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DEVELOPMENT OF AUTOMATIC METHODS BASED ON FLOW TECHNIQUES FOR EVALUATION OF ANTIOXIDANT CAPACITY IN PHARMACEUTICAL AND FOOD PRODUCTS

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Abstract

In the present work, different automatic analytical flow-based methodologies for the determination of antioxidant/radical scavenging capacity were developed. The automation was performed by the computer-controlled multisyringe flow injection analysis (MSFIA) using spectrophotometric and chemiluminometric measurements as detection systems. These methods were applied to pharmaceutical compounds and to several types of food products including red and white wines, blond and dark beers, juices, herbal and tea infusions, and isotonic and soft drinks.

A multisyringe flow injection system was developed for the determination of 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$) scavenging capacity in different reaction media. For this study, a stopped-flow approach was implemented in order to monitor spectrophotometrically the reduction of $DPPH^{\bullet}$ radicals by several recognised antioxidant compounds in different solvents including methanol and ethanolic solution 50% v/v (unbufered, apparent pH 4.1 and apparent pH 7.6). Moreover, the number of $DPPH^{\bullet}$ molecules reduced by one molecule of antioxidant was determined whenever a stable absorbance value was attained within the period of measurement.

An automatic method based on MSFIA system using the spectrophotometric $DPPH^{\bullet}$ scavenging reaction was developed for the determination of antioxidant capacity of several food products. The determination was based on the colour disappearance due to the reduction of $DPPH^{\bullet}$ by antioxidant compounds present in the sample. The influence of initial $DPPH^{\bullet}$ concentration and sample dilution in the performance of the analytical methodology was also studied. The results were expressed as vitamin C equivalent antioxidant capacity, calculated as the equivalent amount of ascorbic acid (mg) present in 100 mL of sample.

For the determination of Folin-Ciocalteu (FC) reducing capacity, a MSFIA system was developed based on the spectrophotometric detection of the blue complexes, formed after the transfer of electrons in alkaline medium from reducing compounds present in the sample to the phosphomolybdic/phosphotungstic acid complexes (FC reagent). Different

strategies for mixing of sample and reagent were tested, through software control without manifold reconfiguration. The proposed method was applied to compounds with known antioxidant/reducing capacity (both phenolic and nonphenolic) and to several types of food products using gallic acid as the standard.

Exploiting the flexibility of flow management associated to computer-control features of the MSFIA systems, the sequential spectrophotometric determination of FC reducing capacity and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{\bullet+}$) scavenging capacity were carried out in the same manifold using gallic acid and trolox as standard compounds. The proposed flow system configuration allowed the performance of each method separately or in tandem by changing the parameters in the controlling software. The determination of FC assay relies on the absorbance increase due to the reduction of FC reagent, whilst the $ABTS^{\bullet+}$ scavenging capacity was assessed by the absorbance decrease due to reduction of colored $ABTS^{\bullet+}$ radical cation by antioxidant compounds. This method was applied to a large number of beverages (n = 72) with recognised antioxidant properties and the results were compared within and between methods.

A chemiluminometric automatic flow methodology for in vitro determination of hypochlorous acid (HOCl) scavenging capacity, under pH and concentration conditions similar to those found in vivo, was developed. The determination was based on the inhibition of chemiluminescence reaction of HOCl and luminol after prior scavenging reaction of antioxidant compounds towards the oxidant species (HOCl). The proposed method was applied to nonsteroidal anti-inflammatory drugs of different chemical families, and to positive controls (cysteine, gallic acid, lipoic acid). The HOCl scavenging capacity at different pH values (7.4 and 10.0, for comparison purposes) was evaluated.

Finally, the results obtained by the developed automatic methodologies were similar to those reported in the literature as well as statistically comparable with those provided by batch procedures. The advantages and limitations of the proposed flow systems are discussed in detail.

Resumo

Neste trabalho, foram desenvolvidas várias metodologias automáticas de fluxo para a determinação da capacidade antioxidante. Para tal, recorreu-se à análise por injecção em fluxo baseada em multi-seringa com detecção baseada em espectrofotometria na zona do visível ou em quimioluminescência. As metodologias desenvolvidas foram aplicadas a compostos farmacêuticos e a diferentes produtos alimentares, tais como vinhos (tintos e brancos), cervejas (louras e pretas), sumos, infusões e bebidas isotónicas.

Visando a determinação da capacidade antioxidante baseada na captura do radical 2,2-difenil-1-picrilhidrazilo ($DPPH^{\bullet}$) em diferentes meios reaccionais, foi desenvolvido um sistema automático de fluxo baseado em multi-seringa que incluiu a paragem da mistura reaccional no detector para monitorização espectrofotométrica da redução do radical $DPPH^{\bullet}$ provocada por compostos antioxidantes. Foram testados diferentes solventes, nomeadamente metanol e soluções etanólicas a 50% v/v (sem ajuste de pH, pH 4,1 e pH 7,6). A metodologia desenvolvida foi aplicada a vários compostos reconhecidos como antioxidantes e permitiu determinar o número de moléculas de $DPPH^{\bullet}$ reduzidas por uma molécula de antioxidante quando a absorvância atingiu um valor estável dentro do intervalo de medição.

Com base nos resultados anteriores, foi desenvolvido um sistema automático de fluxo baseado em multi-seringa recorrendo à mesma reacção para a determinação da capacidade antioxidante de vários produtos alimentares. A determinação foi baseada na diminuição da absorvância devido à redução do radical $DPPH^{\bullet}$ por reacção com os compostos antioxidantes presentes na amostra. Foram estudadas a influência da concentração inicial do radical $DPPH^{\bullet}$ e da diluição da amostra na performance da metodologia analítica. Os resultados foram expressos como a quantidade de ácido ascórbico equivalente (mg) presente em 100 mL de amostra.

No sistema de fluxo desenvolvido para a determinação da capacidade redutora de Folin-Ciocalteu (FC), a metodologia baseou-se na detecção espectrofotométrica dos complexos azuis formados após a transferência de electrões em meio alcalino a partir dos compostos

redutores presentes na amostra para os complexos oriundos dos ácidos fosfotungstico e fosfomolíbdico (reagente FC). Foram estudadas diferentes estratégias de mistura entre amostra e o reagente FC recorrendo ao controlo do sistema por computador, sem necessidade de qualquer reconfiguração física. O ácido gálico foi usado como composto padrão e a metodologia proposta foi aplicada a compostos com reconhecida capacidade antioxidante (fenólicos e não fenólicos) e a vários tipos de produtos alimentares.

A determinação sequencial da capacidade redutora da amostra usando o reagente FC e da capacidade de captura do radical catiónico 2,2′-azinobis(3-etilbenzotiazolina-6-sulfonato) ($ABTS^{\bullet+}$) foi realizada recorrendo ao mesmo sistema de fluxo usando o ácido gálico e o trolox como compostos padrão. A elevada flexibilidade da gestão de fluidos associada ao controlo por computador nos sistemas multi-seringa permitiu a realização dos métodos de uma forma isolada ou sequencial apenas pela modificação dos parâmetros no programa de controlo do sistema. A determinação das propriedades antioxidantes no método Folin-Ciocalteu baseou-se no aumento de absorvância devido à redução do reagente FC, enquanto que a capacidade de captura do radical $ABTS^{\bullet+}$ foi determinada pela diminuição de absorvância devido à redução do radical corado pelos compostos antioxidantes. A aplicação a um elevado número de bebidas (n = 72) com reconhecidas propriedades antioxidantes permitiu a realização de um estudo comparativo entre os dois métodos.

Para a determinação *in vitro* da capacidade sequestrante para o ácido hipocloroso (HOCl) em condições reaccionais próximas das encontradas *in vivo* no que diz respeito ao pH e à concentração, foi desenvolvido um sistema automático de fluxo com detecção quimioluminométrica. A determinação baseou-se na inibição da reacção de quimioluminescência entre o HOCl e o luminol após reacção prévia entre os compostos antioxidantes e a espécie oxidante (HOCl). A metodologia proposta foi aplicada a fármacos anti-inflamatórios não esteróides de diferentes famílias químicas e a vários controlos positivos (cisteína, ácido gálico, ácido lipóico). Com o propósito de comparação, a capacidade sequestrante para o HOCl foi avaliada a diferentes valores de pH (7,4 e 10,0).

As metodologias automáticas propostas forneceram resultados concordantes com os dados disponíveis na literatura e também estatisticamente comparáveis com os obtidos usando os procedimentos discretos. As vantagens e limitações dos sistemas propostos são discutidas.

Résumé

Ce travail décrit le développement de plusieurs méthodes automatiques de flux pour la détermination de la capacité antioxydant. Ainsi, nous avons eu recours à l'analyse avec injection en flux avec multi seringue et avec détection par spectrophotométrie dans le visible et par chimiluminescence. Les méthodes développées ont été appliquées à quelques composés pharmaceutiques et à différents produits alimentaires, tels que vins (rouges et blancs), bières (blondes et brunes), jus, infusions et boissons isotoniques.

Un système automatique de flux avec multi seringue incluant un arrêt du mélange réactionnel dans le détecteur pour contrôler spectophotométriquement la réduction du radical 2,2-diphényl-1-picrylhidrazyl ($DPPH^{\bullet}$), provoquée par les composés antioxydants, dans différents milieux réactionnels a été développé. Différents solvants ont été testés, à savoir: méthanol et solutions éthanoliques à 50% (v/v) sans contrôle du pH, à pH 4,1 et 7,6. La méthode développée a été appliquée à plusieurs composés reconnus comme étant antioxydants et a permis de déterminer le nombre de molécules de $DPPH^{\bullet}$ réduites par une molécule de antioxydant quand l'absorbance avait atteint une valeur stable.

Basé sur les résultats précédents, un système automatique de flux avec multi seringue utilisant la même réaction pour la détermination de la capacité antioxydant de différents produits alimentaires a été développé. La détermination était basée sur la diminution de l'absorbance due à la réduction du radical $DPPH^{\bullet}$ par réaction avec les composés antioxydants présents dans l'échantillon. L'influence de la concentration initiale du radical $DPPH^{\bullet}$ et l'influence de la dilution de l'échantillon sur la performance da la méthodologie analytique ont été étudiées. Les résultats ont été exprimés en quantité d'acide ascorbique équivalent (mg) présent dans 100 mL d'échantillon.

En ce qui concerne le système de flux développé pour la détermination de la capacité réductrice de Folin-Ciocalteu (FC), la méthodologie était basée sur la détection spectrophotométrique des complexes bleus formés après transfert d'électrons en milieu alcalin à partir des composés réducteurs présents dans l'échantillon vers les complexes provenant des acides phosphotungstique et phosphomolybdique (réactif FC). Les

différentes stratégies de mélange de l'échantillon et du réactif FC ont été étudiées recourant au contrôle du système par ordinateur, sans la nécessité de quelconque reconfiguration physique. L'acide gallique a été utilisé comme composé étalon et la méthodologie proposée a été appliquée à des composés dont l'activité antioxydant est reconnue (phénoliques et non phénoliques) et à plusieurs types de produits alimentaires.

La détermination séquentielle de la capacité réductrice de l'échantillon utilisant le réactif FC et la capacité de capture du radical cationique $ABTS^{\bullet+}$ (acide 2,2'-azinobis(3-éthylbenzothiazoline-6-sulfonique)) a été réalisée recourant au même système de flux utilisant l'acide gallique et le trolox comme composés étalons. La grande flexibilité de la gestion des fluides associée au contrôle par ordinateur des systèmes avec multi seringue a permis de réaliser les méthodes de manière isolée ou séquentielle simplement en modifiant les paramètres du programme de contrôle du système. La détermination des propriétés antioxydants de la méthode Folin-Ciocalteu est basée sur l'augmentation de l'absorbance due à la réduction du réactif FC. La capacité de capture du radical $ABTS^{\bullet+}$, quant à elle, a été déterminée par la diminution de l'absorbance due à la réduction du radical coloré par les composés antioxydants. L'application à un nombre élevé de boissons (n = 72) a permis la réalisation d'une étude comparative entre les deux méthodes.

Pour la détermination *in vitro* de la capacité sequestrante pour l'acide hypochloreux (HOCl) en conditions réactionnelles proches de celles rencontrées *in vivo* quant au pH et à la concentration, un système automatique de flux avec détection chimiluminométrique a été développé. La détermination est basée sur l'inhibition de la réaction de chimiluminescence entre HOCl et le luminol après une première réaction entre les composés antioxydants et l'espèce oxydante (HOCl). La méthodologie proposée a été appliquée à des composés pharmaceutiques anti-inflammatoires non stéroïdiens de différentes familles chimiques et à plusieurs contrôles positifs (cystéine, acide gallique, acide lipoïque). Dans un but de comparaison, la capacité sequestrante pour HOCl a été évaluée à différentes valeurs de pH (7,4 et 10,0). Les méthodologies automatiques proposées ont fourni des résultats concordants avec ceux disponibles dans la littérature et aussi statistiquement comparables avec ceux obtenus utilisant les procédés discrets. Les avantages et les limitations des systèmes proposés sont discutés.

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Framework and objectives

During the past decade, the formation of reactive of oxygen species (ROS) and reactive nitrogen species (RNS) have been implicated in the oxidative deterioration of food products as well as in the pathogenesis of several human diseases such as atherosclerosis, diabetes mellitus, chronic inflammation, neurodegenerative disorders and certain types of cancer. On the other hand, antioxidant compounds present in food and in biological systems play an important role in the protection of biomolecules from these free radical redox-reactions. In this regard, in a broad spectrum of areas, including biomedical, nutrition and agrochemical, the assessment of antioxidant/radical scavenging capacity has become a topic of increasing attention. This situation demands the availability of simple, convenient, rapid and reliable in vitro analytical methodologies.

In this context, analytical chemistry researchers have been devoted efforts to develop and/or to improve the antioxidant capacity assays. Thus, in the last few years, several in vitro analytical methods for the assessment of scavenging capacity against specific ROS/RNS using different target/probe species and reaction conditions were developed. The scavenging capacity assays against stable and non-biological chromogen radicals as well as the evaluation of total reduction capacity have also been implemented. At present moment, a validated in vitro assay that can reliably measure the *total antioxidant capacity* is not yet available. Among the present methods, undoubtedly the most widely used especially for screening/routine purposes are (i) the 2,2-diphenyl-1-pycrylhydrazyl radical ($DPPH^{\bullet}$) assay; (ii) the 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonate) radical cation ($ABTS^{\bullet+}$) assay, and (iii) the Folin-Ciocalteu reducing capacity. In fact, the last two assays abovementioned have been recently proposed as standardized methods for measurement of antioxidant capacity of food products and dietary supplements. Despite their high applicability to food and biological samples, in general they are time-consuming, laborious, and costly, especially when routine work concerning large number of samples has to be performed as it happens within food industry and clinical field. In addition, these

assays are also susceptible to operational errors, such as inadequate sample/reagent mixing or poor reproducibility of time events.

These limiting aspects can be overcome by automation. In this way, flow injection analysis and its predecessor computer-controlled flow procedures represent a useful analytical tool to improve antioxidant methodologies mainly due to the strict control of reaction conditions, the reproducible contact between oxidant and antioxidant/scavenger molecule and the high determination rate. The later flow procedures present greater potentialities, as it allows more versatile flow management including sample manipulation, improved precision on timing events and capacity to accommodate a wide variety of assays in the same manifold by changing the parameters in the controlling software.

Considering all these features, the objective of the present work was the development of automatic flow systems based on multisyringe flow injection analysis for the automation of the most widely used antioxidant capacity assays, using either chromogen radicals or reactive species found in vivo. Study of the influence of reaction media including solvent and pH in the assessment of antioxidant capacity was aimed. Implementation of different antioxidant methodologies using the same equipment through software control for comparison purposes in real time was proposed. Determination of scavenging capacity of reactive species under reaction conditions closer to those found in vivo including, reaction time, pH value and concentration of both oxidant and antioxidant compounds was also considered. Finally, it was aimed to develop faster methods providing results statistically comparable with those attained by the batch procedures.

Organisation of the dissertation

This dissertation is organised in eight chapters.

Chapter 1 is a general overview about the analytical methods used for the determination of antioxidant capacity. For this, a brief description regarding the oxidants and antioxidants species found in biological systems is given. The chemistry principles, some of its variants, recent applications and the merits and the disadvantages of the most common in vitro analytical methods used for the assessment of this property are discussed in detail. Particular emphasis is given to flow-based methods developed for determination of antioxidant capacity. Furthermore, the current state-of-the-art of these flow-based methodologies as well as a survey and critical discussion about their features and limitations to assess the antioxidant capacity in food and biological samples are also discussed in this chapter.

In Chapter 2, the materials and methods that were used throughout the experimental work are presented. The devices used to assemble the flow injection systems are also fully described. In addition, the assessment of antioxidant capacity and expression of this parameter are also described in detail in this chapter.

The following five chapters (from Chapter 3 to Chapter 7) describe the flow-based methods developed in the present work. They are presented under the format of papers published in international peer reviewed journals. Although each chapter is organised in different way, according to the requirements of the respective journal, in general all of them are composed by four parts: i) *introduction*, referring to general aspects concerning that particular determination, to the related methods already described in the literature and also to the objectives that are proposed; ii) *materials and methods (experimental section)*, where the preparation of reagents and solutions, the manifold devised and the instrumentation used, the functioning and the analytical flow protocol sequence as well as the batch comparative procedures used are fully described; iii) *results and discussion*, including the development of the flow manifold, the study of chemical and physical

parameters, the analytical features of the developed method, the application to food products or to pharmaceutical compounds and the comparison of the results obtained by the proposed methodology with that attained by batch procedures as well as with results reported in the literature; and iv) *conclusions*, where the key findings of the research are included and the main characteristics and advantages of the developed automatic antioxidant capacity assays are highlighted.

Finally, a chapter containing the general conclusions (Chapter 8) of the work developed ends this dissertation. The most important analytical features of the developed flow methodologies and the main contributions to the assessment of antioxidant capacity are summarised and critically discussed. The perspectives and future research trends in this analytical field are also given.

List of acronyms and respective names

Acronym	Name
AAPH	2,2′-Azobis(2-amidinopropane) dihydrochloride
ABTS	2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
$ABTS^{\bullet+}$	2,2′-Azinobis-(3-ethylbenzothiazoline-6-sulphonate) radical cation
AH	Antioxidant compound(s)
AMVN	2,2′-Azobis(2,4-dimethylvaleronitrile)
α_1-AP	α_1-Antiproteinase
AUC	Area under curve
BODIPY 581/591	4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-*s*-indacene-3-undecanoic acid
BPEA	9,10-Bis-(phenylethynyl)anthracene
BSA	Bovine serum albumin
Carboxy-PTIO	2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide
CCPO	2-Carbopentyloxy-3,5,6-trichlorophenyl oxalate
CL	Chemiluminescence
CLA	2-Methyl-6-phenyl-3,7-dihydroimidazo[1-2-a]pyrazin-3-one
$CO_3^{\bullet-}$	Carbonate radical anion
CV	Cyclic voltammetry
DAF-2	4,5-Diaminofluorescein
DAF-2T	Triazolofluorescein
DCF	Dichlorofluorescein
DCFH-DA	Dichlorofluorescein-diacetate
DHBA	Dihydroxybenzoic acid
DHR	Dihydrorhodamine 123

Acronym	**Name**
DMPO	5,5-Dimethyl-1-pyrroline-*N*-oxide
$DPPH^{\bullet}$	2,2-Diphenyl-1-picrylhydrazyl radical
DPPH	2,2-Diphenyl-1-picrylhydrazyne
DTNB	5,5-Dithiobis(2-nitrobenzoic acid)
EC_{50}	Efficient concentration
EDTA	Ethylenediamine tetraacetic acid
ESR	Electron spin resonance
ET	Electron transfer
FIA	Flow injection analysis
FC	Folin-Ciocalteu
FRAP	Ferric reducing antioxidant power
GC-FID	Gas chromatography with flame ionization detector
HAT	Hydrogen atom transfer
HPLC	High performance liquid chromatography
HPLC-ED	High performance liquid chromatography with electrochemical detection
$HO^{\bullet}$	Hydroxyl radical
H_2O_2	Hydrogen peroxide
HOBr	Hypobromous acid
HOCl	Hypochlorous acid
HRP	Horseradish peroxidase
HVA	Homovanillic acid
Ia	Intensity of the anodic current
IC_{50}	Concentration of the antioxidant compound that correspond to 50% of the blank analytical signal
KMBA	α-Keto-γ-methiolbutyric acid
LDL	Low-density lipoproteins

Acronym	**Name**
LPO	Lactoperoxidase
MCLA	2-Methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazo[1,2-a]pyrazin-3-one
MPFS	Multipumping flow systems
MSFIA	Multisyringe flow injection analysis
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NBT	Nitroblue tetrazolium
$NDPO_2$	Disodium 3,3'-(1,4-naphthalene)bispropionate
$NO^{\bullet}$	Nitric oxide radical
$NO_2^{\bullet}$	Nitrogen dioxide radical
N_2O_3	Dinitrogen trioxide
NOC-7	3-(2-Hydroxy-1-methyl-ethyl-2-nitrosohydrazino)-*N*-methyl-1-propanamine
NOSs	Nitric oxide synthases
NSAIDs	Non-steroidal anti-inflammatory drugs
$^1O_2$	Singlet oxygen
$O_2^{\bullet -}$	Superoxide anion radical
O_3	Ozone
OCl^-	Hypochlorite anion
$ONOO^-$	Peroxynitrite anion
ONOOH	Peroxynitrous acid
$ONOOCO_2^-$	Nitrosoperoxycarbonate anion
ORAC	Oxygen radical absorbance capacity
PABA	p-Aminobenzoic acid
β-PE	Phycoerythrin
PH	Oxidizable target

Acronym	**Name**
PMS	Phenazine methosulphate
POCL	Peroxyoxalate chemiluminescence
$R^{\bullet}$	Carbon-centered radicals
R–N=N–R	Thermolabile azo-compounds
RNS	Reactive nitrogen species
$RO^{\bullet}$	Alkoxyl radicals
$ROO^{\bullet}$	Peroxyl radicals
ROOH	Hydroperoxides
ROS	Reactive oxygen species
RSD	Relative standard deviation
RSE	Radical scavenging efficiency
S	Area under the anodic wave
SIA	Sequential injection analysis
SIN-1	3-Morpholinosydnonimine *N*-ethylcarbamide
–SH	Thiol group
TBARS	Thiobarbituric acid reactive substances
TCHQ	Tetrachlorohydroquinone
T_{EC50}	Time needed to reach steady state with EC_{50} concentration
SOD	Superoxide dismutase
TEAC	Trolox equivalent antioxidant capacity
TNB	5-Thio-2-nitrobenzoic acid
TOSC	Total oxyradical scavenging capacity
TPTZ	2,4,6-Tripyridyl-*s*-triazine
TRAP	Total radical-trapping antioxidant parameter
VCEAC	Vitamin C equivalent antioxidant capacity
XOD	Xanthine oxidase

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CHAPTER 1

General introduction

1.1. Oxidants and antioxidants in biological systems

Chemically, any compound that can accept electrons is an oxidant or oxidizing agent, and the chemical reaction is defined as a reduction. In contrast, a substance that donates electrons is a reductant or reducing agent, and the chemical reaction is defined as an oxidation (Petrucci *et al.*, 2006). An oxidation is impossible without a reduction elsewhere, and when such reactions characterize a chemical mechanism, it is called redox reaction. Such reactions are the heart of numerous biochemical pathways and cellular chemistry, biosynthesis, and regulation (Kohen and Nyska, 2002). They are also important for understanding the oxidative stress phenomena and radical/antioxidant effects. While oxidant and reductant are chemical terms, in biological environments they are usually termed as pro-oxidant and antioxidant, respectively (Cao and Prior, 1998). Pro-oxidant is a substance that can induces oxidative damage to various biological targets such as nucleic acids (e.g. base modification, single and double-strand breaks), lipids (e.g. peroxidation, fatty acid loss), and proteins (e.g. oxidation of specific amino-acid residues, formation of carbonyls). An antioxidant is a substance that can efficiently reduce a pro-oxidant with concomitant formation of products having no or low toxicity. Indeed, a broader definition of antioxidant was suggested by Halliwell *et al.* (1995) as "any substance that when present at low concentrations, compared to those of an oxidizable substrate significantly delays or prevents oxidation of that substrate". The term "oxidizable substrate" includes almost everything found in biological systems including nuclei acids, lipids, and proteins. Therefore, according to this definition, not all reductants involved in a chemical reaction are antioxidants; only those compounds which are capable of protecting the biological target meet these criteria.

In general these pro-oxidants are referred to as reactive oxygen species (ROS) and reactive nitrogen species (RNS) that can be classified into two groups of substances, radicals and non-radicals (Table 1.1). Depending on the site and the concentration generated, these species are well recognised for playing a dual role, as both beneficial and deleterious effects have been established (Valko *et al.*, 2007). Radicals are chemical species capable of independent existence, possessing one or more unpaired electrons. Among these species,

hydroxyl radical ($HO^{\bullet}$) is considered the most powerful oxidizing radical formed in biological systems. Due to its high reactivity, it can react at the site of its production with most organic and inorganic molecules (Halliwell and Gutteridge, 1999). The group of non-radical compounds contains a large variety of substances, some of which are extremely reactive. For instance, the protonated form of peroxynitrite (ONOOH) is a powerful oxidizing agent that may cause depletion of thiol groups (-SH) and oxidation of many biomolecules causing damage similar to that observed when $HO^{\bullet}$ is involved.

Table 1.1. Pro-oxidants: most common reactive oxygen species (ROS) and reactive nitrogen species (RNS).

Reactive oxygen species (ROS)		**Reactive nitrogen species (RNS)**	
Radicals			
Alkoxyl radicals	$RO^{\bullet}$	Nitric oxide radical	$NO^{\bullet}$
Peroxyl radicals	$ROO^{\bullet}$	Nitrogen dioxide radical	$NO_2^{\bullet}$
Hydroxyl radical	$HO^{\bullet}$		
Superoxide anion radical	$O_2^{\bullet -}$		
Non-radicals			
Hydrogen peroxide	H_2O_2	Dinitrogen trioxide	N_2O_3
Hypobromous acid	HOBr	Peroxynitrite anion	$ONOO^-$
Hypochlorous acid	HOCl	Peroxynitrous acid	ONOOH
Singlet oxygen	$^1O_2$	Nitrosoperoxycarbonate anion	$ONOOCO_2^-$

The major pathways for the production of ROS and RNS in vivo are illustrated in Fig. 1.1.

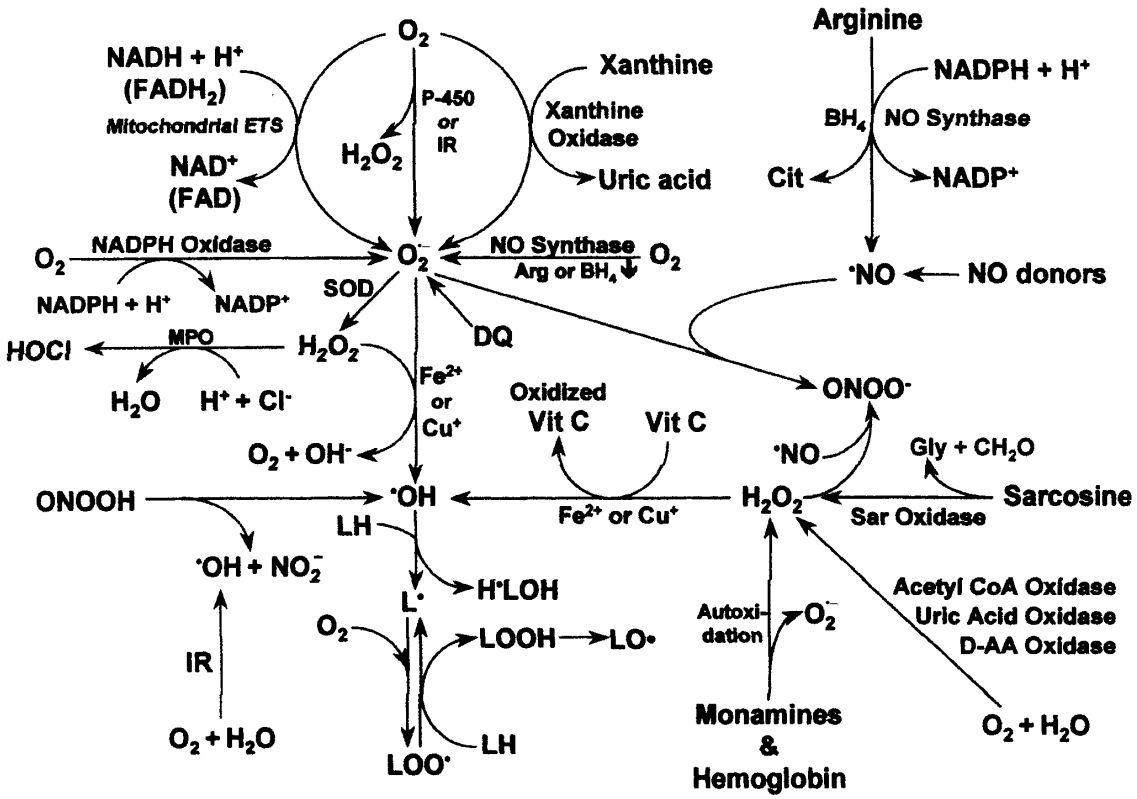


Figure 1.1. Production of reactive oxygen and nitrogen species in mammalian cells (Fang *et al.*, 2002). AA, amino acid; Arg, L-arginine; BH_4, (6R)-5,6,7,8,-tetrahydro-L-biopterin; CH_2O, formaldehyde; Cit, L-citrulline; DQ, diquat; ETS, electron transport system; FAD, flavin adenine dinucleotide (oxidized); $FADH_2$, flavin adenine dinucleotide (reduced); Gly, glycine; H_2O_2, hydrogen peroxide; HOCl, hypochlorous acid; H$^{\bullet}$LOH, hydroxy lipid radical; IR, ionizing radiation; L$^{\bullet}$, lipid radical; LH, lipid (unsaturated fatty acid); LO$^{\bullet}$, lipid alkoxyl radical; LOO$^{\bullet}$, lipid peroxyl radical; LOOH, lipid hydroperoxide; MPO, myeloperoxidase; NAD^+, nicotinamide adenine dinucleotide (oxidized); NADH, nicotinamide adenine dinucleotide (reduced); $NADP^+$, nicotinamide adenine dinucleotide phosphate (oxidized); NADPH, nicotinamide adenine dinucleotide phosphate (reduced); NO$^{\bullet}$, nitric oxide; $O_2^{\bullet-}$, superoxide anion radical; $^{\bullet}$OH, hydroxyl radical; $ONOO^-$, peroxynitrite anion; ONOOH, peroxynitrous acid; P-450, cytochrome P-450; Sar, Sarcosine; SOD, superoxide dismutase; Vit C, vitamin C.

These reactive species, radicals and non-radicals, can be easily formed from exogenous and endogenous sources (Kohen and Nyska, 2002). The exogenous sources include exposure of biological systems to γ- or UV-irradiation that results in the production of a wide range of non-radical and radical species such as hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\bullet}$), and superoxide anion radical ($O_2^{\bullet-}$). Ozone (O_3), which presence in the upper atmosphere is essential in scavenging deleterious UV-irradiation, is also used as disinfection agent by the food industry to destroy food-borne pathogens. Nevertheless, it can oxidize biomolecules yielding the formation of various reactive species. Additionally, a large variety of xenobiotics (e.g. drugs, pollutants, toxins, pesticides, and herbicides) produce ROS/RNS as a by-product of their in vivo metabolism. Although the exposure to exogenous sources is high, the exposure to endogenous sources is much more important and extensive, because it is a continuous process. The main endogenous sources are related with the mitochondrial electron-transport chain, and also with the activity of some enzymes, such as nitric oxide synthases (NOSs) and xanthine oxidase (XOD) that catalyzes the production of nitric oxide radical ($NO^{\bullet}$) and $O_2^{\bullet-}$, respectively. Moreover, activated phagocytes produce a variety of reactive oxygen, halogen and nitrogen species that play an important role in the mechanism of defense against infectious agents (Halliwell, 2006).

To counteract the assault of these ROS/RNS species, living cells have developed a complex defense system composed of enzymatic and non-enzymatic antioxidants that convert them to harmless species. Enzymatic antioxidant defenses include superoxide dismutase (SOD) that detoxifies the $O_2^{\bullet-}$ to water and H_2O_2, catalase which converts H_2O_2 to oxygen and water, and glutathione peroxidase whose function is to detoxify cellular peroxides to alcohols and water. Non-enzymatic antioxidants are represented by dietary antioxidants such as ascorbic acid (vitamin C), α-tocopherol (vitamin E), carotenoids, and polyphenolic compounds. Uric acid, glutathione, bilirubin, albumin, and other proteins (transferrin, ceruloplasmin, myoglobin and ferritin) are considered as endogenous antioxidants for protecting essential biological targets against ROS/RNS action.

In a normal biological system, there is an appropriate pro-oxidant/antioxidant balance. However, this balance can be shifted towards the pro-oxidant agents when there is an overproduction of ROS/RNS or when levels of antioxidant protection are diminished. This

state is called 'oxidative stress', and it can be triggered by several factors such as diseases, diet, lifestyle, and environmental conditions (Kohen and Nyska, 2002). As mentioned before, the excess of ROS/RNS can oxidize cellular lipids, proteins, or nucleic acids, inhibiting their normal function. Because of this, oxidative stress has been implicated in the pathogenesis of several human diseases, including atherosclerosis, cancer, cardiovascular diseases, diabetes mellitus, inflammatory diseases, ischemia/reperfusion injury, and neurodegenerative disorders (Alzheimer's and Parkinson's diseases) as well as in the ageing process (Valko *et al.*, 2007). Oxidation can also affect foods, where it is one of the major causes of chemical spoilage, resulting in rancidity and/or deterioration of the nutritional quality, colour, flavour, texture and safety of foods (Frankel, 1996).

Regarding the protective effects of antioxidants against these deleterious oxidative-induced reactions, interest in antioxidant research has become a topic of increasing attention in the last few years, especially within biological, medical, nutritional, and agrochemical fields. In fact, a literature search performed on the *ISI Web of Knowledge* search engine for articles containing the expression "antioxidant or antioxidants", revealed that the number of publications has increased about 345% in the past decade (16080 until 1996, while between 1997 and 2006 around 55493 papers were published). This situation demands the existence of simple, convenient, and reliable in vitro analytical methodologies for the fast determination of antioxidant capacity of pure compounds or in complex matrices, such as foods and biological samples (Sánchez-Moreno, 2002; Huang *et al.*, 2005; Prior *et al.*, 2005; Roginsky and Lissi, 2005; Wood *et al.*, 2006).

Analytical methods for evaluation of antioxidant capacity of pure compounds are applied to investigate the structure-activity relationship (Kim and Lee, 2004), to separate and detect specific dietary components and to determine their contribution to the total antioxidant composition (Tsao and Deng, 2004). However, separating each antioxidant compound and studying it individually is costly and inefficient due to the high number, diversity, and complexity of antioxidants present in food and biological samples. Moreover, the possible interactions (additive, synergistic, or inhibitory effects) that may exist between different antioxidants imply that the sum of the antioxidant capacity from each compound isolated may not exactly reflect the overall action (Jia *et al.*, 1998). For this reason, analytical

methods that allow the evaluation of antioxidant capacity in the whole sample, taking into consideration the interactions between all compounds present in the matrix, may offer an additional advantage when compared to those based on separation techniques that are time-consuming, expensive, and often not suitable for screening/routine determinations.

Assessment of antioxidant capacity in food products is of utmost importance to determine the antioxidant effectiveness for food protection against oxidative damage, for food quality monitoring over a product shelf life, and for commercialization of nutritional-added-value products. Moreover, these methods could be a useful tool to make a selection among different species, varieties, maturation degree, and culture conditions, in order to obtain high content of antioxidants in foods (Kaur and Kapoor, 2001). In the case of biological samples (e.g. plasma, serum, urine), measurement of antioxidant status is essential for diagnostic and treatment monitoring, especially during supplementation trials for boosting plasmatic antioxidant levels (Wood *et al.*, 2006). In fact, as Huang *et al.* (2005) stated "a valid in vitro assay is an invaluable tool for clinical studies if it is combined with bioavailability data and valid oxidative stress biomarker assays". However, at present time a validated in vitro assay that can reliably measure the *total antioxidant capacity* of foods and biological samples is not yet available. This situation is due to several reasons. Firstly, the term *total antioxidant capacity* is not a measurable parameter because it is a broader definition that covers: i) inhibition of generation and scavenging capacity against ROS/RNS; ii) reducing capacity; iii) metal chelating capacity; iv) activity of antioxidative enzymes; v) and inhibition of oxidative enzymes. Secondly, different antioxidants act by different mechanisms and even the same compound can have different ways of actuation. In this regard, no single assay provides all of the information desired because it does not reflect the complexity of the interactions of antioxidants. Thus, the evaluation of overall antioxidant capacity requires multiple assays to generate an "antioxidant profile". Finally, the methods currently used to assess this property differ from each other in terms of reaction conditions, considering the oxidant species, and the target/probes applied. Even when only one of these assays is considered, different forms for expressing the results are found, besides the application of different antioxidant standard compounds, solvents, reaction time, and pH conditions (Huang *et. al*, 2005). This makes the comparison of

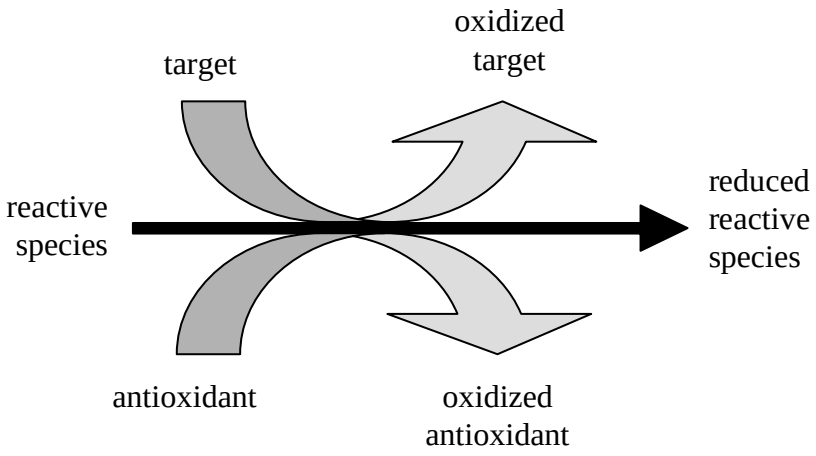
results from different studies difficult and stresses the necessity to standardize antioxidant methods to bring some order to the present chaos in this field.

