -52.8 | 0.93 ± 0.12 | 0.93 ± 0.00 | 0.8 |
| | 9 | 1.15 ± 0.06 | 1.46 ± 0.12 | 26.5 | 2.49 ± 0.31 | 1.37 ± 0.08 | -45.2 | 0.9 ± 0.00 | 1.25 ± 0.00 | 38.6 |
| | 10 | 1.22 ± 0.09 | 1.3 ± 0.12 | 6.2 | 2.3 ± 0.10 | 1.11 ± 0.00 | -51.7 | 1.04 ± 0.11 | 1.55 ± 0.11 | 48.9 |
| | 11 | 1.16 ± 0.07 | 1.03 ± 0.12 | -11.3 | 3.18 ± 0.41 | 3.06 ± 0.65 | -3.9 | 0.77 ± 0.02 | 1 ± 0.00 | 29.1 |
| | 12 | 1.47 ± 0.07 | 1.3 ± 0.12 | -11.8 | 3.69 ± 1.44 | 1.74 ± 0.00 | -52.9 | 1.03 ± 0.10 | 1.35 ± 0.14 | 30.6 |
| C | 1 | 0.38 ± 0.01 | 0.39 ± 0.08 | 4.1 | 3.08 ± 0.37 | 4.33 ± 0.00 | 40.6 | 0.86 ± 0.04 | 0.82 ± 0.00 | -5.3 |
| | 2 | 0.3 ± 0.07 | 0.21 ± 0.08 | -28.5 | 3.01 ± 0.32 | 2.17 ± 0.19 | -27.9 | 0.98 ± 0.03 | 1.02 ± 0.00 | 3.8 |
| | 3 | 0.24 ± 0.25 | 0.25 ± 0.00 | 4 | 1.61 ± 0.22 | 1.83 ± 0.00 | 13.7 | 1.16 ± 0.08 | 1.43 ± 0.00 | 22.9 |
| | 4 | 0.34 ± 0.03 | 0.25 ± 0.00 | -25.7 | 4.82 ± 0.27 | 3.75 ± 0.00 | -22.2 | 1.2 ± 0.08 | 1.39 ± 0.12 | 16.1 |
| | 5 | 0.44 ± 0.05 | 0.32 ± 0.10 | -26.7 | 5.03 ± 0.51 | 4.25 ± 0.28 | -15.5 | 0.87 ± 0.07 | 1.03 ± 0.12 | 18.2 |
| | 6 | 0.77 ± 0.03 | 0.51 ± 0.00 | -33.8 | 3.91 ± 0.23 | 4.48 ± 0.36 | 14.6 | 0.63 ± 0.06 | 0.65 ± 0.10 | 2.5 |
| | 7 | 0.84 ± 0.03 | 0.97 ± 0.00 | 16.1 | 3.29 ± 0.45 | 4.48 ± 0.18 | 36.2 | 0.5 ± 0.03 | 0.6 ± 0.00 | 19.7 |
| | 8 | 0.84 ± 0.07 | 0.97 ± 0.00 | 16.1 | 2.04 ± 0.13 | 0.84 ± 0.07 | -58.9 | 0.66 ± 0.03 | 0.71 ± 0.00 | 7 |
| | 9 | 0.98 ± 0.08 | 0.97 ± 0.00 | -0.6 | 1.58 ± 0.14 | 0.9 ± 0.00 | -42.9 | 0.68 ± 0.06 | 0.75 ± 0.00 | 10.9 |
| | 10 | 0.87 ± 0.03 | 0.97 ± 0.00 | 11.2 | 1.5 ± 0.21 | 0.56 ± 0.00 | -62.3 | 0.62 ± 0.03 | 0.9 ± 0.14 | 44.2 |
| | 11 | 0.82 ± 0.02 | 0.81 ± 0.15 | -1.7 | 2.38 ± 0.21 | 1.74 ± 0.00 | -27 | 0.6 ± 0.01 | 0.55 ± 0.11 | -8.1 |
| | 12 | 1.02 ± 0.22 | 1.24 ± 0.00 | 22.1 | 1.76 ± 0.47 | 1.04 ± 0.11 | -40.6 | 0.77 ± 0.01 | 0.8 ± 0.11 | 4.1 |

A – sample extract analysed without dilution;
B – sample extract analysed after dilution (dilution factor of 1.4),
C – sample extract analysed after dilution (dilution factor of 2).

**Table 5.3.** Linear relationships obtained for the analysis of different samples in the SI-LOV and the comparison method for HRP activity, with limits of the 95% confidence interval.

| Sample | Sample dilution | Linear relationship (n=12) |
|---|---|---|
| **Watercress** | A | $Act_{developed\ meth.}$ = 1.068 (± 0.574) $Act_{comp.\ meth.}$ + 0.132 (± 0.857) |
| | B | $Act_{developed\ meth.}$ = 1.250 (± 0.408) $Act_{comp.\ meth.}$ - 0.275 (± 0.404) |
| | C | $Act_{developed\ meth.}$ = 1.212 (± 0.289) $Act_{comp.\ meth.}$ - 0.135 (± 0.205) |
| **Spinach** | A | $Act_{developed\ meth.}$ = 0.914 (± 0.841) $Act_{comp.\ meth.}$ - 0.639 (± 4.431) |
| | B | $Act_{developed\ meth.}$ = 1.002 (± 2.273) $Act_{comp.\ meth.}$ - 0.444 (± 0.635) |
| | C | $Act_{developed\ meth.}$ = 1.077 (± 1.512) $Act_{comp.\ meth.}$ - 0.521 (± 0.491) |
| **Green beans** | A | $Act_{developed\ meth.}$ = 0.959 (± 0.180) $Act_{comp.\ meth.}$ + 0.313 (± 0.355) |
| | B | $Act_{developed\ meth.}$ = 1.452 (± 0.478) $Act_{comp.\ meth.}$ - 0.157 (± 0.517) |
| | C | $Act_{developed\ meth.}$ = 1.191 (± 0.316) $Act_{comp.\ meth.}$ - 0.060 (± 0.260) |

A – sample extract analysed without dilution;
B – sample extract analysed after dilution (dilution factor of 1.4);
C – sample extract analysed after dilution (dilution factor of 2).

The activity obtained in the developed and in the comparison method by fresh weight of sample (Act/g) is presented on Fig.5.2. It can be concluded that the activity obtained for all samples is characteristic of the sample and not dependent on the weight of sample used. It can be seen that green beans – the vegetables that are less susceptible to undesirable alterations in texture, flavours and colours – are the vegetables that have lower peroxidase activity per weight of sample. On the other hand, watercress, which usually is a fresh product and has a short shelf life, has higher peroxidase activity per weight of sample.

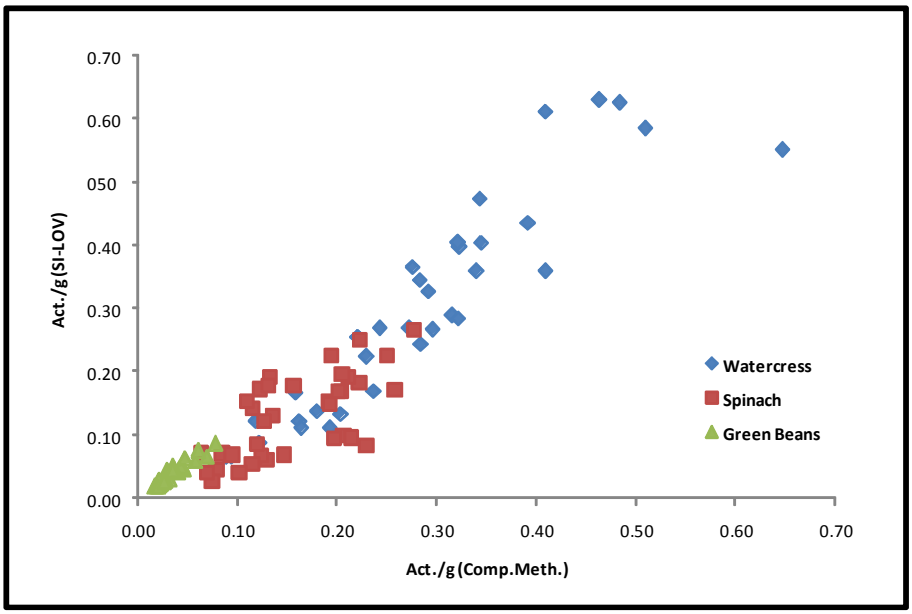


**Fig. 5.2.** Activity of peroxidase per fresh weight of the sample obtained by SI-LOV and by the comparison method.

When considering all the samples analysed within one type of commodity, and the activity values obtained for this commodity with different dilution rates, it is possible to conclude that the peroxidase concentrations in the diluted samples are not significantly different from the ones obtained without dilution. F values calculated were: 0.27 for watercress, 0.70 for spinach, and 1.3 for green beans; with a corresponding critical F value of 3.28. This result can be justified by the wide dispersion of the values itself within the studied commodities. However, if some of the individual samples are considered, a clear tendency can be verified, indicating an apparent matrix effect. This behaviour is referred as a frequent pitfall of spectrophotometric methods for enzyme activity assays in natural samples, resulting in the necessity of a strict control in the experimental conditions for batch

assays. This also underlines the advantages of using flow methods with inherent assay reproducibility for enzymatic determinations.

### 5.3.3.2 Thermal inactivation of peroxidase in solutions

Taking advantage of the versatility of the system, it was possible to perform on-line the monitoring of the thermal inactivation of peroxidase aiming also to provide a valuable tool for evaluation of the thermal blanching process effectiveness (Icier et al., 2006; Doğan et al., 2007).

The thermal inactivation of peroxidase enzyme in a solution of 2 mg/L was studied in the temperature range of 65 to 85 ºC. The results obtained for the developed and for the comparison method are presented in Fig.5.3. The results of the developed method are in good agreement with the comparison method. It can also be concluded that with the rise of the temperature from 65 to 75 ºC, it is possible to reduce the procedure time at 200 s to achieve a 20% of remaining activity. When the blanching temperature is 85 ºC it is possible to reduce the time of the process in a total of 470 s, to attain the 20% of remaining activity. A higher standard deviation is verified when the blanching is carried out at 65 ºC (Fig.5.3I), this can be due to that the enzyme is thermal stable up to 60 ºC (Nunc Brand, 2008; Rani and Abraham, 2006) and the temperature of blanching is close to this limit.