In this context, Prior *et al.* (2005) have proposed some guidelines for consideration in method selection for standardization. Thus, a standardized method for determination of antioxidant capacity in routine analysis should meet the following requirements/criteria: (i) measurement of the chemistry actually occurring in potential applications; (ii) utilization of biological relevant molecules; (iii) technically simple; (iv) with a defined endpoint and chemical mechanism; (v) readily available instrumentation; (vi) good repeatability and reproducibility; (vii) adaptable for assay of both hydrophilic and lipophilic antioxidants; (viii) and adaptable to high-throughput analysis.

In this regard, knowledge of the chemistry principles of the available methodologies is of utmost importance to select the adequate technique(s). Generally, the in vitro analytical methods for determination of antioxidant capacity rely on two different approaches, named here as competitive or non-competitive scheme (Fig. 1.2).

In the competitive scheme, the target species, defined here as a compound that represents a biomolecule which may be attacked in vivo, and the antioxidant compounds compete for the reactive species (radical or non-radical). The assessment of antioxidant capacity is based on the quantification of a compound that facilitates the analytical measurement, defined here as the probe. In most of the competitive assays, the probe is the target species or its oxidized form. Nevertheless, the probe can also be a compound added after the above mentioned reaction that allows the quantification of the remaining reactive species or target molecules. In these assays, the antioxidant capacity of tested compounds is dependent on: i) the rate of reaction between them and the reactive species, ii) the rate of reaction between the target molecule and the reactive species, and iii) the concentration ratio between antioxidants and target. Among the requirements for these type of assays, the following should be highlighted: i) the probe must be reactive with oxidants at low concentration; ii) there must be a dramatic spectroscopic change between the native and oxidized probe (to maximize the sensitivity); iii) no radical chain reaction beyond probe oxidation should occur, and iv) the antioxidant should not react with the target species.

- **Competitive scheme**



- **Non-competitive scheme**

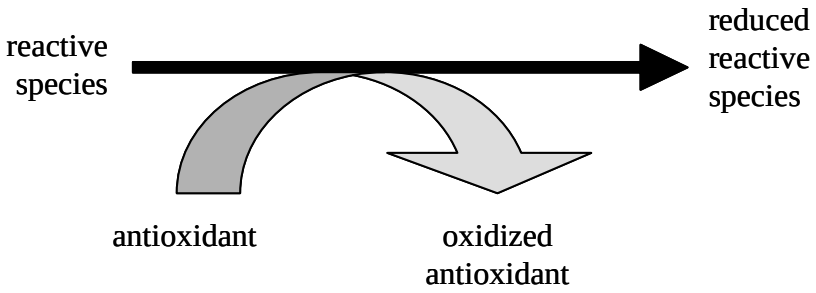


Figure 1.2. Principle of competitive and non-competitive schemes for the antioxidant capacity assays.

In the non-competitive assays, putative antioxidant compounds react with reactive species without the presence of any other competing target molecule. In this way, these assays involve two components in the initial reaction mixture: the antioxidant compound(s) and the reactive species, which may also be the probe for reaction monitoring. Otherwise, the remaining reactive species may be measured after addition of some derivative reagent.
In this context, some of the most commonly used methods for in vitro determination of antioxidant capacity are reviewed in the following sections, where the chemical principles, some of its variants, recent applications as well as the advantages and shortcomings are outlined. These assays were roughly divided into two categories according to the type of the oxidant species: i) scavenging capacity assays against specific ROS/RNS; ii) scavenging capacity assays against stable, non-biological radicals and evaluation of total reducing capacity. The determination of the activity of antioxidative enzymes (superoxide dismutase, catalase, and glutathione peroxidase), or of biomarkers of oxidative stress is out of the scope of this review.

1.2. Scavenging capacity assays against specific ROS/RNS

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are regularly produced in food and biological systems and may damage biomolecules (Valko *et al.*, 2007). Non-enzymatic dietary antioxidant compounds and the complex physiological antioxidant systems protect these oxidation-induced reactions by inhibition of generation and/or scavenging the ROS/RNS. All of these harmful reactive species participate in the oxidative processes, thus a comprehensive profile of antioxidant capacity can only be elucidated by the application of different assays, utilizing specific ROS/RNS species, which will be discussed further. The analytical features of these assays are summarized in Table 1.2 (page 37).

1.2.1. Peroxyl radical ($ROO^{\bullet}$) scavenging capacity assays

Peroxyl radicals ($ROO^{\bullet}$) are commonly found in food and biological substrates and they are formed during lipid oxidation chain reactions, such as the oxidation of polyunsaturated fats (Halliwell and Gutteridge, 1999). They have harmful effects on health and they are also associated to quality deterioration of foods. Their impact on these areas foster the existence of several methods for determining the peroxyl radical scavenging capacity, which were subject of review recently (Laguerre *et al.*, 2007; Roginsky and Lissi, 2005).
In general, methods for examination of $ROO^{\bullet}$ scavenging capacity measure the ability of an antioxidant to scavenge peroxyl radicals by hydrogen atom transfer (HAT) reactions. In these assays a competitive scheme is applied, where antioxidants or target molecules react with $ROO^{\bullet}$. Hence, the assay system is composed of three components: i) thermolabile azo-compound (R–N=N–R), which yields carbon-centered radicals ($R^{\bullet}$) that react fast with O_2 to give a steady flux of $ROO^{\bullet}$ radicals; ii) oxidizable target (PH); and iii) antioxidant compound(s) (AH), as represented schematically in the following equations (*1.1–1.5*):

$$R\text{–}N{=}N\text{–}R \rightarrow 2\,R^{\bullet} + N_2 \qquad (1.1)$$

$$R^{\bullet} + O_2 \rightarrow ROO^{\bullet} \qquad (1.2)$$

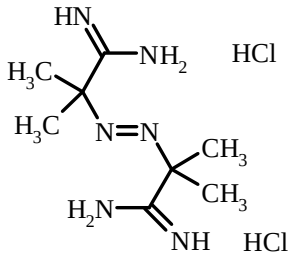
$$ROO^{\bullet} + PH \rightarrow ROOH + P^{\bullet} \qquad (1.3)$$

$$ROO^{\bullet} + AH \rightarrow ROOH + A^{\bullet} \qquad (1.4)$$

$$ROO^{\bullet} + A^{\bullet} \rightarrow ROOA \qquad (1.5)$$

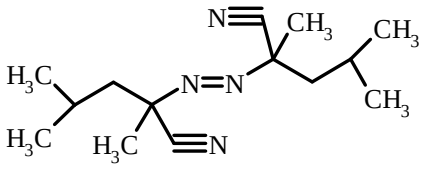
The most frequently applied peroxyl radical generators are the water-soluble 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) and the lipid-soluble 2,2′-azobis(2,4-

dimethylvaleronitrile) (AMVN); their molecular structure are represented in Fig. 1.3. The rate of their spontaneous decomposition and production of $ROO^{\bullet}$ radicals is primarily determined by temperature of the reaction medium (Niki, 1990).



AAPH

2,2′-azobis(2-amidinopropane) dihydrochloride



AMVN

2,2′-azobis(2,4-dimethylvaleronitrile)

Figure 1.3. Molecular structure of thermolabile azo-compounds used for generation of peroxyl radicals.

In these competitive assays, the presence of antioxidant compounds inhibits or retards the rate of target/probe oxidation induced by peroxyl radicals (eq. *1.3*). Therefore, in the beginning of the assay, insignificant spectroscopic changes of the target/probe would be observed (induction period or lag phase) because the antioxidants protect the target/probe from $ROO^{\bullet}$-oxidation. As the reaction proceeds, the antioxidants are consumed by the constant flux of $ROO^{\bullet}$ and the oxidation of the target/probe would progress at a slower rate when compared with the control (absence of antioxidant compounds/samples). Finally, when the antioxidants are depleted, the reaction rate of oxidation of the target/probe is similar to that obtained for the control. As mentioned before, the beginning and duration of these three phases is dependent on the rate of reaction between antioxidant and $ROO^{\bullet}$, the rate of reaction between the target/probe and $ROO^{\bullet}$, and the concentration ratio between antioxidant and target/probe.

Although the competitive scheme applied resembles in vivo conditions, the concentration of the target species is usually smaller than the concentration of antioxidants. This is in contradiction with the "definition of antioxidant" (Halliwell *et al.*, 1995) and with what is found in real situations, where the antioxidant concentration is much smaller than that of the oxidizable substrate (lipids or proteins, for instance). Moreover, these assays apply a $ROO^{\bullet}$ reaction without taking into account the essential propagation step in lipid autoxidation, such as the breakdown of hydroperoxides (ROOH) yielding peroxyl and alkoxyl radicals ($RO^{\bullet}$) (Huang *et al.*, 2005). Finally, most of these assays are rather time-consuming and their application requires a significant expertise and experience in chemical kinetics. As a consequence, HAT-assays are commonly not suitable for routine determinations.

The analytical methods comprising these features include the oxygen radical absorbance capacity (ORAC) assay, the total radical-trapping antioxidant parameter (TRAP) assay, and the crocin bleaching assay. The inhibition of low-density lipoproteins (LDL) oxidation, the total oxyradical scavenging capacity (TOSC), and the chemiluminescence-based assays employing peroxyl radicals may also be included. The major difference among these assays lies mostly in the approach used for quantification of antioxidant capacity: the ORAC assay applies the area under curve (AUC) that represents the oxidation of the target along time, the TRAP assay relies on the "lag time", that corresponds to the time period between the beginning of the assay and the beginning of the oxidation of the target, whilst the crocin-bleaching assay utilises the initial oxidation rate of target species.

1.2.1.1. Oxygen radical absorbance capacity (ORAC assay)

The assay is based on the intensity of fluorescence decrease of the target/probe along time under reproducible and constant flux of peroxyl radicals, generated from the thermal decomposition of AAPH in aqueous buffer. In the presence of a sample that contains chain-breaking antioxidants, the decay of fluorescence is inhibited (Glazer, 1990). Initially, the protein isolated from *Porphyridium cruentum*, β-phycoerythrin (β-PE), was used as the

fluorescent target/probe, which reacted with $ROO^{\bullet}$ to form a nonfluorescent product (Cao *et al.*, 1993). Nevertheless, some shortcomings were observed such as large lot-to-lot variability, photobleaching of the β-PE after exposure to excitation light, and interaction with polyphenols by non-specific protein binding. To overcome these limitations, the synthetic, non-protein fluorescein has been used as the fluorescent target/probe, instead of the original β-PE (Ou *et al.*, 2001). The application to both lipophilic and hydrophilic chain-breaking antioxidants was carried out using a mixture of acetone/water containing 7% of randomly methylated β-cyclodextrin as a water solubility enhancer (Huang *et al.*, 2002a). Lipophilic compounds were also quantified by ORAC assay using either organic media or liposomes, AMVN as a lipophilic peroxyl radical generator, and 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-*s*-indacene-3-undecanoic acid (BODIPY 581/591 C_{11}) as a fluorescent target/probe (Naguib, 1998). To improve the throughput, Huang *et al.* (2002b) developed a high-throughput assay using a multichannel liquid handling system coupled with a microplate fluorescence reader in 96-well format.

In the ORAC assay, the reaction is monitored for extended periods (≥ 30 min) and the quantification is based in the area under curve (AUC) that represents the oxidation of the probe along time. The protective effect of antioxidants is evaluated from the net integrated area under the fluorescence decay curves ($AUC_{sample} - AUC_{blank}$) and results are expressed as μM of trolox equivalents. The advantage of the AUC approach is that it can be applied for antioxidants that exhibit distinct lag phases and to those samples that have no lag phases. Moreover, it takes into account the initial reaction rate and the total extent of inhibition, which includes the action of slow-reacting or secondary antioxidant products formed. The principles of the ORAC assay can be adapted to determine the action against other reactive oxygen species (Ou *et al.*, 2002a). The main limitation of the ORAC assay is that the oxidative deterioration and antioxidant protection of fluorescent target/probe (fluorescein) does not necessarily mimic a critical biological substrate (Frankel and Meyer, 2000). The assay is also time-consuming and has technical limitations related to the source of peroxyl radicals; the compounds applied are highly sensitive to temperature and likely to undergo spontaneous decay.

This methodology has provided substantial information regarding the antioxidant capacity of lipophilic and hydrophilic antioxidant compounds (Cho *et al.*, 2007), food products (Wu *et al.*, 2004), animal tissues (Cao *et al.*, 1996), and in bioavailability studies (Mertens-Talcott *et al.*, 2006; Prior *et al.*, 2007).

1.2.1.2. Total radical-trapping antioxidant parameter (TRAP assay)

The total radical-trapping antioxidant parameter (TRAP) assay was introduced by Wayner *et al.* (1985), for the determination of the antioxidant status of human plasma. This method was based on the measurement of the time period in which oxygen uptake was inhibited by plasma during a controlled $ROO^{\bullet}$ peroxidation reaction induced by the thermal decomposition of an azo-compound. In this assay, the target was the human plasma while the oxygen consumed in the oxidation of plasma material was the probe used to follow the action of antioxidants. The measurement is based on the "lag time" that corresponds to the time period between the beginning of the assay and the beginning of the oxidation of the target molecules. Later, the same research group evaluated the relative contributions of the main antioxidants of human plasma to the TRAP value (Wayner *et al*., 1987).

One of the major problems with the original TRAP assay lies in the utilization of the oxygen electrode as detector, since it may not maintain its stability over the period of time required (Rice-Evans and Miller, 1994). To overcome this limitation, this assay was later improved using β-phycoerythrin (β-PE) as the fluorescent target/probe, and the ability of the plasma to protect β-PE from peroxyl radical oxidation was fluorimetrically monitored (DeLange and Glazer, 1989). Ghiselli *et al.* (1995), proposed some modifications in order to circumvent interferences from plasma proteins, lipids and metal ions. They also evaluated the contribution and synergistic effects of main antioxidant compounds, and the effect of plasma storage in the TRAP values. Valkonen and Kuusi (1997) applied dichlorofluorescein-diacetate (DCFH-DA) as the fluorescent oxidizable substrate. The oxidation of DCFH-DA by peroxyl radicals yields the formation of highly fluorescent dichlorofluorescein (DCF) product. In this case, the presence of antioxidant compounds

competitively inhibits the increase of fluorescence signal. For the analysis of lipophilic samples as olive and seed oils, peroxyl radicals were generated by the lipophilic AMVN azo compound (Cabrini *et al.*, 2001). Due to the lower solubility of fluorescent target/probes in the reaction medium, the TRAP value was determined by measuring the time period during which oxygen uptake was inhibited.

Disregarding the different variations discussed above, the quantification is based on the lag phase duration, in which oxidation is inhibited by the antioxidants, compared to the lag phase of trolox. The antioxidant capacity expressed as trolox equivalents (X_{AO}) is calculated by the following equation:

$$X_{AO} = (C_{Trolox}/T_{Trolox}) * T_{AO} \qquad (1.6)$$

where C_{Trolox} is the trolox concentration, whilst T_{Trolox} and T_{AO} are the lag time of the kinetic curve of target oxidation in the presence of trolox or in the presence of antioxidant/sample, respectively; X_{AO} is then multiplied by 2.0, the stoichiometric factor of trolox, and by the dilution factor of the sample to give the TRAP value (μM).

The main shortcoming of this assay is the use of the lag phase for quantifying antioxidant capacity, since not every antioxidant possesses an obvious lag phase and also the antioxidant capacity profile after the lag phase is totally ignored. Moreover, the application of different criteria for establishing the endpoint makes difficult the comparison between laboratories. Another important limitation of this assay, and also of the ORAC assay, is that the oxidative deterioration and antioxidant protection of fluorescent target/probe does not necessarily mimic a critical biological substrate.

This assay has been applied to in vitro evaluation of antioxidant capacity of foods (Pellegrini *et al.*, 2003a), and to assess the plasma status after ingestion of food-rich antioxidant products (Dalla-Valle *et al.*, 2007).

1.2.1.3. Crocin bleaching assay

This assay measures the ability of antioxidant compounds to protect the naturally occurring carotenoid derivative crocin (Fig. 1.4) from oxidation by $ROO^{\bullet}$ radicals formed through thermolysis of AAPH (Bors *et al.*, 1984). The reaction is initiated by the addition of AAPH and the bleaching rate (absorbance decrease/time) of crocin is monitored at 443 nm during 10 min. Antioxidants compete with crocin for $ROO^{\bullet}$, and the degree of inhibition of crocin oxidation depends on the antioxidant capacity of tested samples. The quantification of antioxidant capacity is based on the ratio of initial crocin bleaching rates in absence (V_0) and in presence (V) of antioxidants, and is given by the Stern-Volmer-like relation:

$$V_0/V = 1 + (k_{AO}/k_C)*([AO]/[C]) \qquad (1.7)$$

where [AO] and [C] are the concentrations of antioxidant and crocin, respectively, k_{AO} and k_C are the rate constants for the reaction of the peroxyl radicals with antioxidant and crocin, respectively. After measuring V_0/V value at known ratio of [AO] to [C], k_{AO}/k_C is given by the slope value obtained from eq. (*1.7*) and indicates the relative peroxyl radical scavenging capacity. A microplate-adapted crocin bleaching assay based on the inhibition percentage at a fixed time instead of kinetic analysis have also been reported (Lussignoli *et al.*, 1999).

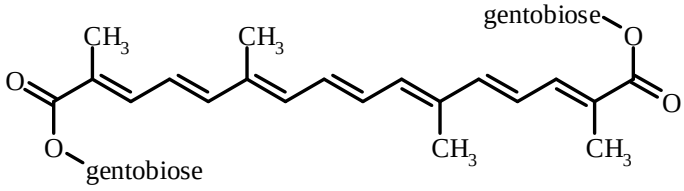


Figure 1.4. Chemical structure of crocin, a natural carotenoid compound formed from diester of the disaccharide gentobiose and the dicarboxylic acid crocetin; when dissolved in water, it forms an orange solution (λ_{max} = 443 nm).

This assay was used to determine the structure-antioxidant activity relationships of flavanones present in Citrus fruit (Di Majo *et al.*, 2005). Nevertheless, it has limited applications in food samples since many food pigments, such as carotenoids, absorb at the same wavelength of the determination. Besides this drawback, crocin is a natural food pigment extracted from saffron, which may confer a low reproducibility between assays. In fact, Chatterjee *et al.* (2005) described a more affordable crocin assay using the Indian spice saffron instead of the commercial chemical product and found that the antioxidant capacity values of pure natural compounds, plant extracts, and human plasma from healthy individuals using either approach were similar.

Recently, Ordoudi and Tsimidou (2006a) have examined the crocin bleaching assay performance and validation procedures. The studies were focused on target/probe and test compound characteristics, conditions for peroxyl radical generation, reaction monitoring, and expression of results. They observed that any authentic commercial saffron can be used for target/probe preparation given that: interferences, such as tocopherols, are removed; the concentration of working solution is adequately adjusted; and the changes of the stock target/probe solution during storage are not neglected. Results are expressed as "percent inhibition of crocin bleaching value" instead of the ratio of initial crocin bleaching rates. This assay has been applied to structure-activity relationship studies of selected phenolic compounds (Ordoudi and Tsimidou, 2006b).

1.2.1.4. Inhibition of low-density lipoproteins (LDL) oxidation

In this assay the low-density lipoproteins (LDL), isolated from blood samples, are the oxidizable target. The peroxidation of LDL is initiated by thermal decomposition of AAPH or by Cu(II) and assessed through the formation of conjugated dienes, determined spectrophotometrically at 234 nm after HPLC separation (Handelman *et al.*, 1999). Sánchez-Moreno *et al.* (2000) studied the oxidation of LDL induced by Cu(II) and proposed several oxidizability indexes to measure the antioxidant activity of dietary polyphenols. The concentration of antioxidant that increases the lag time by 50%

compared to the control (CLT_{50}) was determined graphically upon the representation of the ratio lag time antioxidant/lag time control as a function of antioxidant concentration. In this assay the use of peroxyl radicals and LDL as oxidant and target species, respectively, allows a strong resemblance to oxidative reactions that might occur in biological systems. This assay has been applied to evaluate the capacity to inhibit in vitro LDL oxidation of pure compounds, plant foods and beverages (Saura-Calixto and Goni, 2006). Recently, Gomes-Ruiz *et al.* (2007) used this method to determine the antioxidant capacity of different compounds present in coffee or which are produced as a result of the metabolism of this beverage; 1-methyluric acid was particularly effective at inhibiting oxidation of LDL. However, the major limitation is that the LDL has to be isolated on a regular basis from different individuals, and therefore a high inter-batch variation is verified. Moreover, problems with this assay arise because it is difficult to measure the small lag times that occur, and many substances also absorb at the wavelength of the determination.

1.2.1.5. Total oxyradical scavenging capacity (TOSC assay)

In the original total oxyradical scavenging capacity (TOSC) assay, peroxyl radicals generated by thermal homolysis of AAPH cause the oxidation of α-keto-γ-methiolbutyric acid (KMBA) to ethylene, which is monitored by gas chromatographic analysis of head space from the reaction vessel (Winston *et al.*, 1998). The antioxidant capacity of the compounds tested is quantified by their ability to inhibit ethylene formation relatively to a control reaction. Later, Regoli and Winston (1999) extended this assay to other two potent oxidants found in vivo, $HO^{\bullet}$ and $ONOO^{-}$. These oxidants were generated by Fe^{3+}/H_2O_2/ascorbate system (Fenton reaction) and by 3-morpholinosydnonimine *N*-ethylcarbamide (SIN-1), respectively. The assay conditions were established in such a way that the control reactions give similar yields of ethylene from each of the oxidants studied. Therefore, the relative efficiency of various antioxidants could be compared. This improved version of the TOSC assay is a useful and robust way for distinguishing the reactivity of various relevant biological oxidants and the relative capacities of antioxidants

to scavenge different oxidants. In the TOSC assay, the approach for quantification of antioxidant capacity is similar to that applied for the ORAC assay. In this case it is based in the area under the curve that represents the inhibition of ethylene formation as a function of time. Nevertheless, there is not a linear relationship between AUC and antioxidant concentration or dilution factor of the sample. Therefore, the concentrations or dilution factors that correspond to TOSC values of 20, 50, and 80% are calculated, and DT_{50} is also determined, which is the first derivative of the curve at a TOSC of 50% (Lichtenthäler and Marx, 2005a). The long reaction time (>100 min), the short shelf-life of the assay solutions, and the necessity of multiple manual injections from the same sample into a gas chromatograph to monitor the formation of ethylene, make this assay difficult to implement to routine analysis. To overcome this last drawback, Lichtenthäler and Marx (2003) monitored the time course of ethylene formation resorting to automated headspace gas chromatography. Later, the same research group applied this automated version for a comparative and detailed survey of the antioxidant capacities of 14 common European fruit and vegetable juices (Lichtenthäler and Marx, 2005a). The antioxidant capacity of methanol and ethanol seed extracts (Rodrigues *et al.*, 2006) and fruit pulp (Lichtenthäler *et al.*, 2005b) from *Euterpe oleracea Mart.* (açai) were also studied with the TOSC assay in a modified and automated version.

1.2.1.6. Chemiluminescence-based assays

The general principle of chemiluminescence (CL) assays is based on the reaction of oxidants (ROS/RNS) with chemiluminogenic probes (luminol or lucigenin) to produce excited state species that emit light. A recent outlook on chemiluminescent methods for ROS is given by Lu *et al.* (2006). These assays are based on competitive or on non-competitive reaction schemes. In the first case, the CL target/probe and sample (containing antioxidant compounds) compete for the reactive species, while in the second approach the CL probe is added after a prior contact between the reactive species and sample. In both strategies, the addition of sample containing antioxidant/radical scavenger compounds

results in inhibition of the light production and the intensity of CL-quenching is directly proportional to the antioxidant capacity of the sample. Nevertheless, it should be stressed that the antioxidant/sample may reduce or scavenge the reactive species as well as the radicals involved in the target/probe CL-oxidation (Fig. 1.5). The interference on CL reaction will depend both on the rate of reaction of antioxidants with ROS/RNS and with radicals formed during CL-probe oxidation. In this regard, these interferences may be minimized and reliable results are obtained with a non-competitive approach, since the antioxidants react with oxidants before the addition of CL-probe. This strategy is easily attained using the flow-based methods, which are discussed further in this chapter.

Peroxyl radical scavenging capacity assays have also been implemented using CL as detection system. The principles of $ROO^{\bullet}$ induced luminol-CL assays were described in detail by Alho and Leinonen (1999). Lissi *et al.* (1992 and 1995) evaluated the effect of different recognised antioxidant compounds (ascorbic acid, cysteine, uric acid and trolox) and biological fluids (urine and blood plasma) on the intensity of luminol induced CL by $ROO^{\bullet}$. In this assay, the addition of the antioxidant/sample induces a CL-lag phase (time during which CL emission was not detected) whose magnitude was directly related to the antioxidant concentration. The application of CL-based methods is frequent but it should be pointed out that other ROS, including H_2O_2, $HO^{\bullet}$, $O_2^{\bullet-}$ and $ONOO^-$, also induces luminol CL. Hence, luminol-based methods are not specific, unless the generation of ROS is controlled and unless the test sample do not present any other ROS. Furthermore, even when only one type of ROS is present, the inhibition of CL-signal may be due to its scavenging or due to the scavenging of luminol-derived radicals. Indeed, the effect elicited by antioxidants at low concentrations is most likely due to trapping of luminol-derived radicals after the fast scavenging of the $ROO^{\bullet}$ radicals by luminol (Lu *et al.*, 2006).

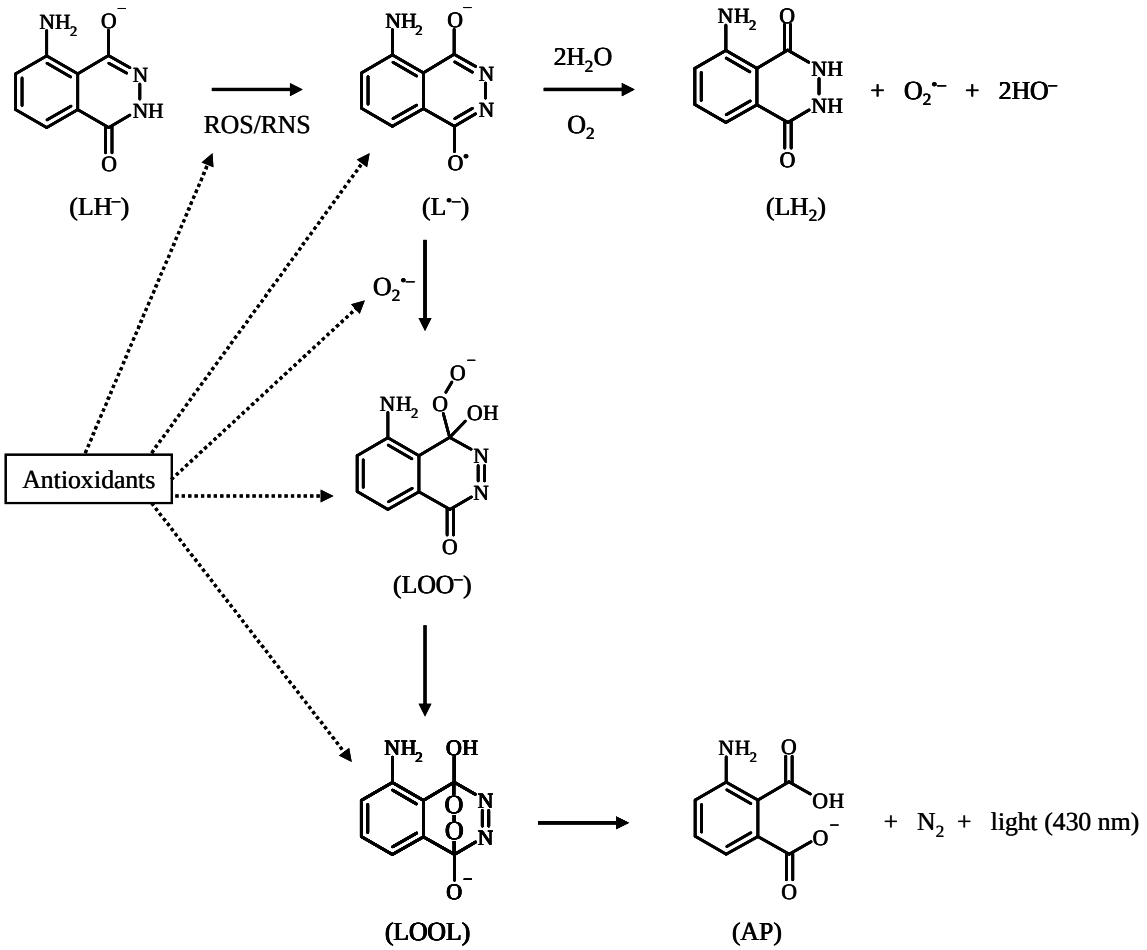


Figure 1.5. Mechanism of luminol oxidation induced by reactive species (ROS/RNS) and antioxidant inhibition pathways (Giokas *et al.*, 2007). At alkaline pH, monodissociate luminol (LH^-) reacts with ROS/RNS to yield the diazasemiquinone radical ($L^{\bullet-}$). The later reduces O_2 to superoxide anion ($O_2^{\bullet-}$) and is oxidised to 5-aminohyphenthalazine-1,4-dione (LH_2). Diazasemiquinone radical and $O_2^{\bullet-}$ yield the carbon-centered hydroperoxide anion (LOO^-) that rearranges to a transient endoperoxide (LOOL), which decomposes to give light emission and products (N_2 and aminophthalate (AP)). Antioxidants may react with ROS/RNS as well as with the radicals formed during luminol oxidation pathway.

1.2.2. Superoxide radical anion ($O_2^{\bullet-}$) scavenging capacity assays

Superoxide radical anion ($O_2^{\bullet-}$) is produced as a result of the donation of one electron to oxygen. This radical arises either from several metabolic processes or following oxygen activation by irradiation (Fig. 1.1). Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase from activated neutrophils generates $O_2^{\bullet-}$ in the respiratory burst necessary for bacteria destruction (Halliwell, 2006). Moreover, under oxidative stress conditions, xanthine oxidase (XOD) transfers electrons to oxygen and produces $O_2^{\bullet-}$ and hydrogen peroxide. Most of the $O_2^{\bullet-}$ generated in vivo undergoes a dismutation reaction catalysed by the antioxidative enzyme superoxide dismutase to give hydrogen peroxide. Despite the low reactivity of $O_2^{\bullet-}$, it is considered the "primary" ROS that interacts with other molecules to generate "secondary" ROS, either directly or prevalently through enzyme- or metal-catalysed processes. In the last case, it originates the production of the highly reactive hydroxyl radical.

The analytical methods for determination of $O_2^{\bullet-}$ scavenging capacity make use of the system XOD/hypoxanthine or xanthine at pH 7.4 to generate superoxide radical anion. To a minor extent, $O_2^{\bullet-}$ is also generated using a non-enzymatic reaction of phenazine methosulphate (PMS) in the presence of nicotinamide adenine dinucleotide (NADH). In both generation systems, $O_2^{\bullet-}$ may reduce nitroblue tetrazolium (NBT) into formazan, which is spectrophotometrically monitored at 560 nm (Aruoma, *et al.* 1993; Fernandes *et al.*, 2003; Floriano-Sanchez *et al.*, 2006). Antioxidant compounds compete with NBT for $O_2^{\bullet-}$ and decrease the rate of reaction. Another widely used probe for $O_2^{\bullet-}$ is cytochrome c. The kinetic analysis of reduction of ferricytochrome c to ferrocytochrome c was monitored at 550 nm (Aruoma, *et al.* 1993; Quick *et al.*, 2000). In fact, Aruoma *et al.* (1993) observed that inhibition of NBT reduction was generally greater than that of cytochrome c reduction. This is because $O_2^{\bullet-}$ reacts much faster with cytochrome c than it does with NBT, so a given concentration of added $O_2^{\bullet-}$ scavenger competes less efficiently in the cytochrome c system and exerts less inhibition. This is a clear example of the influence of the nature of the probe in the assessment of antioxidant capacity. Wang and Jiao (2000) used hydroxylammonium chloride, with consequent formation of nitrite that was determined

spectrophotometrically at 530 nm after addition of sulfanilic acid and α-naphthylamine (Griess reaction) (Elstner and Heupel, 1976). This assay was applied for determining the $O_2^{\bullet-}$ scavenging capacity of fruit juices and the results were expressed as μmol of α-tocopherol equivalent per 10 g of fresh weight. The scavenging capacity towards $O_2^{\bullet-}$, using XOD/hypoxanthine generating system, has also been measured by reaction with KMBA to produce ethylene, which is measured by gas chromatography (Lavelli *et al.*, 1999). The scavenging capacity against this radical can also be measured by using electron spin resonance (ESR) spectrometry (Calliste *et al.*, 2001). Here, the $O_2^{\bullet-}$ is trapped by 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), and the resultant DMPO-OOH adduct is detected by ESR.

The CL-based determination of $O_2^{\bullet-}$ scavenging capacity has also been described. Luminol or lucigenin are frequently applied as target compounds (Lu *et al.*, 2006), but neither of them is selective towards $O_2^{\bullet-}$ (Oosthuizen and Greyling, 1999; Li *et al.*, 1998). CLA (2-methyl-6-phenyl-3,7-dihydroimidazo[1-2-a]pyrazin-3-one) and MCLA [2-methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazo[1,2-a]pyrazin-3-one], analogs of coelenterazine, have also been described as more specific targets of $O_2^{\bullet-}$ but their application is more focused to in vivo monitoring of superoxide formation (Lu *et al.*, 2006). Ogawa *et al.* (1999) developed an HPLC system with indirect luminol-based CL for screening individual antioxidants present in extracts of green tea leaves (essentially catechins and flavones). The determination of antioxidants was based on the decrease of CL intensity derived from luminol and $O_2^{\bullet-}$ (generated from the hypoxanthine/XOD system). Epicatechin was detected as the main antioxidant among the various intrinsic substances present in green tea extracts. The $O_2^{\bullet-}$ scavenging capacity of some therapeutic compounds (NSAIDs, β-blockers) and medicinal plant extracts have also been measured by monitoring the $O_2^{\bullet-}$ induced lucigenin chemiluminescence (Costa *et al.*, 2006a; Gomes *et al.*, 2006; Abreu *et al.*, 2006; Chen and Yen, 2007).

All these methodologies may provide erroneous values if controls are not established for substances that inhibit or interfere with $O_2^{\bullet-}$ generation, or if the sample itself directly reduces NBT or ferricytochrome c, for instance. This is an important issue as far as ferricytochrome c is concerned, as it is easily reduced by ascorbic acid. Furthermore, the

reduced antioxidant formed by attack of $O_2^{\bullet-}$ could also reduce NBT or ferricytochrome c (Halliwell *et al.*, 1995). The nonfluorescent hydroethidine has also been used as the target/probe for measuring $O_2^{\bullet-}$ scavenging capacity (Zhao *et al.*, 2003). The target is oxidized by $O_2^{\bullet-}$ (generated from XOD/xanthine system) to form a species that exhibits a strong fluorescence signal (2-hydroxyethidium). This approach can circumvent the problem of direct reduction of the target/probe by antioxidants, but possible inhibition of xanthine oxidase by antioxidants/sample remains an issue.

1.2.3. Hydrogen peroxide (H_2O_2) scavenging capacity assays

Hydrogen peroxide (H_2O_2) is generated in vivo, under physiological conditions by peroxisomes, by several oxidative enzymes including glucose oxidase and D-amino acid oxidase, and by dismutation of superoxide radical, catalysed by superoxide dismutase. Additionally, H_2O_2 produced in the respiratory burst of activated phagocytes is known to play an important role in killing of several bacterial and fungal strains (Halliwell, 2006). Hydrogen peroxide is very diffusible within and between cells but it is rather inert at low concentrations and reacts quite slowly with most biological compounds. Nevertheless, its oxidant power is associated to the presence of transition metals ions, especially Fe(II) or Cu(I), generating the potent oxidant hydroxyl radical (Fig. 1.1).

Generally, the analytical methods for determination of H_2O_2 scavenging capacity employ peroxidase-based reactions. The most common assay employs horseradish peroxidase (HRP), which uses H_2O_2 to oxidize scopoletin into a non-fluorescent product (Corbett, 1989). In the presence of putative scavenger compounds, the oxidation of scopoletin is inhibited and the scavenging reaction can be fluorimetrically monitored. Another fluorimetric method widely used is based on homovanillic acid (HVA), whose fluorescent biphenyl dimer is more stable than scopoletin (Pázdzioch-Czochra and Widénska, 2002). The presence of substances with H_2O_2 scavenging capacity prevents the oxidation of homovanillic acid. However, these peroxidase-based approaches do not allow determining whether the antioxidant is reacting directly with H_2O_2, or reacting with intermediates

formed from enzyme and H_2O_2 (it is possible that the superoxide radical is produced during enzyme activity). Moreover, antioxidants such as ascorbic acid, quercetin dihydrate, and thiols can be a substrate for the peroxidase enzyme, introducing errors in the determination of scavenging capacity. Therefore, if the compound does interfere with peroxidase-based system, other assays for H_2O_2 should be used. For instance, the direct reaction of H_2O_2 and titanium(IV) was applied as it originates a complex Ti-H_2O_2 that is dissolved in acidic medium and further measured spectrophotometrically measured at 410 nm. This approach was applied to evaluate H_2O_2 scavenging capacity of fruit juices and results were expressed as μmol of ascorbate equivalent per 10 g of fresh weight (Wang and Jiao, 2000). A valid alternative to these methods was proposed by Arnous *et al.* (2002). The enzyme-free methodology implemented relied on the peroxyoxalate chemiluminescence (POCL) using 9,10-diphenylanthracene and imidazole as a fluorophore (probe) and catalyst, respectively. Briefly, POCL involves hydrogen peroxide imidazole-catalysed oxidation of an aryl oxalate ester yielding a high-energy intermediate (dioxetanedione) that transfers its energy to the fluorophore. The transition of the excited state of the fluorophore to its ground state causes the emission of light. Therefore, any compound with capacity to scavenge H_2O_2 would lead in CL inhibition. In this way, several antioxidants including β-carotene, α-tocopherol, butylated hydroxytoluene, quercetin, and L-ascorbic acid were investigated and the results were compared to other in vitro tests. Later, this assay was applied to investigate a wide range of natural antioxidants (cinnamic and benzoic acids) and to examine possible structure-H_2O_2 scavenging capacity relationships (Mansouri *et al.*, 2005). Due to the non-polar environment employed (ethyl acetate/acetonitrile (9:1)), this method was proposed to evaluate primarily the H_2O_2 scavenging capacity of lipophilic antioxidants. As a major shortcoming of this assay is the fluorophore employed (9,10-diphenylanthracene), which is suspected to be a carcinogen. Costa *et al.* (2005) developed a microplate H_2O_2-chemiluminescence based assay. The H_2O_2 induced the oxidation of lucigenin and the scavenging capacity was measured as the percentage inhibition of the intensity of the CL signal. This method was applied to evaluate the scavenging capacity of several non-steroidal anti-inflammatory drugs (NSAIDs).

1.2.4. Hydroxyl radical ($HO^{\bullet}$) scavenging capacity assays

The hydroxyl radical ($HO^{\bullet}$) is extremely reactive in vivo (rate constants > 10^9 M^{-1} s^{-1}) and it can hydroxylate any molecule found in the food matrix or in living cells (including proteins, polyunsaturated fatty acids, sugars, and nucleic acids). Biologically, the $HO^{\bullet}$ radical can be generated by several mechanisms: i) homolytic fission of oxygen-hydrogen bonds in water driven by continuous exposure to background ionizing radiation; ii) reaction between Fe^{2+} released under stress conditions and H_2O_2 (eq. *1.8*, Fenton reaction); iii) Haber-Weiss reaction involving superoxide radical (eq. *1.9*); and iv) reaction of HOCl with $O_2^{\bullet-}$ (Halliwell, 2006).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^{\bullet} + HO^{-} \qquad (1.8)$$

$$O_2^{\bullet-} + H_2O_2 \rightarrow O_2 + HO^{\bullet} + HO^{-} \qquad (1.9)$$

Due to the high reactivity of hydroxyl radicals, almost anything in biological systems can be regarded as an $HO^{\bullet}$ scavenger. Hence, this task is not performed by any specific molecule or enzyme. Thus, the evaluation of direct scavenging of $HO^{\bullet}$ may be irrelevant for evaluation of antioxidant action of a compound or matrix, simply because very high concentrations of scavenger are required to compete with adjacent molecules in vivo or in the food matrix for any $HO^{\bullet}$ generated. For these reason, it is more relevant and useful to quantify the capacity of putative antioxidants to scavenge or block the formation of its precursors ($O_2^{\bullet-}$, H_2O_2, HOCl) and/or to sequester free metal ions related to $HO^{\bullet}$ formation. Scavenger compounds that act in this way would behave as preventive antioxidants.

Despite this remark, several in vitro methodologies for determination of $HO^{\bullet}$ scavenging capacity are available, mostly based on Fe^{3+} + EDTA + H_2O_2 + ascorbic acid system to generate a constant flux of $HO^{\bullet}$ radicals. Those radicals attack the sugar 2-deoxy-D-ribose (used as target), degrading it into a series of fragments, some or all of which react upon heating with thiobarbituric acid at low pH to give a pink chromogen (Halliwell *et al.*, 1987). If a $HO^{\bullet}$ scavenger is added to the reaction mixture, it will compete with

deoxyribose for $HO^{\bullet}$ radicals, inhibiting the degradation of the target species. It should be stressed that the substance(s) under test may interfere with the generation system of hydroxyl radicals. Thus, compounds may inhibit the $HO^{\bullet}$ generation by reacting directly with H_2O_2 or by chelating the metal ion. In this way, the performance of the deoxyribose assay without EDTA allows the identification of compounds which chelate metal ions (Hagerman *et al.*, 1998). In this case, iron(III) ions are chelated by deoxyribose causing "site specific" hydroxyl radical damage, and when the test substances are iron-chelating agents the hydroxyl radical damage of deoxyribose is inhibited. On the other hand, compounds (such as ascorbic acid) can reduce Fe^{3+} to Fe^{2+} enhancing the generation of hydroxyl radicals and acting as pro-oxidant agents. Indeed, Hagerman *et al.* (1998) also modified the deoxyribose method by omitting ascorbic acid to evaluate the potential of certain tannins to behave as pro-oxidants.

Zhu *et al.* (2000) reported a metal-independent, organic Fenton reaction. The mixture of tetrachlorohydroquinone (TCHQ) and H_2O_2 hydroxylates salicylic acid and this process is inhibited by $HO^{\bullet}$ scavenging agents. The oxidation reaction was not affected by several iron chelators. Thus, this metal-free TCHQ/H_2O_2 system provides the generation of $HO^{\bullet}$ with less redox species involved, which makes it more specific for evaluating the $HO^{\bullet}$ scavenging capacity. Ou *et al.* (2002a) developed a fluorimetric assay to evaluate "hydroxyl radical prevention capacity" using fluorescein as the target/probe. In this assay, $HO^{\bullet}$ radical is generated by a Co^{2+}–mediated Fenton-like reaction and the $HO^{\bullet}$ scavenging capacity is mainly due to the metal-chelating capability of the compounds. The quantification approach is the same as that of the ORAC assay except that gallic acid is used as the reference standard compound. This assay has been recently applied to evaluate and compare the antioxidant properties of pomegranate peel extract with pomegranate pulp extract (Li *et al.*, 2006) and the antioxidant properties of aqueous methanolic extracts of different barley cultivars (Madhujith and Shahidi, 2007).

The CL-based determination of scavenging capacity against $HO^{\bullet}$ using luminol has also been described (Hirayama and Yida, 1997; Yildiz and Demiryurek, 1998a). In this case, $HO^{\bullet}$ is generated from the reaction between ferrous iron with molecular oxygen, which induces luminol CL. However, other ROS, including $O_2^{\bullet-}$ and H_2O_2 are generated at the

same time, so it is difficult to detect $HO^{\bullet}$ specifically with this system. The $HO^{\bullet}$ scavenging capacity of three standard antioxidants, ascorbic acid, quercetin, and trolox, were evaluated using the Co(II)/EDTA-induced luminol chemiluminescence (Parejo *et al.*, 2000). This CL assay was further applied to investigate the radical scavenging capacity of different plant extracts (Parejo *et al.*, 2003).

1.2.5. Hypochlorous acid (HOCl) scavenging capacity assays

Stimulated polymorphonuclear leukocytes contain and secrete the enzyme myeloperoxidase, which catalyzes the oxidation of chloride ions by H_2O_2 into the powerful nonspecific chlorinating and oxidizing agent, hypochlorous acid (HOCl) (Weiss, 1989). Production of HOCl contributes to the mechanism of defense against foreign micro-organisms; nevertheless HOCl may also attacks amines and sulphydryl groups of several biomolecules causing damage to the host cell structures (Halliwell, 2006). One of the most important targets attacked by HOCl in vivo is α_1-antiproteinase (α_1-AP), which is the major proteolytic inhibitor in body fluids, protecting cells against attack by proteinases such as elastase.

In the analytical methods for in vitro determination of HOCl scavengers, this oxidant is obtained from the enzymatic system myeloperoxidase/H_2O_2/Cl^- or by acidifying commercial sodium hypochlorite to pH 6.2 with sulphuric acid (Aruoma, 1997). The former approach can be applied if the sample species do not interfere with HOCl generation (e.g. inhibition of myeloperoxidase activity or direct reaction with H_2O_2). In the second, the determination of the concentration of HOCl solution must be performed daily (Halliwell *et al.*, 1995).

The elastase assay (Haenen and Bast, 1991), measures the ability of a compound to protect the α_1-AP against inactivation by HOCl. This is assessed after addition of elastase, which is inactivated by any remaining α_1-AP. The elastase activity is subsequently measured using an elastase substrate (*N*-t-BOC-L-alanine *p*-nitrophenol ester) and monitoring the absorbance increase at 410 nm. In this assay, the different target (α_1-AP) and probe

(elastase) species occur in vivo. Furthermore, the analytical process also mimics an in vivo situation and therefore the HOCl scavenging capacity of compounds/samples under study occurs at biologically significant conditions. Nevertheless, this method has been criticized to be time-consuming and compounds which have inhibitory effects on the activities of either enzyme could falsely be interpreted to be HOCl scavengers (Halliwell *et al.*, 1995). Later, the same research group developed a method in which the compound 5-thio-2-nitrobenzoic acid (TNB) was oxidized by HOCl into 5,5-dithiobis(2-nitrobenzoic acid) (DTNB). The absorbance decrease at 412 nm is inhibited by putative HOCl scavenger compounds (Ching *et al.*, 1994; Fernandes *et al.*, 2003). However, it was observed that compounds containing free thiol groups, such as dihydrolipoic acid, cysteine, and glutathione, interfered in this method, yielding an excess of TNB. To overcome these limitations, Yan *et al.* (1996) developed a novel application of the protein carbonyl assay. The method is based on the observation that bovine serum albumin (BSA) carbonyl content is increased upon oxidation by HOCl, and that this increase is inhibited in the presence of HOCl scavengers. In the assay developed by Gatto *et al*. (2002), scavenger compounds inhibit the HOCl-oxidation of human serum albumin. These effects were evaluated by reversed-phase HPLC with spectrophotometric detection at 280 nm. A fluorimetric competition assay based on para-aminobenzoic acid chlorination was developed to determine the HOCl scavenging rate constants and applied to some non-steroidal anti-inflammatory drugs (Antwerpen *et al.*, 2004). The specificity of the system was improved by chromatographic separation of the drugs and the oxidation products. Finally, methods based on luminol-elicited CL have also been proposed, where HOCl scavengers promote the decrease of analytical signal (Yildiz *et al.*, 1998b; Mouithys-Mickalad *et al.*, 2000; Costa *et al.*, 2006a).

1.2.6. Singlet oxygen ($^1O_2$) scavenging capacity assays

Singlet oxygen ($^1O_2$) is an excited state of molecular oxygen that has no unpaired electrons and is therefore not classified as a radical. It is known to be a powerful oxidizing agent,

reacting directly with a wide range of biomolecules that are unreactive with the ground-state of oxygen molecule, such as proteins (Davies, 2004). The formation of $^1O_2$ in vivo occurs under the presence of light and photosensitizers and it has been linked to UV light damage to skin and to cataract formation of the eye lens (Baier *et al.*, 2007). Its generation in the absence of light is thought to be the result of the spontaneous dismutation of the superoxide anion and to occur during peroxidase-catalyzed reactions. $^1O_2$ is also formed after the reaction of hypochlorite with hydrogen peroxide (eq. *1.10*). In addition, eosinophil peroxidase oxidizes Br^- generating hypobromous acid (HOBr), and OBr^- also reacts with H_2O_2 yielding singlet oxygen (Halliwell, 2006).

$$OCl^- + H_2O_2 \rightarrow Cl^- + H_2O + {}^1O_2 \qquad (1.10)$$

Due to its decay to the lower energy ground state, $^1O_2$ emits characteristic phosphorescence at 1270 nm. Wilkinson *et al.* (1995) measured the $^1O_2$ scavenging ability of several compounds through the decay rates of the light intensity. Nevertheless, the intensity of luminescence based on the self-emission of $^1O_2$ is often insufficient to provide reproducible quantitative information, even in aqueous medium. A more sensitivity method based on monitoring the scavenging of singlet oxygen delayed fluorescence of tetra-*tert*-butylphthalocyanine was developed (Fu *et al.*, 1997). The assay was applied to compounds which are well-documented to be singlet oxygen quenchers such as beta-carotene, α-tocopherol, and lauric acid. This method is not widely applied but it is useful for measurement of rate constants for $^1O_2$ quenching, requiring commonly available equipment and also applicable to systems where the 1270 nm luminescence is difficult to detect.

Recently, a fluorescence-based microplate screening assay for evaluating $^1O_2$ scavenging activity was developed by Costa *et al.* (2007). The $^1O_2$, selectively generated by the thermal decomposition of the endoperoxide disodium 3,3'-(1,4-naphthalene)bispropionate ($NDPO_2$), oxidize the highly sensitive probe dihydrorhodamine 123 (DHR) to the fluorescent form rhodamine 123. The assay was successfully applied for screening scavenging activity of several recognised antioxidant compounds against $^1O_2$.

1.2.7. Nitric oxide radical ($NO^{\bullet}$) scavenging capacity assays

In 1992, due to its extraordinary in vivo properties, nitric oxide radical ($NO^{\bullet}$) was acclaimed as the "molecule of the year" in the *Science Magazine* (Koshland, 1992). The $NO^{\bullet}$, which is biologically synthesized through the conversion of L-arginine to L-citrulline by a group of enzymes called nitric oxide synthases (NOSs), has a pivotal role in the regulation of diverse physiological and pathophysiological processes (Pacher *et al.*, 2007). Three distinct NOS enzymes have been identified and characterized, namely NOS neuronal (nNOS), NOS inducible (iNOS), and NOS endothelial (eNOS). The production of $NO^{\bullet}$ at physiological levels (at the nanomolar range) is involved in the homeostatic biochemical processes such as signal transduction, neurotransmission, smooth muscle relaxation, peristalsis, inhibition of platelet aggregation, blood pressure modulation, immune system control, learning, and memory (Moncada *et al.*, 1991). In contrast, under pathophysiological conditions as endotoxin shock and chronic inflammation, the iNOS enzymes present in immune-competent cells may lead to the continuous production of micromolar amounts of $NO^{\bullet}$ (1–10 μM). The reactivity/toxicity of $NO^{\bullet}$ is enhanced by oxygen due to the production of reactive intermediates, including nitrogen dioxide radical ($NO_2^{\bullet}$) and dinitrogen trioxide (N_2O_3) which exert many damaging effects on lipids, proteins and nucleic acids. Moreover, the sustained release of $NO^{\bullet}$ yield an overproduction of peroxynitrite, after reaction with superoxide anion radicals. For this reason, analytical methods that allow the determination of $NO^{\bullet}$ scavenging capacity of pure compounds/samples are of great interest to prevent these harmful oxidative reactions.

Vriesman *et al.* (1997) developed a relatively simple method for the quantification of $NO^{\bullet}$ scavenging capacity of sulfur-containing compounds in aqueous solution using an amperometric $NO^{\bullet}$ sensor. $NO^{\bullet}$ is added to buffered solutions of the scavenger (glutathione, glutathione disulfide, S-methyl glutathione, N-acetyl cysteine, lipoic acid and dihydrolipoic acid) and its concentration is followed as a function of time. The natural logarithm of the $NO^{\bullet}$ concentration and time are linearly related. After correction for the spontaneous degradation of $NO^{\bullet}$, second-order rate kinetics of the scavenging reaction were determined. They observed that only those compounds which contained a thiol group

displayed a considerable $NO^{\bullet}$ scavenging capacity. In this method there is a non-competitive reaction mechanism, since only the reactive species ($NO^{\bullet}$) and the scavenger molecule(s) are present in the reaction medium.

The assessment of $NO^{\bullet}$ scavenging capacity has also been performed using ESR spectrometry (Nishibayashi *et al.*, 1996; Asanuma *et al.*, 2001). The $NO^{\bullet}$ generated from the donor 3-(2-hydroxy-1-methyl-ethyl-2-nitrosohydrazino)-*N*-methyl-1-propanamine (NOC-7) was oxidized to NO_2 by the target 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide, named as carboxy-PTIO with the formation of carboxy-PTI spin adduct, that was measured by ESR. The method was applied to non-steroidal anti-inflammatory drugs and a semi-competitive scheme was adopted as the drug was incubated with the $NO^{\bullet}$ generator for 30 minutes, prior to the addition of carboxy-PTIO. The main limitation of this method, considering also the detection technique that is not easily available, is the long reaction time (120 min). Recently, Peréz *et al.* (2007) used a non-competitive reaction method for evaluation of the $NO^{\bullet}$ scavenging capacity of eight NSAIDs, using spermine NONOate as the $NO^{\bullet}$ donor in the absence (control) or presence of scavenger sample. The amount of remaining nitric oxide was measured through the quantification of the total accumulated $NO^{\bullet}$ oxidation products using the Griess reaction. For this, nitrate was converted to nitrite using NADH-dependent nitrate reductase. The interference of NADH was eliminated by addition of lactate dehydrogenase and pyruvate. The chromophoric azo derivative formed from nitrite is then measured spectrophotometrically at 540 nm. Standard curves were generated using sodium nitrite and results were expressed as percentage change from control response. Compared to other methods, this methodology is not straightforward, requiring the addition of several enzymatic reagents.

The fluorescent target/probe 4,5-diaminofluorescein (DAF-2), widely used for in vivo $NO^{\bullet}$ detection and imaging, has also been applied for screening of $NO^{\bullet}$ scavenging capacity (Nagata *et al.*, 1999). In this assay, DAF-2 does not react directly with $NO^{\bullet}$, but react with an active intermediate formed in the course of the auto-oxidation of $NO^{\bullet}$, probably dinitrogen trioxide, obtained by the following reactions (eq. *1.11*, *1.12*):

$$2\ NO^{\bullet} + O_2 \rightarrow 2\ NO_2 \qquad (1.11)$$

$$NO_2 + NO^{\bullet} \leftrightarrow N_2O_3 \qquad (1.12)$$

The nitrosation of the weak fluorescent target/probe DAF-2 by derivative $NO^{\bullet}$ species yield the formation of the strong green-fluorescent triazolofluorescein product (DAF-2T, Fig. 1.6). The fluorescence quantum efficiency is increased more than 100 times after transformation into the triazole form.

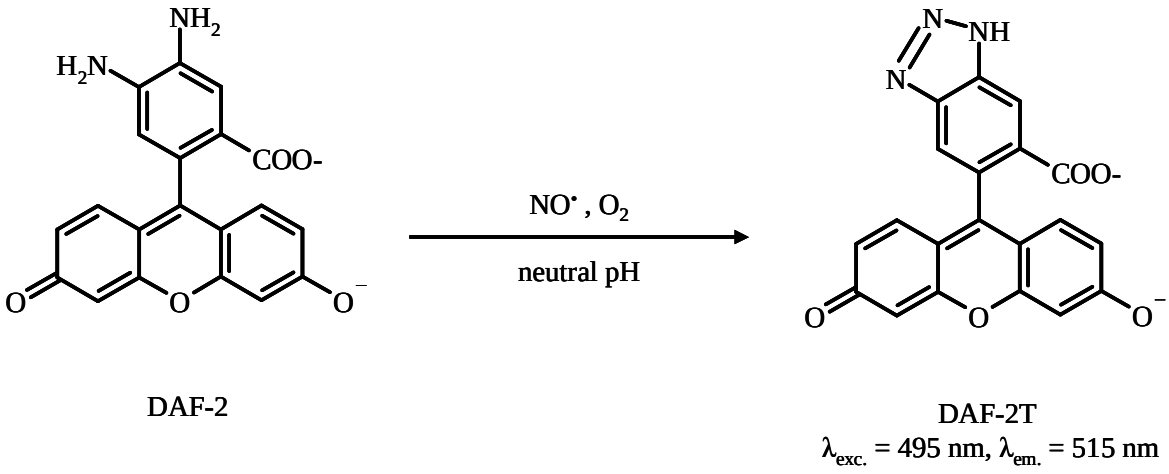


Figure 1.6. Formation of the highly fluorescent compound triazolofluorescein (DAF-2T) from 4,5-diaminofluorescein (DAF-2) and $NO^{\bullet}$ in the presence of O_2.

Recently, this assay was adapted to 96-well microplate reader to examine the $NO^{\bullet}$ scavenging capacity of pharmaceutical β-blockers compounds (Gomes *et al.*, 2006). The $NO^{\bullet}$ scavenging capacity was determined by the ability of compounds to prevent the $NO^{\bullet}$–induced nitrosation of DAF-2 and results are expressed as the percentage of inhibition of the DAF-2 oxidation as a function of concentration of the scavenger compound. Nevertheless, the results obtained using DAF-2 as a target/probe for $NO^{\bullet}$ quantification

should be interpreted with caution, since some antioxidant compounds (dehydroascorbic and ascorbic acid) may react directly with DAF-2 and form fluorescent compounds with emission spectra similar to that of DAF-2T (Zhang *et al.*, 2002).

1.2.8. Peroxynitrite ($ONOO^-$) scavenging capacity assays

Cells of the immune system produce both the superoxide anion and nitric oxide radical during the oxidative burst triggered by pathologic processes (Halliwell, 2006). Under these conditions, they react ($k > 10^9\ M^{-1}\ s^{-1}$, eq. *1.13*) to produce significant amounts of peroxynitrite anion. Under physiological pH, the ratio of peroxynitrite and peroxynitrous acid (ONOOH), a very strong oxidant, is 4 to 1.

$$O_2^{\bullet -} + NO^{\bullet} \rightarrow ONOO^- \qquad (1.13)$$

The main route of damage caused by these species is the nitration of aromatic amino acids, particularly tyrosine, yielding the formation of the biomarker 3-nitrotyrosine. Moreover, $ONOO^-$ can also react with carbon dioxide present in the body fluids with the formation of an adduct, nitrosoperoxycarbonate anion ($ONOOCO_2^-$) (Squadrito and Pryor, 2002). This adduct may decompose to carbon dioxide and nitrate (about 70%) and also to free radicals as nitrogen dioxide radical ($NO_2^{\bullet}$) and carbonate radical anion ($CO_3^{\bullet -}$) (about 30%). Taking into account the high carbon dioxide concentration in physiological conditions, it is probable that most of $ONOO^-$ damage effects in vivo are mediated by these reactive intermediates.

Methodologies for measuring the $ONOO^-$ scavenging capacity usually depend either on tyrosine nitration (Pannala *et al.*, 1997) or on dihydrorhodamine 123 (DHR) (Kooy *et al.*, 1994). The first method is based on the formation of 3-nitrotyrosine, that is detected spectrophotometrically along with the unreacted tyrosine after HPLC separation. The concentration of 3-nitrotyrosine is measured after incubation of tyrosine with varying concentrations of putative $ONOO^-$ scavengers and the inhibition percentage of 3-

nitrotyrosine formation is determined. In the second method, the non-fluorescent DHR is oxidized by peroxynitrite to the fluorescent rhodamine 123. In the presence of $ONOO^-$ scavengers the fluorescence intensity is lower than that of the control and the inhibition percentage of DHR oxidation is assessed. This assay has been adapted to a microplate reader to determine the $ONOO^-$ scavenging capacity of arylpropionic acid NSAIDs (Costa *et al.*, 2006b), β-blockers compounds (Gomes *et al.*, 2006), herb extracts (Choi *et al.*, 2002) and medicinal tincture from *Pedilanthus tithymaloides* (Abreu *et al.*, 2006). Luminol-enhanced chemiluminescence has also been used to estimate the $ONOO^-$ scavenging capacity (Yildiz *et al.*, 1998b).

Table 1.2. Summary of analytical features of some in vitro scavenging capacity assays against specific ROS/RNS.

Reactive species	Assay	Target compound	Detection probe	Principle of measurement	Quantification	Ref.
Peroxyl radical ($ROO^{\bullet}$)	ORAC	β-PE	β-PE	Fluorescence decay along time due to oxidation of probe is delayed/inhibited by antioxidants	Net AUC expressed as trolox equivalents	Cao *et al.*, 1993
		BODIPY 581/591	BODIPY 581/591			Naguib, 1998
		Fluorescein	Fluorescein			Ou *et al.*, 2001
	TRAP	Human plasma	Oxygen	Oxygen consumption due to oxidation of plasma material is delayed by antioxidants	Lag time expressed as trolox equivalents	Wayner *et al.*, 1985
		β-PE	β-PE	Fluorescence decay along time due to oxidation of β-PE is delayed by antioxidants		DeLange and Glazer, 1989
		DCFH-DA	DCF	Fluorescence increase along time due to formation of DCF from oxidation of DCFH-DA is delayed by antioxidants		Valkonen and Kussi, 1997
	Crocin bleaching	Crocin	Crocin	Absorbance decrease along time due to oxidation of crocin is inhibited by antioxidants	Ratio of initial crocin bleaching rates in absence and in presence of test sample	Bors *et al.*, 1984
		Crocin	Crocin	Absorbance decrease due to oxidation of crocin is inhibited by antioxidants and measured after a fixed time period	Inhibition percentage	Lussignoli *et al.*, 1999

AUC, area under the curve; BODIPY 581/591, 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-*s*-indacene-3-undecanoic acid; DCF, dichlorofluorescein; DCFH-DA, dichlorofluorescein-diacetate; ORAC, oxygen radical absorbance capacity; β-PE, β-phycoerythrin; TRAP, total radical-trapping antioxidant parameter.

Table 1.2. Summary of analytical features of some in vitro scavenging capacity assays against specific ROS/RNS (continuation).

Reactive species	Assay	Target compound	Detection probe	Principle of measurement	Quantification	Ref.
Peroxyl radical ($ROO^{\bullet}$)	LDL oxidation	LDL	Conjugated dienes	Absorbance increase due to formation of conjugated dienes from oxidation of LDL is inhibited by antioxidants	Lag time	Handelman *et al.*, 1999
	TOSC	KMBA	Ethylene	Ethylene, formed due to oxidation of KMBA, is measured along time using GC-FID	Relative AUC	Winston *et al.*, 1998
	CL-based	Luminol	Luminol (oxidized)	CL emission due to oxidation of luminol is inhibited by antioxidants	Lag time expressed as trolox equivalents	Lissi *et al.*, 1992

AUC, area under curve; CL, chemiluminescence; GC-FID, gas chromatography with flame ionization detector; KMBA, α-keto-γ-methiol-butyric acid; LDL, low-density lipoproteins; TOSC, total oxyradical scavenging capacity.

Table 1.2. Summary of analytical features of some in vitro scavenging capacity assays against specific ROS/RNS (continuation).

Reactive species	Target compound	Detection probe	Principle of measurement	Quantification	Ref.
Superoxide radical anion ($O_2^{\bullet-}$)	n.a.	Formazan	Absorbance increase along time due to formation of formazan from reduction of NBT is inhibited by antioxidants	Inhibition percentage	Aruoma *et al.*, 1993
	n.a.	Ferrocytochrome c	Absorbance increase along time due to formation of ferrocytochrome c from reduction of ferricytochrome c is inhibited by antioxidants	Inhibition percentage	Aruoma *et al.*, 1993
	n.a.	Nitrite	Absorbance increase after nitrite derivatisation using Griess reaction	Inhibition percentage expressed as α-tocopherol equivalents	Wang and Jiao, 2000
	KMBA	Ethylene	Ethylene, formed due to oxidation of KMBA, is measured along time using GC-FID and its formation is inhibited by antioxidants	Inhibition percentage expressed as trolox equivalents	Lavelli *et al.*, 1999
	DMPO	DMPO-OOH	Formation of DMPO-OOH adduct is measured by ESR and it is inhibited by antioxidants	Relative inhibition	Calliste *et al.*, 2001
	Hydroethidine	2-hydroxyethidium	Fluorescence increase due to oxidation of hydroethidine	Direct quantification of superoxide	Zhao *et al.*, 2003
	Luminol	Luminol (oxidized)	CL emission due to oxidation of luminol is inhibited by antioxidants	Inhibition percentage	Oosthuizen and Greyling, 1999
	Lucigenin	Lucigenin (oxidized)	CL emission due to oxidation of lucigenin is inhibited by antioxidants	Inhibition percentage	Oosthuizen and Greyling, 1999

CL, chemiluminescence; DMPO, 5,5-dimethyl-1-pyrroline-N-oxide; ESR, electron spin resonance; GC-FID, gas chromatography with flame ionization detector; KMBA, α-keto-γ-methiolbutyric acid; NBT, nitroblue tetrazolium.

Table 1.2. Summary of analytical features of some in vitro scavenging capacity assays against specific ROS/RNS (continuation).

Reactive species	Target compound	Detection probe	Principle of measurement	Quantification	Ref.
Hydrogen peroxide (H_2O_2)	Scopoletin	Scopoletin	Fluorescence decay along time due to oxidation of scopoletin is inhibited by antioxidants	Inhibition percentage expressed as trolox equivalents	Corbett, 1989
	HVA	Biphenyl dimer of HVA	Fluorescence increase due to oxidation of HVA is inhibited by antioxidants and measured after a fixed time period	Inhibition percentage	Pazdzioch-Czochra and Widénska, 2002
	n.a.	Ti-H_2O_2 complex	Absorbance increase due to formation of Ti-H_2O_2 complex is inhibited by antioxidants and measured after a fixed time period	Inhibition percentage expressed as ascorbate equivalents	Wang and Jiao, 2000
	Aryl oxalate ester	9,10-Diphenylanthracene	CL emission due to fluorophore excitation is inhibited by antioxidants	Reciprocal of inhibition ratio	Arnous *et al.*, 2002
	Lucigenin	Lucigenin (oxidized)	CL emission due to oxidation of lucigenin is inhibited by antioxidants	Inhibition percentage	Costa *et al.*, 2005
Hydroxyl radical ($HO^{\bullet}$)	Deoxyribose	Pink chromogen (TBARS)	Absorbance increase due to reaction of thiobarbituric acid with oxidation products from deoxyribose is inhibited by antioxidants and measured after a fixed time period	Inhibition percentage	Halliwell *et al.*, 1987
	Salicylic acid	2,3-DHBA and 2,5-DHBA	DHBA, formed due to oxidation of salicylic acid, is measured using HPLC-ED and its formation is inhibited by antioxidants	Quantity of DHBA produced	Zhu *et al.*, 2000
	Fluorescein	Fluorescein	Fluorescence decay along time due to oxidation of fluorescein is inhibited by antioxidants	Net AUC expressed as gallic acid equivalents	Ou *et al.*, 2002a
	Luminol	Luminol (oxidized)	CL emission due to oxidation of luminol is inhibited by antioxidants	Inhibition percentage	Yildiz and Demiryurek, 1998a

AUC, area under curve; CL, chemiluminescence; DHBA, dihydroxybenzoic acid; HPLC-ED, high performance liquid chromatography with electrochemical detection; HVA, homovanillic acid; TBARS, thiobarbituric acid reactive substances.

Table 1.2. Summary of analytical features of some in vitro scavenging capacity assays against specific ROS/RNS (continuation).

Reactive species	Target compound	Detection probe	Principle of measurement	Quantification	Ref.
Hypochlorous acid (HOCl)	α_1-AP	Elastase substrate	Absorbance increase due to activity of non-inhibited elastase, using a colour forming substrate	Elastase activity as ΔA_{410} min^{-1}	Haenen and Bast, 1991
	TNB	TNB	Absorbance decrease due to oxidation of TNB to DTNB is inhibited by antioxidants and measured after a fixed time period	Inhibition of percentage	Ching *et al.*, 1994
	BSA	BSA (carbonyl groups)	The carbonyl content of BSA increases due to oxidation by HOCl and it is inhibited by antioxidants	Inhibition of percentage	Yan *et al.*, 1996
	PABA	PABA	Fluorescence decay along time due to oxidation of PABA is inhibited by antioxidants	Rate constants of reaction between PABA and HOCl	Antwerpen *et al.*, 2004
	Luminol	Luminol (oxidized)	CL emission due to oxidation of luminol is inhibited by antioxidants	Inhibition percentage	Yildiz *et al.*, 1998b
Singlet oxygen ($^1O_2$)	n.a.	$^1O_2$	Intensity of luminescence from self-emission of $^1O_2$ decreases in the presence of antioxidants	Rate constants of luminescence quenching	Wilkinson *et al.*, 1995
	Tetra-tert-butylphthalocyanine	Tetra-tert-butylphthalocyanine	Fluorescence increase along time due to tetra-tert-butylphthalocyanine excitation is delayed/inhibited by antioxidants	Rate constants of luminescence quenching	Fu *et al.*, 1997
	DHR 123	Rhodamine 123	Fluorescence increase due to oxidation of DHR 123 is inhibited by antioxidants	Inhibition percentage	Costa *et al.*, 2007
	Luminol	Luminol (oxidized)	CL emission due to oxidation of luminol is inhibited by antioxidants	Inhibition percentage	Oosthuizen and Greyling, 1999

α_1-AP, α_1-antiproteinase; BSA, bovine serum albumin; CL, chemiluminescence; DHR, dihydrorhodamine; DTNB, 5,5-dithiobis(2-nitrobenzoic acid); PABA, p-aminobenzoic acid; TNB, 5-thio-2-nitrobenzoic acid.

Table 1.2. Summary of analytical features of some in vitro scavenging capacity assays against specific ROS/RNS (continuation).

Reactive species	Target compound	Detection probe	Principle of measurement	Quantification	Ref.
Nitric oxide radical ($NO^{\bullet}$)	n.a.	$NO^{\bullet}$	$NO^{\bullet}$ concentration is measured amperometrically along time and the rate of its disappearance is increased by antioxidants	Second-order rate constant of $NO^{\bullet}$ scavenging	Vriesman *et al.*, 1997
	n.a.	Carboxy-PTI spin adduct	Formation of carboxy-PTI spin adduct is measured by ESR and it is inhibited by antioxidants	Inhibition percentage	Asanuma *et al.*, 2001
	DAF-2	DAF-2T	Fluorescence increase due to oxidation of DAF-2 is inhibited by antioxidants	Inhibition percentage	Nagata *et al.*, 1999
Peroxynitrite ($ONOO^{-}$)	Tyrosine	3-Nitrotyrosine	3-Nitrotyrosine, formed due to oxidation of tyrosine, is measured using HPLC-UV/Vis and it its formation is inhibited by antioxidants	Inhibition percentage	Pannala *et al.*, 1997
	DHR 123	Rhodamine 123	Fluorescence increase due to oxidation of DHR 123 is inhibited by antioxidants	Inhibition percentage	Kooy *et al.*, 1994
	Luminol	Luminol (oxidized)	CL emission due to oxidation of luminol is inhibited by antioxidants	Inhibition percentage	Yildiz *et al.*, 1998b

Carboxy-PTI, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl; CL, chemiluminescence; DAF-2, 4,5-diaminofluorescein; DAF-2T, triazolofluorescein; DHR, dihydrorhodamine; ESR, electron spin resonance.

1.3. Scavenging capacity assays against stable, non-biological radicals and evaluation of total reduction capacity

These antioxidant capacity assays rely on electron transfer (ET) reactions that measure the capacity of a putative antioxidant compound(s) to reduce an oxidant, which is a stable radical or metal ion. The oxidant is also the probe for monitoring the reaction and its reduction is accompanied by a change on its absorption spectra at the visible region. Therefore, degree of color change is proportional to the antioxidant/reducing capacity. Generally, these assays are based on a non-competitive reaction scheme (Fig 1.2).