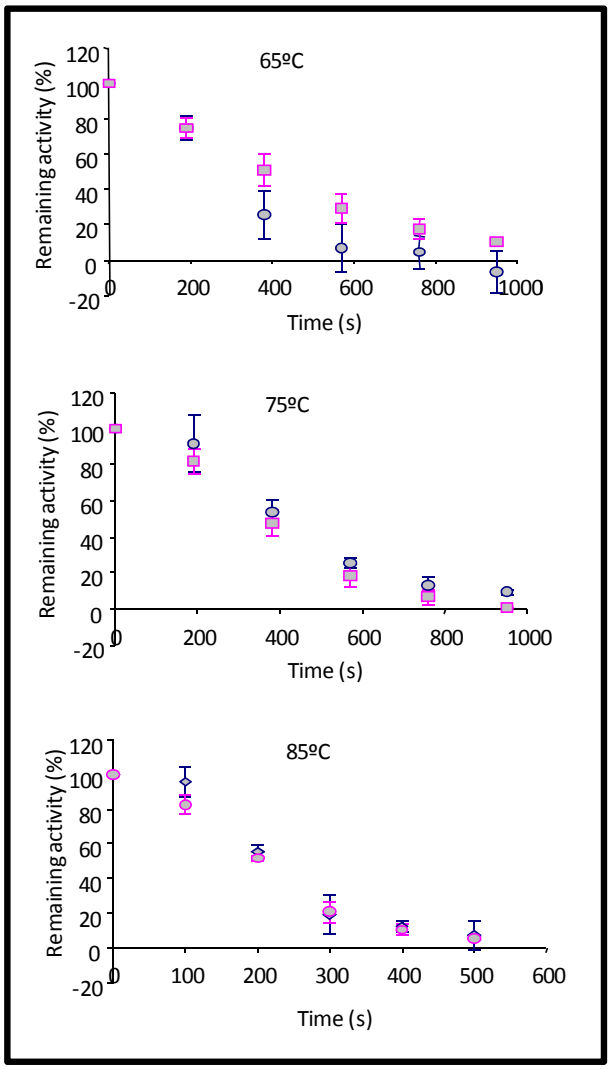


Fig. 5.3. Variation of remaining activity (%) during a thermal inactivation process in solutions of HRP at different temperatures. Data presented as the means of five independent experiments for the SI-LOV and three independent experiments for the comparison method; the error bars denote ± standard deviation.

## 5.4. CONCLUSIONS

In conclusion, the use of a lab-on-valve format with reduced injection volumes proved to be a useful tool for the development of the method for the determination of peroxidase in vegetables. The results obtained for the analysis of real samples are in good agreement with the results obtained for the comparison method, with lower sample and reagent consumptions. It was also possible to achieve a linear range up to 2 mg/L of HRP with a significant (50%) increase of the determination rate in comparison with the batch method. Using the same system, it was also possible to monitor on-line the thermal inactivation of the enzyme in solutions, which is a fundamental parameter for an efficient evaluation of the thermal blanching process.

# CHAPTER 6

## DETERMINATION OF IRON IN WINE SAMPLES EXPLOITING THE BEAD INJECTION APPROACH

## 6.1. INTRODUCTION

The chemical composition of wine is very variable and complex; besides the main organic compounds as ethanol, sugars, organic acids, polyphenols and proteins, wine also contains several inorganic species dissolved (Pohl, 2007; Galani-Nikolakaki et al., 2002; Pyrzyńska, 2004). Some trace metals can also affect the organoleptic characteristics of the wine, including the flavour, freshness, aroma, colour, and taste (Pohl, 2007). Moreover, the content of some metals can be used to prove wine authenticity and quality. It is known that concentrations higher than 5 mg/L can cause several changes on the stability of the product and can promote oxidation and wine aging (Costa and Araújo, 2001; Lasanta et al., 2005). The natural concentration in wine depends principally on the type of the soil where the grapes are produced (Galani-Nikolakaki et al., 2002; Amerine and Ough, 1974; Volpe et al., 2009; Lara et al., 2005). The official method recommended for the determination of iron in alcoholic beverages by OIV and AOAC is based on atomic absorption spectrometry (AAS), either with flame or furnace atomisation, and is the most widely used method for this determination (Pyrzyńska, 2004; Santalad et al., 2007).

Molecular absorption spectrophotometry has been the most common detection method used to quantify chemical components (Matsuoka and Yoshimura, 2010), but not so common in wine analysis. This might be due to a number of reasons associated with the complexity of the wines. To begin with, metals can be present in wines in different forms, either free or bound to different ligands. Therefore, if the

total content is aimed, metals have to be released before or during the colour reaction, either by simple acidification for labile species or by carrying out drastic mineralisation procedures. There are also potential interferences in the colour reactions with a major difficulty arising from the intrinsic colour of the wine. Different flow analysis approaches, like reversed flow (Johnson and Petty, 1982), merging zones (Ferreira et al., 1996), multi-site detection (Oliveira et al., 2005), or use of in-line dialysis (Rangel and Tóth, 2000), have been proposed to tackle this problem. Even so, if the background absorption is very high, the first two approaches may not be efficient, while the use of in-line dialysis might induce a too high dilution. Additionally, in dialysis flow analysis systems, it is difficult to match the diffusion yield of standards and samples, which make difficult to obtain accurate calibration procedures.

Solid phase extraction can be an attractive alternative to the above-mentioned processes, because it might provide the pre concentration of the analyte and the discard of interfering agents. This is quite feasible using sequential injection, lab-on-valve approach (SI-LOV) (Růžička, 2000). The present work employs a commercial available “Nitrilotriacetic Acid (NTA) Superflow resin” which can isolate and concentrate dissolved iron at low pH (Lohan et al., 2005). This resin was originally designed for high throughput sample clean-up procedures based on the affinity chromatography concept. Recently, NTA Superflow resin was applied for the preconcentration of total iron in sea (Lohan et al., 2005; Jong et al., 2008) and river (Páscoa et al., 2009) water samples.

The herein proposed methodology exploits the concept of solid phase spectrometry in a SI-LOV format to circumvent some difficulties associated to complex matrices in spectrophotometric measurements, namely a high background sample absorption; it was applied to the determination of iron (III) in wines. At low pH (ca. 2) the Fe (III) ions are retained in the bead column and react with thiocyanate reagent. As a result of the reaction, an intense red colour is produced and is spectrophotometrically monitored at 480 nm.

## 6.2. EXPERIMENTAL

### 6.2.1. Reagents and solutions

The thiocyanate solution was prepared by dissolving 11.4 g of $NH_4SCN$ in 100 mL of water. The hydrogen peroxide solution (0.3 M) was prepared from the stock solution (Perhydrol, 30% $H_2O_2$). The iron (III) stock standard solutions (up to 5 mg/L) were prepared by diluting commercial 1000 mg/L iron atomic absorption standards (iron standard solution, iron (III) nitrate, VWR-Spectrosol) in 0.01 MHCl solution. The iron (II) stock standard solution (100 mg/L) was prepared by dissolving $(NH_4)_2Fe(SO_4)_2.6H_2O$ in 0.01 MHCl solution.

The bead suspension used was a dilution in water to half (w/w) of the commercial stock solution (NTA Superflow resin, highly cross-linked 6% agarose, 60 to 160 µm of bead diameter, 50% suspension in 30% ethanol, 30510, Qiagen)

### 6.2.2. Samples

The wine samples (red table, white table and port wine) were purchased in a local market. When necessary, dilution was carried out as sample pre-treatment. The other samples, olive and soybean brines were purchased in a local market, and had no other sample pre-treatment than filtration followed by dilution, when necessary.

### 6.2.3. Manifold and flow procedure

The manifold for sequential injection lab-on-valve system for the determination of iron in wine using bead injection approach is depicted in Fig. 6.1.A schematic representation of the bead column built between the two optical fibres and a plug (small piece of yellow PEEK tubing, # 1536, Upchurch Scientific) is also depicted (Fig. 6.1.B).

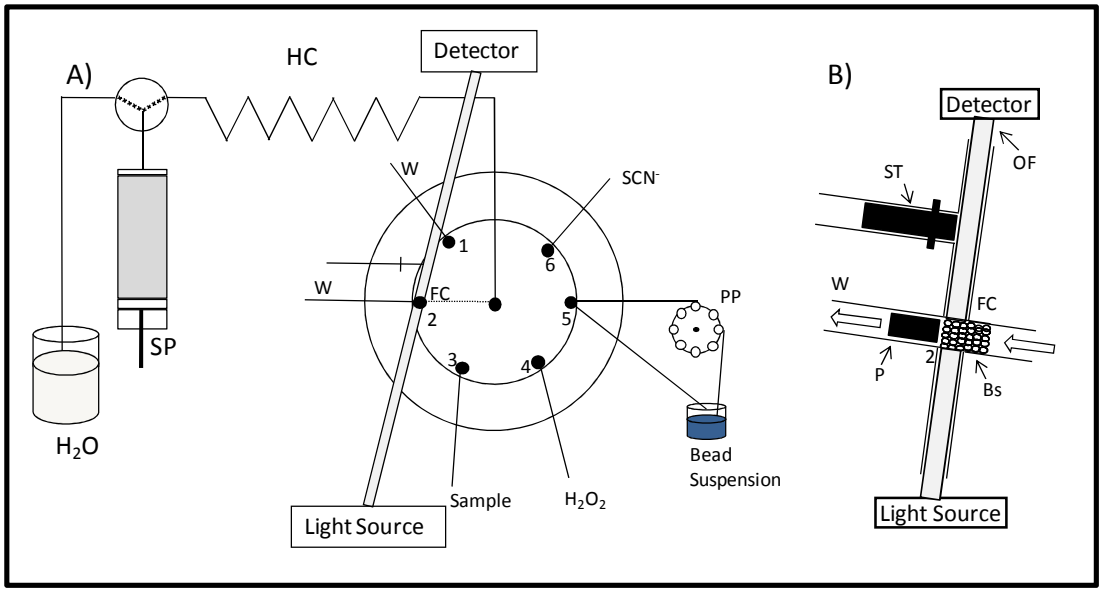


**Fig. 6.1 A)** Configuration of the SI-BI-LOV manifold for the determination of iron. **B)** Position of the bead column in the flow cell. SP, syringe pump; HC, holding coil; FC, flow cell; $SCN^-$, 1.5 M; $H_2O_2$, 0.3 M; W, waste; ST, PTFE stopper, P, PEEK plug; Bs, beads; FO, fibre optics.

The sequential injection bead injection lab-on-valve (SI-BI-LOV) flow procedure is summarised in Table 6.1. The initial steps of A to C, consisted in the preparation of the bead column in the flow cell, where 40 µL of the bead suspension is packed in the flow cell by 80 µL of carrier at 10 µL/s. Afterwards, the sample and the oxidant were aspirated in the following sequence: 10 µL of $H_2O_2$; 250 µL of sample; 10 µL of $H_2O_2$; 250 µL of sample; 10 µL of $H_2O_2$. Then, the sample mixture perfuses the bead column in the flow cell; afterwards the reference scan was performed to establish the absorbance baseline. The change in the absorbance was monitored at 480 nm while 10 µL of $SCN^-$ drove though the bead column. After the measurement, the

physical regeneration of the flow-through sensor was carried out by the aspiration of the beads to the holding coil and subsequent discharging to waste.

**Table 6.1.** Flow protocol sequence of the SI-BI-LOV method for the determination of iron.

| Step | Description | Volume (µL) | Flow rate (µL/s) | Selection valve position |
|---|---|---|---|---|
| **A** | Aspirate beads to HC | 40 | 20 | 5 |
| **B** | Propel beads to FC | 80 | 10 | 2 |
| **C** | Aspirate $H_2O_2$ to HC | 10 | 50 | 4 |
| **D** | Aspirate sample to HC | 250 | 50 | 3 |
| **E** | Aspirate $H_2O_2$ to HC | 10 | 50 | 4 |
| **F** | Aspirate sample to HC | 250 | 50 | 3 |
| **G** | Aspirate $H_2O_2$ to HC | 10 | 50 | 4 |
| **H** | Send sample mixture to FC | 1100 | 9 | 2 |
| **I** | Reference scan, Absorbance scanning | - | - | - |
| **J** | Aspirate $SCN^-$ to HC | 10 | 50 | 6 |
| **K** | Send $SCN^-$ to FC | 75 | 3 | 2 |
| **L** | Aspirate beads from FC, by reversed flow | 100 | 300 | 2 |
| **M** | Dispense beads to waste | 425 | 300 | 1 |

For cleaning the sample circuit, the analytical cycle was stopped and diluted acid- and base solutions were sequentially passed through the flow cell, followed by water for rinsing. At the end of the washing cycle, the blank absorbance was registered and, if this value approached the original one, the system was assumed to be cleaned.