The reactivity in ET-methods is based primarily on ionization potential and on deprotonation of the reactive functional group in a particular pH milieu (Prior *et al.*, 2005). In general, the ionization potential decreases with increasing pH, reflecting increased electron-donating capacity with deprotonation. Thus, in acidic conditions the reducing capacity may be suppressed due to protonation of functional groups of antioxidant compounds, whereas in basic conditions, proton dissociation would enhance the reducing capacity of the sample (Foti *et al.*, 2004). For this reason, the pH values of ET-assays have an important effect on the reducing capacity of the test compounds/samples. These reactions are usually slow, requiring a long time to reach completion and results can vary tremendously upon the time scale of analysis. Moreover, the presence of trace contaminants, metals in particular, has been accounted for high variability of results (Prior *et al.*, 2005).

In these assays, it may be questionable whether the results obtained give quantitative information of the antioxidant capacity of a sample, since not all reductants that are able to reduce the oxidant (probe) are physiologically antioxidants (Prior and Cao, 1999). Therefore, to make the assumption that the reducing capacity of a sample is a global/comprehensive parameter, reflecting one aspect of its antioxidation property, the data obtained with ET-based methods should be regularly correlated with the data obtained by HAT-methods (Roginsky and Lissi, 2005).

In the following sections, the widely used ET-based assays designated trolox equivalent antioxidant capacity (TEAC) assay, 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) assay, ferric

reducing antioxidant power (FRAP) assay, and Folin-Ciocalteu (FC) assay, will be critically discussed. The total reducing capacity estimated by electrochemical methods will also be mentioned. The analytical features of these assays are summarized in the Table 1.3 (page. 55).

1.3.1. Scavenging of 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonate) radical cation ($ABTS^{\bullet+}$) or Trolox equivalent antioxidant capacity (TEAC) assay

The TEAC assay involves the generation of the long-lived radical cation chromophore 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonate) ($ABTS^{\bullet+}$) which has absorption maxima at 414, 645, 734, and 815 nm. The original TEAC assay, developed by Miller *et al.* (1993), was based on the activation of metmyoglobin, acting as peroxidase, with H_2O_2 to generate ferrylmyoglobin radical, which then reacted with ABTS to form the $ABTS^{\bullet+}$ radical cation. In this strategy, the sample to be tested is added previously to the formation of the $ABTS^{\bullet+}$. The test compounds/samples reduce the $ABTS^{\bullet+}$ radicals formed and the lag phase, which corresponds to the delay time in radical formation, is measured. This method has been commercialized by Randox Laboratories (San Francisco, USA) as the world's first kit for the standardization of total antioxidant status measurement in an individual's serum or plasma. Nevertheless, this commercial kit for the TEAC assay is expensive; the reagent cost per sample estimated in the Randox-TEAC assay is approximately nine times that in the ORAC assay (Cao and Prior, 1998).

The order of addition of reagents and sample was then criticized as a major pitfall, because the antioxidants (quercetin, for instance) can react with H_2O_2 and/or with derivate oxidizing species that inhibit the $ABTS^{\bullet+}$ radical formation and that lead to overestimation of antioxidant capacity (Strube *et al.*, 1997). Therefore, a post-addition assay or decolorization strategy was proposed to prevent the interference of antioxidant compounds with radical formation, making the assay more reliable and less susceptible to artefacts. In this case, the sample to be tested was added after generation of a certain amount of $ABTS^{\bullet+}$

radical cation and the remaining $ABTS^{\bullet+}$ concentration after reaction with antioxidant compound/sample was then quantified (Re *et al.*, 1999).

In terms of assay conditions, different strategies have been implemented for $ABTS^{\bullet+}$ generation (Fig 1.7), reaction time applied, detection wavelength used for monitoring the reaction, and the reference antioxidant chosen. $ABTS^{\bullet+}$ radical cation can be generated by chemical reaction using manganese dioxide (Miller *et al.*, 1996), AAPH (Van den Berg *et al.*, 1999), or potassium persulfate (Re *et al.*, 1999), by enzymatic reaction using metmyoglobin (Miller *et al.*, 1993) or horseradish peroxidase (Cano *et al.*, 1998), or by electrochemical generation (Alonso *et al.*, 2002). Reaction times ranging from 1 to 30 min have been adopted throughout the works described in the literature. Concerning the wavelength of detection, the determination at 734 nm is preferred because the interference from other absorbing components and from sample turbidity is minimized (Arnao, 2000).

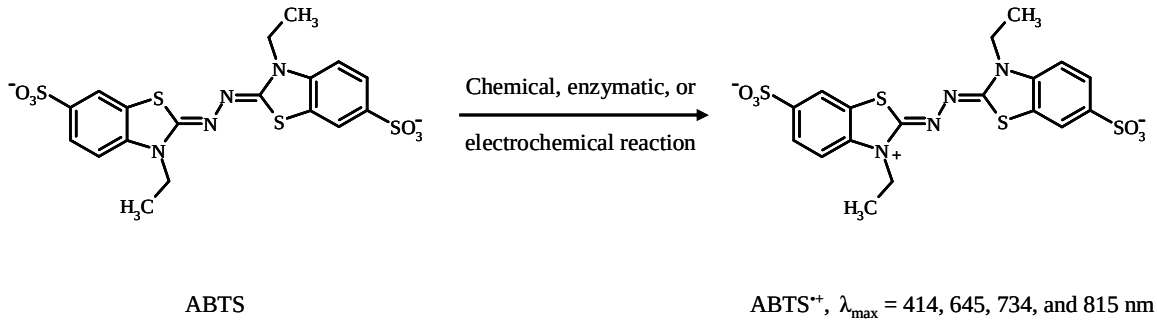


Figure 1.7. Generation of 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonate) radical cation ($ABTS^{\bullet+}$) from 2,2'-azinobis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) by chemical, enzymatic or electrochemical reaction.

In terms of quantification, the absorbance value, proportional to the remaining $ABTS^{\bullet+}$ concentration, is measured after a fixed reaction time. Results are expressed as trolox equivalents, that is, the concentration of trolox solution (mM) with an antioxidant capacity

equivalent to that found for 1.0 mM of the substance under investigation. To express the antioxidant capacity in a more familiar and easily understood manner, ascorbic acid was suggested as the reference compound instead of trolox and results were given as mass of ascorbic acid per 100 g or per 100 mL of test sample, designated as VCEAC – vitamin C equivalent antioxidant capacity (Kim *et al.*, 2002).

This spectrophotometric assay is technically simple, which accounts for its application for screening and routine determinations. The $ABTS^{\bullet+}$ scavenging can be evaluated over a wide pH range, which is useful to study the effect of pH on antioxidant mechanisms. Furthermore, the $ABTS^{\bullet+}$ radical is soluble in water and organic solvents, enabling the determination of antioxidant capacity of both hydrophilic and lipophilic compounds/samples. However, the results provided by this assay have been criticized as the $ABTS^{\bullet+}$ radical is not representative of biomolecules and not even found in any biological system. Thermodynamically, any compound that has a redox potential lower than that of $ABTS^{\bullet+}$ may react with the radical. Despite these concerns, the $ABTS^{\bullet+}$ assay has been extensively used to measure the antioxidant capacity of lipophilic and hydrophilic antioxidant compounds (Pulido *et al.*, 2003), complex samples such as medicinal plants (Surveswaran *et al.*, 2007), food or beverages (Pellegrini *et al.*, 2003; Saura-Calixto and Goni, 2006) and blood samples (Fischer *et al.*, 2005; Dalla-Valle *et al.*, 2007).

1.3.2. Scavenging of 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$ assay)

In this assay, the purple chromogen radical 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$, Fig. 1.8) is reduced by antioxidant/reducing compounds to the corresponding pale yellow hydrazine (Blois, 1958). The scavenging capacity is generally evaluated in organic media by monitoring the absorbance decrease at 515-528 nm until the absorbance remains constant (Brand-Williams *et al.*, 1995) or by electron spin resonance (Calliste *et al.*, 2001). Recently, the determination of antioxidant capacity based on the amperometric reduction of $DPPH^{\bullet}$ at a glassy carbon electrode was proposed by Milardovic *et al.* (2006). The resulting current the electrode polarized at fixed potential was proportional to the residual

concentration of DPPH$^{\bullet}$ after reaction with the antioxidant(s). A biamperometric method using DPPH/DPPH$^{\bullet}$ redox couple and two identical glassy carbon disc electrodes was also presented (Milardovic *et al.*, 2005).

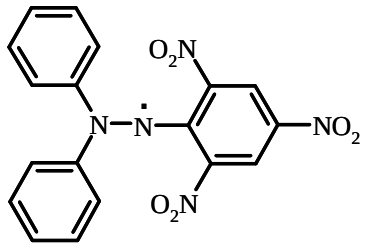


Figure 1.8. Structure of 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$) radical that absorbs at the visible region of the spectra (λ_{max} = 515–528 nm).

In opposition to what was initially believed, the reaction mechanism is based on an ET-reaction whilst the hydrogen atom abstraction is a marginal reaction pathway, because it occurs slowly in strong hydrogen-bond-accepting solvents, such as methanol and ethanol (Foti *et al.*, 2004). As occurs in other ET-based assays, the scavenging capacity against DPPH$^{\bullet}$ radical is strongly influenced by the solvent and the pH of reaction (Magalhães *et al.*, 2007a). Indeed, Stasko *et al.* (2007) studied the suitable conditions and limits for water as a component of a mixed water-ethanol solvent of DPPH$^{\bullet}$ radical assay. They concluded that the 50% (v/v) aqueous/ethanol solutions are a suitable choice for lipophilic and hydrophilic antioxidants and the reaction rate between DPPH$^{\bullet}$ and the antioxidant may increase considerably with increasing water ratios. However, at water ratios over 60% (v/v) the antioxidant capacity decreased, since a part of the DPPH$^{\bullet}$ coagulates and it is not easily accessible to the reaction with antioxidant(s).

Generally, the results are reported as the efficient concentration (EC_{50}), that is the amount of antioxidant necessary to decrease by 50% the initial DPPH$^{\bullet}$ concentration (Brand-

Williams *et al.*, 1995). The time needed to reach the steady state with EC_{50} concentration was calculated from the kinetic curve and defined as T_{EC50}. In recognition of the effect of both parameters the "antiradical efficiency" may be determined by calculating the reciprocal of $EC_{50} \times T_{EC50}$ (Sánchez-Moreno *et al.*, 1998). Therefore, the lower EC_{50} and T_{EC50}, the higher is the "antiradical efficiency". A conceptually similar parameter designated as radical scavenging efficiency (RSE) was suggested by De Beer *et al.* (2003). RSE is calculated as the ratio of the reaction rate (obtained during the first minute) and the EC_{50} value. The main limitation of EC_{50} determination is that the percentage of radical scavenged is dependent of the initial concentration of $DPPH^{\bullet}$ radical (Magalhães *et al.*, 2006a). For this reason, it is more accurate to use the absorbance variation (or concentration of $DPPH^{\bullet}$ consumed) rather than the percentage of the radical consumed. This absorbance value is further interpolated in a dose-response curve of a standard antioxidant such as ascorbic acid or trolox and the results are expressed as equivalent concentration.

The steric accessibility of $DPPH^{\bullet}$ radical is a major determinant of the reaction, since small molecules that have better access to the radical site have relatively higher antioxidant capacity (Huang *et al.*, 2005). On the other hand, many large antioxidant compounds that react quickly with peroxyl radicals may react slowly or may even be inert in this assay. The inexistence of $DPPH^{\bullet}$ or similar radicals in biological systems is also a shortcoming. In addition, the spectrophotometric measurements can be affected by compounds, such as carotenoids, that absorb at the wavelength of determination as well as by the turbidity of the sample. In this way, the electrochemical detection proposed by Milardovic *et al* (2005, 2006) may be a valid alternative to analyse colored and/or turbid samples with low content of antioxidant compounds. The $DPPH^{\bullet}$ assay is not suitable for measuring the antioxidant capacity of plasma, because proteins are precipitated in the alcoholic reaction medium. Finally, the $DPPH^{\bullet}$ scavenging reaction is time-consuming and it may take 20 min up to 6 h (Brand-Williams *et al.*, 1995). Recently, Magalhães *et al.* (2006a) applied a mathematical model to the data collected within the first 3 min of $DPPH^{\bullet}$ scavenging reaction to estimate the total $DPPH^{\bullet}$ consumed. This approach allowed a considerable

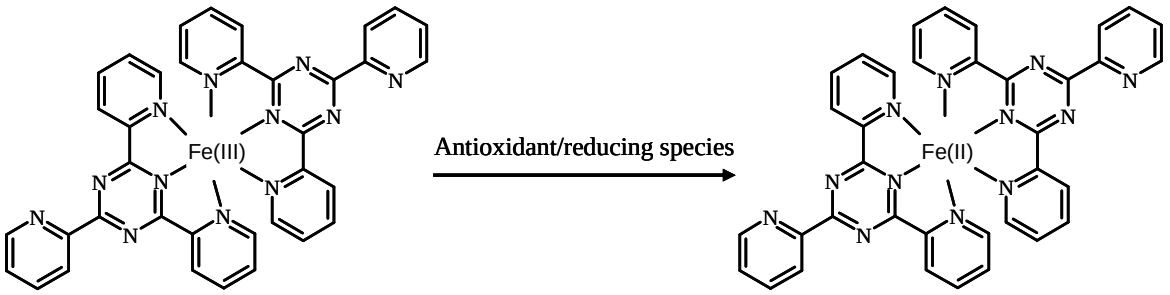
reduction of the time taken for a single analysis for samples containing or originating slow reacting antioxidant compounds.

Despite the limitations abovementioned, the $DPPH^{\bullet}$ is considered a valid, easy and useful assay with regard to screening/measuring the antioxidant capacity of pure compounds, fruit and vegetable juices or extracts (Antolovich *et al.*, 2002; Chinnici *et al.*, 2004; Surveswaran *et al.*, 2007;) as the radical compound is stable, commercially available, and does not have to be generated before assay like $ABTS^{\bullet+}$. The $DPPH^{\bullet}$ assay is also technically simple, just requiring a UV-vis spectrophotometer to be performed.

1.3.3. Ferric reducing antioxidant power (FRAP assay)

The FRAP assay measures the ability of antioxidants to reduce the ferric 2,4,6-tripyridyl-*s*-triazine complex $[Fe(III)\text{-}(TPTZ)_2]^{3+}$ to the intensely blue coloured ferrous complex $[Fe(II)\text{-}(TPTZ)_2]^{2+}$ in acidic medium (Fig. 1.9) (Benzie and Strain, 1996 and 1999). FRAP values are calculated by measuring the absorbance increase at 593 nm and relating it to a ferrous ion standard solution or to an antioxidant standard solution (ascorbic acid, for instance). This method has also been adapted to 96-well microplate reader, giving better reproducibility and higher sample throughput (Tsao *et al.*, 2003).

Concerning its limitations, any compound (even without antioxidant properties) with redox potential lower than that of the redox pair Fe(III)/Fe(II), can theoretically reduce Fe(III) to Fe(II), contributing to the FRAP value and inducing falsely high results. On the other hand, not all antioxidants reduce Fe(III) at a rate fast enough to allow its measurement within the observation time (typically 4 min). Indeed, Pulido *et al.* (2000) observed that dietary polyphenols react more slowly and require longer reaction times ($\geq$ 30 min) for total quantification and depending on the analysis time the order of their reactivity is changed. The polyphenols with such behaviour include caffeic acid, ferulic acid, quercetin, and tannic acid.



$[Fe(III)\text{-}(TPTZ)_2]^{3+}$ $[Fe(II)\text{-}(TPTZ)_2]^{2+}$, λ_{max} = 593 nm

Figure 1.9. FRAP assay based on the reduction of the complex $[Fe(III)\text{-}(TPTZ)_2]^{3+}$ to the intensely colored $[Fe(II)\text{-}(TPTZ)_2]^{2+}$.

As the FRAP assay measures the reducing capacity based upon reduction of ferric ion, antioxidants that act by radical quenching (HAT reaction), particularly thiols and carotenoids, will not be determined (Pulido *et al.*, 2000; Ou *et al.*, 2002b). Another point to take into consideration is the concomitant production of Fe(II), which is a well-known pro-oxidant and may result in the generation of additional radicals in the reaction medium, as $OH^{\bullet}$ from H_2O_2. Finally, compounds that absorb at the wavelength of the determination may interfere, causing overestimation of the FRAP value. For instance, Benzie and Strain (1999) reported an unusually high FRAP value for bilirubin (twice that of trolox and ascorbic acid) because it was oxidized to beliverdin, which absorbs considerably at 593 nm. The low pH (3.6) necessary for this assay may lead to some protein precipitation, as casein in milk samples (Chen *et al.*, 2003).

Despite the low pH value applied when compared to physiological conditions (pH 7.4), this method has been extensively applied to measure the reducing power of foods and plant extracts, plasma and other biological fluids (Pulido *et al*., 2003; Surveswaran *et al.*, 2007; Haldar *et al.*, 2007). The ferric-reducing ability measured may indirectly reflect the antioxidant capacity, although the results should be compared with that obtained by an

HAT-based assay. For instance, Cao and Prior (1998) observed a weak but significant linear correlation between serum FRAP and serum ORAC, while Ou *et al.* (2002b) analysed a total of 927 freeze-dried vegetable samples and the results indicated that the FRAP and ORAC values did not correlate well. In summary, the FRAP assay is simple, inexpensive, and may offer a putative index of antioxidant capacity.

1.3.4. Folin-Ciocalteu reducing capacity (FC assay)

The exact chemical nature of the Folin-Ciocalteu reagent is not known, but it is accepted that it contains phosphomolybdic/phosphotungstic acid complexes (Singleton and Rossi, 1965). The chemistry behind the FC assay relies on the transfer of electrons in alkaline medium from phenolic compounds and other reducing species to molybdenum, forming blue complexes that can be detected spectrophotometrically at 750–765 nm (Singleton *et al.*, 1999). Generally, gallic acid is used as the reference standard compound and results are expressed as gallic acid equivalents (mg L^{-1}). It should be stressed that, the blue complexes formed are independent of the structure of phenolic compounds, therefore ruling out the possibility of coordination complexes formed between the metal and the phenolic compounds (Singleton *et al.*, 1999).

The FC reagent is non-specific to phenolic compounds as it can be reduced by many nonphenolic compounds (e.g. aromatic amines, sulfur dioxide, ascorbic acid, Cu(I), Fe(II), etc) and for that reason is not suitable for determination of "total phenolic content", unless interfering species are considered or removed (Singleton *et al.*, 1999 and Prior *et al.*, 2005). Therefore, the FC assay was recently proposed for the measurement of total reducing capacity of samples (Huang *et al*., 2005). Excellent linear correlations between FC assay and other ET-based assays (TEAC and $DPPH^{\bullet}$, for instance) have been established (Roginsky and Lissi, 2005). Indeed, in a recently reported work the FC reducing capacity of a large number of beverages (n = 72) were correlated with TEAC assay; a good correlation was found (R > 0.9) for red wines, herbal and tea infusions, and beers (Magalhães *et al.*, 2007b). Nevertheless, the contribution from other dietary

antioxidant compounds with non-ET mechanism of action (such as β-carotene) may not be assessed by FC assay (Magalhães *et al.*, 2006b). Despite that, the relationship between the FC method and ORAC, a HAT-based assay, is usually good (Prior *et al.*, 2005). These correlations confirm the value, usefulness of Folin-Ciocalteu reducing capacity for the assessment of antioxidant capacity of food samples. In addition, the FC assay is operationally simple, reproducible and convenient for assessment of dietary antioxidant capacity since the reagent is commercially available, the procedure is rather standardized, and the absorption of the product at a long-wavelength minimizes interferences from the sample matrix. Nevertheless, the original assay is time-consuming (2 h), which makes its implementation difficult for routine analysis. Moreover, it is performed in aqueous phase, thus it is not applicable for lipophilic compounds/matrices.

1.3.5. Total reducing capacity estimated by electrochemical methods

Electrochemical properties of pure compounds, foods, and biological samples may be used for the evaluation of their reducing/antioxidant capacity, since the oxidation potential is conceptually related with the expected antioxidant capacity. Indeed, the oxidation potential and antioxidant capacity are inversely related, so that when oxidation potential is low it means that analytes could be oxidized easily or, in other words, they have higher reducing/antioxidant capacity.

Among the electrochemical methods, the cyclic voltammetry (CV) technique has been adapted to the evaluation of the overall reducing capacity of low molecular weight antioxidants in plasma (Chevion *et al.*, 1997), tissue homogenates (Shohami *et al.*, 1999; Kohen *et al.*, 1999), and plant extracts (Chevion *et al.*, 1999). In fact, this methodology has been used in a variety of clinical situations and pathological disorders including diabetes, brain degenerative diseases, as well as in studies concerning the ageing process (Kohen and Nyska, 2002). Typically, the reducing capacity of tested compound/sample is measured through a three-electrode system, constituted by the working electrode (glassy carbon), the reference electrode (Ag/AgCl), and the auxiliary electrode (platinum wire).

Following the introduction of the sample into the tested well, the potential is linearly applied to the working electrode at a constant rate either toward the positive potential or toward the negative potential. The resulting current vs potential is recorded to produce a cyclic voltammogram. CV tracings are usually recorded from – 0.5 to + 1.5 V versus the reference electrode at a scan rate of 100 or 400 mV s^{-1} (Chevion *et al.*, 2000).

The reducing capacity of a sample is analysed from three parameters obtained through the CV tracings: i) the oxidation potential, $E_{1/2}$, which reflects the specific reducing capacity, the lower the $E_{1/2}$, the higher the ability of the tested sample to donate electrons to the working electrode; ii) the intensity of the anodic current (*Ia*); and iii) the area under the anodic wave (*S*). Both *Ia* and *S* are related to the concentration of the reducing species present in the sample. Nevertheless, as an anodic wave in complex matrices, such as biological and food samples, often represents more than a single component, each of which could donate electron(s) around the same potential, it was proposed the *S* parameter for estimation of the total reducing capacity rather than the *Ia* (Chevion *et al.*, 1999; Kilmartin *et al.*, 2002). Moreover, changes in *S* value better reflect a change in a single component within an anodic wave than the corresponding change in *Ia*.

In this context, the CV methodology allows rapid screening of the electrochemical profile of samples and is especially suitable for screening studies. Furthermore, the CV profile can be obtained in aqueous medium as well as in organic solvents like acetonitrile, water/acetonitrile, and acetonitrile/methanol mixtures provided that there are redox-active components and enough electrolytes in the solution to support redox reactions on the electrode surface. Recently, Martinez *et al.* (2006) compared the CV results obtained on human and horse plasma with the results of two spectrophotometric radical assays, the $DPPH^{\bullet}$ and the Randox-TEAC. They observed a positive linear correlation ($r^2 > 0.964$) between $DPPH^{\bullet}$ results and CV data of the first anodic wave, that reflects the antioxidant capacity of the two major water-soluble antioxidants in plasma, ascorbic and uric acid. On the other hand, they also observed a poor correlation between CV/$DPPH^{\bullet}$ results and the Randox-TEAC results.

These assays based on the electrochemical properties of the compound/sample do not require the use of reactive compounds, since it is based on electrochemical behaviour and,

consequently on their chemico-physical properties. Moreover, turbid and/or intensely colored samples can be determined without prior sample preparation. The shortcoming of these methodologies is related to the fact that some biologically relevant antioxidants (e.g. glutathione, cysteine and other thiol-containing compounds) show a low response when glass carbon electrodes are applied. In this case, other electrodes such as an Au/Hg electrode are needed for glutathione measurement (Kohen *et al.*, 1999). Furthermore, an important practical limitation is that the working electrode has to be frequently cleaned to remove residues of sample from its surface and to maintain its sensitivity. For instance, in the work developed by Blasco *et al.* (2005) a glassy carbon electrode cleaning procedure was described. Hence, between each work session the electrode was cleaned by physical, chemical and electrochemical treatments, while during the session the electrode was electrochemically cleaned, whenever necessary, using cyclic voltammetry.

Table 1.3. In vitro scavenging capacity assays against stable, non-biological radicals and evaluation of total reducing capacity.

Assay	Principle of measurement	Quantification	Ref.
TEAC	$ABTS^{\bullet+}$ radical cation is reduced by antioxidants, causing absorbance decrease at 734 nm	Trolox equivalents (μM), ascorbic acid equivalents (mg/100 mL or 100 g)	Miller *et al.*, 1993
$DPPH^{\bullet}$	$DPPH^{\bullet}$ radical is reduced by antioxidants, causing absorbance decrease at 515-528 nm	EC_{50}, RSE, trolox equivalents (μM), ascorbic acid equivalents (mg/100 mL or 100g)	Brand-Williams *et al.*, 1995
FRAP	The ferric 2,4,6-tripyridyl-s-triazine complex is reduced by antioxidants, causing absorbance increase at 593 nm	Ferrous ions equivalents, ascorbic acid equivalents	Benzie and Strain, 1996
Folin-Ciocalteu reducing capacity	Tungstate-molybdate complexes are reduced by antioxidants, causing absorbance increase at 750-765 nm	Gallic acid equivalents (mg L^{-1})	Singleton *et al.*, 1999
Electrochemical total reducing capacity	The intensity of anodic current is increased due to oxidation of antioxidant compounds at the surface of the electrode	Oxidation potential ($E_{1/2}$), intensity of the anodic current (*Ia*), area under the anodic wave (*S*)	Chevion *et al.*, 2000

$ABTS^{\bullet+}$, 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonate) radical cation; $DPPH^{\bullet}$, 2,2-diphenyl-1-picrylhydrazyl radical; EC_{50}, sample concentration that inhibit 50% of the blank analytical signal; FRAP, ferric reducing antioxidant power; RSE, radical scavenging efficiency; TEAC, trolox equivalent antioxidant capacity.

1.4. Flow-based methods for determination of antioxidant capacity

The assays described in the previous sections are usually characterized by a low level of automation. In fact, they are based on manual discrete methods involving the use of a single reaction cell or a multiwell microplate detector whereby the sample/reagent(s) are added successively. The analytical signal is usually obtained after a pre-selected delay time from the instant of mixing, and it can be an end-point value or an area corresponding to the integral over a given period of time. In general, these determinations are time-consuming, laborious, and costly, especially when routine work in a large number of samples has to be performed as it happens with food and biological samples. In addition, it is also susceptible to operational errors, such as inadequate sample/reagent mixing or poor reproducibility of time events. Furthermore, the implementation of antioxidant assays suffers from an additional difficulty, as short-living and highly reactive species (ROS/RNS) are involved and strict control/reproducible reaction conditions are required to attain reliable results. On the other hand, there is a strong demand from the food industry as well as from the clinical field to develop automatic, fast, robust and reliable analytical methods for routine/screening determination of antioxidant capacity.

Automation can be a sound alternative to overcome these limiting aspects and to implement routine assays. In this context, flow injection analysis (FIA) and its predecessor computer-controlled techniques have proven to be a valuable analytical tool to improve batch methodologies, owing to the three principles implicit to these automatic flow techniques: i) reproducible sample injection or insertion in a carrier/reagent solution; ii) the controlled dispersion of the sample zone; and iii) the reproducible timing of its movement from the injection/insertion point to the detection system (Ruzicka and Hansen, 1988). In addition, the signal detected is transient because neither physical equilibrium (homogenisation of sample and carrier/reagent) nor chemical equilibrium (reaction completeness) is attained. Therefore, the determinations occur under non-equilibrium conditions and are based on the dispersion/reaction kinetic processes. In this regard, automation of antioxidant capacity assays based on flow analysis, offers the following features:

i) enhanced sample throughput (important in routine analysis and for attaining real-time information)
ii) sample and reagents saving, with concomitant reduction in waste generation
iii) reproducible mixture between oxidant and antioxidant/scavenger compound
iv) strict control of reaction time which is particularly important when highly reactive species are present
v) reaction conditions closer to those found in vivo, as ROS/RNS are scavenged immediately after its generation
vi) performance of various antioxidant assays using the same flow manifold
vii) minimization of effects caused by oxygen present in the ambient air as well as solvent evaporation, as in the case of organic-solvent methods, since the reactions occur in a controlled environment.

Nowadays, beyond FIA (Ruzicka and Hansen, 1975), various computer-controlled flow analysis techniques with potential to improve automatic antioxidant methods are available, namely sequential injection analysis (SIA) (Ruzicka and Marshall, 1990), multisyringe flow injection analysis (MSFIA) (Cerdá *et al.*, 1999), multipumping flow systems (MPFS) (Lapa *et al.*, 2002), within others. All these flow modalities present advantages and drawbacks inherent of its mode of operation and/or instrumentation applied. The selection of the most adequate will depend on the specific analysis and features associated to the determination as well as the main purposes to be achieved.

Regarding to FIA (Ruzicka and Hansen, 1975 and 1988), samples are introduced into the system through an injection valve and then dispersed in the carrier inside the tubes conduit. Most commonly, the reagent is continuously added through a confluence point located after the sample injection port and before a coil where the reaction takes place. Finally, the reaction product reaches the flow-through detector where the detection signal is acquired. The main advantages of this flow procedure are the high sample throughput attained and its easy implementation, since it is not necessary the computer control. Nevertheless, the dispensed solutions (carrier, reagents) as well as the waste production are higher when compared with the more recent computer-controlled flow procedures.

In the subsequent flow techniques, computer control is essential for their operation. Although these flow modalities are based on the same principles of FIA (precise sample introduction, controlled dispersion, and reproducible timing), they present greater potentialities, as it allows more versatile sample manipulation inside the flow network as well as improved precision on timing events. Indeed, any changes concerning the sample volume, reagent/sample ratio, and reaction times are accomplished via software programming rather than by physical reconfiguration of the manifold. Reagents and sample saving is another advantage pointed out to computer controlled-techniques, because just the required amounts are aspirated/propelled and carrier is not pumped continuously. Moreover, computer control is a further step in automation and reduces human errors.

Sequential injection analysis (SIA) was introduced by Ruzicka and Marshall (1990) as a feasible and mechanically simpler alternative to FIA. The most basic system comprises a single bi-directional pump, a holding coil, a multiposition selection valve, a reaction coil and a detection system. Aliquots of sample and reagent(s) are sequentially aspirated into the holding coil from reservoirs connected to the lateral ports of the selection valve. The flow is then reversed, and these stacked zones are propelled and mutually dispersed. Owing to combined axial and radial dispersion, these zones are mixed while pass through the reaction coil, where the reaction product is formed and directed to the detector. Compared to FIA, the major difference concerns the way that sample and carrier/reagent are mixed inside the tubes. In FIA, solutions are most commonly mixed in confluence points, giving rise to a concentration gradient of analyte in a constant background of reagent, while in SIA the mixture occurs essentially during the flow reversal forming a partial overlap of sample and reagent zones. Indeed, the mixture takes place essentially at the boundaries of each segment which can be a limitation to attain an efficient overlap when considering reactions involving four or more segments (three reagents + sample, for instance) (Segundo and Rangel, 2002).

In order to overcome this specific drawback of SIA systems, and also to improve the mixing between solutions in flow systems, Reis *et al.* (1994) introduced the concept of multicommutation in flow analysis associated with the binary sampling approach. This flow technique is characterized by the use of individual commutation devices (solenoid

valves) operating in a simultaneous or a sequential way, where solutions can be accessed randomly. Introduction of sample and reagents into the flow network can be performed by aspiration through a single pump channel placed after the detector, and by selecting the positions of the respective valves. On the other hand, the introduction of solutions in the flow manifold can be performed by placing the propulsion device before the commutation valves; in this configuration a multichannel pump is required and solutions are propelled into the flow network or re-circulate to their own-vessel, according to the position of the solenoid valve. In this regard, the multisyringe flow injection analysis (MSFIA), developed by Cerdá *et al*. (1999), can be considered as a multicommutation operation modality in which the multisyringe burette comprises both the propulsion system and the commutation devices. The multisyringe burette is a multiple channel piston pump, composed by four syringes whose pistons are connected to the same bar. At the head of each syringe a three-way commutation valve is placed, connecting the content of each syringe to the flow system or to the solution vessel. From a general point of view, flexibility is the main advantage of multicommutation over the other flow modalities.

Automation resorting to multipumping flow system (MPFS) relies on several solenoid actuated micropumps as the only active component in the manifold (Lapa *et al.*, 2002). The micropumps act as both fluid drivers and commutation units, promoting sample/reagent insertion and mixing. In this way, these active devices encompass the liquid propelling units, the sample insertion devices, and the commutation elements usually present in the abovementioned flow systems. Additionally, in contrast to typical laminar flow conditions attained with previous flow procedures, the micropumps actuation produce a pulsed flow characterised by a chaotic movement of the solutions, which contributes to a fast sample/reagent homogenisation with low axial dispersion and, thus, favouring reaction development. The main features of these flow systems regarding to other flow methodologies are the high simplicity and portability.

1.4.1. Present situation of automatic antioxidant flow-based methods

The development of automatic antioxidant capacity assays based on flow analysis is a quite recent topic in analytical chemistry research. In the past ten years, about 34 scientific papers dealing with this subject have been published for the determination of antioxidant capacity of pure compounds as well as of complex matrices (Fig. 1.10).

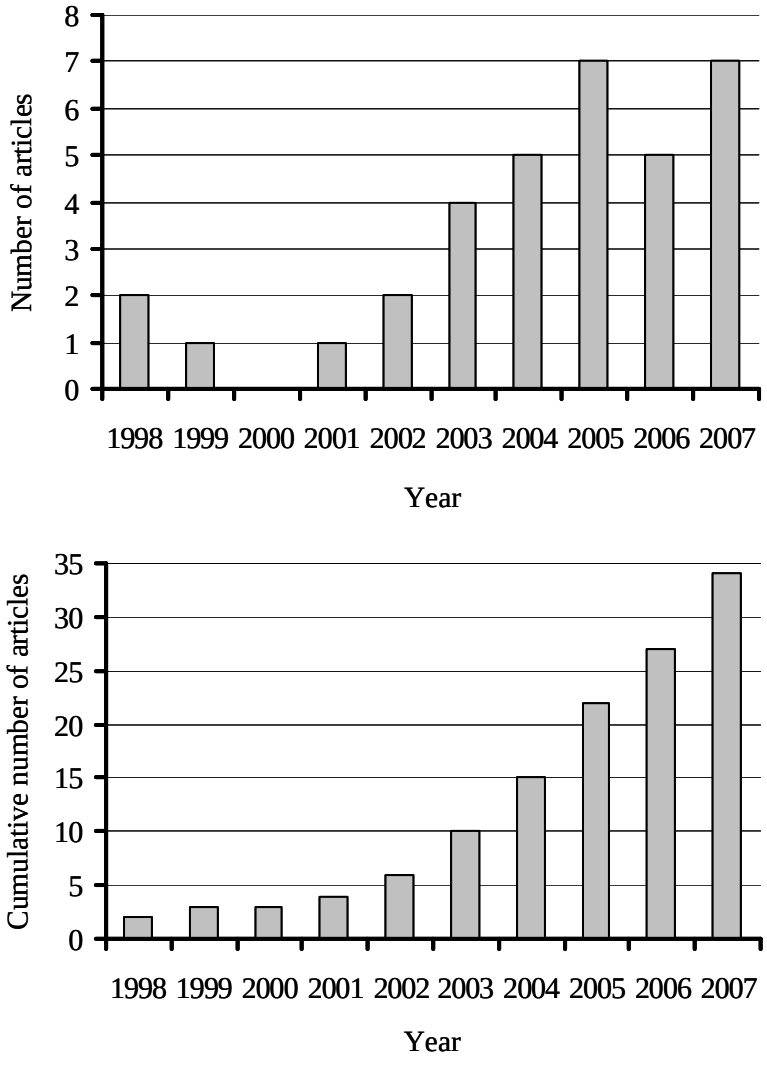


Figure 1.10. Number of articles dealing with the development of flow-based methods for determination of antioxidant capacity published over the past decade (until August 2007).

More than half of these papers (56%) were published in the past three years (until August 2007), indicating that the implementation of antioxidant assays in flow-based techniques is just beginning. Considering all publications dealing with this subject, about 47% were dedicated for determination of scavenging capacity of specific ROS/RNS, while flow-based methods for determination of scavenging capacity of stable radicals $DPPH^{\bullet}$ or $ABTS^{\bullet+}$ and determination of total reducing capacity represent approximately 32 and 21%, respectively.

Different flow-based procedures including FIA, SIA, MSFIA, and MPFS have been applied to develop/implement automatic antioxidant assays (Fig. 1.11). Undoubtedly, flow injection analysis (FIA) is clearly the most used flow-approach accounting for about 70% of the systems described in the literature. The reason for this choice may be the fact that FIA is the flow procedure more common in research laboratories, when compared to other flow-approaches, due to its extremely simple manifold implementation, easy operation and low-cost. In addition, the high sample throughput attained by FIA is an attractive feature to implement rapid automatic antioxidant assays.

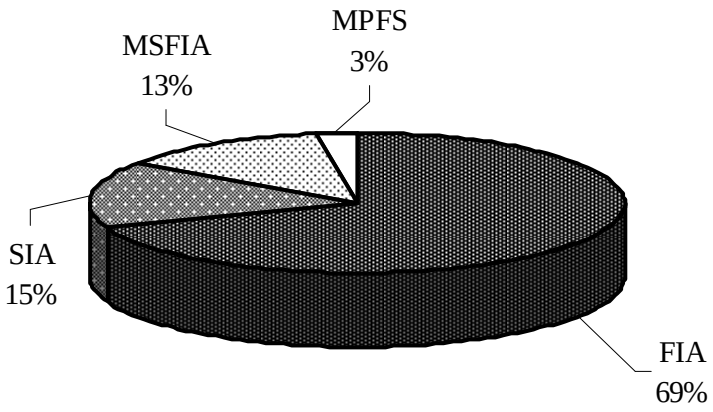


Figure 1.11. Distribution of published articles by flow-based procedure. FIA, flow injection analysis; MPFS, multipumping flow system; MSFIA, multisyringe flow injection analysis; SIA, sequential injection analysis.