### 6.2.4. Reference procedure

A reference method was performed to corroborate the results obtained by the developed method. The method used was a Portuguese reference method (NP2280, 1988) for the determination of iron content in alcoholic beverages and spirits by atomic absorption spectrophotometry.

## 6.3. Results and Discussion

### 6.3.1. Study of the flow system

Several physical parameters like sample and reagents volumes, the aspiration sequence and the flow rates used in the flow system, were studied.

The repeatability of bead packing is an essential parameter to assure reproducible and good quality data. The first study was to select the strategy of how to introduce the bead suspension in the flow system. The option was made to keep the bead suspension circulating in a closed circuit connected to the port 5 (Fig. 6.1) of the LOV system. It is essential to maintain a well-stirred bead suspension, since aspiration of beads from an unstirred sediment results in blockage of the bead aspiration channel. As the nature of the beads can tolerate magnetic stirring, this was the chosen procedure to maintain the beads suspended. A rotating container (placed in a Vortex shaker) was also tested and the repeatability showed no

significant difference when compared to the magnetic stirring. The NTA Superflow beads have a bead structure with relatively high percentage of agarose, this composition offers the beads low flow resistance, and easy handling possibilities inside the flow system. Still, the physical integrity of the beads had to be followed during the method development.The beads in agitation and circulation were kept under visual control, with the use of a microscope (Fig.6.2.), and only after 4 days were possible to observe about 7% more of broken beads when comparing with the freshly prepared suspension. Therefore the replacement of the bead suspension could be done weekly.

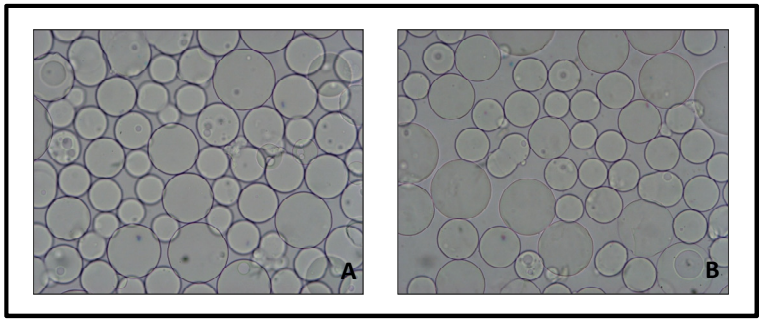


**Fig. 6.2.** Microscopic view of the NTA superflow beads. **A)** New suspension and **B)** suspension after one day of recirculation (100x).

Some physical parameters of the beads packing like: volume of the beads suspension, carrier volume and flow rate were studied, since the light-path geometry must allow sufficient light to penetrate the bead layer and at the same time the target molecules must be on the optical path. The volume of beads

suspension was studied in a range from 20 to 40 µL, while the carrier volume was studied in a range from 45 to 100 µL with a flow rate of bead packing of 2, 5 and 10 µL/s. This study was carried out based on monitoring the increase of absorbance at 225 nm. The minimum volume of bead suspension used to construct the bead column was 25 µL; up to this volume of suspension there were no sufficient beads to make a reproducible sensor. The best loading conditions for the column preparation was selected based on the time required for establishing a reproducible bead column; it was set as 80 µL of carrier used to propel 40 µL of bead suspension at 10 µL/s.

As this work is based on a solid phase reaction, the easiest way to establish the maximum binding capacity of the beads was to study the effect of the sample loading volume. The volume of sample was varied in a range from 100 to 600 µL. This study was performed with a 10 mg/L $Fe^{3+}$ standard solution, 1.5 M of $SCN^-$ and 0.3 M of $H_2O_2$. Over 500 µL of sample, there was no significant increase on the analytical signal; therefore, 500 µL was the volume of sample chosen for the work. In this case, the maximum load of $Fe^{3+}$ corresponds to 250 mg $Fe^{3+}$/L of commercial beads suspension.

The flow rate used to propel the sample towards the bead column was studied in a range from 3 to 15 µL/s. The analytical signal increased with the increase of the flow rate up to 9 µL/s. When the flow rate was augmented from 9 to 11 µL/s, it was possible to observe an increase on the analytical signal of 10%, but at the same time the relative standard deviation of the signal increased from 2% to 5%, therefore as

compromise between increase on the analytical signal and repeatability 9 µL/s was chosen for further work.

With the physical conditions defined, the effect of the concentration of the reagents on the analytical signal was studied. Calibration curves up to 8 mg/L of $Fe^{3+}$ were performed. The concentration of $SCN^-$ was set at 1.5 M and the volume of the reagent was altered to study its effect on the sensitivity of the method. When the quantity is augmented from 7.5 to 15 µmol per assay the sensitivity increased about 25%. Over this value, the linearity of the calibration curve is deteriorated; therefore the chosen quantity was 15 µmol per assay.

Given that the thiocyanate reacts with iron (III), it is necessary to ensure that the iron is in the oxidized form, thus an in-line oxidation is performed. Therefore, three steps were added to the analytical cycle, in order to sandwich the sample between oxidant (hydrogen peroxide) plugs. Since the plug of sample is long (500 µL) it was divided into two, and the oxidant was aspirated before, in the middle, and after the sample. The volumes of oxidant were tested for 10 and 20 µL per plug. Different aspiration sequences were studied using a standard of 4.8 mg/L $Fe^{3+}$ and 0.4 M hydrogen peroxide. It was possible to reduce the volume of the oxidant to 10 µL per plug since no significant loss on the analytical signal was observed. The concentration of oxidant was also studied in a range from 0.04 to 0.5 M and the analytical signal increased with the concentration of hydrogen peroxide up to 0.3 M. The analytical cycle was repeated with a 4.8 mg/L of $Fe^{2+}$ solution and the results

were similar to those obtained with a standard of 4.8 mg/L of $Fe^{3+}$, therefore the 0.3 M concentration was chosen for further work.

The standard solutions of iron were prepared at pH 2. As wine samples have pH values around 3.5, the effect of the sample acidity on the reaction was also studied. Calibration curves were performed with standards solutions prepared in acidic medium at pH of 2 and at 3.5. The values obtained for the sensitivity of the curves at pH 2 and 3.5 showed no significant difference.

### 6.3.2. Study of interfering species

The study of potential interfering species was performed considering the typical composition of wine samples. The effect of ethanol was studied in a range up to 20% (v/v) and there was no significant variation of the sensitivity of the method. Since wine has a complex matrix, the standards were prepared in a wine model solution containing 10% (v/v) of ethanol, 3 g/L of sugars (Glucose:Fructose; 40:60%), 7 g/L of glycerol, 100 mg/L of $Mg^{2+}$, 100 mg/L of $Ca^{2+}$, 50 mg/L of $Na^{+}$ and 4 g/L of tartaric acid, to evaluate the effect matrix interference in the determination. There was no significant difference found on the sensitivity obtained in the analysis of standards prepared in water and for the ones prepared in wine model solution, as it can be concluded from the values of the sensitivity obtained, $0.134 \pm 0.008$ and $0.137 \pm 0.010$, respectively.

### 6.3.3. Figures of merit of the flow system

The performance of the developed method was evaluated in terms of reagent and sample consumption, determination rate and dynamic range. The method presented a sample consumption of 500 µL per assay and a consumption of $SCN^-$ and $H_2O_2$ of 15 µmol and 9 µmol per assay, respectively. It was possible to achieve a linear range up to 5.0 mg/L with a detection and quantification limits (Miller and Miller, 1993) of 0.02 and 0.09 mg/L, respectively. These figures of merit are summarized in Table 6.2.

**Table 6.2.** Figures of merit of the SI-BI-LOV method for the determination of iron.

| Parameter | Value |
|---|---|
| Reagent consumption per assay | |
| $SCN^-$ | 15 µmol |
| $H_2O_2$ | 9 µmol |
| Sample | 500 µL |
| Waste production per assay | 2.5 mL |
| Application range | 0.09 - 5.0 mg/L |
| Determination rate | 20 det./h |
| LOD | 0.02 mg/L |
| LOQ | 0.09 mg/L |

The operational stability of the developed system was calculated by performing the calibration procedure under identical physical and chemical conditions between different working days. A calibration curve: Absorbance = 0.138 (± 0.006) × $[Fe^{3+}]$

(mg $L^{-1}$) - 0.004 (± 0.024); the values in parentheses are the standard deviation for n=8 calibration curves obtained from 5 standards injected 5 times each and performed in a period of five months. Stability can be considered satisfactory, as this data reflects different batches of beads and reagents and daily preparation of standard solutions.

### 6.3.4. Application to samples

A total of twelve (five white and seven red) table wines and four Port (two white and two tawny) wine samples were analysed. No other sample pre-treatment than dilution was required, for some of the samples. To evaluate the accuracy of the developed method, the reference method was also performed. The results obtained in the sample analysis are presented in Table 6.3.

**Table 6.3**. Comparison of the results obtained for the analysis of iron in wine samples by the reference and the SI-BI-LOV procedures.

| Sample | Ref Met. (mg/L)[a] | SI-LOV (mg/L)[b] | RD%[c] | Off-line treatment |
|---|---|---|---|---|
| White table wine 1 | 1.2 ± 0.1 | 1.4 ± 0.1 | 16.7 | none |
| White table wine 2 | 2.1 ± 0.1 | 2.1 ± 0.1 | 0.0 | none |
| White table wine 3 | 9.0 ± 0.4 | 9.2 ± 0.7 | 2.2 | dil.4x |
| White table wine 4 | 6.1 ± 0.2 | 5.4 ± 0.1 | -11.5 | dil.4x |
| White table wine 5 | 2.2 ± 0.1 | 1.9 ± 0.2 | -13.6 | none |
| Red table wine 1 | 4.0 ± 0.2 | 4.1 ± 0.6 | 2.5 | dil.4x |
| Red table wine 2 | 5.2 ± 0.4 | 5.0 ± 0.1 | -3.8 | dil.4x |
| Red table wine 3 | 5.5 ± 0.2 | 4.9 ± 0.6 | -10.9 | dil.4x |
| Red table wine 4 | 5.5 ± 0.4 | 5.4 ± 0.4 | -1.8 | dil.4x |
| Red table wine 5 | 5.4 ± 0.1 | 5.0 ± 0.1 | -7.4 | dil.4x |
| Red table wine 6 | 5.3 ± 0.4 | 6.0 ± 0.8 | 13.2 | dil.4x |
| Red table wine 7 | 4.9 ± 0.2 | 4.3 ± 0.5 | -12.2 | dil.4x |
| Port, white 1 | 2.7 ± 0.2 | 2.6 ± 0.3 | -3.7 | none |
| Port, white 2 | 2.7 ± 0.4 | 2.6 ± 0.1 | -3.7 | none |
| Port, tawny 1 | 4.1 ± 0.4 | 3.9 ± 0.2 | -4.9 | dil.2x |
| Port, tawny 2 | 2.0 ± 0.2 | 2.1 ± 0.8 | 5.0 | none |

[a] mean and standard deviation for n=3.
[b] mean and standard deviation for n=5.
[c] Relative Deviation.