The remaining flow procedures, besides being more recent, require experience in the connection of apparatus with computers for controlling all the instrumentation and for flow management (namely flow direction, flow rates, and volumes). Nevertheless, since 2004 until the present moment (August 2007) the FIA systems and the recent computer-controlled systems account each 50% of the published papers, while before 2004 only FIA systems were reported.

Chemiluminescence (CL) is the most used detection type, accounting for about 40% of the flow-based methods described in the literature (Fig. 1.12). The reason for this prevalence relatively to the other detection systems could be explained by the high reactivity of ROS/RNS with chemiluminogenic reagents, as luminol, plus the demand to attain fast analytical methods due to the short-life of these reactive species. In fact, about 78% of the flow methods developed for ROS/RNS rely on CL as detection system, while only one work applied this type of detection for assessing the total antioxidant capacity (Costin *et al.*, 2003). The other two detection types more used are UV-vis (26%) and amperometry (18%) applied essentially for measurement of $DPPH^{\bullet}$ or $ABTS^{\bullet+}$ radical scavenging capacity and total reducing capacity, respectively. Fluorimetric determinations of some ROS/RNS represent about 11%, while ESR spectrophotometry and potentiometry both correspond to 5%.

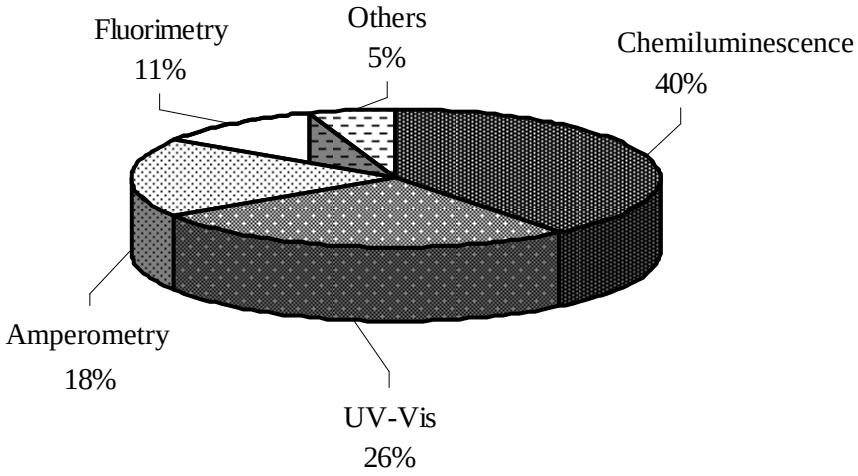


Figure 1.12. Distribution of published articles by type of detection system employed.

From the flow-based methodologies developed only one work was applied to biological samples, human and rat plasma (Bompadre *et al.*, 2004) (Fig. 1.13). Pharmaceutical samples, including multivitamin supplements, NSAIDs, and some ascorbic acid formulations represent 9% of the type of the samples analysed. On the other hand, beverages including beers, coffee, juices, teas, milk, and wines represent 53%, while extracts of medicinal plants and edible food, account for about 29%. This high application to food products (82%) can be explained by the increasing demand from the food industry, verified during the last decade, for rapid and reliable analytical methods for determining antioxidant capacity, since this becomes a significant parameter for commercialization of nutritional-added-value products. As a matter of curiosity wines (34%) and teas (24%) are the most frequently analysed beverages followed by juices (17%), beers (12%) and coffee (7%).

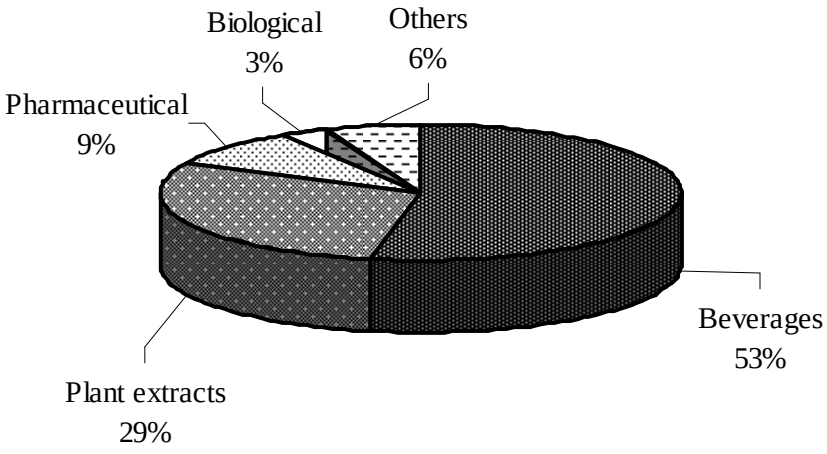


Figure 1.13. Distribution of published articles by type of sample.

The automation of antioxidant capacity assays based on principles of flow injection analysis (FIA) and the computer-controlled techniques (SIA, MSFIA, MPFS) will be discussed in the following sections concerning their chemistry, features of the flow

systems, applications, and the advantages and shortcomings. This discussion is divided according to the type of the reactive species/property measured including: i) flow-based methods for determination of scavenging capacity of specific ROS/RNS (Table 1.4); ii) flow-based methods for determination of scavenging capacity of stable, non-biological radicals ($DPPH^{\bullet}$ and $ABTS^{\bullet+}$) (Table 1.5); and iii) flow-based methods for determination of total reducing/antioxidant capacity (Table 1.6).

1.4.2. Flow-based methods for determination of scavenging capacity of specific ROS/RNS

In 1998, Choi *et al.* developed a simple FIA-CL method for the measurement of radical scavenging capacity ($O_2^{\bullet-}$ and $HO^{\bullet}$). In this work, the carrier stream was a phosphate buffer (pH 7.4) solution containing 50% methanol, cytochrome c, and luminol. The mechanism of CL generation was not clear, but it was indicated that CL appears during the oxidation of luminol mediated by free radicals, probably $O_2^{\bullet-}$ and $HO^{\bullet}$, generated from the reaction of H_2O_2 and cytochrome c. Hence, a standard solution of H_2O_2 was injected into the carrier stream for generation of reactive species (control), while a mixture of H_2O_2 and sample was injected for measuring the radical scavenging capacity. Compounds such as gallic acid scavenge free radicals and the reduction of CL intensity is proportional to the concentration and capacity of the scavengers. Nevertheless, taking into consideration that, the mixture between H_2O_2 and scavenger sample was performed off-line, the possibility of scavenging H_2O_2 should not be discarded. Afterwards, the same research group applied this FIA-CL method to elucidate the relation between radical scavenging effect and the structure of flavonoids and also to study the radical scavenging capacities of various Chinese herbal ingredients (Choi *et al.*, 2000).

Among the flow methods developed for determination of scavenging capacity against specific ROS/RNS, a versatile and simple FIA-chemiluminescent system developed by Sariahmetoglu *et al.* (2003) should be highlighted. The three-channel flow injection system was used to propel the chemiluminogenic reagent (luminol), the oxidant, and the carrier

stream which transported the injected antioxidant sample. According to this manifold configuration, the injected sample was firstly mixed with the oxidant and then the combined solution was merged with luminol just before it enters in the CL-flow cell. Thus, the FIA-design exploits the consumption of oxidant by antioxidant(s), which results in the appearance of negative CL signal proportional to the scavenging ability of the compound(s). Using the proposed FIA-CL system, the authors investigated the scavenging capacity of various recognised antioxidants (ascorbic acid, catalase, dimethyl sulfoxide, mannitol, methionine, SOD, uric acid) against several ROS/RNS (Table 1.4). In fact, changing the composition of the oxidant stream, the scavenging capacity was evaluated for several reactive species including $O_2^{\bullet-}$ (generated from XOD–xanthine enzymatic system), H_2O_2 (luminol-oxidation catalysed with addition of Co^{2+}), $HO^{\bullet}$ (generated from O_2–$FeSO_4$–buffer), hypochlorite anion (OCl^-), and $ONOO^-$ (freshly synthesized from the reaction between $NaNO_2$ and H_2O_2). In this work, the reaction between ROS/RNS and antioxidant (~5 s) occurred before the CL reaction which has been calculated to occur in about 1.8 s. Thus, the inhibition of luminol-CL is principally due to direct reaction between the antioxidant and the ROS/RNS, even though the interference of antioxidant with the luminol-oxidant CL reaction should also be considered (Fig. 1.5).

For the determination of scavenging capacity against superoxide radical anion ($O_2^{\bullet-}$), Tang *et al.* (2004) developed a FIA spectrofluorimetric method using a novel fluorescent target/probe: 2-(2-pyridil)-benzothiazoline. In this assay, the $O_2^{\bullet-}$ (generated from alkaline $Na_2S_2O_4$ solution) oxidize the target/probe to yield the strong fluorescent compound 2-(2-pyridil)-benzothiazol. Hence, in the FIA-assembly, the target/probe compound was injected into the carrier stream and then mixed with alkaline $Na_2S_2O_4$ and finally the SOD solution (standard) or antioxidant sample was added. The scavenger compounds compete with 2-(2-pyridil)-benzothiazoline for $O_2^{\bullet-}$, and the inhibition of fluorescence signal was proportional to the scavenging capacity. Despite the fact that the authors have proposed this flow methodology for the determination of SOD activity, it can be applied to screening studies for compound/samples possessing $O_2^{\bullet-}$ scavenging capacity. A major shortcoming of this method is the utilisation of the unstable alkaline $Na_2S_2O_4$ solution as source of $O_2^{\bullet-}$ because this solution must be replaced every 2 h.

The scavenging capacities of catechins and flavones towards both $O_2^{\bullet-}$ (generated from the enzymatic reaction of XOD and hypoxanthine) and H_2O_2 were determined by flow injection analysis with luminol-based CL detection (Toyo'oka *et al.* 2003). The scavenging capacity was detected by a suppression of the CL-background signal. For the $O_2^{\bullet-}$ scavenging assay, the authors added catalase to XOD solution in order to remove the contribution of H_2O_2, formed from XOD activity and from spontaneous dismutation of $O_2^{\bullet-}$ radicals.

Regarding to hydrogen peroxide, Pinto *et al*. (2005) developed a SIA fluorimetric procedure for the assessment of H_2O_2 scavenging capacity of wines. In this assay, the homovanillic acid (HVA) was oxidised to its fluorescent dimer in the presence of H_2O_2 and peroxidase enzyme. Antioxidant(s) inhibit the oxidation of HVA by a competitive reaction scheme and the reduction in the intensity of the fluorescence signal was proportional to the antioxidant capacity. As discussed above (section 1.2.3), besides scavenging H_2O_2, the putative antioxidant(s) may react with intermediates formed from the action of peroxidase enzyme upon H_2O_2 and/or they may inhibit the activity of this enzyme.

The multipumping flow system (MPFS) developed by Meneses *et al.* (2005), exploit the features of this flow procedure (mixing potential, versatility, and operational simplicity) for the chemiluminometric determination of antioxidant capacity. In this flow system, the only active components were the solenoid micropumps, which enabled the insertion and efficient mixing of sample and reagents as well as the transportation of the sample zone toward the CL-flow cell. The proposed flow method relies on the ability of antioxidant compounds (ascorbic acid, resveratrol, and trolox) to inhibit the CL reaction of luminol or lucigenin with hydrogen peroxide. In fact, the authors evaluated the analytical performance of the methodology using luminol or lucigenin as chemiluminogenic reagents. It was observed that the procedure involving luminol was much more sensitive than that using lucigenin; this took place because the oxidation of luminol and lucigenin exhibited different reaction rates, which was higher for luminol. Finally, it should be highlighted the high throughput attained: 70 or 160 determinations per hour when the chemiluminogenic reagent was lucigenin or luminol, respectively.

Recently, Amatatongchai *et al.* (2007) developed a microfluidic-based flow method for estimating the H_2O_2 scavenging capacity based on a modified peroxyoxalate chemiluminescence assay (Arnous *et al.*, 2002). Chemiluminescence was generated from the reaction of 2-carbopentyloxy-3,5,6-trichlorophenyl oxalate (CCPO) with hydrogen peroxide in the presence of the fluorophore 9,10-bis-(phenylethynyl)anthracene (BPEA) and sodium salicylate used as a catalyst. Thus, when an antioxidant (β-carotene, quercetin, and α-tocopherol) was present in the assay mixture it scavenged the H_2O_2 and quenched the production of light. In the 2-inlet microfluidic system devised, the antioxidant plugs were injected into the hydrogen peroxide stream, which afterwards was merged with the flow stream containing CCPO / BPEA / sodium salicylate. In fact, this assay has features of FIA, since the sample plug was injected into a flowing reagent stream followed by controlled and reproducible dispersion and detection after a fixed time. However, unlike most used FIA-based methods that use capillaries or tubing, these microchips use planar microchannels enabling potentially faster mixing and also facilitates planar integration of optical detectors.

The flow-based methods developed for determining the hydroxyl radical ($HO^{\bullet}$) scavenging capacity represent a suitable analytical tool due to the short-life and high reactivity of this ROS. In fact, $HO^{\bullet}$ radicals are the strongest known oxidant present in vivo, therefore the possibility to evaluate the scavenging effects of putative antioxidants against $HO^{\bullet}$ radical immediately after its generation represent a considerable improvement when compared to batch procedures. Moreover, the introduction of flow-based techniques led to the $HO^{\bullet}$ generation and its capture took place in controlled environment, reducing the contact of $HO^{\bullet}$ with oxygen and other substances in the environment.

In this context, Tang *et al.* (2005) developed a FIA spectrofluorimetric method for detecting $HO^{\bullet}$ using sodium terephthalate as the fluorescent target/probe. In the FIA manifold devised, the H_2O_2 sampled in the loop was consecutively mixed with sodium terephthalate and with Co^{2+} yielding $HO^{\bullet}$ (Fenton-like reaction). Then, the generated $HO^{\bullet}$ reacted with sodium terephthalate and the highly fluorescent product sodium 2-hydroxyterephtalate was formed by aromatic hydroxylation (a stop-flow period of 3 min was required for reaction development). The relative fluorescence intensity was

proportional to the amount of $HO^{\bullet}$ generated. Scavenger compounds/samples were added to previous mixture and compete with fluorescent target/probe for $HO^{\bullet}$ and the inhibited fluorescence signal was directly related to $HO^{\bullet}$ scavenging capacity. The authors used sodium terephthalate instead of sodium benzoate, a common trap-compound of $HO^{\bullet}$, because with the former only one product, without any isomers, was formed. This method was applied to pure compounds (mannitol, thiourea), food extracts and maize pollen polysaccharide. Although the authors had referred that Fe^{2+} had little interference in the determination (tolerance ratio in mol = 2), the presence of this metal ion in food products could be an interference causing an overproduction of hydroxyl radical through the Fenton reaction.

Later, the same research group presented a similar FIA spectrofluorimetric method using ninhydrin as the fluorescent target/probe (Gao *et al.*, 2006). Ninhydrin had no fluorescence, but when attacked by $HO^{\bullet}$, a product of aromatic hydroxylation was obtained which has strong fluorescence. In this assay, the oxidation of the target/probe was less susceptible to interference from Fe^{2+} ion (tolerance ration in mol = 50), compared to the previous work in which terephthalate was used. In addition, this method had higher sampling rate (Table 1.4) and it was applied to the determination of scavenging effects of thiourea and vitamin C as well as to aqueous extracts of some foods (walnut, sunflower seed, black and white sesame, garlic, ginger, peanut, and soybean). Nevertheless, in both methodologies the antioxidant compounds may interfere with the generation system of $HO^{\bullet}$, by reacting directly with H_2O_2 and/or by chelating Co^{2+} (see section 1.2.4).

Recently, Giokas *et al.* (2007) introduced an analytical FIA-CL procedure that minimizes the antioxidant-oxidant (H_2O_2) interactions while favours the inhibition effects of antioxidants on the free radicals generated in situ ($HO^{\bullet}$ and $O_2^{\bullet-}$). In this way, the hydrogen peroxide was previously mixed with luminol-Co^{2+}-EDTA solution for the generation of steady flux of free radicals (mostly $HO^{\bullet}$ and $O_2^{\bullet-}$), while sample was added just before the inlet of the CL-flow cell. In the presence of metal chelator (EDTA), there is an equilibrium regime between the Co^{2+} and the $[Co(EDTA)_2]$ complex, which allowed a constant source of $HO^{\bullet}$ radicals by catalytic oxidation of hydrogen peroxide. Hence, a prolonged CL-signal was produced which was stable for about 30 s. In the present flow conditions, the low

H_2O_2 concentrations and the excess of Co^{2+}-EDTA ensures the consumption of the oxidant before it enters in contact with the sample. Furthermore, because of the very high reactivity of the free radicals generated and the reduced time of exposition of sample, the direct reaction between antioxidants and hydrogen peroxide was minimized. In this way, the interference of H_2O_2 is ignored and the CL inhibition reflects the scavenging capacity of antioxidants toward the free radicals, improving the sensitivity and the reproducibility. The application of the proposed method was restricted to pure compounds (ascorbic acid, glutathione, and uric acid).

The automation of hypochlorous acid scavenging capacity has been developed using luminol-CL as detection system. Beyond the work described before (Sariahmetoglu *et al.*, 2003), a SIA system for measuring the antioxidative activity against hypochlorite ion (OCl^-) was developed by Nakamura *et al.* (2004). In this assay, the stacked zones of OCl^- /scavenger were merged with luminol solution, which was continuously added by an auxiliary pump to the channel that connects the selection valve to the detector. The application of this assay was restricted to antioxidant standard compounds (ascorbic acid, α-tocopherol, and trolox). In addition, the scavenging reaction was carried out at pH 9.5 which is far different from the physiologic pH (7.4). In this regard, recently Magalhães *et al.* (2007c) proposed a chemiluminometric automatic flow methodology for the in vitro determination of hypochlorous acid scavenging capacity, under pH and oxidant concentration conditions similar to those found in vivo. For this, a manifold based on MSFIA was developed to perform in-line the reaction of HOCl and the putative scavenger molecule at physiological pH (or at pH 10 of CL detection, for comparison purposes) prior to the reaction of remaining HOCl with luminol at alkaline conditions. The HOCl scavenging capacity was evaluated through the decrease in the CL emission. In this MSFIA method, the time taken between the contact of HOCl and putative scavenger compounds plus CL detection was about 3 s. This is an advantage because the scavenger compounds that react fast, closer to the time frame of generation of HOCl in vivo, are determined. Moreover, the interference of antioxidant compounds in the CL-reaction is minimized, since the previous mixture HOCl/antioxidant was added to luminol solution just before CL measurement. The proposed MSFIA method was applied to non-steroidal

anti-inflammatory drugs representative of various chemical families and to well-known HOCl scavenger compounds (cysteine, gallic acid, and lipoic acid). A high determination throughput was also attained (Table 1.4). The results showed that the pH of scavenging reaction affects the ability of some compounds to react with HOCl, indicating that the conditions of in vitro testing should be as close as possible to those found in vivo.

As described before, singlet oxygen ($^1O_2$) may be formed from the reaction of OCl^- or OBr^- with hydrogen peroxide (section 1.2.6). In this regard, Miyamoto *et al.* (2006) developed a SIA system with CL detection for the screening of compounds possessing scavenging capacity against $^1O_2$. Hence, lactoperoxidase (LPO) enzyme was used to catalyse the reaction between bromide ion and H_2O_2 to generate $^1O_2$, through the following mechanisms:

$$Br^- + H_2O_2 \xrightarrow{LPO} OBr^- + H_2O \qquad (1.14)$$

$$OBr^- + H_2O_2 \longrightarrow {}^1O_2 + H_2O + Br^- \qquad (1.15)$$

The chemiluminogenic reagent (luminol), used as an indicator for $^1O_2$, was introduced to the main stream through an extra peristaltic pump just before the CL detector and the maximum signal intensity was obtained at the following order of aspiration: dimethyl sulfoxide (DMSO), H_2O_2, LPO and NaBr. The attenuation of luminol CL due to scavenging of $^1O_2$ by antioxidant compounds was measured. By using this SIA-CL method, the scavenging capacity of well known antioxidants such as vitamins C and E, trolox and sodium azide as well as multivitamin supplements were determined. However, some considerations about the efficiency of this methodology should be discussed. Thus, besides scavenging $^1O_2$, the antioxidant compounds may react directly with hydrogen peroxide and/or inhibit the activity of LPO enzyme. The formation of OBr^- as an intermediate species may also be a drawback as this powerful oxidizing agent may react with antioxidants present in the sample (Halliwell, 2006). Finally, as in the other SIA

method reported to evaluate the OCl^- scavenging capacity (Nakamura *et al.*, 2004), dimethyl sulfoxide was used to dissolve the standard antioxidants as well as for acquiring the blank signal. Considering that recent literature (for instance, Floriano-Sanchez *et al.*, 2006) described the scavenging capacity of dimethyl sulfoxide toward HOCl, the choice of this solvent may not be appropriate, under penalty of decreasing the sensitivity. Recently, Tsukagoshi *et al.* (2006) described a micro-reactor consisting of a micro-channel where sodium hypochlorite and hydrogen peroxide were delivered by syringe pumps providing a laminar flow liquid-liquid interface. The collapse of the interface through molecular diffusion originated CL from the singlet oxygen. The scavenger compounds (sodium azide, histidine, and nitroblue tetrazolium) were previously mixed off-line with hypochlorite solution and the $^1O_2$ scavenging capacity was evaluated by the inhibition of CL intensity. The extremely small amounts of reagents expended, the CL profiles and the characteristics obtained with these micro-reactors represent a considerable improvement. In this context, and as the work reported by Amatatongchai *et al*. (2007), it is expected that these microfluidic-based methods may create fully portable systems for field screening of antioxidant capacity of food, pharmaceutical supplements as well as biological samples.

Table 1.4. Flow-based methods for determination of scavenging capacity against specific ROS/RNS.

Reactive species	Flow method	Target compound	Generation of reactive species	pH value	Detection system	Type of sample	Det. rate (h^{-1})	RSD (%)	Ref.
$O_2^{\bullet-}$ and $HO^{\bullet}$	FIA	Luminol	Cytochrome c – H_2O_2	7.4	CL	Plant extracts	144	<1.9	Choi *et al.*, 1998
$O_2^{\bullet-}$	FIA	Luminol	XOD/xanthine	10.0	CL	–	–	–	Sariahmetoglu *et al.*, 2003
	FIA	Luminol	XOD/hypoxanthine	8.0	CL	–	–	–	Toyo'oka *et al.*, 2003
	FIA	2-(2-pyridil)-benzothiazoline	Alkaline Na_2SO_4	9.2	Fluorimetry	Garlic, onion and scallion	55	<0.3	Tang *et al.*, 2004
H_2O_2	FIA	Luminol	–	10.0	CL	–	–	–	Sariahmetoglu *et al.*, 2003
	FIA	Luminol	–	8.0	CL	–	–	–	Toyo'oka *et al.*, 2003
	SIA	HVA	–	7.5	Fluorimetry	Wines	15	<1.8	Pinto *et al.*, 2005
	MPFS	Lucigenin	–	7.4	CL	Pharmaceutical formulations and tea	70	<2.0	Meneses *et al.*, 2005
		Luminol		Alkaline			160		
	FIA-microfluidic	BPEA	–	–	CL	–	–	<1.5	Amatatongchai *et al.*, 2007

BPEA, 9,10-bis-(phenylethynyl)anthracene; CL, chemiluminescence; FIA, flow injection analysis; $HO^{\bullet}$, hydroxyl radical; H_2O_2, hydrogen peroxide; HVA, homovanillic acid; MPFS, multipumping flow system; $O_2^{\bullet-}$, superoxide anion radical; SIA, sequential injection analysis; XOD, xanthine oxidase.

Table 1.4. Flow-based methods for determination of scavenging capacity against specific ROS/RNS (continuation).

Reactive species	Flow method	Target compound	Generation of reactive species	pH value	Detection system	Type of sample	Det. rate (h^{-1})	RSD (%)	Ref.
$HO^{\bullet}$	FIA	Luminol	$O_2/FeSO_4$/buffer	10.0	CL	–	–	–	Sariahmetoglu *et al.*, 2003
	FIA	Terephthalate	H_2O_2/Co^{2+}	7.4	Fluorimetry	Food extracts and maize pollen polysaccharide	16	<0.6	Tang *et al.*, 2005
	FIA	Ninhydrin	H_2O_2/Co^{2+}	7.27	Fluorimetry	Food extracts	22	<1.0	Gao *et al.*, 2006
	FIA	Luminol	H_2O_2/Co^{2+}/EDTA	9.0	CL	–	120	<3.1	Giokas *et al.*, 2007
HOCl	FIA	Luminol	–	10.0	CL	–	–	–	Sariahmetoglu *et al.*, 2003
	SIA	Luminol	–	9.5	CL	–	45	<2.5	Nakamura *et al.*, 2004
	MSFIA	Luminol	–	7.4 and 10.0	CL	NSAIDs	92	<2.3	Magalhães *et al.*, 2007c
$^1O_2$	SIA	Luminol	Lactoperoxidase-H_2O_2-bromide ion system	4.5	CL	Multivitamin supplements	40	<1.4	Miyamoto *et al.*, 2006
$ONOO^-$	FIA	Luminol	$NaNO_2/H_2O_2$	10.0	CL	–	–	–	Sariahmetoglu *et al.*, 2003

CL, chemiluminescence; EDTA, ethylenediaminetetraacetic acid; FIA, flow injection analysis; $HO^{\bullet}$, hydroxyl radical; H_2O_2, hydrogen peroxide; HOCl, hypochlorous acid; MSFIA, multisyringe flow injection analysis; NSAIDs, non-steroidal anti-inflammatory drugs; $^1O_2$, singlet oxygen; $ONOO^-$, peroxynitrite anion; SIA, sequential injection analysis.

1.4.3. Flow-based methods for determination of scavenging capacity of stable, non-biological radicals ($DPPH^{\bullet}$ and $ABTS^{\bullet+}$)

Among the antioxidant assays, the most commonly used for their simplicity and throughput are those involving chromogen compounds of a radical nature, as $DPPH^{\bullet}$ or $ABTS^{\bullet+}$, to simulate ROS/RNS formed in vivo. These stable free radicals are widely used for the evaluation of the radical scavenging capacity of pure compounds and complex samples (see section 1.3). For this reason, the automation of these assays has been a relevant topic of research in the last five years.

The $DPPH^{\bullet}$ assay was initially automated using a FIA system and ESR spectrometry as detection system (Ukeda *et al.*, 2002). In this method, the $DPPH^{\bullet}$ solution was continuously fed into the flow-through flat cell and a constant ESR signal corresponding to the baseline was measured. When the radical scavenger compound/sample solution was injected into the carrier stream, the signal was suppressed and a negative peak appeared due to the reduction of radical $DPPH^{\bullet}$. This signal was proportional to the concentration of the radical scavenger. The low determination rate (13 h^{-1}) was due to the low resistance to pressure of the flow cell (flow rates higher than 0.32 mL min^{-1} were not examined). The results obtained for pure compounds (ascorbic acid, cysteine, trolox) as well as for beverages were compared with the HPLC-$DPPH^{\bullet}$ method described by Yamaguchi *et al.* (1998). The lower values found for some samples was caused by the difference in the reaction time applied in the HPLC method (20 min) and in the FIA method (< 2 min). Later, Polasek *et al.* (2004) developed a SIA-$DPPH^{\bullet}$ methodology suitable for rapid and routine screening of natural samples to search for those with pronounced antioxidant/radical scavenging capacity. In this assay, the $DPPH^{\bullet}$ zone was sandwiched between two zones of the antioxidant test solution. Thereafter, the stacked zones were mixed by moving the carrier back and forth; after a delay time of 20 s the reaction mixture was propelled into the spectrophotometer. The applicability of this SIA system was proved for pure compounds (ascorbic acid, caffeic acid, catechin, epicatechin, and rutin) and for routine screening tests for the presence of antioxidant compounds in a large series of lyophilised herbal and mushroom extracts. The results were compared to the batch

procedure and, despite the lower SIA results attained for two samples, this method seems to be still acceptable for screening purposes.

Recently, an automatic DPPH$^{\bullet}$ assay was developed using a flow procedure based on multisyringe flow injection analysis and spectrophotometric detection (Magalhães *et al.*, 2006a). A stopped flow approach was implemented to monitor the absorbance decrease due to the scavenging of DPPH$^{\bullet}$ radical by antioxidant compound(s). The authors evaluated the influence of initial DPPH$^{\bullet}$ concentration and sample dilution on the analytical performance of the methodology. Concerning the first topic, it was verified that the amount of DPPH$^{\bullet}$ consumed by antioxidant standards (ascorbic and caffeic acids) was independent of the initial concentration of DPPH$^{\bullet}$ radical except for situations where DPPH$^{\bullet}$/antioxidant molar ratio was lower than the stoichiometric value. For this reason, it was suggested to express the antioxidant capacity as the absorbance variation (or concentration of radical consumed) rather than the percentage of radical consumed since in the last case the absorbance decrease is divided by the initial absorbance. In addition, as the time to attain the reaction end-point will decrease for lower antioxidant concentrations, the influence of sample dilution was assessed within the time period of measurement. As expected, the dilution did not influence the results obtained for samples which were composed mainly by fast scavengers, whilst for samples containing or originating slow reacting compounds, the dilution played an important role to achieve results similar to that obtained by the end-point time-consuming batch method. Furthermore, for samples that do not exhaust its scavenging capacity within the period of measurement, a mathematical model was applied to estimate the total DPPH$^{\bullet}$ consumed. A similar MSFIA system was developed to evaluate the DPPH$^{\bullet}$ scavenging reaction of recognised antioxidant compounds in different reaction conditions (Magalhães *et al.*, 2007a). In this way, using the same configuration, the reaction conditions were adjusted in-line and it was applied to study the influence of pH (unbufered, 4.1, and 7.6) and solvent (methanol and ethanolic solution 50% v/v) during the first 3 min of the DPPH$^{\bullet}$ scavenging reaction. For those situations in which a stable value of absorbance was attained within time period of absorbance monitoring, the number of DPPH$^{\bullet}$ molecules reduced per molecule of antioxidant was calculated.

The automation of $ABTS^{\bullet+}$ assay was firstly implemented by Pellegrini *et al.* (2003b). For this, the authors used an HPLC pump to propel the mobile phase ($ABTS^{\bullet+}$ in ethanol) through a loop injector, a single bead string reactor filled with acid-washed silanized beads, a delay coil and a photodiode array UV-vis detector. After injecting the antioxidant(s), a negative peak representing the decolorization of $ABTS^{\bullet+}$ was obtained. Its area was proportional to the concentration of $ABTS^{\bullet+}$ radical reduced by antioxidant(s). The proposed flow injection system was applied for the evaluation of antioxidant capacity of pure compounds (ascorbic acid, caffeic acid, ferulic acid, gallic acid, naringenin, quercetin, α-tocopherol, and vanillic acid) and results were compared to the batch assay. In general, the two set of results were in good agreement, with exception of naringenin that exhibited a TEAC value 50% lower than that reported using the original spectrophotometric assay. The applicability of the technique was tested by measuring the antioxidant capacity of several common beverages (beer, coffee, cola, fruit juices, and tea) and results were not statistically different from the batch assay.

According to Bompadre *et al.* (2004), the previous FIA-$ABTS^{\bullet+}$ assay partially failed in obtaining good repeatability when more complex biological samples were analysed. Therefore, they proposed a novel system to improve this assay and to extend its application to blood plasma samples. In fact, when plasma samples were analyzed, a poor correlation was found between the proposed FIA method and the previously reported by Pellegrini *et al.* (2003b) as well as with those obtained with batch assay. The authors concluded that the temperature may be a critical aspect in the measurement of plasma antioxidant capacity whilst its influence may be less important in the assay of non-complex biological samples (mouthrinse, white wines). For this reason, the temperature and time/way of exposure of the active compounds present in the samples with $ABTS^{\bullet+}$ radical were strictly controlled. The temperature of the reaction coil was maintained at 35 ºC whilst the reaction time was fixed at 1.3 min. The improved FIA-$ABTS^{\bullet+}$ method was useful to screen rapidly, without dilution, and with high repeatability the antioxidant capacity of both non-complex biological mixtures and plasma samples.

The two FIA-$ABTS^{\bullet+}$ systems previously described are based on single-line manifolds and achieve sample throughputs up to 30 samples h^{-1}. Labrinea and Georgiou (2005) presented

a double line FIA system for determination of $ABTS^{\bullet+}$ scavenging capacity. This system exploits the concentration gradients formed along the injected sample bolus to obtain information on the reaction kinetics of $ABTS^{\bullet+}$ scavenging through a single injection. As the flow injection signals are the output of two kinetic processes occurring simultaneously, i. e. physical dispersion and chemical reaction, the authors observed that the TEAC values obtained for fast-reacting scavengers as ascorbic acid and caffeic acid were independent from the end-point time of measurement. Physical dispersion was the only process that influenced the analytical signal. On the opposite, the TEAC values of slow-reacting scavengers, such as (+)-catechin, (–)-epicatechin, ferulic acid, and gallic acid increased using the peak tail readings because of the longer reaction time allowed. The same authors had previously developed a stopped flow method using a similar manifold to study the effect of reaction time (10, 360, and 600 s) and pH (4.6, 5.4, and 7.4) on the $ABTS^{\bullet+}$ scavenging reaction (Labrinea and Georgiou, 2004). They concluded that the TEAC values were dependent on reaction time as well as on pH value for almost all studied antioxidant compounds, due to the differences in the rate of reaction for antioxidants and for trolox (reference compound). Due to the variation of TEAC with pH, they suggested that it is important to measure the $ABTS^{\bullet+}$ scavenging capacity at the pH of the system to which the results are going to be applied.

This dependence of $ABTS^{\bullet+}$ assay on the reaction time and on the pH was also reported by Lima *et al.* (2005). The proposed SIA–$ABTS^{\bullet+}$ manifold incorporates a well-stirred mixing chamber placed in a side port of the selection valve. This approach allowed a thorough mixture between the sample and $ABTS^{\bullet+}$ radical and made possible to use one single standard solution to perform the calibration procedure (based on aspiration of variable volumes of sample). By changing the carrier solution, different pH values were studied (non-buffered, 5.4 and 7.4). For almost all antioxidants tested, the authors verified that higher sensitivity values were obtained for higher pH values. This is not surprising taking into account that this assay is based on ET-mechanism (section 1.3), which is favoured under alkaline conditions.

Exploiting the idea that more than one method should be used for the evaluation of antioxidant capacity and taking advantage of the high versatility of computer-controlled

automatic methods, the $ABTS^{\bullet+}$ assay has been implemented with other methodologies in the same manifold without the need for system reconfiguration. Moreover, the fact of performing the determinations sequentially has also the advantage of processing the sample at the same time, avoiding errors that may arise due to sample modification over time. In the work described by Pinto *et al.* (2005), the SIA system developed accommodates in the same manifold the spectrophotometric $ABTS^{\bullet+}$ assay and the fluorimetric H_2O_2 scavenging capacity assay (described in section 1.4.2). The proposed method was applied to several pure compounds such as ascorbic acid, caffeic acid, catechin, gallic acid, and taxifolin as well as to Portuguese white and red wines. Both methodologies were developed using trolox as standard compound and results were expressed as TEAC (mM). Regarding the application to wine samples, a higher dilution level was necessary for the $ABTS^{\bullet+}$ assay. Thus, in order to introduce the same sample in the system and sequentially analyse by the two methods, an additional dilution was carried out in-line through two dilution coils incorporated in the side ports of the selection valve. Despite this, the developed method presented an acceptable determination throughput of 30 determinations per hour, accounting for 15 determinations for each assay.

Recently, an automatic flow procedure based on MSFIA was developed for the sequential spectrophotometric determination of $ABTS^{\bullet+}$ scavenging capacity and FC reducing capacity (Magalhães *et al.*, 2007b). The proposed method was applied to a large number of beverages (n = 72) divided in six groups, namely red wines, white wines, juices, herbal infusions, tea infusions and beers. The aim of this work was to provide an automatic flow method for routine assessment of reducing and antioxidant capacities of food products as well as for evaluation of the correlation between these two antioxidant assays. In this flow method, the same sample could be introduced in the system and analysed sequentially by the two methodologies without the need of an additional dilution. The results were compared within and between methods and showed that the correlation between these assays may vary according to the type of sample analysed.

The automatic $ABTS^{\bullet+}$ assays described above relies on the radical cation preformed off-line by chemical or enzymatic oxidation of ABTS (Table 1.5). This step is time-consuming because the kinetics of ABTS oxidation is slow and a few hours are required for reaction

completeness (16 or 3 h for chemical or enzymatic generation, respectively). To avoid this time-consuming step and make a step further toward the development of a fully automatic method, the $ABTS^{\bullet+}$ radical cation has been generated in-line through electrochemical oxidation and enzymatic reaction. In this context, Ivekovic *et al.* (2005) proposed a new manifold of the spectrophotometric $ABTS^{\bullet+}$ assay, where the radicals were generated in-line by electrochemical oxidation of ABTS in a flow-through electrolysis cell, incorporated into the FIA system. This flow-through cell was operated under constant current conditions, with the working electrode potential fixed at 700 ± 5 mV, and the amount of the $ABTS^{\bullet+}$ radical generated in-line was determined by the flow rate and the value of the current imposed on the cell. The applicability of the method was tested in nineteen pure compounds and several common beverages (coffee, red wine, and teas).

The in-line enzymatic generation of $ABTS^{\bullet+}$ radical for determination of antioxidant capacity was recently implemented by Milardovic *et al.* (2007a). The FIA method developed is based on the continuous flow of ABTS/H_2O_2 solution through a tubular flow-through bioreactor containing the enzyme horseradish peroxidase, which catalyses the oxidation of ABTS. The $ABTS^{\bullet+}$ radical generated was further merged with antioxidant compound/sample and the residual reduced concentration of $ABTS^{\bullet+}$ was measured in a biamperometric detector containing an interdigitated electrode. The current intensity obtained was proportional to the $ABTS^{\bullet+}$ radical scavenging capacity. To minimize the possible reaction between unreacted H_2O_2 and antioxidant(s), the authors reduced the inlet of H_2O_2 concentration from 120 μM (optimization studies) to 30 μM (application to food samples). Since it was verified that the ABTS/H_2O_2 solution was unstable during a working day, the same authors proposed later an FIA system in which the $ABTS^{\bullet+}$ radical was bienzymatically produced by glucose-oxidase and horseradish peroxidase enzymes, separately immobilized in tubular flow-through reactors (Milardovic *et al.* 2007b). In this case, the H_2O_2 necessary for ABTS oxidation was continuously formed in-line through the oxidation of glucose instead of continuous flow of ABTS/H_2O_2 solution. Despite the authors had developed the bienzymatic $ABTS^{\bullet+}$ method to minimize also the interference of H_2O_2, possibly occurring in monoenzymatic approach, it was not demonstrated that the H_2O_2 formed in the first enzymatic reaction has been completely consumed in the second

enzymatic reaction ($ABTS^{\bullet+}$ radical generation). Hence, the antioxidant(s) added to the $ABTS^{\bullet+}$ flow stream may still react with residual H_2O_2 and therefore this interference should not be discarded.

Table 1.5. Flow-based methods for determination of scavenging capacity of stable, non-biological radicals ($DPPH^{\bullet}$ and $ABTS^{\bullet+}$).

Reactive species	Flow method	Generation of reactive species	pH value	Detection system	Type of sample	Det. rate (h^{-1})	RSD (%)	Ref.
$DPPH^{\bullet}$	FIA	–	–	ESR	Coffee, red wine, tea	13	<3.2	Ukeda *et al.*, 2002
	SIA	–	unbufered and 4.8	UV-Vis	Herbal and mushroom extracts	45	<1.8	Polasek *et al.*, 2004
	MSFIA	–	–	UV-Vis	Beers, juices, tea, wines	13	<1.0	Magalhães *et al.*, 2006a
$ABTS^{\bullet+}$	FIA	Chemical	–	UV-Vis	Beer, coffee, cola, juices, tea	30	<1.7	Pellegrini *et al.*, 2003b
	FIA	Chemical	7.4	UV-Vis	Mouthrinse, white wine and plasma	–	<2.7	Bompadre *et al.*, 2004
	FIA	Enzymatic	4.6	UV-Vis	Honeys and wines	120	<2.7	Labrinea and Georgiou, 2005
	FIA	Electrochemical in-line	7.4	UV-Vis	Coffee, red wine, tea	32	<1.95	Ivekovic *et al.*, 2005
	FIA	Enzymatic in-line	7.4	Biamperometry	Juices, tea, wine	42	–	Milardovic *et al.*, 2007a
	FIA	Bienzymatic in-line	7.4	Biamperometry	Spirits and wines	–	–	Milardovic *et al.*, 2007b

$ABTS^{\bullet+}$, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonate) radical cation; $DPPH^{\bullet}$, 2,2-diphenyl-1-picrylhydrazyl radical; ESR, electron spin resonance; FIA, flow injection analysis; MSFIA, multisyringe flow injection analysis; SIA, sequential injection analysis; UV-Vis, ultra-violet-visible spectrophotometry.

Table 1.5. Flow-based methods for determination of scavenging capacity of stable, non-biological radicals ($DPPH^{\bullet}$ and $ABTS^{\bullet+}$) (continuation).

Reactive species	Flow method	Generation of reactive species	pH value	Detection system	Type of sample	Det. rate (h^{-1})	RSD (%)	Ref.
$ABTS^{\bullet+}$	SIA	Chemical	unbufered, 5.4 and 7.4	UV-Vis	Beer, juices, milk, tea, yoghurt	–	<0.3	Lima *et al.*, 2005
	SIA	Chemical	7.5	UV-Vis	Wines	15	<2.4	Pinto *et al.*, 2005
	MSFIA	Enzymatic	4.6	UV-Vis	Beers, juices, tea, wines	18	<3.1	Magalhães *et al.*, 2007b

$ABTS^{\bullet+}$, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonate) radical cation; MSFIA, multisyringe flow injection analysis; SIA, sequential injection analysis; UV-Vis, ultra-violet-visible spectrophotometry.

1.4.4. Flow-based methods for determination of total reducing/antioxidant capacity

As discussed in section 1.3.5., the oxidation potential (reducing capacity) of specific compound(s) or sample is conceptually related to their expected antioxidant capacity. Indeed, oxidation potential and antioxidant capacity are inversely related, so that when the oxidation potential of an analyte is low it means that this analyte could be oxidized easily or, in other words, more easily the compound(s) will donate an electron and higher is its antioxidant capacity. In food samples, with few exceptions such as carotenoids and vitamins C and E, the most readily oxidizable compounds are those containing (poly)phenolic groups. In fact, polyphenols are the most abundant antioxidants in our diet, since the average daily intake is about 1 g, which is almost 10-fold the intake of vitamin C, 100-fold the intake of vitamin E, and 500-fold the intake of carotenoids (Scalbert and Williamson, 2000). For this reason, the assessment of "total phenolic" content has gained enormous attention and has been directly related with the reducing/antioxidant capacity of food products.

In this context, in the last few years some flow-based methods have been developed to improve the measurement of overall reducing/antioxidant capacity of pure compounds and of a wide variety of food products (Table 1.6). This property has been determined through a redox reaction between the reductant compounds present in the sample and an oxidizing reagent (such as acidic potassium permanganate, ferric ion, or tungstate-molybdate). Amperometric methods, based on the measurement of current intensity obtained at a fixed potential have also been described.

Phenolic compounds commonly found in plants and derived foods, such as wine, generate chemiluminescence upon oxidation with acidic (pH 2) potassium permanganate in the presence of sodium polyphosphate (Hindson and Barnett, 2001). In this regard, Costin *et al.* (2003) developed a FIA system for the determination of the total phenolic/antioxidant levels in wine using acidic potassium permanganate CL detection. In this work, the sample injected into the carrier stream merged with acidic potassium permanganate just before the flow-through cell and the intensity of CL peak was measured. The proposed method was applied to pure compounds (caffeic acid, catechin, epicatechin, ferulic acid, gallic acid, 4-

hydroxycinnamic acid, quercetin, rutin, and vanillin) and several red and white wine samples. The CL response showed a good correlation with the batch $DPPH^{\bullet}$ radical scavenging assay.

Shpigun *et al.* (2006) developed a flow injection potentiometric method for the evaluation of antioxidant capacity based upon ferric-reducing ability. This method is based on the redox reaction between water-soluble antioxidants and the oxidant $[Fe(CN)_6]^{3-}$. The change in the composition of the carrier solution ($[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ redox-reagent) originated a negative potentiometric signal. The height of this transient negative signal was proportional to the reducing/antioxidant capacity of the compound/sample and a linear relationship between negative peak heights versus logarithm of the antioxidant concentration was attained. The proposed FIA-potentiometric method was applied to pure compounds (ascorbic acid, caffeic acid, chlorogenic acid, L-cysteine, gallic acid, pyrocatechol, pyrogallol, tannic acid, uric acid, and trolox) and a wide variety of food samples. As this assay relies on a chemistry similar to that applied in the FRAP assay, based on the reduction of ferric ion to ferrous ion, the presence of well known pro-oxidant species (Fe^{2+}) in the reaction medium may be considered as a shortcoming.

The automation of Folin-Ciocalteu assay intended for determination of total reducing capacity, measured as the cumulative capacity of phenolic and nonphenolic compounds to reduce the FC reagent (tungstate-molybdate acid complexes) was recently implemented by MSFIA system using spectrophotometric detection (Magalhães *et al.*, 2006b). In this work, different strategies for mixing of sample and reagent were exploited (continuous flow of FC reagent, merging zones, and intercalated zones approach), in order to attain lower reagent consumption and higher determination throughput. These studies were performed by changing the working conditions through software control without manifold reconfiguration. In addition, the carbonate buffer solution used for pH adjustment in the batch procedure was replaced by sodium hydroxide solution, since the rate of reduction of FC reagent is increased in higher alkaline medium. The MSFIA method was applied to several phenolic and nonphenolic compounds as well as to food products (wines, beers, teas, soft drinks and juices) and results were expressed as gallic acid equivalents (mg L^{-1}). Finally, it should be stressed that the results obtained by the proposed MSFIA method

using 4 min of reaction were in agreement with those attained by the time-consuming (2 h) batch method proposed for standardization (Singleton *et al.*, 1999). As mentioned before, this assay was also implemented in a MSFIA manifold that enabled the sequential determination of this parameter and the $ABTS^{\bullet+}$ scavenging capacity (Magalhães *et al.*, 2007b).

The flow methods abovementioned use reactive species, oxidants, to evaluate the reducing/antioxidant capacity of pure compounds and samples. In contrast, the flow methods using amperometric detection rely on the chemico-physical properties of the molecule(s) present in the sample, essentially polyphenols, and do not require the use of reactive species, radicals or non-radicals. This is an important issue as the antioxidant capacity assessed is strongly dependent on the oxidant species applied (Frankel and Meyer, 2000). The combination of single-line FIA systems with amperometric detection allowed high sample throughputs as well as high repeatability and reproducibility which are important features for routine determinations.

In 1998, Mannino *et al.* developed a simple FIA system for the determination of the antioxidant capacity in several red and white wines with an electrochemical detector operating at a fixed potential. The authors observed that at the lower potential established (+ 0.4 V *vs* Ag/AgCl) only some polyphenolic compounds, having particular structural features like three phenolic groups or two phenolic groups in *o*- or *p*-position, can be oxidised. These compounds showed a relevant antioxidant capacity when evaluated by other methodologies.

In this way, the same research group extended later this procedure for the evaluation of the "total antioxidant power" of olive oils as a model of lipophilic samples (Mannino *et al.*, 1999). The olive oils samples were injected into the FIA system with an electrochemical detector operating at a potential of + 0.5 V (*vs.* Ag/AgCl). This method offered an attractive alternative to the normally used Rancimat method that, owing to the severity of the oxidation conditions used, is not useful for the prediction of olive oil shelf-life. It was demonstrated that, molecules with an oxidation potential lower than + 0.6 V (*vs.* Ag/AgCl) possess good antioxidant capacity whereas molecules with higher values showed moderate or no antioxidant capacity. The results obtained by the FIA-electrochemical method were

compared to those of the $ABTS^{\bullet+}$ assay and a good correlation was found. Buratti *et al.* (2001) investigated the electrochemical properties of lipophilic compounds present in vegetables, such as carotenoids, chlorophylls, tocopherols, and capsaicin as well as several vegetable extracts. *β*-Carotene was used as standard compound and results were expressed as mg of *β*-carotene equivalents per kg of sample. These electrochemical-lipophilic assays rely on the use of high toxic solvents, such as chloroform, methyl tert-butyl ether, and methanol. In these cases, the implementation of flow-based methods can be an advantage in relation to the batch procedures since the exposition to operator is minimized.

The antioxidant capacities of low-molecular-weight fractions of whey were measured by FIA amperometric system using an oxidation potential of + 0.7 V (*vs.* Ag/AgCl) with trolox as standard compound (Chen *et al.*, 2002). This FIA procedure was later applied to an extended range of whey samples and the results obtained were compared to those provided by spectrophotometric batch methods: $ABTS^{\bullet+}$ and FRAP assays (Chen *et al.*, 2003). A limitation to the application of this methodology is that only low-molecular-weight redox substances were measurable. In fact, the possible current response to larger molecules (such as proteins) would be very low due to their low diffusion coefficient. On the other hand, the high-protein content of these samples caused fouling problems due to protein adsorption at the electrode surface, with concomitant decrease of its area. Hence, proteins had to be removed off-line prior to determination.

Blasco *et al.* (2005) have proposed a new screening electrochemical protocol to determine total polyphenolics in foods. They introduced the concept of the "Electrochemical Index", defined as the total polyphenolic content obtained by electrochemistry. For this, a conventional FIA system with amperometric detection using a glassy carbon disk electrode was employed. Basically, the electrochemical protocol consisted in the measurement of the amperometric current at neutral pH (7.5) and at different oxidation potentials. Since the selectivity increases after a decrease of the oxidation potential, different degrees in the total polyphenolic fractions could be attained. In this way, it was suggested that the total polyphenolics measured under no selective oxidation conditions (+ 0.8 V) represent the "electrochemical index" while the total polyphenolics measured under more selective conditions (+ 0.5 V) represent the high antioxidant polyphenolic fraction. The present

electrochemical protocol was applied to several food products (peel, pulp and juices of apple and pear, wines, and fresh and processed green beans). The results obtained using the electrochemical protocol were well correlated with "total phenolics" obtained using a spectrophotometric method (FC assay).

Later, the same research group exploited the analytical possibilities of the "electrochemical index" to estimate the antioxidant capacity in honey samples (Ávila *et al.*, 2006). The results obtained at both potentials applied (+ 0.5 and + 0.8 V) exhibited a high correlation with the $DPPH^{\bullet}$ assay. Recently, Buratti *et al.* (2007) using a FIA system with an electrochemical detector operating at a potential of + 0.5 V (*vs.* Ag/AgCl) evaluated the antioxidant capacity of honeybee products (honey, propolis and royal jelly) and similar conclusions were attained.

In general, the reported FIA-electrochemical methods have several features, such as simplicity, versatility, low cost, and high sample throughput, necessary for rapid and reliable analytical methodologies for determination of reducing/antioxidant capacity in complex matrices as food products. Nevertheless, the different manifolds developed until now apply different oxidation potentials and different pH values (Table 1.6). In addition, the electrochemical properties of samples have been quantified using different reference standard compounds such as caffeic acid (Maninno *et al.*, 1998), *β*-carotene (Buratti *et al.*, 2001), (+)-catechin (Maninno *et al.*, 1998; Blasco *et al.*, 2005), galangin (Buratti *et al.* 2007), gallic acid (Ávila *et al.*, 2006) and trolox (Chen *et al.*, 2002). As reported by Ávila *et al.* (2006), the values obtained for honey samples were strongly influenced by the oxidation potential, the pH and the reference compound used. Therefore, continued efforts to standardize the reaction conditions are unequivocally necessary in order to obtain comparable results within and between methods.

Table 1.6. Flow-based methods for determination of total reducing/antioxidant capacity.

Assay	Flow method	pH value	Detection system	Type of sample	Det. rate (h^{-1})	RSD (%)	Ref.
Total phenolics	FIA	2.0	CL	Wines	120	<0.8	Costin *et al.*, 2003
Reduction of redox reagent	FIA	≥ 7.0	Potentiometry	Fruit extracts, herbal infusions and tea	100	<1.8	Shipgun *et al.*, 2006
FC reducing capacity	MSFIA	–	UV-Vis	Beers, juices, tea, wines	12	<1.3	Magalhães *et al.*, 2006b
Reducing capacity	FIA	–	Amperometry (+0.4 V)	Wines	–	–	Mannino *et al.*, 1998
Reducing capacity	FIA	–	Amperometry (+0.5 V)	Extra virgin olive oils and olive oils	90	<3.5	Mannino *et al.*, 1999
Reducing capacity	FIA	–	Amperometry (+0.5 V)	Vegetable extracts	60	<3.5	Buratti *et al.*, 2001
Reducing capacity	FIA	4.6	Amperometry (+0.7 V)	LMW fractions of whey	60	<2.7	Chen *et al.*, 2002
Reducing capacity	FIA	7.5	Amperometry (+0.8 V, +0.5 V, +0.3 V)	Apple, pear, green beans, juices, wines	–	<7	Blasco *et al.*, 2005

CL, chemiluminescence; FC, Folin-Ciocalteu; FIA, flow injection analysis; LMW, low molecular weight; MSFIA, multisyringe flow injection analysis; UV-Vis, ultra-violet-visible spectrophotometry.

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CHAPTER 2

General materials and methods

2.1. Introduction

In this chapter, reagents, standards and samples that were used throughout the work are described. The devices used to assemble the flow injection systems are also fully described. Furthermore, several aspects related to the automatic antioxidant capacity assays, namely the optimization procedures and the expression of this parameter, and the statistical treatment used to assess the quality of the results are also described in detail in the following sections.

2.2. Reagents, solutions and samples

All chemicals used were of analytical reagent grade with no further purification. For the preparation of all solutions, water from Milli-Q system (resistivity > 18 MΩ cm), ethanol and methanol pro analysis were used throughout the work.

Standard stock solutions were obtained by weighing the respective reagent in a Mettler Toledo analytical balance (model AG 285), followed by dissolution in the appropriate solvent (namely water, buffer solution, ethanol or methanol). The working standards were obtained by rigorous dilution of the stock solution using glass pipettes, Gilson micropipettes and volumetric flasks (class A) of different volumes. Micropipettes (models P100, P200, P1000, and P5000, with corresponding maximum capacities of 100, 200, 1000, and 5000 μL) were regularly calibrated with deionised water.

When necessary, pH of solutions was measured using a combined glass pH electrode (Crison 52-02) and a Crison model GLP 22 milivoltimeter.

All food samples analysed during this work were purchased at local markets. Herbal and tea infusions were prepared by pouring 200 mL of deionized water at 90 ºC into a glass with herbal or tea bag and by brewing for a fixed time. Carbon dioxide from white wines and beers was removed by stirring. Food samples were diluted with water just before measurement (Chapters 5 and 6). In Chapter 4, samples were first diluted 50% using ethanol in order to attain a final concentration of 50% (v/v) in ethanol. For wine and beer

samples, the initial alcohol content was considered and these samples were diluted with ethanol and water. Samples were further diluted using ethanolic solution 50% (v/v).

2.3. Multisyringe flow injection systems

The automatic flow procedures developed for the determination of antioxidant capacity throughout the present work were attained by multisyringe flow injection analysis (MSFIA). Described for the first time by Cerdà *et al.* (1999), these flow systems combine the multi-channel operation of peristaltic pumps with the possibility of selecting only the exact volume of the sample and reagent required for analysis by means of commutation valves. Recently, Segundo and Magalhães (2006) discussed the characteristics (apparatus, manifold design, and operation mode) of MSFIA systems and presented a survey of applications. The MSFIA systems developed were basically constituted by a multisyringe burette and a flow network connected to a detection system (Fig. 2.1). The flow management and data acquisition were defined through computer control. The devices used during this work will be described below in detail.

2.3.1. Multisyringe burette

A multisyringe burette was used throughout the work for aspiration/propulsion of solutions through the flow network (Fig. 2.1). This device is a multiple channel piston pump, containing up to four syringes (Hamilton), where all pistons are connected to the same bar, which is driven by a single motor of an usual automatic burette. The movement (direction and speed) of the motor is controlled by computer software through a serial port. The multisyringe module also comprises a three-way commutation valves connected to the head of each syringe, allowing optional coupling to the flow network or to the solution reservoir. The exchange options of the commutation valves are often classified in off/on

lines, where the "off" line was assigned to the solution reservoirs (reagents, carrier or waste) and the "on" line was reserved for the flow network (Fig. 2.1).

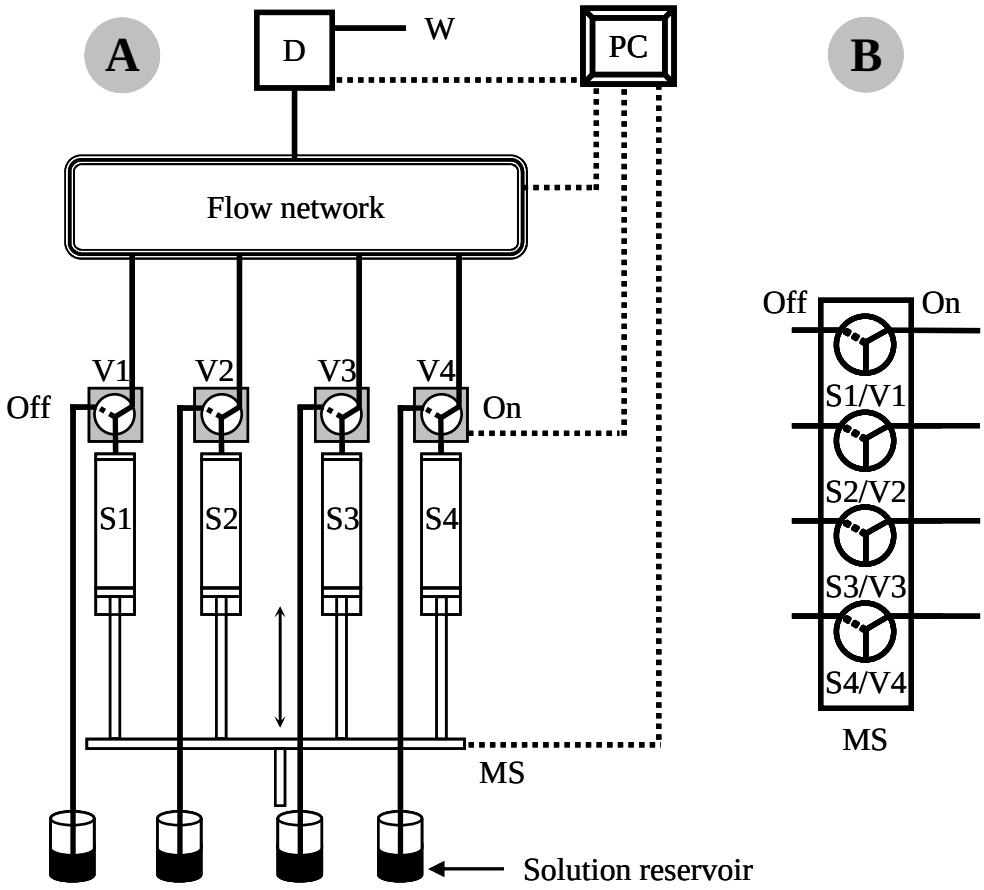

Figure 2.1. A) Schematic representation of multisyringe flow injection systems developed for determination of antioxidant capacity: MS, multisyringe burette; S1–S4, syringes; V1–V4, three-way commutation valves; D, detector; PC, personal computer; W, waste. **B)** Simplified schematic representation of the multisyringe burette. The "off" line was assigned to the solution reservoir, and the "on" line was reserved for the flow network.

Considering that all pistons move at once in the same direction either delivering liquids (dispense operation) or loading the syringes (pickup operation) and that each commutation valve may be switched to two positions (on/off), there are four possibilities for flow management, as schematically depicted in Fig. 2.2.

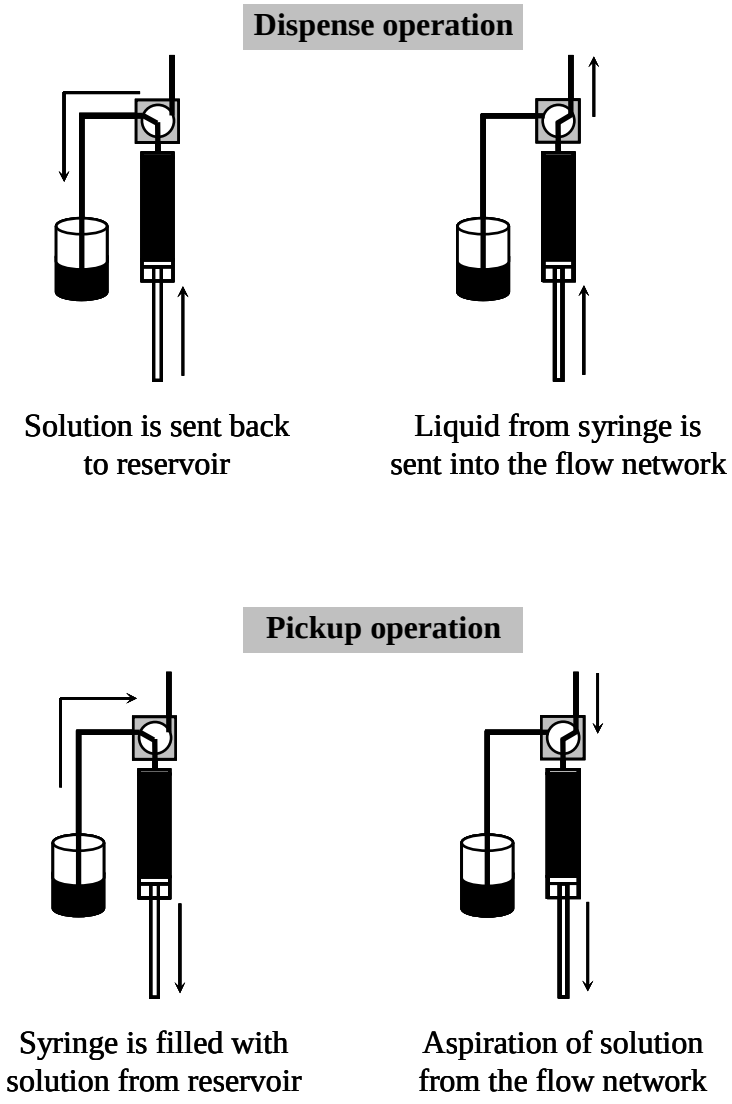

Figure 2.2. Flow management possibilities for one syringe during the operation of a multisyringe apparatus.

Therefore, when the pistons are moving upwards, it is possible to send liquid back to respective reservoir, or dispense into the flow network direction. This feature enables that only the necessary amount of reagent/carrier solution is introduced in the flow network. Furthermore, when the pistons are moving downwards, it is possible to refill the syringes with solutions present in the respective reservoir or to aspirate reagents and/or samples from the flow network. Segundo *et al*. (2005), studied the order of movements of piston bar during the analytical cycle and verified that any alteration of the flow direction affected the volume delivered in the subsequent step. For this reason, the introduction of a "dummy" step was recommended whenever the flow direction was changed before the aspiration/propulsion of precise volumes.

A wide range of flow rates can be obtained with this apparatus, depending on the motor speed and the volumes of the available syringes. For instance, for a 10 mL syringe, flow rates from 0.57 up to 30 mL min^{-1} can be attained (Miró *et al.*, 2002). Nevertheless, once the flow rate and volume is fixed for one syringe, it is also defined for other channels, depending on the ratio between syringes capacities. The main disadvantage of this propulsion unit is that the forward movement must be stopped to reload the syringes, decreasing the determination frequency.

2.3.2. Flow network

All the tubing connecting the different components of the multisyringe flow injection systems was made of polytetrafluoroethylene of 0.8 mm i.d., except for tubes between flasks and syringes, which were of 1.5 mm i.d. in order to avoid back pressure or vacuum at high flow rates. End-fittings and connectors were also used. Laboratory made acrylic three-way connectors were used as confluences throughout the work, while four-way connectors were also used in the systems described in Chapters 5, 6, and 7.

In MSFIA systems it is not feasible to introduce the sample into the system by filling one of the available syringes, since it would take a long time for washing operations to avoid carry-over effect (Miró *et al.*, 2002). Moreover, the amount of sample required for those

washing steps would be considerably large, which is not compatible with samples that are scarce, toxic or expensive. Hence, other devices such as extra commutation valves (Chapters 3–6) and a 10-port multiposition selection valve (Chapter 7) were incorporated in the manifolds in order to provide access to the reagents, antioxidant standards, or samples. In Chapter 6, the flow assembly included two 8-port multiposition selection valves, disposed in the same module, for accommodating the six reaction coils; three for FC assay and the other three for TEAC assay. This type of valves acts as a stream selector, connecting just one side port to the central one. The number of possible positions depends on the number of the side ports.

Throughout the work, the time-based mode was applied for fluid insertion, rather than the volume-based approach (Segundo *et al.*, 2005), since in the former any alteration in the reagent/sample volume can be performed through software control, without any physical reconfiguration of the manifold. It was based on the aspiration (Chapters 3–6) of a certain volume, defined by the flow rate and time during which the multisyringe burette was activated. In Chapter 7, the time-based sampling was performed by aspirating a large volume of sample through a selection valve, followed by the introduction of precise aliquots for further determination. This strategy enhances the sample throughput, since it is not necessary to aspirate sample or to refill the syringes between each determination.

2.3.3. Detectors

For the spectrophotometric determination described in Chapters 3, 4, and 5, the spectrophotometer models used were the Jenway 6100 and 6105. The wavelength was set at 517 nm (Chapters 3 and 4) whilst the absorbance measurements in Chapter 5 were carried out at 750 nm.

In Chapter 6, to perform sequentially the FC and TEAC assays, the absorbance measurements were carried out at 750 and 734 nm, respectively. For this, an Ocean Optics PC 2000-ISA spectrophotometer connected to a 200 μm fiber optic cable and a DH-2000 deuterium-halogen light source from Top Sensor Systems was applied.

A flow-through cell from Hellma (internal volume 80 μL, optical path 10 mm, ref. 178.710-QS) was used for spectrophotometric measurements performed in the flow systems.

In Chapter 7, the detection system was based on lab-made chemiluminescence cell adapted to flow assemblies. The chemiluminescence detector that was previously described for utilization in MSFIA manifolds (Miró *et al.*, 2005) was improved by engraving the flow path on an Acetal-Delrin block, providing a spiral-shaped flow-through cell with an inner volume of 80 μL and an effective emitting surface of approximately 0.85 cm^2. This flow cell was placed and fixed over a photosensor module (Hamamatsu HS5784) window, producing a compact light-tight box (Fig. 2.3).

For the comparative procedures performed in Chapters 4 and 5, the $DPPH^{\bullet}$ assay (Brand-Williams *et al.*, 1995) and the Folin-Ciocalteu assay (Singleton *et al.*, 1999), respectively, were adapted to a microplate reader (Synergy HT, Bio-Tek). In Chapter 4, the reduction in $DPPH^{\bullet}$ concentration due to antioxidant compounds/sample was monitored every 20 s until the absorbance decrease (λ = 517 nm) was inferior or equal to 0.003 units min^{-1}. In Chapter 5, the absorbance increase (λ = 760 nm) due to the reduction of Folin-Ciocalteu reagent by antioxidant compounds/sample was monitored every 60 s during 2 h. In both assays, all experiments were performed in quadruplicate and the temperature was kept at 25.0 ± 0.1 ^{o}C.

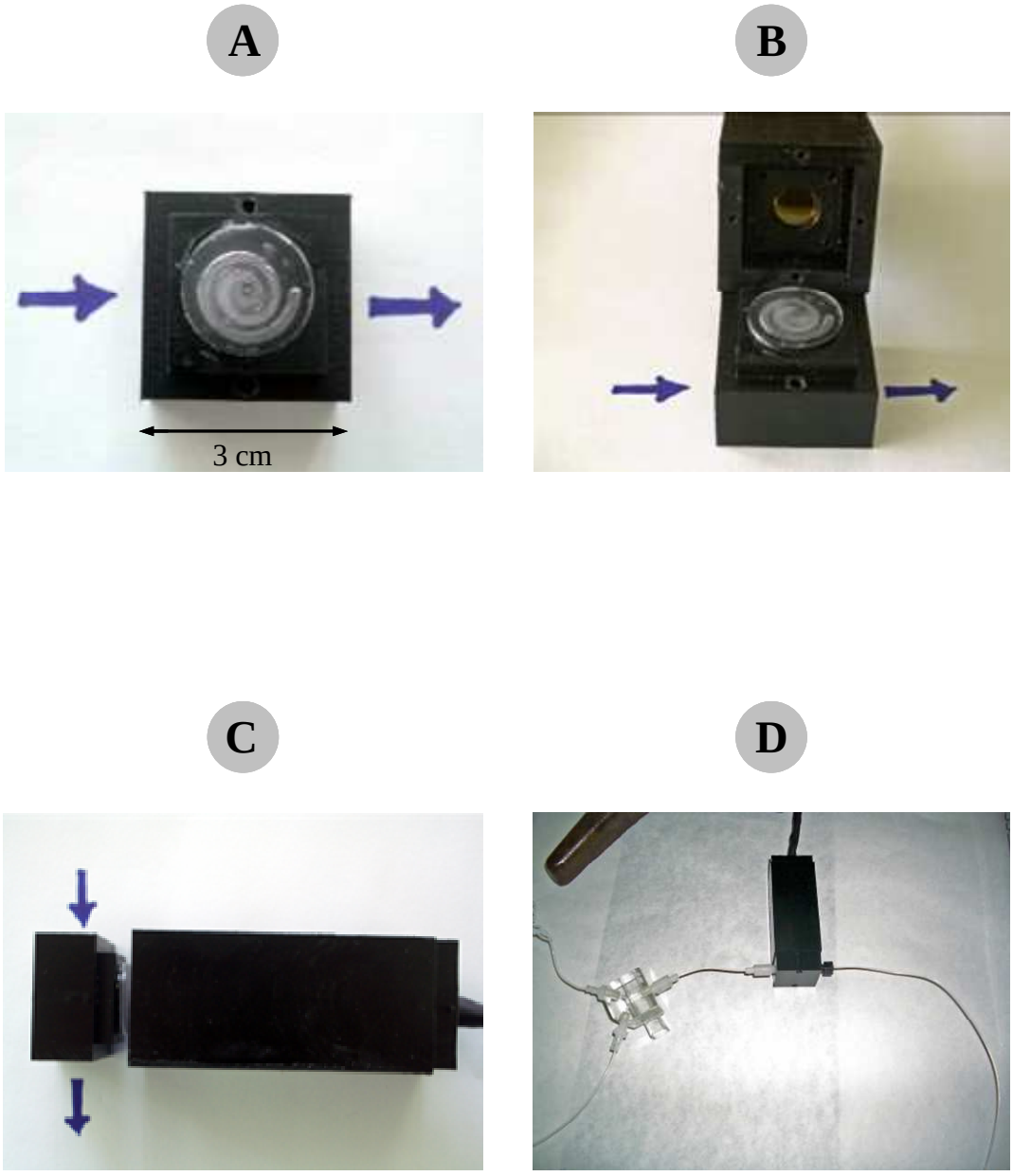

Figure 2.3. A) Frontal view of the custom-built spiral-shaped flow cell. **B)** Frontal and **C)** upper view of the flow cell positioned over the photosensor module, located inside the light-tight box. **D)** Upper view of CL-detector connected to the flow manifold. The arrows indicate the flow direction.

2.3.4. Computer control and data acquisition

The multisyringe operation (number of steps per unit of time and direction of piston displacement), and the positioning of commutation and selection valves was performed through computer control. For most of the systems described (Chapters 3–6), this was attained by a personal computer equipped with an Advantec PCL-711 B interface card, running lab-made software written in QuickBasic 4.5 (Microsoft). The data acquisition at 3 Hz (Chapter 3, 4, and 5) was implemented by connecting the analog output of the spectrophotometer to the interface card and using the same software developed for controlling the other components of the flow system. In Chapter 6, the data acquisition was performed by SpectraWin (version 4.2) software, after an external trigger signal from the interface card. The acquisition frequency was 4 Hz, corresponding to an integration time of 0.023 s and an average of 11 scans. Finally, in Chapter 7, the software package Autoanalysis, written in Delphi (version 5.0) and Visual C++ (version 6.0), was used (Becerra *et al.*, 1999). This software controls the multisyringe and valve operation, and also enables the integration of the experimental data and treatment of the analytical signals. Whenever necessary, the analytical signals were also registered on paper using a Kipp and Zonen BD 111 strip chart recorder.

2.4. Development of the automatic antioxidant capacity assays

During the studies concerning the physical and chemical parameters the univariate method was used, which consisted of varying each parameter under study within a certain interval, while keeping the other parameters fixed. The developed systems were characterised in terms of working concentration range, limit of detection, determination frequency and repeatability.

The working concentration range was determined by injecting a series of standard solutions with different concentrations, and determining the range where the analytical signal was related to the concentration.

The limit of detection was expressed as the concentration (c_L) derived from the smallest measure (x_L) which can be detected with reasonable certainty for a given analytical procedure (IUPAC, 1976; Currie, 1995). The value of x_L is given by the equation $x_L = x_b + 3s_{y/x}$, where x_b is the intercept value of the calibration curve and $s_{y/x}$ is the deviation (or uncertainty) of y values. The minimum detectable concentration can be further calculated by interpolation in the calibration curve (Miller and Miller, 2005).