The results obtained for the developed method are in good agreement with those obtained for the reference method. A linear relationship: $C_{developed\ meth.} = m\ C_{comp.meth.} + b$, was established for the samples analysed, and the values obtained for the intercept ($b$) and for the slope ($m$) were – 0.025 (± 0.470) and 0.973 (± 0.101), respectively. The values presented in parentheses are the limits of the 95% confidence intervals (Miller and Miller, 1993).

The previous table indicates that every red table wine sample had to be diluted. The main reason for this is not the high level of iron present but the natural colour of the matrix. Given that this is a spectrophotometric assay, the baseline absorbance must be established after the sample mixture contacts the bead column. For red table wines, this was not possible due to the natural colour of the matrix. The possibility to use a reduced sample volume as an alternative to the off-line dilution of the sample was tested. A red table wine with a volume of 150 µL, as opposed to 500 µL, was introduced in the system, packed between two plugs of 10 µL of $H_2O_2$. Even though a decrease on the sensitivity of the method of about 50% was observed, the result obtained for the analysis of a red table wine sample for the developed method (1.45 ± 0.19) was in good agreement with the result obtained for the reference method (1.4 ± 0.1). However, for other red table wine samples the same protocol, did not allow to establish the baseline of the measurement. As a further reduction in the sample volume would decrease too much the sensitivity, we concluded that this approach could not be a general solution for every red wine.

As it can be concluded from Table 6.3, samples of red table wines and port wines presented distinct and typical concentration levels, therefore other samples were also tested to assess the adaptability of the method to samples of non-wine origin and in different concentration ranges.A certified reference water sample and two different types of brines, olive and soybean, were analysed and the results obtained for the developed method were comparable to those obtained for the reference method (Table 6.4).

**Table 6.4**. Comparison of the results obtained for the analysis of iron in other food samples by the reference and the SI-BI-LOV method.

| Sample | Ref Met. (mg/L)[a] | SI-LOV (mg/L)[b] |
|---|---|---|
| Water reference SPSSW2 | 0.10 ± 0.00[c] | 0.13 ± 0.01 |
| Olive brine | 15.4 ± 1.9 | 14.4 ± 3.3 |
| Soybean brine | 1.54 ± 0.03 | 1.18 ± 0.06 |

[a] mean and standard deviation for n=3.
[b] mean and standard deviation for n=5.
[c] Certified value.

## 6.4. CONCLUSIONS

The use of a lab-on-valve format with bead injection solid phase extraction proved to be a useful tool for the development of a molecular absorption spectrometry alternative to the atomic absorption spectrometry (AAS) reference method for the determination of metals in complex matrices like wine. The proposed procedure also enabled to quantify the total content of the metal, with much simpler equipment. Based on the results (*viz*: good agreement with the reference atomic absorption procedure), it can be concluded that total iron is determined. This can be also deduced from the experimental conditions: the pH, the presence of an oxidant and a strong complexant and the additional off-line dilution of the samples contributes to the shift of the chemical equilibrium in a way that most of the iron originally present in a complexed form may be released, and made available in the form of $Fe^{3+}$.

This flow system incorporating this optosensor also demonstrated to be a useful strategy to avoid interferences from complex matrices with high background absorption values, in a spectrophotometric method. This was possible by using the bead injection procedure to separate the analyte from the matrix; taking advantage of the use of programmable flow. The chosen NTA resin proved to display characteristics appropriate for bead injection. The overall strategy could be adapted to other spectrophotometric determinations in complex matrices.

The results obtained for the analysis of several different food samples are in good agreement with the results obtained for the reference method. When comparing the present work to similar approaches (Table 6.5) the applicability of the developed SIA-BI approach was illustrated using various sample matrixes and in a highly automated fashion.

**Table 6.5.** Comparison of analytical and operational characteristics of flow-based bead injection methods for the determination of total iron.

| Parameter | Ruedas Rama et al., 2003 | Ampan et al., 2002 | Jitmanee et al., 2002 | Present work |
|---|---|---|---|---|
| Flow method | Flow injection | Flow injection | Flow injection | SI-LOV |
| Samples | River, well water and wine (recovery assays) | Tap, pond and drinking water (recovery assay) | Beer (various type) | Wine (Table, Port), Reference water, brines |
| Reagent | Ferrozine / ascorbic acid | 1, 10-phenantroline / ascorbic acid | 1, 10-phenantroline / ascorbic acid | Thiocyanate/ $H_2O_2$ |
| Beads | Sephadex QAE A-25 | Chelex-100 | Chelex-100 | NTA-Superflow |
| Application range | 0.1-3.0 mg/L | Up to 3.90 mmol/L | Single standard calibration LOD: 0.2 mg/L | 0.09 - 5.0 mg/L |
| Injected volume | 100 mL | 15 mL | 60 mL | 500 mL |
| Sampling frequency | 16 per hour | n.g. | 30 per hour | 20 per hour |

n.g. –notgiven

Regarding the main limitations of the proposed method, we can point out the need for sample dilution if some red wines are analysed. Another difficulty is the cleaning of the flow system after the analysis of red table wines; however, with additional port(s) on the SI-LOV device, the cleaning protocol could be easily incorporated into the analytical cycle.

# CHAPTER 7

## DETERMINATION OF TOTAL PROTEIN CONTENT IN WHITE WINE

## 7.1. INTRODUCTION

Quality is the main concern in winemaking, and is dependent upon various organoleptic properties, including colour and clarity. These characteristics are extremely important as they can influence the product's image, consequently its acceptance by the consumer. In this context, proteins have an important role. The total protein content has been considered as a mixture of grape proteins and proteins from autolysed yeast. These proteins have molecular mass ranging from 9 to 66 kDa and isoelectric points ranging from 3 to 9 (Sauvage et al, 2010) where thaumatin-like proteins (TLP) and chitinases are the predominant proteins present in white wine. Therefore, the total protein content alone can be considered as a poor index of the tendency for the wine to become cloudy; on the other hand, the nature of the proteins responsible for the turbidity in wine remains uncertain, even though their presence is a prerequisite for haze formation. Several studies consider that if the total protein content is high, the tendency for the wine to become unstable also increases (Waters and Colby, 2009; Ferreira et al., 2002). However, other authors (Ferreira et al., 2002; Fusi et al., 2010; Sauvage et al., 2010) reported that the haze formation depends on the presence of specific proteins, TLP and chitinases.

Proteins are usually present in low concentrations in finished wines and their levels differ by variety. For total protein in white wine, Food Composition and Nutrition Tables (Souci et al., 2000) present a range from 0.08 to 0.29 g/L; other authors

present a wider range from 0.01 to 0.50 g/L (Maragon et al., 2011). The reference method usually performed is based on the total nitrogen measurement. The recommended Kjeldahl method measures the total nitrogen in the sample, not only the protein nitrogen; it is time consuming, involves corrosive reagents and needs a relatively large amount of protein (Lindeboom and Wanasundara, 2007). Therefore, the protein content is not obtained directly but rather obtained by calculations, leading to frequent overestimation (Amerine and Ough, 1980). Other methods used for wine protein quantification can be based on spectrophotometric detection such as the Bradford method (Bradford, 1976; Le Bourse et al., 2010). These methods are simple and fast but do not provide an accurate result, considering the response variation to different proteins and due to the absence of a commercial standard of wine proteins. To cope with this problem bovine serum albumin (BSA) is accepted as an alternative standard and is usually used for total protein studies, since it presents a haze formation capacity similar to the major wine proteins (TLP and Chitinase) (Ferreira et al., 2002). Other colorimetric methods such as Lowry (Lowry et al., 1951), Biuret (Gornall et al., 1949) or Smith (Smith et al., 1985) can provide good results; however, the results obtained are not always comparable within these methods due to the presence of a large number of wine components; in this particular determination, it is important to consider the possible interference of phenolic compounds (Lowry et al., 1951; Moreno-Arribas et al., 2002; Sapan et al., 1999; Zaia et al., 1998). Phenolic compounds are crucial components in wine that can react with the Folin-Ciocalteau's reagent (FCr) at alkaline pH, resulting in a false

positive response (Moreno-Arribas et al., 2002; Zaia et al., 1998). Nevertheless, since this method is about 100 times more sensitive than the Buriet test, and about 5 times more sensitive than the Bradford test (Lucarini and Kilikian, 1999), it can be carried out if the interference of the phenolic compounds is neutralized (Lindeboom and Wanasundara, 2007). With the aim of reducing the interferences, some protein quantification methods are based on a prior isolation, usually by gel filtration (Sephadex G-25) (Amerine and Ough, 1980).

As demonstrated before (Chap. 6), the BI approach is easily performed in the SI-LOV system to carry out SPS to avoid interferences. In this scenario, a methodology for the quantification of total protein content in white wine using a SI-LOV system in a BI mode was developed. In this work, the beads chosen as solid support were the commercial available NTA superflow resin, the same one used in the work described in Chap. 6. NTA is an important chelating agent capable of sequestering metal ions, which can be advantageous to employ in the Lowry method. The Lawry method can be divided (Lindeboom and Wanasundara, 2007) in two steps: protein reaction with copper followed by the reduction of FCr. However, the exact role of the copper is not very clear. Some authors reported that $Cu^{2+}$ binds the protein and this process results in the reduction to Cu(I) that then reacts with the FCr (Winters and Minchin, 2005); others claim that the proteins reduce the reagent, whereby copper chelates the peptides structure and facilitates electron transfer from the protein to the reagent (Lindeboom and Wanasundara, 2007).

In the present work, the classical Lowry method was applied to the bead injection SI-LOV format. First the $Cu^{2+}$ is bound by the NTA (Anderegg, 1982) at the surface of the beads. The beads charged by $Cu^{2+}$retain the protein in the form of a chelate, as the sample solution passes though the beads column. When the FCr is added to the copper-protein retained on the solid support, the reduction of the FCr occurs and an increase of the colour intensity can be spectrophotometrically monitored at 500 nm.

## 7.2. Experimental

### 7.2.1. Reagents and solutions

The 100 mM copper solution was prepared from $CuSO_4.5H_2O$. The FCr solution was a dilution of 1/10 (v/v) from the commercial product (Folin-Ciocalteu's phenol reagent, 47641, Fluka) in deionised water. To daily prepare the working standards solutions, 1 g/L of BSA (fraction V, 05484, Fluka) was prepared in NaCl 0.15 M, and this solution was further diluted in water.

The commercial stock suspension (NTA Superflow resin, highly cross-linked 6% agarose, 60 to 160 µm of bead diameter, 50% suspension in 30% ethanol, 30510, Qiagen) was diluted (1:1) in water (w/w).

### 7.2.2. Samples

The analysed samples, white wine, sparkling wine and beer were purchased in a local market. Before its introduction in the system, the samples pH was adjusted to 5.5. No other pre-treatment was required with the exception of the beer sample that was 5 times diluted.

### 7.2.3. Manifold and flow procedure

The manifold for the sequential injection lab-on-valve system for the determination of total protein content in white wine using bead injection approach is depicted in Fig. 7.1. A schematic representation of the bead column built between the two optical fibres and a plug (small piece of yellow PEEK tubing, # 1536, Upchurch Scientific) is also depicted (Fig. 7.1.B).

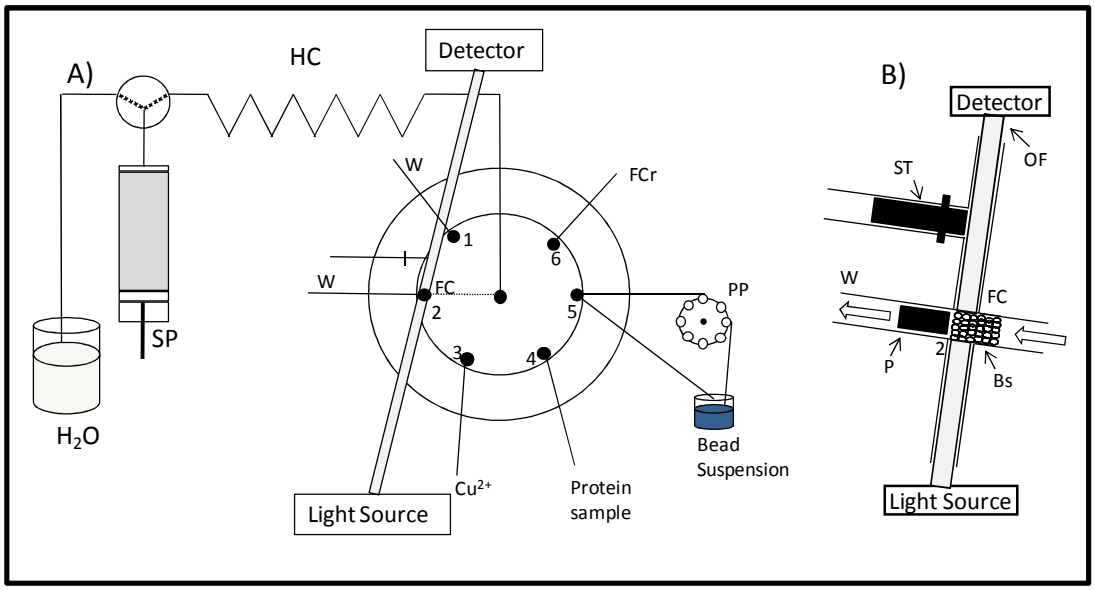


**Fig. 7.1. A)** Configuration of the SI-BI-LOV manifold for the determination of total protein content in white wine samples. **B)** Position of the bead column in the flow cell. SP, syringe pump; HC, holding coil; FC, flow cell; FCr, 25 µL; $Cu^{2+}$, 100 mM; W, waste; ST, PTFE stopper; P, PEEK plug; Bs, beads; FO, fibre optic.

The SI-BI-LOV flow procedure is summarised in Table 7.1.

**Table 7.1.** Flow protocol sequence of the SI-BI-LOV method for the determination of total protein content.

| Step | Description | Volume (µL) | Flow rate (µL/s) | Selection valve position |
|---|---|---|---|---|
| A | Aspirate carrier to SP | 1500 | 250 | - |
| B | Aspirate beads suspension to HC | 40 | 20 | 5 |
| C | Propel beads suspension to FC | 80 | 10 | 2 |
| D | Aspirate $Cu^{2+}$ solution to HC | 100 | 50 | 3 |
| E | Propel $Cu^{2+}$solution to FC | 500 | 9 | 2 |
| E | Aspirate protein sample to HC | 400 | 50 | 4 |
| F | Propel protein sample to FC | 700 | 9 | 2 |
| G | Aspirate FCr to HC | 25 | 50 | 6 |
| H | Reference scan, Absorbance scanning | - | - | - |
| I | Propel FCr to FC | 250 | 6 | 2 |
| J | Aspirate beads from FC, by reversed flow | 200 | 300 | 2 |
| K | Dispense beads to waste | Empty SP | 300 | 1 |

The initial steps of A to C consisted in the preparation of the bead column in the flow cell, where 40 µL of the bead suspension was packed in the flow cell by 80 µL of carrier at 10 µL/s. Afterwards, 100 µL of the $Cu^{2+}$ solution was propelled to the flow cell with 500 µL of carrier at 9 µL/s, followed by 400 µL of sample solution with 700 µL of carrier solution at a flow rate of 9 µL/s. The change in the absorbance was monitored at 500 nm while 25 µL of the diluted FCr drove though the bead column at 6 µL/s. After the measurement, the physical regeneration of the flow-through

sensor was carried out by reversed flow; the beads were aspirated to the holding coil and subsequently discharged to waste.

## 7.3. Results and Discussion

### 7.3.1. Study of the flow system

The preparation of the flow-through sensor was accomplished as described in the previous chapter. Following the packing of the column, it is necessary to charge all the beads present in the sensor with the copper ions; therefore the breakthrough point has to be achieved. The concentration of the metal solution chosen for this determination was 100 mM of $Cu^{2+}$, since it is the concentration recommended for this purpose by the bead manufacturer. The volume of $Cu^{2+}$solution was tested from 50 to 400 µL. It was possible to obtain an increase of 24% on the analytical signal with the increase of the volume from 50 to 100 µL. A further increase in the volume did not reveal a significant difference, therefore 100 µL was the chosen volume; it proved to be sufficient to charge all the beads of the flow-through sensor. The flow rate of this step was also aim of study because it had to provide a residence time long enough to achieve a good efficiency of the process. Therefore, the flow rates tested were 2, 4, 6 and 9 µL/s. As the results obtained were similar, 9 µL/s was the flow rate used to charge the beads. Higher flow rates were not tested

since we wanted to have a sufficient residence time to achieve an efficient procedure of the in-line charge of the beads.

The dilution factor of the FCr was the subsequent step of the optimization study. The volume of FCr was set as 50 µL, and the dilution factor was varied from 0 to 20 (in deionised water). This study was carried out with 75 µL of BSA standard solution 1g/L and the effect on the analytical signal caused by the increase on the dilution factor of the FCr was monitored at 500 nm. A dilution of 10 times of FCr was chosen since there was no loss on the repeatability and reproducibility of the results.

Afterwards, the effect of the volume and the flow rate used to propel the FCr and the protein solution on the sensitivity were studied simultaneously using a $2^4$ randomized experimental design. The lowest and highest values studied were: 25 or 125 µL of FCr propelled at 4 or 9 µL/s, and 50 or 250 µL of protein solution propelled at 2 or 9 µL/s. With the results obtained for this experiment it was possible to verify that 25 µL was the adequate volume of FCr propelled at 9 µL/s. The volume of protein solution was the most important parameter to influence the analytical signal; therefore, this was subject of further study. The volume and the flow rate of FCr defined, the volume of protein was studied in a range from 200 to 500 µL, and the selected volume was 400 µL, since an increase of 30% on the analytical signal was. With a further increase, no significant difference was found.

The developed methodology comprises four steps: (*i*) load of bead column; (*ii*) charging the bead column with $Cu^{2+}$; (*iii*) load of protein sample; and (*iv*) reaction

with the FCr. The next figure represents the change in the absorbance throughout these different steps. For the determination of total protein content in white wine, only the change in the absorbance of the fourth step is monitored. A reference scan is performed after loading of the protein sample and the analytical signal corresponds to the effect of the FCr in the presence of the protein. Fig. 7.2.B also illustrates the increase of the analytical signal with the increase of the concentration of BSA.

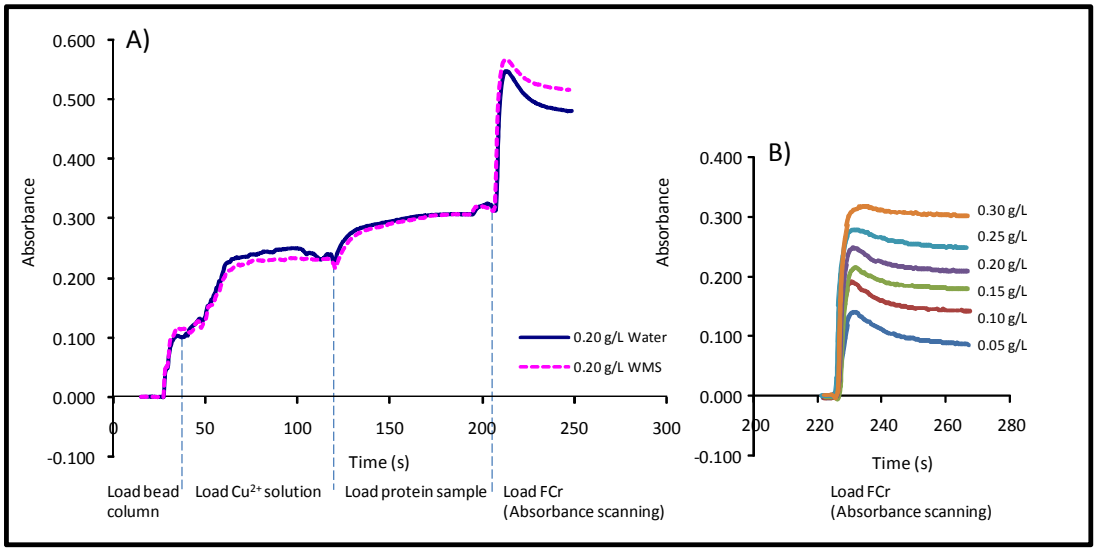


**Fig. 7.2.** Flow register of the determination of the total protein content. **A)** Protein standard 0.20 g/L prepared in water and in a wine model solution, **B)** and variation of the absorbance with the increase of the concentration of BSA.

### 7.3.2. Study of interfering species

The effect of possible interferences present in wine was evaluated by preparing solutions presenting a fixed concentration of BSA (0.20 g/L) and displaying a concentration of interferent normally found in finished white whites. These solutions were tested without any sample pre-treatment.The results obtained were compared using a *t*-test for two samples assuming equal variances (Miller and Miller, 1993), and the obtained values for $t_{calc}$ and $t_{critical}$ are summarized in Table 7.2. Organic acids, sugars, glycerol and ethanol, all main components in finished wine, did not interfere in the determination; the same occurred for other ions normally presented in wine as $Ca^{2+}$ and $Mg^{2+}$.