The determination frequency, expressed as the number of determinations per hour, was the time required for a complete analytical cycle. This was calculated as the summation of the time taken for each operation step performed plus the time required for communication between the computer and the components of the flow systems. The sampling rate was assessed by the ratio between the determination frequency and the number of analytical cycles performed for each sample.

The repeatability, or precision, of the developed method was evaluated by the determination of the relative standard deviation (RSD) values corresponding to consecutive determinations of standard antioxidant solutions (Chapters 4, 5 and 7) and samples (Chapter 6). The standard solutions and samples with different analyte concentrations were selected to provide a reliable estimation of the repeatability of the method along the application range.

2.5. Determination of antioxidant capacity

In Chapter 3, the antioxidant capacity of pure compounds in different reaction media was assessed as the number of $DPPH^{\bullet}$ molecules reduced by one molecule of antioxidant (AH), calculated by the following equation:

$$n = (A_0 - A_f) / (\varepsilon_{DPPH\bullet} \times ([AH]_{st}/D)) \qquad (2.1)$$

where, $(A_0 - A_f)$ is the absorbance decrease due to $DPPH^{\bullet}$ consumption by antioxidant compounds; $\varepsilon_{DPPH\bullet}$ is the molar extinction coefficient of $DPPH^{\bullet}$ in the respective reaction

media used; $[AH]_{st}$ is the concentration of antioxidant in the standard solution; and D is the dispersion coefficient (Ruzicka and Hansen, 1988).

The determination of the amount of $DPPH^{\bullet}$ consumed by antioxidant compounds, described in Chapter 4, was assessed from the difference between the absorbance of $DPPH^{\bullet}$ in the absence of antioxidant compounds and the absorbance of $DPPH^{\bullet}$ measured in the presence of antioxidant compounds after a fixed period of time (120 s). In this way, a calibration curve relating the absorbance decrease after 120 s and the concentration of ascorbic acid (mg/100 mL) was established. The absorbance decrease obtained for samples was interpolated to that of ascorbic acid standard curve and the results were expressed as vitamin C equivalent antioxidant capacity (VCEAC), representing the equivalent amount of ascorbic acid (mg) present in 100 mL of sample. These units were chosen since ascorbic acid is a common antioxidant compound found in food products and the nutritional labelling on food products are indicated per100 mL or per 100g. For samples that did not exhaust its scavenging capacity within the period of measurement, the information gathered during 180 s of absorbance monitoring was used to estimate the total consumption of $DPPH^{\bullet}$. The following double-exponential equation was applied:

$$DPPH^{\bullet} = DPPH_1^{\bullet} \times e^{-\alpha t} + DPPH_2^{\bullet} \times e^{-\beta t} + DPPH_r^{\bullet} \qquad (2.2)$$

where, $DPPH^{\bullet}$ is the radical concentration at any time *t*; α and β are the first-order rate constants for the simple uniexponential decay of the fast and slow step, respectively; $DPPH_1^{\bullet}$ and $DPPH_2^{\bullet}$ represent the concentration of radical consumed in the fast and in the slow step, respectively; $DPPH_r^{\bullet}$ is the remaining radical concentration in the medium after antioxidant depletion. Since $DPPH^{\bullet}$ concentration values were proportional to absorbance values, the VCEAC values of samples were calculated through the sum of absorbance decrease (proportional to radical consumption) in the fast and slow steps ($DPPH_1^{\bullet}$+ $DPPH_2^{\bullet}$).

For the determination of FC reducing capacity, described in Chapters 5 and 6, gallic acid was used as the standard compound. In this method, the absorbance increase due to the reduction of Folin-Ciocalteu reagent was related to the concentration of gallic acid

standard solutions (mg L^{-1}). The FC reducing capacity of pure compounds (Chapter 5) was estimated by the ratio (%) between the slope of the calibration curve determined for the testing compound and the slope of the calibration curve of gallic acid. The FC reducing capacity of food samples (Chapters 5 and 6) was obtained by interpolation of the absorbance value in the calibration curve of gallic acid. Thus, the results were expressed as gallic acid equivalents (mg L^{-1}). Finally, for the determination of TEAC (Chapter 6), a calibration curve relating the absorbance values to the trolox concentration (mM) was established. The absorbance values obtained for samples were interpolated in this calibration curve and results were expressed as trolox equivalents (mM).

In Chapter 7, the HOCl scavenging capacity of different NSAIDs and positive controls was evaluated through the decrease in CL emission. The percentage of CL inhibition was assessed using the following formula:

$$(\%)\ \text{CL inhibition} = ((CL_{blank} - CL_{sample})/CL_{blank}) \times 100 \qquad (2.3)$$

where, CL_{blank} and CL_{sample} represent the light intensity obtained in the absence and in the presence of scavenger compounds, respectively. The percentage of CL inhibition was related to the concentration of the scavenger compound (μM). The sample concentration providing 50% inhibition of CL-blank emission (IC_{50}) was then estimated and used to compare the HOCl scavenging capacity of the compounds tested.

2.6. Validation of the results

In Chapter 3, the number of $DPPH^{\bullet}$ molecules reduced by one molecule of antioxidant obtained for several pure compounds were compared to values previously reported in the literature using conventional batch methods.

In order to evaluate the accuracy of the flow methodologies proposed in Chapters 4 and 5, the results obtained by the developed flow method (C_{MSFIA}) were compared with those obtained by the conventional batch method (C_{batch}). The results obtained from the two

procedures were plotted against each other (C_{MSFIA} versus C_{batch}) and a linear regression was established between the two variables. The parameters of the regression line, intercept (C_0) and slope (S), were calculated. In the ideal situation, or in perfect agreement of the methods, C_0 should be equal to 0 and S to 1. Hence, the methods are statistically comparable if C_0 and S do not differ significantly from 0 and 1, respectively. This was verified by estimating the errors in the intercept and slope values through calculation of their confidence limits at 95% significance level (Miller and Miller, 2005). The relative deviation (RD) values, expressed as percentage, were calculated based on the expression *2.4*.

$$((C_{MSFIA} - C_{batch}) \times 100) / C_{batch} \qquad (2.4)$$

Furthermore, a paired *t*-test was performed on the data obtained for all samples in Chapter 5. Whenever the *t* value calculated was inferior to the reference *t* value ($P = 0.05$), the null hypothesis was verified, indicating that there was no significant difference for the mean concentrations obtained by the two methods (Miller and Miller, 2005).

In Chapter 6, the FC reducing capacity and TEAC of each sample were expressed as the mean ± standard deviation ($n = 3$). Twelve food products of each group, namely red wines, white wines, herbal infusions, tea infusions, juices, and beers were analysed and the mean, maximum, minimum, and interval values were calculated. These results were compared with the data reported in the literature. For each method, one-way analysis of variance (ANOVA) was performed to determine if there was a significant difference between groups at a 95% level. The Levene test was applied to test the homogeneity of variances. Finally, a post hoc comparison test (Tamhane's T2) was applied to determine which group(s) differ from each other. For comparison between methods, linear regression was applied to study the possible correlation between the studied parameters. These statistical analyses were carried out using SPSS (Statistical Package for the Social Sciences) program version 14.0 for Windows.

In Chapter 7, the HOCl scavenging capacity expressed by the IC_{50} values were compared to values reported in the literature, obtained by other methodologies.

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CHAPTER 3

Multi-syringe flow injection system for the determination of the scavenging capacity of the diphenylpicrylhydrazyl radical in methanol and ethanolic media

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Original Paper

Multi-syringe flow injection system for the determination of the scavenging capacity of the diphenylpicrylhydrazyl radical in methanol and ethanolic media

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Abstract. A multi-syringe flow injection system for the determination of 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•) scavenging capacity using methanol or ethanolic solutions as reaction media is presented. A stopped flow approach was implemented in order to monitor the DPPH• consumption using spectrophotometry at 517 nm. The kinetic profile for the first 3 min of reaction was determined for several antioxidant compounds and the number of DPPH• molecules reduced by one molecule of antioxidant was calculated whenever the absorbance value was stable within the period of measurement. For ascorbic acid, trolox, isoeugenol and quercetin in methanol, the values obtained were similar to those reported in the literature. It was possible to perform 14 determinations per hour, consuming 0.34 μmol of DPPH• per determination, which accounts for the application of this system as a screening tool for fast scavenger compounds.

Key words: Multi-syringe flow injection; DPPH•; spectrophotometry; solvent and pH effect.

Antioxidants play a crucial role in the protection of organisms or tissues as well as of nonliving systems against oxidative stress [1]. In the past few years, this subject has been addressed by many research groups in a broad spectrum of areas, including physiology, pharmacology, nutrition and even food processing. The growing importance of this topic of research is supported by evidence that several pathologies such as arteriosclerosis, cataracts and certain types of cancer are caused or accelerated by oxidative stress induced by reactive nitrogen species (RNS) and reactive oxygen species (ROS) [2]. In all these areas of research reliable methods for antioxidant assessment are necessary, and those involving chromogen compounds of a radical nature to simulate RNS and ROS are popular, especially for screening purposes [3].

In this context, a multi-syringe flow injection (MSFIA) system for the determination of 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•) scavenging capacity using different reaction media is proposed. The MSFIA technique was proposed in 1999 [4–7], in order to combine the multi-channel operation of flow injection analysis (FIA) [8] and the flexibility of multi-commutation flow systems [9]. The DPPH• assay has been performed using FIA [10, 11], sequential injection analysis (SIA) [12], and MSFIA [13] systems. Although application to beverages and herbal and mushroom extracts was reported, a systematic study of the reaction conditions was not performed.

Therefore, in the present work, the novel features introduced by MSFIA and the controlled conditions

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(time, dispersion) provided by flow injection methods in general were applied to study the influence of pH and solvent (methanol and ethanol solution) during the first 3 min of reaction for several antioxidant compounds. A stopped flow approach was chosen in order to establish the kinetic profile. For those situations in which a stable value of absorbance was attained within the time period of measurement, the number of DPPH• molecules reduced by one molecule of antioxidant was also calculated.

Material and methods

Reagents and solutions

Water from MilliQ system (resistivity > 18 MΩ cm), ethanol p.a. and methanol p.a. were used for the preparation of all solutions, and all chemicals used were of analytical reagent grade.

The radical 2,2-diphenyl-1-picrylhydrazyl (DPPH•); L-ascorbic acid; 3,4,5-trihydroxybenzoic acid (gallic acid); and 2(3)-*t*-butyl-4-hydroxyanisole (BHA) were purchased from Sigma (St. Louis, USA). 6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (trolox); 2-methoxy-4-propenylphenol (isoeugenol); 2-methoxy-4-(2-propenyl)phenol (eugenol) and di-sodium hydrogen phosphate dihydrate were obtained from Fluka (Buchs, Switzerland). 2-Hydroxy-1,2,3-propanetricarboxylic acid monohydrate (citric acid monohydrate) and 1,2,3-benzenetriol (pyrogallol) were purchased from Riedel-de-Haen (Seelze, Germany). 3-(3,4-Dihydroxyphenyl)-2-propenoic acid (caffeic acid); 3-phenyl-2-propenoic acid (cinnamic acid); 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-1-benzopyran-4-one dihydrate (quercetin dihydrate); quercetin-3-rutinoside hydrate (rutin hydrate); 1,2-benzenediol (catechol) and 1,3-benzenediol (resorcinol) were all obtained from Aldrich (Milwaukee, USA).

For the flow system, methanol p.a. or ethanol solution 50% v/v was used as carrier. For the experiments with pH adjustment, buffer solutions containing ethanol 50% v/v were prepared according to Perrin and Dempsey [14]. Therefore, a citrate-phosphate buffer solution was prepared by placing 20.0 ml of 0.02 mol L^{-1} Na_2HPO_4 solution in a volumetric flask and by making the volume up to 100 mL using 0.01 mol L^{-1} citric acid solution. This solution was further diluted using an equal volume of ethanol and the apparent pH of the final solution was 4.1. For the experiments at apparent pH 7.6, the buffer solution was prepared in a similar way, using 40.0 mL of 0.01 mol L^{-1} citric acid solution and 0.02 mol L^{-1} Na_2HPO_4 to complete the volume up to 100 mL.

Stock solutions of DPPH• containing 8.0×10^{-4} mol L^{-1} were prepared using either methanol or ethanol as solvent. These solutions were kept at 4 °C, protected from light. They were stable at least for 5 days. Working solutions of DPPH• (1.6×10^{-4} mol L^{-1}) were prepared daily by dilution of the prior solutions, using only methanol or ethanol and water in order to achieve a final concentration of 50% v/v in ethanol.

Stock solutions containing each antioxidant compound at 1.0×10^{-2} mol L^{-1} were prepared daily, using either methanol or ethanol solution (50% v/v). Working solutions between 0.60 and 6.0×10^{-4} mol L^{-1} were also prepared using each solvent.

For determination of dispersion coefficient of Ruzicka [8], a bromothymol blue (BTB) solution was prepared from a stock solution (0.50 g L^{-1}) by dilution in methanol or ethanol solution 50% v/v in order to provide an absorbance value of about 0.520 at 428 nm.

Apparatus

Solutions were propelled through the flow system by means of a multi-syringe burette (Crison Instruments, Allela, Spain). This device is a multiple channel piston pump, where all pistons are driven by a single motor, controlled by computer software through a serial port. It also comprises three-way commutation valves (NResearch, Caldwell, NJ, USA) connected to the head of each syringe.

In the present work, the multi-syringe was equipped with three syringes with different volumes: 5 mL in positions (S1) and (S2) and 10 mL in position (S3) (Fig. 1). Two extra commutation valves were included in the module used for introduction of antioxidant solution. For all valves, the exchange options were classified in on/off lines. The "off" line was assigned to the solution flasks and the "on" line was reserved for the flow network in the valves placed at the multi-syringe. For the other valves, the positions "on/off" were chosen to minimize the time during which the valves were switched on in order to avoid over-heating problems.

A personal computer, running lab-made software written in QuickBasic 4.5 (Microsoft®), controlled the multi-syringe operation (number of steps and direction of piston displacement and position of all commutation valves).

As detection system a Jenway 6100 (Essex, UK) spectrophotometer equipped with a Hellma (Mullheim, Baden, Germany) 178.710-QS flow-through cell (internal volume 80 µL; optical path 10 mm) was used and the wavelength was set at 517 nm. For preliminary studies, the analytical signal was recorded using a Kipp and Zonen (Delft, the Netherlands) BD 111 strip chart recorder.

Data acquisition was performed through a PCL-711L interface card at 3 Hz, using the same software developed for controlling the flow system.

Manifold and MSFIA procedure

The system components were arranged as shown schematically in Fig. 1. All connections were made of Omnifit (Cambridge, UK)

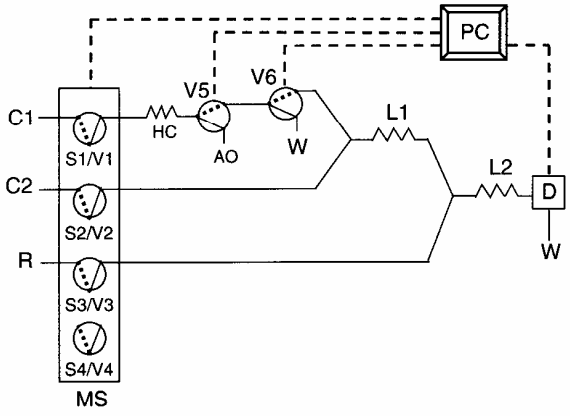

Fig. 1. MSFIA manifold used for the evaluation of scavenging capacity using DPPH• reaction: *MS* multi-syringe; *Si* syringes; *Vi* commutation valves (solid and dotted lines represent the on and off positions, respectively); *HC* holding coil (200 cm); *L1* mixing coil (50 cm); *L2* reaction coil (120 cm); *D* detector ($\lambda = 517$ nm); *C1* carrier (methanol or ethanol solution 50% v/v); *C2* carrier (methanol or ethanol solution 50% v/v or ethanol solution 50% v/v with citrate/phosphate buffer solution (pH 4.1 or 7.6)); *R* DPPH• reagent prepared in C1; *AO* antioxidant solution prepared in C1; *PC* personal computer; *W* waste

Table 1. Protocol sequence for determination of scavenging capacity using the DPPH• reaction. The indicated values for volume and flow rate refer to syringe 3 (10 mL)

Description	Position of commutation valves					Volume/ µL	Time/s	Flow rate/ mL min^{-1}	Direction of piston displacement
	1	2	3	5	6				
Syringes are filled with the respective solutions	off	off	off	off	off	2500	18.75	8.0	pick up
Antioxidant solution is aspirated through HC	on	off	off	on	off	100	3.00	2.0	pick up
Dummy step to change the flow direction	off	off	off	off	off	500	3.75	8.0	dispense
Antioxidant solution, carrier and reagent are sent towards detection system	on	on	on	off	off	775	15.50	3.0	dispense
Flow stop for reaction monitoring with data acquisition	off	off	off	off	off	–	180.00	–	–
Carrier and reagent are sent to wash the system	on	on	on	off	off	1325	26.50	3.0	dispense

PTFE tubing (0.8 mm i.d.) with Gilson (Villiers-le-Bel, France) end-fittings and connectors. Moreover, two laboratory-made acrylic Y-shaped connectors were used as confluences.

The tubing connections designated in Fig. 1 as HC, L1 and L2 were 200 cm, 50 and 120 cm long, respectively. The tubing length between valves V5 and V6 was 4 cm. The connection between valve V6 and the confluence had the same length.

The protocol sequence adopted for determination of scavenging capacity using the DPPH• reaction is presented in Table 1. Before starting the analytical procedure, all syringes were filled with the respective solutions. By activating syringe 1, the flow cell was filled with carrier solution and the absorbance signal was adjusted to zero. After that, the solutions contained in syringes S1, S2 and S3 were propelled to the detector simultaneously. The absorbance value measured (A_0) was due to DPPH• solution from syringe 3 after dilution inside the flow system, corresponding to the absorbance of radical solution in the absence of antioxidant compounds.

The procedure included six steps. The first step consisted of filling the syringes with the respective solutions. Next, 50 µL of antioxidant solution were aspirated through HC. After that, a dummy step where solutions were sent to their flasks was performed in order to change the direction of piston displacement [15]. This was achieved by propelling the solutions placed in the syringes back to the reservoirs. Then, syringes 1, 2 and 3 were activated simultaneously. Hence, antioxidant solution was sequentially merged with a carrier stream and DPPH• solution and propelled through the reaction coil until reaching the flow cell. The flow was then stopped and the absorbance decrease due to radical consumption was monitored during 180 s. Finally, all channels were washed, including the flow cell.

For experiments using pH control, the carrier in syringe 2 was replaced by buffer solution. All measurements were carried out at room temperature (22 ± 2 °C) and performed in triplicate.

Determination of the number of DPPH• molecules reduced by one molecule of antioxidant

The number of DPPH• molecules reduced by one molecule of antioxidant (AO) was calculated as described by Goupy et al. [16] with some modifications. According to these authors, $n = [\text{DPPH}^\bullet]_{\text{consumed}}/[\text{AO}]_{\text{initial}} = (A_0 - A_f)/(\varepsilon_{\text{DPPH}^\bullet} \times [\text{AO}]_{\text{initial}})$, where $(A_0 - A_f)$ is the absorbance decrease due to DPPH• consumption and $\varepsilon_{\text{DPPH}^\bullet}$ is the molar extinction coefficient of DPPH• in the reaction media used.

To apply this equation to flow injection conditions (controlled dispersion), the $[\text{AO}]_{\text{initial}}$ was replaced by $[\text{AO}]_{\text{st}}/D$, where $[\text{AO}]_{\text{st}}$ is the concentration of antioxidant in the standard solution and D is the dispersion coefficient of Ruzicka [8].

The dispersion coefficient was determined by injecting BTB solution ($n = 10$) instead of antioxidant solution and measuring the absorbance at 428 nm. The values obtained were 13.2 for methanol and 20.6 for ethanol solution 50% v/v.

The value of $\varepsilon_{\text{DPPH}^\bullet}$ was also determined in the different reaction media by establishing calibration curves between 1.0 and 8.0×10^{-5} mol L^{-1} of DPPH• using the 80 µL flow cell. The values obtained were 10005 ± 179; 9776 ± 71; 9738 ± 76 and 10240 ± 147 mol^{-1} L cm^{-1} for methanol and ethanol solution 50% v/v (without buffer, apparent pH 4.1 and apparent pH 7.6), respectively.

Results and discussion

Development of MSFIA system for evaluation of scavenging capacity using DPPH• reaction

The first goal of this work was to develop a multi-syringe flow injection system for determination of DPPH• scavenging capacity, through the absorbance decrease at 517 nm due to consumption of DPPH• radical. Hence, a manifold was devised to allow the monitoring of the reaction between antioxidant compounds and DPPH• using a stopped flow approach. The possibility of performing in-line pH adjustment was also considered.

In this case, three syringes were necessary: one of them containing carrier to propel the antioxidant solution, another syringe containing either carrier or buffer

solution and the last one containing DPPH• solution. These three streams were connected sequentially using two confluences in order to allow the mixture of antioxidant solution and buffer/carrier (after the first confluence) prior to reaction with DPPH• (after the second confluence). The capacity of each syringe was chosen in order to provide similar flow rates for the merging streams in each confluence. The reaction was monitored by stopping the flow when the antioxidant solution passed through the flow cell during 180 s.

The antioxidant solution volume was fixed at 50 μL and sampling was based on time and flow rate of its aspiration. Valve V5 provided the access to the antioxidant solution and valve V6 was introduced in the manifold to allow antioxidant solution exchange without disturbing the content of the flow cell. Otherwise, it would have been necessary to send DPPH• solution in order to re-establish the baseline after sample exchange, increasing the reagent consumption. In order to attain an initial value of absorbance near to 0.8 AU, the DPPH• concentration was fixed at 1.6×10^{-4} mol L^{-1}.

The flow rate values for the syringes filling step were fixed at 4.0/4.0/8.0 mL min^{-1} for syringes S1/S2/S3, respectively. For the sampling step, the flow rate applied was 1.0 mL min^{-1}. The total flow rate for the two steps in which all solutions were propelled towards the detector was initially set at 2.0 mL min^{-1} (0.5 + 0.5 + 1.0 from S1, S2 and S3). Using this flow rate value and a length of 50 and 90 cm for coiled reactors in L1 and L2, it was not possible to attain a stable baseline from the mixture of DPPH• solution from syringe 3 and carrier solution (ethanol solution 50% v/v) from syringes 1 and 2. Therefore, in order to improve the mixture conditions, the geometry and length of tubing L2 was varied. These experiments were performed at 4.0 and 6.0 mL min^{-1} (total flow rate), using coiled and knitted reactors with 45, 90 and 120 cm. The stability of the baseline increased for the larger reactors and for higher flow rates. Hence, the knitted reactor with 120 cm of length provided a stable baseline when a flow rate of 6.0 mL min^{-1} was applied and it was used for further studies.

Next, using the conditions stated above, the time during which the solutions were sent to the detector prior to stopping the flow was studied in order to maximize the antioxidant solution portion present in the flow cell. This experiment was carried out using BTB solution instead of antioxidant solution. The flow was stopped during 1 min and the absorbance was measured at 428 nm. The maximum absorbance value was obtained at 15.50 s, while values that were between 86 and 96% of the maximum value were obtained for time values between 14.00 and 17.00 s. Therefore, the value chosen for further studies was 15.50 s. Using these conditions, it was possible to perform about

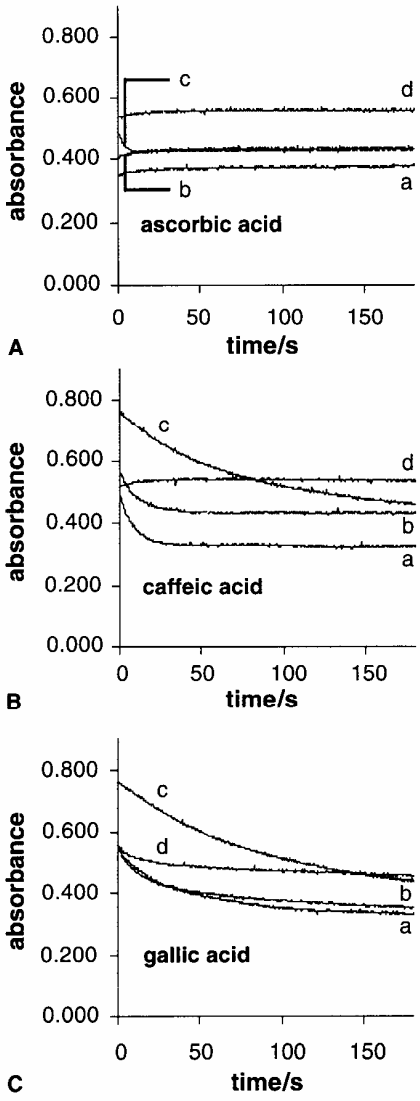

Fig. 2. Profile of DPPH• consumption for ascorbic, caffeic and gallic acids in different reaction media. The DPPH•/AO molar ratio was fixed at 8.0 for gallic acid or at 4.0 for the other two compounds. (*a*) methanol; (*b*) ethanol solution 50% v/v; (*c*) ethanol solution 50% v/v with citrate/phosphate buffer, pH 4.1; (*d*) ethanol solution 50% v/v with citrate/phosphate buffer, pH 7.6

14 analytical cycles per hour, requiring 0.34 μmol of DPPH• per determination.

Application to antioxidant compounds

The DPPH• method is currently carried out in methanol or ethanol solution. In the present work, different solvents were tested, including methanol and ethanol solution 50% v/v (unbuffered, apparent pH 4.1 and apparent pH 7.6). For each reaction media, antioxidant compounds present in plants or food products and/or applied as standards in the determination of antioxidant capacity were tested. Cinnamic acid was chosen as negative control.

The profile of DPPH• consumption for ascorbic, caffeic and gallic acids in the different reaction media at a fixed [DPPH•]/[AO] ratio is represented in Fig. 2. Other concentration ratios were also tested (between 4.0 and 12.0), providing curves with similar shape to those presented in Fig. 2. Two types of kinetic behavior were observed: fast, when the absorbance value stabilized within 180 s (Fig. 2, ascorbic acid) or slow, when the stabilization was not attained within the period of measurement (Fig. 2, caffeic and gallic acids in ethanolic media, pH 4.1). For the negative control (cinnamic acid), the absorbance variation was near zero as expected for all media and concentration ratio tested.

For those situations in which a stable value of absorbance was attained within the time period of absorbance monitoring, the number of DPPH• molecules consumed per molecule of antioxidant was calculated (Table 2). Considering the values obtained when the reaction media was methanol, it was possible to attain values similar to those reported in the literature using time-consuming batch methods. For instance, the values obtained for ascorbic acid (2.15 ± 0.02), trolox (1.94 ± 0.04), isoeugenol (1.03 ± 0.02) and quercetin (4.68 ± 0.07) were in agreement with those provided by other authors (2, 2, 1 and 4.86, respectively) [16, 17]. Moreover, the number of DPPH• molecules consumed per molecule of antioxidant obtained in methanol and ethanol solution 50% v/v were similar, indicating that the replacement of methanol for a less harmful reaction media is possible for the compounds listed in Table 2.

Furthermore, for other compounds the results are in agreement to those expected according to their chemical structures. The number of DPPH• molecules consumed per molecule of antioxidant for catechol (two adjacent hydroxyl groups) and pyrogallol (three adjacent hydroxyl groups) were 2.02 ± 0.04 and 3.40 ± 0.03, respectively, which are in agreement with the previous observation that the increase in adjacent hydroxyl groups increase the number of scavenged DPPH• molecules [18]. For quercetin and rutin, which are flavonoid with similar structure except for the presence of rutinose bonded to the oxygen at C_3 position, the number of DPPH• molecules consumed per molecule of antioxidant obtained in methanol was 4.68 ± 0.07 and 2.07 ± 0.02, respectively. This difference, reported before by Sánchez-Moreno et al. [19], is due to solvent addition in the quercetin molecule at the C_3 position, which do not take place in the rutin molecule since the OH group in this position is not available [20]. This addition enables the regeneration of the catechol group, which can be further oxidized, consuming more DPPH•.

When the results obtained for 50% v/v ethanol media with and without addition of buffer solution were compared, similar kinetic behavior was observed for ascorbic acid and trolox in all ethanolic solutions

Table 2. Number of DPPH• molecules[a] reduced by one molecule of antioxidant in different reaction media

Compound	Methanol	Ethanol 50% v/v	Ethanol 50% v/v, pH 4.1	Ethanol 50% v/v, pH 7.6
Ascorbic acid	2.15 ± 0.02	1.90 ± 0.01	1.86 ± 0.01	1.35 ± 0.00
Caffeic acid	2.40 ± 0.02	2.01 ± 0.02	n.c.	1.54 ± 0.02
Catechol	2.02 ± 0.04	1.88 ± 0.05	n.c.	1.59 ± 0.02
Gallic acid	4.67 ± 0.05	4.50 ± 0.02	n.c.	3.52 ± 0.02
Isoeugenol	1.03 ± 0.02	1.00 ± 0.01	1.00 ± 0.01	0.76 ± 0.03
Pyrogallol	3.40 ± 0.03	3.74 ± 0.06	n.c.	3.09 ± 0.02
Quercetin	4.68 ± 0.07	4.48 ± 0.03	n.c.	4.44 ± 0.01
Rutin	2.07 ± 0.02	1.92 ± 0.03	n.c.	3.24 ± 0.01
Trolox	1.94 ± 0.04	2.05 ± 0.03	1.97 ± 0.03	1.52 ± 0.01

n.c. Not calculated.

[a] Mean ± standard deviation; $n = 3$.

tested. For caffeic acid, catechol, eugenol, gallic acid, isoeugenol, pyrogallol, quercetin and rutin, the reaction was slower at apparent pH 4.1 when compared to the unbuffered solution. For these compounds, when the results obtained at apparent pH 7.6 were compared to those provided by the unbuffered solution, the reaction kinetics was similar for eugenol, isoeugenol, pyrogallol and quercetin; slower for rutin and faster for caffeic acid, catechol and gallic acid. These last results are in agreement with those described by Foti et al. [21] that verified that the presence in the solvent of adventitious acids or bases caused a reduction or an enhancement, respectively, of the observed value of the rate constants for the reaction between DPPH• and antioxidants bearing a phenolic group. Moreover, they also observed that the presence of free carboxylic groups in the phenol structure (as in caffeic and gallic acids) made the observed rate constants strongly dependent on the phenol concentration used in the experiment. These observations and the results obtained in the present study may be due to the contribution of these groups to the pH of the reaction media in the absence of buffer substances or to the effect of the pH of the reaction media in the form of these groups (protonated or ionized).

In conclusion, the MSFIA technique provided controlled and reproducible conditions concerning time, mixture and pH adjustment for monitoring the reaction between antioxidant molecules and DPPH• using different solvents in a contained environment, minimizing the exposure of the analyst. Moreover, it was possible to obtain values for the number of DPPH• molecules consumed per molecule of antioxidant similar to those attained by time-consuming end-point batch methods as long as a stable absorbance value is reached within the time period of measurement. This observation was only possible due to the stopped-flow approach implemented in the present system and could not be achieved in the previously described systems based on single-point measurements [10–12]. Finally, the application of the present automatic flow system for screening purposes can be valuable when searching for scavenger compounds, especially in areas in which a large number of compounds must be tested (as in combinatorial chemistry or in bioassay-guided isolation of active fractions of plant extracts).

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CHAPTER 4

Automatic method for determination of total antioxidant capacity using 2,2-diphenyl-1-picrylhydrazyl assay

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Automatic method for determination of total antioxidant capacity using 2,2-diphenyl-1-picrylhydrazyl assay

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Abstract

In the present work, an automatic method based on multi-syringe flow injection analysis (MSFIA) was developed for the determination of total antioxidant capacity, measured as the cumulative capacity of the compounds present in the sample to scavenge free radicals, using the 2,2-diphenyl-1-picrylhydrazyl (DPPH•) reaction. The determination is based on the colour disappearance due to the scavenging of DPPH• by antioxidant compounds monitored spectrophotometrically at 517 nm.

The influence of initial DPPH• concentration and sample dilution in the present methodology was studied. It was verified that the amount of DPPH• consumed by antioxidant standards (ascorbic and caffeic acids) was independent of the initial concentration of radical except for situations where DPPH•/antioxidant molar ratio was lower than the stoichiometric value. Furthermore, the sample dilution factor plays an important role for achieving results comparable to those from end-point batch method since the exhausting of scavenging ability of the sample should take place during the period of absorbance measurement.

The proposed method was applied to several food products and the total antioxidant capacity was expressed as Vitamin C equivalent antioxidant capacity (VCEAC). The results obtained by the proposed method ranged from 1.1 to 318 mg of ascorbic acid/100 ml and they were statistically comparable to those provided by the batch method. The detection limit was 0.34 mg of ascorbic acid/100 ml and the determination frequency was about 13 h^{-1} with an excellent repeatability (R.S.D. < 1%, $n = 10$).

Keywords: Total antioxidant capacity; DPPH•; Multi-syringe flow injection; Beverages

1. Introduction

There is recent evidence that free radicals induce oxidative damage to biomolecules. This damage has been implicated in ageing and in several human pathologies such as cancer, atherosclerosis, rheumatoid arthritis and other diseases [1]. Furthermore, there is a considerable amount of studies indicating that the active dietary constituents of fresh fruit, vegetables and beverages, prevent these free radical-induced diseases and protect against foodstuff oxidative deterioration [2–4]. These protective effects have been attributed, in large part, to the antioxidants species (Vitamins C and E, carotenoids and polyphenolic compounds) which scavenge free radicals [5,6].

Several methodologies, based on free radical capture or formation suppression, are used to measure the antioxidant capacity of biological material and model compounds [7,8]. The most commonly used for their ease, speed and sensitivity are those involving chromogen compounds of a radical nature to simulate radical oxygen and nitrogen species. The most widely used assays are based on the scavenging of radical cation 2,2-azinobis-(3-ethylbenzothiazoline-6-sulphonate) ($ABTS^{\bullet+}$ assay) [9] or of the stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH• assay) [10,11]. The presence of antioxidant species leads to the disappearance of these radical chromogens which can be followed by spectrophotometric methods.

Recently, the DPPH• assay was implemented using automatic methods based on flow injection analysis (FIA) [12,13], sequential injection analysis (SIA) [14] or HPLC-FIA [15,16], that were applied for screening and evaluation of scavenging capacity of several pure compounds and complex matrices such as plant extracts and beverages. In the HPLC-FIA method, the separated

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analytes react postcolumn with the DPPH• solution [16], and the induced scavenging is detected as a negative peak. These methods, combining separation and evaluation of radical-scavenging capacity, present a major advantage relatively to batch methods as bioassay-guide fractionation of natural or food samples is a time consuming and labour-intensive process. Moreover, the loss in activity for antioxidants due to decomposition during the isolation and purification procedures is avoided.

However, the presence of antioxidant compounds in complex matrices does not necessarily imply that the same antioxidant properties are verified in the whole sample. On the other hand, the synergistic effect that may exist between different antioxidants implies that the sum of antioxidant capacities from each compound isolated may not exactly reflect the overall action [17]. For this reason, automatic methods based on FIA or SIA allowed the evaluation of antioxidant capacity in an environment that enabled the interactions between all compounds present in the matrix.

Recently, novel computer controlled techniques for automatic flow analysis were reported, namely multi-syringe flow injection analysis (MSFIA). This technique was proposed in 1999 [18], in order to combine the multi-channel operation of FIA and the flexibility of multi-commutation flow systems [19]. Therefore, the objective of the present work was the development of an automatic flow system for the assessment of total antioxidant capacity exploiting the features introduced by MSFIA in flow analysis.

The methodology was based on consumption of DPPH• by antioxidant species present in the sample by monitoring the absorbance at 517 nm. A stopped flow approach was chosen in order to follow the reaction development and to assess if total consumption of antioxidant is attained during reaction monitoring. Furthermore, the influence of initial DPPH• concentration and sample dilution are also addressed in the present work.

2. Experimental

2.1. Reagents and solutions

All chemicals used were of analytical-reagent grade with no further purification. For the preparation of all solutions water from Milli-Q system (resistivity > 18 MΩ cm) and ethanol p.a. were used.

2,2-Diphenyl-1-picrylhydrazyl (DPPH•) and ascorbic acid were purchased from Sigma (St. Louis, USA). Caffeic acid was obtained from Aldrich (Milwaukee, USA). A stock solution (5.80×10^{-4} mol l^{-1}) of DPPH• was prepared by dissolving the appropriate amount in ethanol. This solution was kept at 4 °C and protected from light, and it was stable during a week.

For the flow system, the DPPH• working solution containing 1.45×10^{-4} mol l^{-1} was prepared by measuring 50 ml of the stock solution, 50 ml of ethanol and the volume was made up to 200 ml with water. This working solution was prepared daily and protected from light. Ethanol solution 50% (v/v) was used as carrier solution.

Ascorbic and caffeic acid stock solutions (1.00×10^{-2} mol l^{-1}) were prepared by dissolving the appropriate amount of the respective solid in ethanol solution 50% (v/v). Working standard solutions containing either ascorbic or caffeic acid in the concentration range $0.25–10.00 \times 10^{-4}$ mol l^{-1} were prepared by dilution of the respective stock solution using ethanol solution 50% (v/v). These solutions were prepared daily.