**Table 7.2.** Interfering species on the determination of total protein content in white wine.

| **Interfering species** | **Concentration tested** | $t_{calc}$ | $t_{critical}$ |
|---|---|---|---|
| L-(+)-Tartaric Acid | 4 g/L | 2.73 | 2.77 |
| Gallic Acid | 3 mg/L | 0.08 | 2.57 |
| Caffeic Acid | 2 mg/L | 1.55 | 2.77 |
| p-Coumaric Acid | 1 mg/L | 0.77 | 2.57 |
| Ascorcic acid | 1.5 mg/L | 0.10 | 2.77 |
| Glycerol | 10 g/L | 0.18 | 2.77 |
| Sugars (Glucose:Fructose; 40:60%) | 3 g/L | 2.09 | 2.77 |
| Ethanol | 10% (v/v) | 0.63 | 2.45 |
| $Ca^{2+}$ | 100 mg/L | 1.07 | 2.77 |
| $Mg^{2+}$ | 100 mg/L | 2.56 | 2.77 |

Although there was no observed interference when the potential interfering species were separately measured, a wine model solution (WMS) containing 10% (v/v) of ethanol, 3 g/L of sugars (Glucose:Fructose; 40:60%), 7 g/L of glycerol, 100 mg/L of $Mg^{2+}$, 100 mg/L of $Ca^{2+}$, 50 mg/L of $Na^{+}$ and 4g/L of tartaric acid, was prepared and analysed to evaluate the effect of matrix interference in the determination. The results showed a decrease of 50% on the sensitivity of the calibration curve performed with the standards prepared in WMS compared to the sensitivity of the calibration curve with aqueous standards. To tackle this problem and considering that the majority of wine proteins have an isoelectric point (pI) in a range from 4.1 to 5.8 (Waters and Colby, 2009), and the BSA has a pI of 4.7 (Chaiyasut and Tsuda, 2001), a new calibration curve was performed using standards with a pH adjusted to 5.5. In these conditions, there was no significant difference on the sensitivity obtained in the analysis of standards prepared in water and for the ones prepared in WMS, as it can be concluded from the values presented in Table 7.3 for the slopes and intercepts (± standard errors) from each regression (Miller and Miller, 1993). To verify the selectivity of the proposed method, 250 mg/L of gallic acid was added to the WMS prepared (WMS+G).The aim of this study was to revisit the effect of the phenolic compounds (normally present in the matrix) on the method. The authors Winters and Minchin (2005) stated that accurate protein measurements could only be made in the presence of phenolic compounds up to a concentration of 40 mg/L. Once again there was no significant difference found, as it can be confirmed on Fig. 7.3.

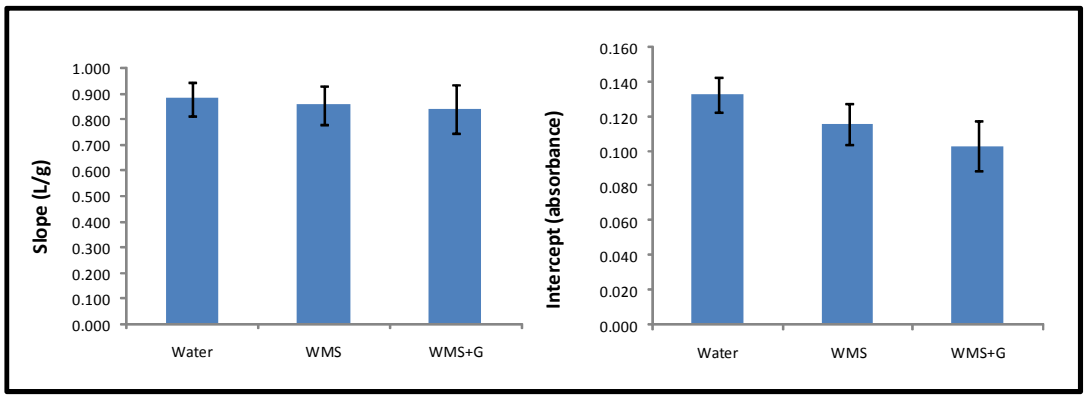


**Fig. 7.3.**Comparison of equation parameters for calibration curves for different matrices. **A)** Slope and **B)** Intercept. Error bars represent the standard error of the parameters.

### 7.3.3. Figures of merit of the flow system

The performance of the developed method was evaluated in terms of reagent and sample consumption, determination rate and dynamic range. The method presented a sample consumption of 400 µL per assay and a consumption of FCr and $Cu^{2+}$ solution of 25 µL and 100 µL per assay, respectively. It was possible to achieve a linear range up to 0.30 g/L with a limit of detection and quantification (Miller and Miller, 1993) of 0.03 and 0.10 g/L, respectively. As for the determination rate, it was possible to perform 9 determinations per hour, which is satisfactory for the Lowry method (Pomory, 2008).These figures of merit of the developed method are summarized in Table 7.3.

**Table 7.3.** Figures of merit of the SI-BI-LOV method for the determination of total protein content.

| Parameter | Value |
|---|---|
| Reagent consumption per assay | |
| Sample | 400 µL |
| FCr | 25 µL |
| $Cu^{2+}$ solution | 100 µL |
| Waste production per assay | 4 mL |
| Determination rate | 9 det. /h |
| Dynamic range | Up to 0.30 g/L |
| LOD | 0.03 g/L |
| LOQ | 0.10 g/L |
| Repeatability | 4.9% (0.10 g/L) (n=4) |
| (RSD) | 4.4% (0.12 g/L) (n=5) |
| | 3.9% (0.16 g/L) (n=4) |
| | 1.9% (0.22 g/L) (n=4) |

The reproducibility of the system was assessed by performing the calibration procedure under identical physical and chemical conditions between six different working days (between 24 of March and 01 of April of 2011). The calibration curves were performed using 6 standard solutions; the slope of the calibration curve obtained was 0.847 ± 0.180 corresponding to the mean and to the 95%confidence interval of the estimate. Stability can be considered satisfactory, as this data reflects different batches of beads and reagents and daily preparation of standard solutions.

Within day the flow system presents an acceptable repeatability obtained with a RSD <5% as reported in Table 7.3.

## 7.3.4. Application to samples

The developed method was applied to white table wines, to a sparkling wine and to a beer sample. The results obtained are summarized in Table 7.4. The values of the recovery test were calculated as reported by IUPAC (2002).

**Table 7.4.** Results obtained for the recovery tests for the SI-BI-LOV determination of total protein content.

| Sample | $pH_i$ [a] | Recovery (%) | | |
|---|---|---|---|---|
| | | [BSA] added | | |
| | | 0.10 g/L | 0.15 g/L | 0.20 g/L |
| White table wine 1 | 3.1 | 85.7 ± 4.1 | -- | 105.5 ± 11.8 |
| White table wine 2 | 2.9 | 96.3 ± 4.2 | -- | 100.8 ± 7.0 |
| White table wine 3 | 3.2 | 107.0 ± 6.2 | -- | -- |
| White table wine 4 | 3.3 | 98.4 ± 4.8 | 95.0 ± 4.4 | 87.7 ± 2.6 |
| White table wine 5 | 2.6 | 75.7 ± 4.5 | 104.7 ± 3.9 | 110.0 ± 1.9 |
| Sparkling wine | 3.1 | 102.7 ± 13.6 | 104.1 ± 3.5 | 99.0 ± 9.0 |
| Beer | 4.2 | 102 ± 3.8 | 100.1 ± 7.0 | 95.3 ± 5.1 |

[a] – original pH of the sample

A significant test was used to evaluate if the mean percentages obtained for the recovery test in the chosen ranges were statistically different from 100%.The values of $|t_{calc}|$ for the different concentrations, 0.10, 0.15, and 0.20 g/L, were 1.10, 0.44, and 0.09, respectively- lower than their corresponding value of $|t_{crit}|$ 2.45, 3.18, and

2.57, respectively. Therefore, the recoveries were no statistically different from 100%, within a confidence interval of 95%.

## 7.4. Conclusions

SI-LOV platform proved to be a valuable tool for the development of a methodology for total protein content in white wines and also in a sparkling wine and a beer. When compared with other methodology for this quantification (Chen et al., 2008b) using the same platform (SI-LOV) some similarities can be found: both present a linear range in the same order of magnitude. On the other hand, the method was applied to samples of different nature (human serum, urine, milk and yogurt). Although these samples present a complex matrix, they were subject to a high dilution factor which reduces significantly any interference with the matrix of the sample. In the developed methodology the possible interfering species were excluded from the determination by means of SPS.

# CHAPTER 8

## GENERAL CONCLUSIONS

## 8.1 GENERAL CONCLUSIONS

The SI-LOV system proved to be a valuable tool for the improvement of methodologies for biochemical assays in terms of sensitivity, robustness, and sample and reagent consumption. In fact, this concept was successfully used to develop new methodologies based on: $(i)$ enzymatic and $(ii)$ bead injection assays.

The enzymatic assays were explored and different enzymatic methodologies were developed. In terms of the determination mode, it was possible to quantify the enzymatic assay by a direct or a kinetic measurement. The quantification of ethanol in alcoholic beverages (Chap. 3) was performed by direct measurement and by means of stopped-flow approach, resulting in a kinetic measurement mode. The use of a kinetic mode proved to be a good alternative when the sample presents an intrinsic absorption. This also occurred in the determination of the activity of peroxidase in vegetable extracts (Chap.5); additionally, in this case the sample presented an intrinsic colour similar to the reaction product. In the development of the methodology for the enzymatic determination of hydrogen peroxide in contact lens care (Chap.4), the sample had to be diluted before introduction in the system. As one of our goals was to minimise the sample off-line treatment, a dialysis unit was coupled to the system. With this procedure it was possible to perform an in-line dilution of approximately 80 times, being advantageous when monitoring real processes.

Another highly important issue was the environmental concern on reagents consumption and effluent production. The methodologies described in this dissertation presented a waste production lower than 4.00 mL per assay, even if the cleaning step after every measurement is considered.

To avoid interferences from complex matrices, bead injection analysis proved to be a useful, as demonstrated in Chap. 6 and 7. In fact, despite the renewal of the column beads in every assay, a good repeatability and reproducibility was obtained. One disadvantage that could be attributed to this BIA mode is the waste of the beads in each assay, in contrast to their potential re-use.

I would also like to point out that the same apparatus was used in the determination of different analytes, using various approaches, and applied to different samples, confirming the versatility of the integrated manifold.

One weakness that could be pointed out to the current lab-on-valve design is that it does not provide thermostatic control of the manifold which is a desirable feature for kinetic-based assays (Wu et al., 2001). On the other hand, due to the proximity of all components, if the samples and/or reagents containers are kept at controlled temperature, this potential problem is minimized. That was demonstrated in Chap. 4 and 5, where the assays were performed in different temperature ranges by placing the sample vessel in a temperature controlled reservoir.

As suggestions for future work, several other potentialities could be explored, especially taking advantage of the combination of SI-LOV system with BIA approach. Beads can be functionalized to respond to different analytes for its application in various analytical fields. For example, their use for the immobilisation of different compounds, like antibodies or cells, other chelating agents, can be a useful tool for the development of new methodologies with increased specificity.

Coupling of LOV with various detection systems, including BI-spectroscopy, MS, amperometry or fluorometry, is expected to be well suited to current demands in bioanalytical sciences for rapid screening and diagnostics.Furthermore, some integration could be accomplished to increase the potential and the versatility of the system; instead of coupling a dialysis unit, like it was described in Chap. 4, the separation device could be integrated in the central sample processing unit.

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Susana S. M. P. Vidigal, Ildikó V. Tóth, António O. S. S. Rangel, Exploiting the bead injection LOV approach to carry out spectrophotometric assays in wine: Application to the determination of iron, Talanta, 84 (2011) 1298 – 1303.

## Communications in international scientific symposiums

### Invited Oral Communications

Ildikó V. Tóth, Francisco M. Campos, Karina B. H. Domínguez, Susana S. M. P. Vidigal, António O. S. S. Rangel, Flow Methods for Dynamic System Monitoring: "Old" Approaches to "New" challenges, L36, Flow Analysis XI, International Conference, Pollensa, Mallorca, Spain (September 2009).

### Oral Communications

Susana S. M. P. Vidigal, Ildikó V. Tóth, António O. S. S. Rangel, Sequential Injection Lab-On-Valve System for the Determination of the Activity of Peroxidase in Vegetables, L17, Flow Analysis XI, International Conference, Pollensa, Mallorca, Spain (September 2009).

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### Oral Communications

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# LIST OF PUBLICATIONS

# APPENDIX A

## PRINTOUT OF THE FLOW PROGRAM USED FOR THE PEAK HEIGHT MEASUREMENT OF ETHANOL IN ALCOHOLIC BEVERAGES, CHAPTER 3.

```
' Configure Hardware
Hardware Settings portname (number, string) 1, Waste
Hardware Settings portname (number, string) 2, Flowcell
Hardware Settings portname (number, string) 3, ReagentA
Hardware Settings portname (number, string) 4, ReagentB
Hardware Settings portname (number, string) 5, Dye
Hardware Settings portname (number, string) 6, Carrier
Hardware Settings Wavelength 1 (nm) 340
Hardware Settings Wavelength 2 (nm) 500
Hardware Settings Wavelength 3 (nm) 550
Hardware Settings Wavelength 4 (nm) 800
Hardware Settings Use Wavelength 4 as Reference
Hardware Settings End Settings

' Start Analysis
Loop Start (#) 1
    Analyte New Sample

    ' Aspirate carrier
    Syringe Pump Valve In
```

```
Syringe Pump Flowrate (microliter/sec) 100
Syringe Pump Aspirate (microliter) 1000
Syringe Pump Delay Until Done

' Aspirate buffer
Multiposition Valve  Carrier
Syringe Pump Valve Out
Syringe Pump Flowrate (microliter/sec) 80
Syringe Pump Aspirate (microliter) 50
Syringe Pump Delay Until Done
' Aspirate Sample
 Multiposition Valve  Dye
 Syringe Pump Valve Out
 Syringe Pump Flowrate (µL/sec) 25
 Syringe Pump Aspirate (µL) 15
 Syringe Pump Delay Until Done

' Aspirate reagentA, Enz
 Multiposition Valve   ReagentA
 Syringe Pump Valve Out
 Syringe Pump Flowrate (µL/sec) 25
 Syringe Pump Aspirate (µL) 5
 Syringe Pump Delay Until Done
```

```
' Aspirate reagentB, NAD
Multiposition Valve  ReagentB
Syringe Pump Valve Out
 Syringe Pump Flowrate (µL/sec) 25
Syringe Pump Aspirate (µL) 5
Syringe Pump Delay Until Done

' Aspirate buffer
Multiposition Valve  Carrier
Syringe Pump Valve Out
Syringe Pump Flowrate (µL/sec) 25
Syringe Pump Aspirate (µL) 100
Syringe Pump Delay Until Done

' Send To Flow Cell and Measure Absorbance
Multiposition Valve  Flowcell
Syringe Pump Valve Out
Syringe Pump Flowrate (µL/sec) 15
Syringe Pump Dispense (microliter) 10
Spectrometer Reference Scan
Delay (sec) 30
Spectrometer Absorbance Scanning
Syringe Pump Dispense (microliter) 450
Syringe Pump Delay Until Done
Spectrometer Stop Scanning
```

Syringe Pump Flowrate (microliter/sec) 100

Syringe Pump Empty

Syringe Pump Delay Until Done

Loop End

## PRINTOUT OF THE FLOW PROGRAM USED FOR THE INITIAL RATE MEASUREMENT OF ETHANOL IN ALCOHOLIC BEVERAGES, CHAP. 3

' Configure Hardware

Hardware Settings portname (number, string) 1, Waste

Hardware Settings portname (number, string) 2, Flowcell

Hardware Settings portname (number, string) 3, ReagentA

Hardware Settings portname (number, string) 4, ReagentB

Hardware Settings portname (number, string) 5, Dye

Hardware Settings portname (number, string) 6, Carrier

Hardware Settings Wavelength 1 (nm) 340

Hardware Settings Wavelength 2 (nm) 500

Hardware Settings Wavelength 3 (nm) 550

Hardware Settings Wavelength 4 (nm) 800

Hardware Settings Use Wavelength 4 as Reference

Hardware Settings End Settings

```
' Start Analysis
Loop Start (#) 1
    Analyte New Sample
    ' Aspirate carrier
    Syringe Pump Valve In
    Syringe Pump Flowrate (microliter/sec) 100
    Syringe Pump Aspirate (microliter) 1000
    Syringe Pump Delay Until Done

   ' Aspirate buffer
   Multiposition Valve  Carrier
   Syringe Pump Valve Out
   Syringe Pump Flowrate (microliter/sec) 80
   Syringe Pump Aspirate (microliter) 50
   Syringe Pump Delay Until Done

   ' Aspirate Sample
    Multiposition Valve  Dye
    Syringe Pump Valve Out
    Syringe Pump Flowrate (µL/sec) 25
    Syringe Pump Aspirate (µL) 15
    Syringe Pump Delay Until Done

   ' Aspirate reagentA, Enz
   Multiposition Valve   ReagentA
```

```
Syringe Pump Valve Out
Syringe Pump Flowrate (µL/sec) 25
Syringe Pump Aspirate (µL) 5
Syringe Pump Delay Until Done

' Aspirate reagentB, NAD
Multiposition Valve  ReagentB
Syringe Pump Valve Out
 Syringe Pump Flowrate (µL/sec) 25
Syringe Pump Aspirate (µL) 5
Syringe Pump Delay Until Done

' Aspirate buffer
Multiposition Valve  Carrier
Syringe Pump Valve Out
Syringe Pump Flowrate (µL/sec) 25
Syringe Pump Aspirate (µL) 100
Syringe Pump Delay Until Done

' Send To Flow Cell and Measure Absorbance
Multiposition Valve  Flowcell
Syringe Pump Valve Out
Syringe Pump Flowrate (µL/sec) 15
Syringe Pump Dispense (microliter) 10
Delay (sec) 1
```

Spectrometer Reference Scan

Syringe Pump Dispense (microliter) 120

Spectrometer Absorbance Scanning

Delay (sec) 30

Spectrometer Stop Scanning

Syringe Pump Flowrate (microliter/sec) 100

Syringe Pump Empty

Syringe Pump Delay Until Done

Loop End

# APPENDIX B

## PRINTOUT OF THE FLOW PROTOCOL USED FOR THE MEASUREMENT OF HYDROGEN PEROXIDE WITHOUT IN-LINE DILUTION

' Configure hardware

Hardware Settings portname (number, string) 1, waste

Hardware Settings portname (number, string) 2, Flowcell

Hardware Settings portname (number, string) 3, ABTS1

Hardware Settings portname (number, string) 4, HRP

Hardware Settings portname (number, string) 5, Hydrogen peroxide

Hardware Settings portname (number, string) 6, ABTS2

Hardware Settings Wavelength 1 (nm) 410

Hardware Settings Wavelength 2 (nm) 500

Hardware Settings Wavelength 3 (nm) 550

Hardware Settings Wavelength 4 (nm) 800

Hardware Settings Use Wavelength 4 as Reference

Hardware Settings End Settings

' Start Analysis

Loop Start (#) 5

Peristaltic Pump Clockwise(%) 75

Delay (sec) 30

Peristaltic Pump Off

Loop Start (#) 5

Analyte New Sample

Peristaltic Pump Clockwise(%) 75

' Aspirate Carrier

Syringe Pump Valve In

Syringe Pump Flowrate (microliter/sec) 150

Syringe Pump Aspirate (microliter) 1000

Syringe Pump Delay Until Done

' Aspirate ABTS1

Multiposition Valve  ABTS1

Syringe Pump Valve Out

Syringe Pump Flowrate (microliter/sec) 80

Syringe Pump Aspirate (microliter) 50

Syringe Pump Delay Until Done

Peristaltic Pump Off

' Aspirate hydrogen peroxide

Multiposition Valve Hydrogen peroxide

Syringe Pump Valve Out

Syringe Pump Flowrate (microliter/sec) 25

Syringe Pump Aspirate (microliter) 15

Syringe Pump Delay Until Done

' Aspirate HRP

Multiposition Valve  HRP

Syringe Pump Valve Out

Syringe Pump Flowrate (microliter/sec) 25

Syringe Pump Aspirate (microliter) 15

Syringe Pump Delay Until Done

' Aspirate ABTS2

Multiposition Valve  ABTS2

Syringe Pump Valve Out

Syringe Pump Flowrate (microliter/sec) 80

Syringe Pump Aspirate (microliter) 100

Syringe Pump Delay Until Done

' Send to flowcell and measure absorbance

Multiposition Valve  Flowcell

Syringe Pump Valve Out

Syringe Pump Flowrate (microliter/sec) 15

Syringe Pump Dispense (microliter) 10

Syringe Pump Delay Until Done

Spectrometer Reference Scan

Delay (sec) 1

Spectrometer Absorbance Scanning

Syringe Pump Flowrate (microliter/sec) 15

Syringe Pump Dispense (microliter) 400

Syringe Pump Delay Until Done

Spectrometer Stop Scanning

' Wash flowcell

Syringe Pump Valve Out

Syringe Pump Flowrate (microliter/sec) 150

Syringe Pump Empty

Syringe Pump Delay Until Done

Loop End

Loop End

## PRINTOUT OF THE FLOW PROTOCOL USED FOR THE MEASUREMENT OF HYDROGEN PEROXIDE WITH IN-LINE DILUTION

Configure hardware

Hardware Settings portname (number, string) 1, waste

Hardware Settings portname (number, string) 2, Flowcell

Hardware Settings portname (number, string) 3, ABTS1

Hardware Settings portname (number, string) 4, HRP

Hardware Settings portname (number, string) 5, Hydrogen peroxide

Hardware Settings portname (number, string) 6, ABTS2

Hardware Settings Wavelength 1 (nm) 410

Hardware Settings Wavelength 2 (nm) 500

Hardware Settings Wavelength 3 (nm) 550

Hardware Settings Wavelength 4 (nm) 800

Hardware Settings Use Wavelength 4 as Reference

Hardware Settings End Settings

' Start Analysis

Loop Start (#) 5

Peristaltic Pump Clockwise(%) 75

Delay (sec) 30

Peristaltic Pump Off

Loop Start (#) 5

Analyte New Sample

Peristaltic Pump Clockwise(%) 75

Delay (sec) 10

' Aspirate Carrier

Syringe Pump Valve In

Syringe Pump Flowrate (microliter/sec) 150

Syringe Pump Aspirate (microliter) 1000

Syringe Pump Delay Until Done

' Aspirate ABTS1

Multiposition Valve ABTS1

Syringe Pump Valve Out

Syringe Pump Flowrate (microliter/sec) 80

Syringe Pump Aspirate (microliter) 50

Syringe Pump Delay Until Done

Peristaltic Pump Off

' Aspirate hydrogen peroxide

Multiposition Valve Hydrogen peroxide

Syringe Pump Valve Out

Syringe Pump Flowrate (microliter/sec) 25

Syringe Pump Aspirate (microliter) 15

Syringe Pump Delay Until Done

' Aspirate HRP

Multiposition Valve HRP

Syringe Pump Valve Out

Syringe Pump Flowrate (microliter/sec) 25

Syringe Pump Aspirate (microliter) 15

Syringe Pump Delay Until Done

' Aspirate ABTS2

Multiposition Valve  ABTS2

Syringe Pump Valve Out

Syringe Pump Flowrate (microliter/sec) 80

Syringe Pump Aspirate (microliter) 100

Syringe Pump Delay Until Done

' Send to flowcell and measure absorbance

Multiposition Valve  Flowcell

Syringe Pump Valve Out

Syringe Pump Flowrate (microliter/sec) 15

Syringe Pump Dispense (microliter) 10

Syringe Pump Delay Until Done

Spectrometer Reference Scan

Delay (sec) 1

Spectrometer Absorbance Scanning

Syringe Pump Flowrate (microliter/sec) 15

Syringe Pump Dispense (microliter) 400

Syringe Pump Delay Until Done

Spectrometer Stop Scanning

' Wash flowcell

Syringe Pump Valve Out

Syringe Pump Flowrate (microliter/sec) 150

Syringe Pump Empty

Syringe Pump Delay Until Done

Loop End

Loop End

# APPENDIX C

## PRINTOUT OF THE FLOW PROGRAM USED FOR THE DETERMINATION OF THE ACTIVITY OF VEGETABLES EXTRACTS, CHAPTER 5.

```
' Configure hardware
Hardware Settings portname (number, string) 1, waste
Hardware Settings portname (number, string) 2, Flowcell
Hardware Settings portname (number, string) 3, ABTS1
Hardware Settings portname (number, string) 4, HRP
Hardware Settings portname (number, string) 5, Hydrogen peroxide
Hardware Settings portname (number, string) 6, ABTS2
Hardware Settings Wavelength 1 (nm) 410
Hardware Settings Wavelength 2 (nm) 500
Hardware Settings Wavelength 3 (nm) 550
Hardware Settings Wavelength 4 (nm) 800
Hardware Settings Use Wavelength 4 as Reference
Hardware Settings End Settings

' Start Analysis

Loop Start (#) 3
Peristaltic Pump Clockwise(%) 75
Delay (sec) 30
Peristaltic Pump Off
```

Loop Start (#) 2

Analyte New Sample

Peristaltic Pump Clockwise(%) 75

' Aspirate Carrier

Syringe Pump Valve In

Syringe Pump Flowrate (microliter/sec) 150

Syringe Pump Aspirate (microliter) 1000

Syringe Pump Delay Until Done

' Aspirate ABTS1

Multiposition Valve ABTS1

Syringe Pump Valve Out

Syringe Pump Flowrate (microliter/sec) 80

Syringe Pump Aspirate (microliter) 50

Syringe Pump Delay Until Done

Peristaltic Pump Off

' Aspirate hydrogen peroxide

Multiposition Valve Hydrogen peroxide

Syringe Pump Valve Out

Syringe Pump Flowrate (microliter/sec) 25

Syringe Pump Aspirate (microliter) 15

Syringe Pump Delay Until Done

' Aspirate HRP

Multiposition Valve HRP

Syringe Pump Valve Out

Syringe Pump Flowrate (microliter/sec) 25

Syringe Pump Aspirate (microliter) 15

Syringe Pump Delay Until Done

' Aspirate ABTS2

Multiposition Valve ABTS2

Syringe Pump Valve Out

Syringe Pump Flowrate (microliter/sec) 80

Syringe Pump Aspirate (microliter) 100

Syringe Pump Delay Until Done

' Send to flowcell and measure absorbance

Multiposition Valve Flowcell

Syringe Pump Valve Out

Syringe Pump Flowrate (microliter/sec) 15

Syringe Pump Dispense (microliter) 10

Syringe Pump Delay Until Done

Spectrometer Reference Scan

Spectrometer Absorbance Scanning

Delay (sec) 30

Syringe Pump Dispense (microliter) 110

```
Syringe Pump Delay Until Done

Spectrometer Stop Scanning


' Wash flowcell

Syringe Pump Valve Out

Syringe Pump Flowrate (microliter/sec) 150

Syringe Pump Empty

Syringe Pump Delay Until Done


Loop End


Loop End
```

# APPENDIX D

## PRINTOUT OF THE FLOW PROGRAM USED FOR THE DETERMINATION OF IRON IN WINE, CHAPTER 6.

```
' Hardware settings
Hardware Settings portname (number, string) 1, Waste
Hardware Settings portname (number, string) 2, Flowcell
Hardware Settings portname (number, string) 3, Standard1
Hardware Settings portname (number, string) 4, Oxidant
Hardware Settings portname (number, string) 5, Beads
Hardware Settings portname (number, string) 6, Colour Reagent
Hardware Settings Wavelength 1 (nm) 480
Hardware Settings Wavelength 2 (nm) 250
Hardware Settings Wavelength 3 (nm) 225
Hardware Settings Wavelength 4 (nm) 800
Hardware Settings Use Wavelength 4 as Reference
Hardware Settings End Settings

'Recirculate beads
Peristaltic Pump Clockwise(%) 50
Peristaltic Pump On

'Start Analysis
```

```
Analyte New Sample

Analyte Name Standard 1


Loop Start (#) 5


Syringe Pump Valve In

Syringe Pump Flowrate (microliter/sec) 250

Syringe Pump Aspirate (microliter) 1000

Syringe Pump Delay Until Done

Syringe Pump Valve Out


'Aspiration of beads suspension

Multiposition Valve  Beads

Syringe Pump Flowrate (microliter/sec) 20

Syringe Pump Aspirate (microliter) 40

Syringe Pump Delay Until Done


'Bead packing

Multiposition Valve  Flowcell

Syringe Pump Flowrate (microliter/sec) 10

Syringe Pump Dispense (microliter) 80

Syringe Pump Delay Until Done


'Oxidant

Multiposition Valve  Oxidant
```

Syringe Pump Flowrate (microliter/sec) 50

Syringe Pump Aspirate (microliter) 10

Syringe Pump Delay Until Done

'Sample

Multiposition Valve  Standard1

Syringe Pump Flowrate (microliter/sec) 50

Syringe Pump Aspirate (microliter) 250

Syringe Pump Delay Until Done

'Oxidant

Multiposition Valve  Oxidant

Syringe Pump Flowrate (microliter/sec) 50

Syringe Pump Aspirate (microliter) 10

Syringe Pump Delay Until Done

'Sample

Multiposition Valve  Standard1

Syringe Pump Flowrate (microliter/sec) 50

Syringe Pump Aspirate (microliter) 250

Syringe Pump Delay Until Done

'Oxidant

Multiposition Valve  Oxidant

Syringe Pump Flowrate (microliter/sec) 50

```
Syringe Pump Aspirate (microliter) 10

Syringe Pump Delay Until Done


'Send sample to flowcell

Multiposition Valve  Flowcell

Syringe Pump Flowrate (microliter/sec) 9

Syringe Pump Dispense (microliter) 1100

Syringe Pump Delay Until Done


Spectrometer Reference Scan

Spectrometer Absorbance Scanning


'Colour reagent

Multiposition Valve  Colour Reagent

Syringe Pump Flowrate (microliter/sec) 50

Syringe Pump Aspirate (microliter) 10

Syringe Pump Delay Until Done

Multiposition Valve  Flowcell

Syringe Pump Flowrate (microliter/sec) 3

Syringe Pump Dispense (microliter) 75

Syringe Pump Delay Until Done


Spectrometer Stop Scanning
```

'Discard beads: Reversed Flow

Syringe Pump Flowrate (microliter/sec) 300

Syringe Pump Aspirate (microliter) 100

Syringe Pump Delay Until Done

Multiposition Valve  Waste

Syringe Pump Empty

Syringe Pump Delay Until Done

'Clean Flowcell

Syringe Pump Valve In

Syringe Pump Flowrate (microliter/sec) 250

Syringe Pump Aspirate (microliter) 1000

Syringe Pump Delay Until Done

Syringe Pump Valve Out

Multiposition Valve  Flowcell

Syringe Pump Flowrate (microliter/sec) 250

Syringe Pump Empty

Syringe Pump Delay Until Done

Loop End

Peristaltic Pump Off

# APPENDIX E

## PRINTOUT OF THE FLOW PROGRAM USED FOR THE DETERMINATION OF TOTAL PROTEIN CONTENT IN WINE, CHAPTER 7.

```
' Hardware settings
Hardware Settings portname (number, string) 1, Waste
Hardware Settings portname (number, string) 2, Flowcell
Hardware Settings portname (number, string) 3, Metal Solution
Hardware Settings portname (number, string) 4, Protein Solution
Hardware Settings portname (number, string) 5, Beads Suspension
Hardware Settings portname (number, string) 6, Colour Reagent
Hardware Settings Wavelength 1 (nm) 500
Hardware Settings Wavelength 2 (nm) 435
Hardware Settings Wavelength 3 (nm) 450
Hardware Settings Wavelength 4 (nm) 800
Hardware Settings End Settings

'Start Analysis
Loop Start (#) 5
Analyte New Sample
'Recirculate beads
Peristaltic Pump Clockwise(%) 50
Peristaltic Pump On
```

Syringe Pump Valve In

Syringe Pump Flowrate (microliter/sec) 250

Syringe Pump Aspirate (microliter) 1500

Syringe Pump Delay Until Done

Syringe Pump Valve Out

Peristaltic Pump Off

'Aspiration of beads suspension

Multiposition Valve  Beads Suspension

Syringe Pump Flowrate (microliter/sec) 20

Syringe Pump Aspirate (microliter) 40

Syringe Pump Delay Until Done

Peristaltic Pump On

'Bead packing

Multiposition Valve  Flowcell

Syringe Pump Flowrate (microliter/sec) 10

Syringe Pump Dispense (microliter) 80

Syringe Pump Delay Until Done

'Load metal solution

Multiposition Valve  Metal Solution

Syringe Pump Flowrate (microliter/sec) 50

Syringe Pump Aspirate (microliter) 100

Syringe Pump Delay Until Done

```
Multiposition Valve  Flowcell
Syringe Pump Flowrate (microliter/sec) 9
Syringe Pump Dispense (microliter) 500
Syringe Pump Delay Until Done

'Aspirate Protein Sample
Multiposition Valve  Protein Solution
Syringe Pump Flowrate (microliter/sec) 50
Syringe Pump Aspirate (microliter) 400
Syringe Pump Delay Until Done
Multiposition Valve  Flowcell
Syringe Pump Flowrate (microliter/sec) 9
Syringe Pump Dispense (microliter) 700
Syringe Pump Delay Until Done

'Aspirate Colour reagent
Multiposition Valve  Colour Reagent
Syringe Pump Flowrate (microliter/sec) 50
Syringe Pump Aspirate (microliter) 25
Syringe Pump Delay Until Done

Spectrometer Reference Scan
Spectrometer Absorbance Scanning

Multiposition Valve  Flowcell
```

Syringe Pump Flowrate (microliter/sec) 6

Syringe Pump Dispense (microliter) 250

Syringe Pump Delay Until Done

Spectrometer Stop Scanning

'Discard beads: Reversed Flow

Syringe Pump Flowrate (microliter/sec) 300

Syringe Pump Aspirate (microliter) 200

Syringe Pump Delay Until Done

Multiposition Valve  Waste

Syringe Pump Empty

Syringe Pump Delay Until Done

'Clean Flowcell

Syringe Pump Valve In

Syringe Pump Flowrate (microliter/sec) 250

Syringe Pump Aspirate (microliter) 1000

Syringe Pump Delay Until Done

Syringe Pump Valve Out

Multiposition Valve  Flowcell

Syringe Pump Flowrate (microliter/sec) 300

Syringe Pump Empty

Syringe Pump Delay Until Done

Peristaltic Pump Off

Loop End