For the analysis using the batch method, a DPPH• solution (1.25×10^{-4} mol l^{-1}) in ethanol (50%, v/v) was prepared. Working standard solutions containing ascorbic acid in the concentration range $0.25–2.00 \times 10^{-4}$ mol l^{-1} were prepared as described above.

In order to evaluate the influence of DPPH• concentration on the establishment of calibration curves, different DPPH• concentrations were prepared in ethanol solution 50% (v/v). The concentrations studied were 1.500, 0.938, 0.750 and 0.375×10^{-4} mol l^{-1} providing initial absorbance values of 0.840, 0.528, 0.426 and 0.208, respectively.

All samples were purchased at local markets. The green tea extracts were prepared by pouring 200 ml deionised water at 90 °C into a glass with tea bag (1.49–1.77 g of leaves) and by brewing during 3 min [20].

Before introduction into the flow system or analysis using the batch method, samples were first diluted 50% using ethanol in order to attain a final concentration of 50% (v/v) in ethanol. For wine and beer samples, the initial alcohol content was considered and these samples were diluted with ethanol and water. Some samples were further diluted using ethanol solution 50% (v/v). The sample dilutions used for the flow system varied from 1:2 to 1:333 and for the batch method varied between 1:2 and 1:200.

For determination of dispersion coefficient of Ruzicka and Hansen [21], a bromothymol blue (BTB) solution was prepared from a stock solution (0.50 g l^{-1}) by dilution in ethanol solution 50% (v/v) in order to provide an absorbance value of about 0.520 at 428 nm.

2.2. Apparatus

Solutions were propelled through the flow network by means of a multi-syringe burette (Crison Instruments, Allela, Spain). For this application, the multi-syringe was equipped with 5 ml syringes in positions 1 and 2 while 10 ml syringes were placed in the other two positions (Fig. 1). Three extra commutation valves were included in the module used. For all valves, the exchange options were classified in on/off lines. The "off" line was assigned to the solution flasks and the "on" line was reserved for the flow network in the valves placed at the multi-syringe. For the other valves, the positions "on/off" were chosen to minimise the time during which the valves were switched on in order to avoid over-heating problems.

A personal computer, running lab-made software written in QuickBasic 4.5 (Microsoft), controlled the multi-syringe operation (number of steps and direction of piston displacement and the position of all commutation valves).

As detection system, a Jenway 6105 (Essex, UK) UV–vis spectrophotometer equipped with a thermostatic cell holder and a flow-through cell from Hellma (80 μl, ref. 178.710-QS, Mullheim/Baden, Germany) was used and the absorbance measurements were carried out at 517 nm. The cell holder was connected

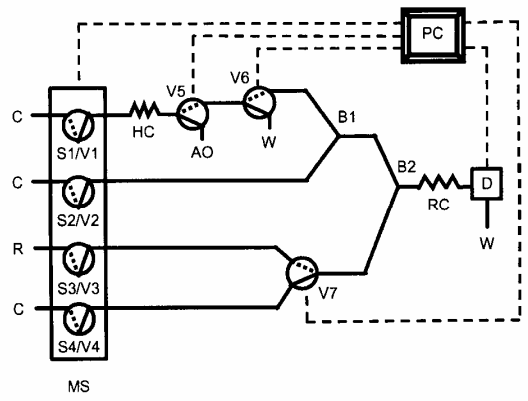

Fig. 1. MSFIA manifold used for the determination of total antioxidant capacity using DPPH• assay: MS, multi-syringe; S*i*, syringes; V*i*, commutation valves (solid and dotted lines represent the position on and off, respectively); B1 and B2, confluences; HC, holding coil (200 cm); RC, reaction coil (120 cm); D, detector; C, carrier (ethanol solution 50%, v/v); R, 2,2-diphenyl-1-picrylhydrazyl reagent prepared in C; AO, antioxidant standard solution or sample; PC, personal computer; W, waste.

to a Tectron S-543 (Altrincham, UK) thermostatic bath. The data acquisition was performed through a PCL-711B interface card at 3 Hz, using the same software developed for controlling the flow system. The data obtained were analysed using Origin 6.1 software.

2.3. MSFIA manifold and procedure

The system components were arranged as shown schematically in Fig. 1. All connections were made from Omnifit (Cambridge, UK) PTFE tubing (0.8 mm i.d.) with Gilson (Villiers-le-Bel, France) end-fittings and connectors. Two laboratory-made acrylic Y-shaped connectors were used as confluences.

The connections between the multi-syringe and the valve V7 were 200 cm long. The holding coil (HC) had the same length. The tubing length between the valves V5 and V6 was 4 cm long. The connection between this valve and the confluence B1 had the same length. The connection between confluences B1 and B2 was 50 cm long while the connection between the valve V7 and confluence B2 was 10 cm long. The reaction coil (RC) was 120 cm long.

The protocol sequence for the determination of total antioxidant capacity using DPPH• assay is listed in Table 1. Before starting the analytical procedure, syringe 4 was filled with carrier solution and further propelled to detection system. Thus, the flow-through cell was filled with it and the absorbance signal was adjusted to zero. After that, syringe 3 was filled with radical solution while the syringes 1 and 2 were filled with carrier solution and further propelled to the detector. The absorbance value measured corresponded to the absorbance of radical solution in the absence of antioxidant compounds.

The procedure included six steps. The first step consisted of filling syringes with the respective solutions. Then, 50 μl of standard/sample was aspirated into the holding coil (HC). After a dummy step, applied to change the flow direction [22], the sample, carrier and reagent were sent towards the detection system. During this step the sample plug was pushed by carrier up to confluence B1, where it was diluted by carrier solution from syringe 2. Subsequently, the diluted sample was mixed with DPPH• solution, after confluence B2. This mixture was further propelled until it reached the detection system. Then, the flow was stopped and the absorbance at 517 nm was measured during 180 s (temperature = 25 ± 1 °C). Finally, the flow-through cell was washed with carrier and reagent solution to re-establish the baseline. After this last step, the flow system was ready for a new analytical cycle.

For the determination of intrinsic absorption of sample the same procedure was performed, except for steps 4 and 6. In these steps, the valve V3 was in position off and the valves V4 and V7 were in position on in order to replace the DPPH• solution by carrier solution. Moreover, the absorbance measurements were performed during 30 s instead of 120 s.

For the determination of DPPH•/AO molar ratio in the flow cell, the DPPH• concentration in the flow cell was calculated by the ratio between absorbance value of radical solution in the absence of antioxidant compounds and the respective molar absorption coefficient at 517 nm ($\varepsilon = 11\,071 \pm 58\ \mathrm{mol^{-1}\,l\,cm^{-1}}$). The antioxidant concentration in the flow cell

Table 1
Protocol sequence for the determination of total antioxidant capacity using DPPH• assay

Step	Description	Position of the commutation valves							Volume (μl)	Time (s)
		1	2	3	4	5	6	7		
1	Syringes are filled with the respective solutions	F	F	F	F	F	F	F	3000	22.50
2	Sample is aspirated	N	F	F	F	N	F	F	100	3.00
3	Dummy step to change the flow direction	F	F	F	F	F	F	F	500	3.75
4	Sample, carrier and reagent are sent towards detection system	N	N	N	F	F	F	F	750	15.00
5	Flow stop for reaction monitoring with data acquisition	F	F	F	F	F	F	F	–	180.00
6	Carrier and reagent are sent to wash the system	N	N	N	F	F	F	F	1850	37.00

The indicated values for volume refer to syringe 3 (10 ml). N and F represent the positions on and off, respectively.

was calculated as the ratio between the concentration of the solution introduced in the system and the dispersion coefficient.

2.4. End-point batch method

The DPPH• method described by Brand-Williams et al. [11] was applied with some modifications [15] and it was adapted to a microplate reader (Synergy HT, Bio-Tek, Vermont, USA). The absorbance measurements were carried out at 517 nm. Hence, 50 μl of ascorbic acid solution or sample and 200 μl of DPPH• solution were placed in each well. To evaluate the radical absorbance in the absence of antioxidant species, 50 μl of ethanol solution 50% (v/v) was added in place of antioxidant solutions. To evaluate the intrinsic absorption of sample, 200 μl of ethanol solution 50% (v/v) was added to 50 μl of sample. The reduction in DPPH• concentration was monitored every 20 s until the absorbance decrease was inferior or equal to 0.003 units min^{-1} [23]. All experiments were performed in quadruplicate and the temperature was kept at 25.0 ± 0.1 °C.

3. Results and discussion

3.1. Development of the MSFIA system for determination of total antioxidant capacity

The configuration of the MSFIA system was designed to allow the determination of total antioxidant capacity in several samples using DPPH• scavenging reaction and spectrophotometric detection. Since the reaction kinetics is strongly influenced by the type of antioxidant compound present in the sample a stopped flow approach was implemented. Hence, the absorbance was monitored during 180 s. The DPPH• consumption was assessed from the difference between the absorbance of DPPH• in the absence of antioxidant compounds (A_0) and absorbance of DPPH• measured in the presence of antioxidant compounds after a fixed period of time (A_f).

In multi-syringe flow systems it is not feasible to introduce the sample into the system through one of the available syringes, as it would take a long time of washing steps for avoiding carry-over effect. Hence, in the present system the valve V5 was included to provide access to the antioxidant standard solution or sample. The valve V6 was included to allow sample exchange without disturbing the content of the flow cell. Otherwise, it would have been necessary to send DPPH• solution in order to re-establish the baseline after sample exchange, increasing the reagent consumption.

As some samples can absorb radiation at the same wavelength of detection, the manifold was adapted to accommodate in-line measurement of sample blank. Therefore, valve V7 was included to allow the replacement of the DPPH• solution by carrier, in order to perform the determination of intrinsic absorption of sample. In this way, after confluence B2 the sample plug was mixed with either DPPH• or carrier solution depending on the position of valve V7 (off or on, respectively).

The confluence B1 was included in the manifold to increase the sample dilution. Moreover, this confluence could be used to allow the adjustment of pH or ionic strength by replacing the content of the syringe 2. The reaction coil (RC) disposed in knitted form was set at 120 cm long.

Studies concerning the sample volume and the DPPH• concentration were carried out in order to evaluate the influence of these parameters in the analytical performance. The conditions used and the results obtained are discussed in the following paragraphs.

For the different sample volumes tested (25, 50, 100 and 200 μl), preliminary studies were performed in order to adjust the time during which solutions were sent towards the detector (Table 1, step 4). This was necessary to guarantee that the flow was halted after maximum absorbance was achieved. These experiments were performed using bromothymol blue dissolved in ethanol solution 50% (v/v), as sample, and the procedure described in Table 1 was applied. The wavelength was set at 428 nm. The time applied in step 4 for each sample volume was selected in order to attain at least 90% of the highest signal (peak maximum). Therefore, the time during which solutions were sent towards the detector was set to 14.5, 15.0, 16.5 and 19.0 s for 25, 50, 100 and 200 μl, respectively. Moreover, the dispersion coefficient [21] for each sample volume was calculated in order to determine the sample dilution inside the system. The values found were 44.2, 22.3, 13.0 and 7.8 for the above-mentioned volumes.

Two antioxidant compounds with similar DPPH• consumption in molar basis, but with different kinetic behaviours were studied: ascorbic acid (rapid kinetics) and caffeic acid (rapid-intermediate kinetics) [24]. The DPPH• concentration was set at 1.50×10^{-4} mol l^{-1} providing an absorbance value of 0.813 ± 0.005 after dilution inside the flow system. The concentration of antioxidant standards used varied from 0.25 to 10.00×10^{-4} mol l^{-1}. The absorbance values obtained during 180 s after flow halting for 50 μl of ascorbic and caffeic acid solutions are depicted in Fig. 2.

The absorbance of radical solution in the absence of antioxidant compounds (Fig. 2A and F) was stable during the period of absorbance measurement. As expected, the antioxidant compounds reduced the radical DPPH•, resulting in absorbance decrease. Furthermore, the amount of radical reduced increased with antioxidant concentration.

For the period during which the absorbance was monitored, the value was constant because the reaction between ascorbic acid and DPPH• occurred before absorbance measurement took place (Fig. 2B–E). Therefore, the absorbance decrease provoked by a given ascorbic acid concentration was equal at any time after flow halting. On the other hand, as caffeic acid presents slower kinetic behaviour than ascorbic acid, the absorbance values continued to decrease during the interval of absorbance measurement for some of the solutions tested (Fig. 2H–J). Similar results were obtained for other sample volumes studied.

For each sample volume studied, a calibration curve was established by plotting the absorbance decrease (Δ absorbance) as a function of antioxidant concentration ($R \geq 0.9991$). The absorbance decrease was calculated from the difference between the absorbance of DPPH• in the absence of antioxidant compounds (A_0) and absorbance of DPPH• measured in the presence of antioxidant compounds after 120 s (A_f).

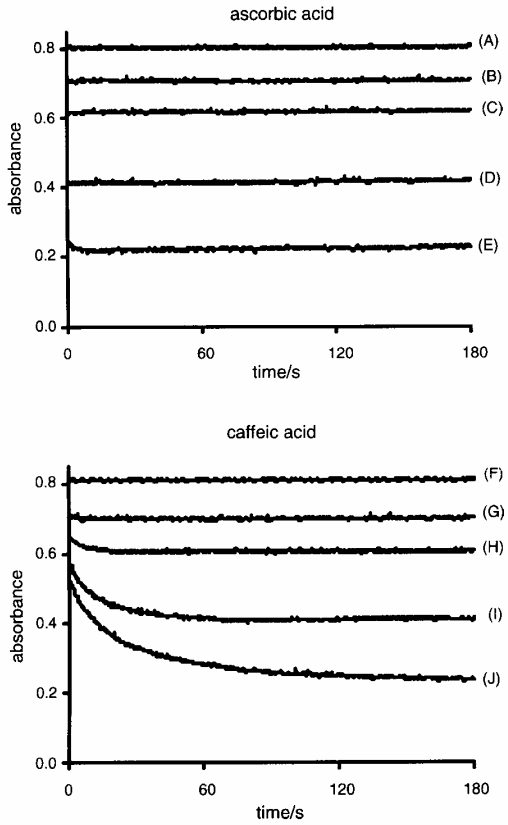

Fig. 2. Absorbance measurements during 180 s after flow halting obtained for 50 μl of ascorbic and caffeic acid solutions. The DPPH• concentration was fixed at 1.50×10^{-4} mol l^{-1}. The antioxidant concentration is expressed in 10^{-4} mol l^{-1}: (A, F) 0.00; (B, G) 1.00; (C, H) 2.00; (D, I) 4.00; (E, J) 6.00.

It should be emphasised that for higher concentration of antioxidant with slow kinetic behaviour, such as caffeic acid, the reaction may not be completed within 120 s (Fig. 2J). Nevertheless, such situations can be easily spotted in the calibration curve as they would originate points deviated from the line established by the other points corresponding to lower antioxidant concentrations.

The sensitivity, estimated through the slope of calibration curve (absorbance decrease per concentration of antioxidant compound) increased with sample volume. In fact the values obtained for 25, 50 and 100 were 17, 36 and 60% of the value obtained for 200 μl. Nevertheless, the sample volume of 50 μl was selected for further studies because the amount of reagent expended to re-establish the baseline (Table 1, step 6) was inferior to that obtained for larger sample volumes (100 and 200 μl). Moreover, the determination frequency was enhanced.

Using the experimental conditions described above, the influence of initial concentration of DPPH• was assessed. The absorbance decrease values obtained for ascorbic acid solutions using different concentrations of DPPH• are listed in Table 2.

Table 2
Values of absorbance decrease obtained for ascorbic acid for different initial concentration of DPPH• ($n = 3$; S.D. ≤ 0.004)

[Ascorbic acid] (10^{-4} mol l^{-1})	[DPPH•] (10^{-4} mol l^{-1})		
	1.500	0.750	0.375
1.00	0.094 (16.9)	0.093 (8.6)	0.092 (4.2)
1.50	0.135 (11.3)	0.133 (5.7)	0.133 (2.8)
2.00	0.178 (8.5)	0.175 (4.3)	0.173 (2.1)
4.00	0.367 (4.2)	0.359 (2.1)	0.196 (1.0)[a]
6.00	0.560 (2.8)	0.388 (1.4)[a]	0.197 (0.7)[a]

The values in parenthesis correspond to the DPPH•/antioxidant molar ratio in the flow cell.
[a] DPPH•/antioxidant molar ratio lower than the stoichiometric value.

The values of DPPH•/antioxidant molar ratio in the flow cell are also indicated.

The DPPH• consumption by antioxidant compound was independent of the initial concentration of radical except for situations where DPPH•/antioxidant molar ratio was lower than the stoichiometric value (which is 2 for ascorbic acid). This observation may have some implications concerning the way of antioxidant properties are expressed. In the literature it is usually found the expression of antioxidant capacity as percentage of consumed DPPH•, referred to as "inhibition" or "quenching", which is calculated as the ratio between absorbance decrease and initial absorbance of DPPH•. As absorbance decrease is independent of initial absorbance of DPPH•, the values of percentage of consumed DPPH• will be different for the same amount of antioxidant. For example, for antioxidant concentration of 2.00×10^{-4} mol l^{-1} the absorbance decrease was similar, independently of radical concentration, but the percentage of inhibition or quenching was 21.2, 41.1 and 83.2% when the DPPH• concentration was 1.500, 0.750 and 0.375×10^{-4} mol l^{-1}, respectively. For this reason, when evaluating the total antioxidant capacity, it is more accurate to use the absorbance variation rather than the percentage of radical consumed. It is also important to assure an excess of radical DPPH• with the intention of exhausting the scavenging ability of the antioxidant, i.e. the DPPH•/antioxidant molar ratio should be superior to the stoichiometric value.

3.2. Evaluation of the MSFIA method and its application to samples

The proposed MSFIA method was applied to determine the total antioxidant capacity of several samples including fruit juices, isotonic and soft drinks, tea, beers and wines. This was expressed as Vitamin C equivalent antioxidant capacity (VCEAC) calculated as the equivalent amount of ascorbic acid (mg) present in 100 ml of sample. These units were chosen since nutritional labelling on food products are indicated/100 ml or 100 g [25]. Therefore, a calibration curve relating the absorbance decrease after 120 s and the concentration of ascorbic acid (Vitamin C) was established ($R \geq 0.9996$). The standard concentration varied between 0.44 and 10.57 mg of ascorbic acid/100 ml

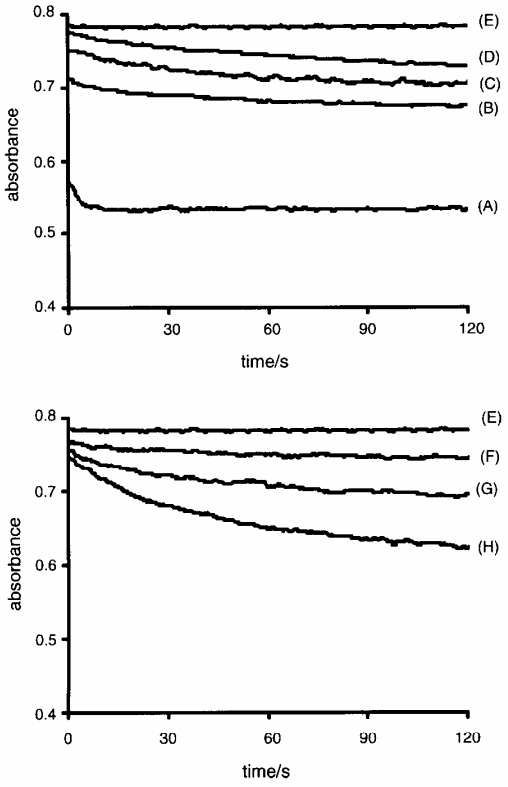

Fig. 3. Profile of DPPH• consumption for some samples, diluted prior to introduction into the flow system: (A) orange juice A 1:2; (B) green tea 1:50; (C) dark beer 1:20; (D) white wine 1:10; (E) ethanol solution 50% (v/v); (F) red wine A 1:333; (G) red wine A 1:100; (H) red wine A 1:50.

(corresponding to 0.25 and 6.00×10^{-4} mol l^{-1}). Thus, the absorbance decrease obtained for samples was related to that of ascorbic acid and the total antioxidant capacity was expressed as Vitamin C equivalents in mg/100 ml. This result was multiplied by the respective dilution factor.

Some samples can absorb or originate compounds that also absorb at the wavelength of measurement (517 nm). In both situations, the absorbance decrease of DPPH• solution can be underestimated [26]. On the other hand, the absorbing species present in the sample can also react with DPPH•, changing its contribution to the total absorbance measured along the reaction. For this reason, the sample blank should not be included when calculating DPPH• consumption. Nevertheless, in the present work, sample blank was measured and values <0.006 were found for all samples tested. As these values represent <1% of initial DPPH• absorbance, their contribution was not significant whether the coloured species were bleached or not during determination.

The absorbance decrease of radical solution obtained for some of the samples tested are presented in Fig. 3. For samples such as orange juices (Fig. 3A), in which ascorbic acid is the mainly antioxidant compound, the radical was immediately scavenged and the absorbance value stabilised after a few seconds of absorbance measurement. However, for samples such as wine (Fig. 3D) or beer (Fig. 3C) where the components mainly responsible for scavenging the DPPH• radical are polyphenolic compounds, a biphasic kinetic response was obtained. In these cases the reaction occurred rapidly in the first seconds and then slowed, as described by Goupy et al. [27]. This slower step was due to the antioxidant properties of slow reacting components originally present in the sample and/or due to reaction products formed during the rapid phase.

The VCEAC value was calculated for all samples using the proposed MSFIA system and the end-point batch method. Similar results (R.D. < 10%) were found for samples for which a stable value of absorbance was attained before 120 s, indicating that the scavenging capacity was exhausted during the period of absorbance measurement. For samples that showed a biphasic kinetic response, MSFIA results were significantly lower than those provided by the end-point batch method as the reaction continued after the period of absorbance monitoring.

Therefore, in order to reach end-point values within 2 min, the influence of sample dilution was assessed as the time to attain the reaction end-point will decrease for lower antioxidant concentrations [24,28]. This approach was applied to all samples tested and, as expected, the dilution did not influence the results obtained for samples which were composed mainly by fast scavengers (Table 3, isotonic drink A). On the other hand, for samples such as red wine A (Table 3 and Fig. 3F–H), the dilution plays an important role to achieve the exhaustion of scavenging ability within the MSFIA conditions. For this sample, VCEAC values obtained by MSFIA were approximately 67, 75, 90 and 97% of those obtained by batch method when the dilution factor was 1:50, 1:100, 1:200 and 1:333, respectively. This increase in VCEAC values for higher dilution factors was also observed in the batch method when the DPPH• consumption was determined using the absorbance values after 2 min of reaction (Table 3). This effect was not noticed for end-point values, indicating that the differences reported before are an artifact due to the reaction time employed. They would probably be minimised by using a large period for absorbance monitoring.

Moreover, dilution of sample did not provide results comparable to those of the end-point batch method for all samples. In fact, for other wine samples, the VCEAC values determined by MSFIA were about 60% of those given by the end-point batch method. Furthermore, this approach has a serious limitation as the dilution factor cannot be increased indefinitely since the DPPH• consumption should be higher than that provided by the lowest ascorbic acid standard.

Therefore, another approach was tried for these samples, based not only on the absorbance measurement at 120 s after flow stop but in all the information gathered during 180 s of absorbance monitoring in order to estimate the total consumption of DPPH•. For this, the double-exponential model for DPPH• consumption proposed by Espín et al. [28] was applied, based on the following equation:

$$DPPH^{\bullet} = DPPH_1^{\bullet}\, e^{-\alpha t} + DPPH_2^{\bullet}\, e^{-\beta t} + DPPH_r^{\bullet}$$

Table 3
VCEAC values (mg of ascorbic acid/100 ml) obtained by MSFIA and by batch method (after 2 min of reaction and at end-point) applying different dilution factors

Sample	Dilution factor	MSFIA[a] (2 min)	Batch[b] (2 min)	Batch[b] (end-point)	End-point time (min)
Isotonic drink A	10	10.8 ± 0.2	9.7 ± 0.3	10.0 ± 0.3	5
	6.6	10.1 ± 0.1	9.5 ± 0.1	9.7 ± 0.1	5
	5	10.1 ± 0.2	9.7 ± 0.1	9.9 ± 0.2	5
Soft drink	40	31.9 ± 0.7	25.6 ± 1.3	27.4 ± 1.3	5
	20	28.0 ± 1.1	23.7 ± 0.6	26.8 ± 0.4	10
	13.3	27.7 ± 0.4	22.5 ± 0.6	27.7 ± 0.3	20
Red Wine A	333	215 ± 6	155 ± 16	221 ± 16	15
	200	199 ± 6	140 ± 11	222 ± 9	25
	100	165 ± 1	131 ± 6	217 ± 4	40
	50	148 ± 3	–	–	

Each value corresponds to the mean ± S.D.
[a] (n = 3).
[b] (n = 4).

where DPPH• is the radical concentration at any time, and α and β are the first-order rate constants for the simple uniexponential that form the double exponential equation, one corresponding to the fast step and the other to the slow step. The parameters $DPPH_1$• and $DPPH_2$• are the radical concentrations at time zero for the simple uniexponential equations that form the double exponential, and represent the DPPH• consumed in the fast step ($DPPH_1$•) and that consumed in the slow step ($DPPH_2$•). Finally, $DPPH_r$• is the remaining DPPH• concentration in the medium after antioxidant depletion.

The DPPH• consumption can be calculated from the difference between the initial concentration of DPPH• and $DPPH_r$• or from the sum of DPPH• consumed in the fast step ($DPPH_1$•) and that consumed in the slow step ($DPPH_2$•). Since DPPH• concentration values were proportional to absorbance values, the experimental data (absorbance versus time) obtained for three samples were fitted by non-linear regression ($R^2 > 0.996$), and the absorbance values corresponding to $DPPH_r$•, $DPPH_1$•, and $DPPH_2$• were obtained. The VCEAC values were calculated (Table 4) and similar values to those obtained by the end-point batch method were attained using estimate values for DPPH• consumption in the fast and slow steps. This was not verified when using the estimate value for remaining DPPH•, which provided an underestimated consumption. This situation may be due to the utilisation of data concerning only the first 3 min of reaction while it took about 35 min to reach the end-point. Therefore, we propose the application of $DPPH_1$• and $DPPH_2$• values for calculation of VCEAC when samples do not exhaust its scavenging capacity within 3 min of reaction monitoring.

The results obtained by the proposed methodology (C_{MSFIA}) and by the batch method (C_{batch}) for the determination of total antioxidant capacity in all samples are summarised in Table 5. In order to evaluate the accuracy of the proposed methodology, a linear relationship between the results provided by the two methods was established. The equation $C_{MSFIA} = 1.009\ (\pm 0.025) C_{batch} - 0.9\ (\pm 2.9)$ was obtained, where the values in parentheses are the limits of the 95% confidence intervals ($R = 0.9994$). From these data, it is clear that the calculated slope and intercept do not differ significantly from the values 1 and 0, respectively [29]. The precision of the MSFIA method was estimated by calculating the relative standard deviation from 10 consecutive determinations of two standard ascorbic acid solutions (3.52 and 10.57 mg/100 ml), providing values of 0.9 and 0.4%, respectively.

The detection limit was calculated as the concentration corresponding to the intercept value plus three times the statistic $s_{y/x}$ [29]. For five different calibration curves, the calculated detection limit was about 0.17 mg/100 ml. However, as the samples had to be diluted 1:2 in order to attain a final concentration of ethanol 50% (v/v), the detection limit was in fact 0.34 mg/100 ml.

Considering that the time required for a complete analytical cycle is not merely the summation of the time taken for each step performed because data transference between the computer and the multi-syringe must also be accounted, the whole analytical

Table 4
VCEAC values (mg of ascorbic acid/100 ml) obtained for samples presenting slow kinetic behaviour using end-point batch method and MSFIA

Sample	MSFIA[a]			End-point batch method[b]
	Single point (120 s)	Kinetic ($DPPH_r$•)	Kinetic ($DPPH_1$• + $DPPH_2$•)	
White wine	13.0 ± 0.3 (1:20)	21.3 ± 1.7 (1:10)	24.8 ± 0.6 (1:10)	22.6 ± 0.5 (1:20)
Red wine A	215 ± 6 (1:333)	187 ± 10 (1:50)	226 ± 8 (1:50)	217 ± 4 (1:100)
Red wine B	183 ± 9 (1:200)	209 ± 11 (1:133)	318 ± 2 (1:133)	311 ± 7 (1:200)

Each value corresponds to the mean ± S.D.
[a] (n = 3).
[b] (n = 4).

Table 5
VCEAC values (mg of ascorbic acid/100 ml) obtained by MSFIA methodology (C_{MSFIA}) and by the batch method (C_{batch}) for the determination of total antioxidant capacity

Sample	C_{MSFIA}[a]	C_{batch}[b]	R.D. (%)
Fruit juice	36.2 ± 0.3 (1:10)	36.2 ± 1.9 (1:80)	0.0
Orange juice A	9.9 ± 0.1 (1:2)	9.9 ± 0.4 (1:10)	0.0
Orange juice B	27.6 ± 0.3 (1:20)	25.2 ± 1.5 (1:40)	+9.5
Soft drink	27.7 ± 0.4 (1:13.3)	27.7 ± 0.3 (1:13.3)	0.0
Isotonic drink A	10.1 ± 0.2 (1:5)	9.9 ± 0.2 (1:5)	+2.0
Isotonic drink B	1.1 ± 0.1 (1:2)	1.2 ± 0.1 (1:2)	−8.3
Green tea	111 ± 1 (1:100)	119 ± 1 (1:100)	−6.7
Dark beer	36.1 ± 0.8 (1:66.6)	39.1 ± 0.4 (1:20)	−7.7
Lager beer	10.2 ± 0.3 (1:10)	11.8 ± 0.2 (1:10)	−13.6
White wine[c]	24.8 ± 0.6 (1:10)	22.6 ± 0.5 (1:20)	+9.7
Red wine A	215 ± 6 (1:333)	217 ± 4 (1:100)	−0.9
Red wine B[c]	318 ± 2 (1:133)	311 ± 7 (1:200)	+2.3

Each value corresponds to the mean ± S.D. The values in parentheses are the sample dilution performed prior to introduction into the flow system. R.D. = relative deviation between the two methods.
[a] (n = 3).
[b] (n = 4).
[c] MSFIA results based on kinetic information.

cycle listed in Table 1 took 273 s. Therefore, in this case, the determination frequency was approximately 13 h^{-1}.

4. Conclusions

The automatic system proposed in the present work allowed the performance of DPPH• assay using strictly controlled reaction conditions with reduced handling of the sample, control of temperature and excellent repeatability. The contact of radical and antioxidant species with oxygen and other substances in the working environment is reduced. Moreover, the solvent loss due to evaporation is also reduced when compared to batch methods.

Considering the automatic flow systems previously described using the DPPH• assay, the present system relies on less expensive and common detection system when compared to electron spin resonance (ESR) spectrophotometer used previously [12,13]. Moreover, the antioxidant capacity was calculated through the absorbance decrease which is independent of initial absorbance of DPPH• solution rather than the percentage of consumed radical [14]. This aspect is essential for standardization and for obtaining comparable and reliable results for studies performed in different laboratories.

The present work offers a fast alternative to the end-point batch method, also providing qualitative information about the kinetic behaviour of antioxidant compounds initially present in the samples or formed during the reaction with DPPH•. For samples containing compounds that rapidly scavenge the radical DPPH•, such juices containing ascorbic acid, the total antioxidant capacity can be determined using a single absorbance measurement after flow stop. Moreover, the controlled conditions concerning time and mixture of reagents provided by the automatic flow system allowed the application of a mathematical model to the data collected within the first 3 min of reaction to estimate the total DPPH• consumption for samples containing or originating slow reacting compounds. In this case, the combination of automation and mathematical modelling resulted in a considerable reduction of the time taken for a single analysis, providing results comparable to those attained using the end-point batch method.

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CHAPTER 5

Automatic method for the determination of Folin-Ciocalteu reducing capacity in food products

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Automatic Method for the Determination of Folin–Ciocalteu Reducing Capacity in Food Products

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In the present work, an automatic flow procedure based on multi-syringe flow injection analysis was developed for the assessment of Folin–Ciocalteu reagent (FCR) reducing capacity in several types of food products using gallic acid as the standard. Different strategies for mixing of sample and reagent were tested (continuous flow of FCR, merging zones, and intercalated zones approaches); lower reagent consumption and higher determination throughput were attained for the merging zones approach (100 μL of sample + 100 μL of FCR). The application of the proposed method to compounds with known antioxidant activity (both phenolic and nonphenolic) and to samples (wines, beers, teas, soft drinks, and fruit juices) provided results similar to those obtained by the conventional batch method. The detection limit was 0.6 mg L^{-1}, and the determination frequency was about 12 h^{-1}. Good repeatability was attained (RSD < 1.3%, $n = 10$).

KEYWORDS: Multi-syringe flow injection; Folin–Ciocalteu reducing capacity; phenolic compounds

INTRODUCTION

Free radicals, reactive oxygen species (ROS), and reactive nitrogen species (RNS) derived either from normal metabolic processes or from external sources are directly related with oxidation in food and biological systems. They are also implicated in the oxidative rancidity, which is one of the most critical factors affecting the shelf life of processed food, and in the development of several human diseases such as neurological degeneration, cataracts, diabetes, cardiovascular diseases, and certain types of cancer (*1*, *2*). Interest in antioxidant nutrients has increased in the light of recent evidence regarding their protective effects against these free radical-induced reactions (*3*, *4*). With a few exceptions (such as carotenoids, vitamin C, and vitamin E), the most important dietary antioxidants are the phenolic compounds (*5*). For this reason, the assessment of total phenolic content has gained enormous attention in the last few years, especially within the food, biological, and agrochemical fields.

Many analytical procedures have been developed for quantification of total phenolic content in foods (*6*, *7*). Although separative methods such as capillary electrophoresis and high-performance liquid chromatography with diode array detection are powerful techniques for the isolation and identification of phenolic compounds in complex samples, their application to estimate the total phenolic content may be inaccurate (*8*). Moreover, the separative techniques are time-consuming, expensive, and often not suitable for routine determinations.

For quantification of total phenolic content, most of the available methods are based on the reaction of phenolic compounds with a colorimetric reagent, thus allowing their measurement in the visible region of the spectra (*7*). Among these methods, the Folin–Ciocalteu assay (FC assay) is frequently applied (*9*, *10*), and recent studies have shown that total phenols determined by this method can be correlated to antioxidant activity determined by different methods ($ABTS^{+\bullet}$ and $DPPH^{\bullet}$ assays, for instance) (*11*). For this reason, the method described by Singleton and Rossi (*9*) has been proposed recently as a standardized method for use in the routine quality control and measurement of antioxidant capacity of food products and dietary supplements (*12*). Moreover, the novel designation "FC reagent reducing capacity" was suggested (*13*).

For routine analysis, the automation of FC assay has been described using flow injection analysis (FIA) (*14*–*17*) and sequential injection analysis (SIA) (*18*) for the determination of total polyphenols index of wine and beer samples. However, these methodologies replaced the recommended gallic acid reference standard with oenological tannin (*14*), coumaric acid (*15*), or tannic acid (*16*–*18*).

Therefore, the objective of the present work was the development of an automatic flow procedure based on multi-syringe flow injection analysis (MSFIA) (*19*, *20*) for the assessment of FC reagent reducing capacity using gallic acid as standard. MSFIA was introduced in 1999 in order to combine the multichannel operation of flow injection analysis to the flexible

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flow management offered by the multi-commutation technique. These features were exploited in the present work for evaluation of different strategies for mixing of sample and reagent. Furthermore, the application of the proposed method to samples and compounds with known antioxidant activity (both phenolic and nonphenolic) was also evaluated. The results were compared with the conventional batch method proposed for standardization.

MATERIALS AND METHODS

Chemicals. All chemicals used were of analytical-reagent grade with no further purification. Folin−Ciocalteu reagent (FCR), gallic acid, ascorbic acid, resorcinol, butylated hydroxyanisole (BHA), quercetin, and ferrous sulfate were purchased from Sigma (St. Louis, MO). Caffeic acid, catechol, propyl gallate, ferulic acid, and cinnamic acid were obtained from Aldrich (Milwaukee, WI). Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), β-carotene, sinapic acid, ellagic acid, and (−)-epicatechin were obtained from Fluka (Buchs, Switzerland). Pyrogallol and tannic acid were purchased from Riedel-de-Haën (Seelze, Germany). Citric acid, sodium sulfite, D-(+)-glucose, sodium carbonate, and sodium hydroxide were obtained from Merck (Darmstadt, Germany).

Reagents and Samples. Water from Milli-Q system (resistivity > 18 MΩ cm) and ethanol absolute pro analysis were used for the preparation of all solutions.

The stock solutions were prepared by dissolving gallic acid, ascorbic acid, citric acid, ferrous sulfate, sodium sulfite, and D-(+)-glucose in water. Resorcinol, catechol, BHA, trolox, pyrogallol, caffeic acid, propyl gallate, tannic acid, ferulic acid, sinapic acid, and cinnamic acid were dissolved in ethanol solution 50% (v/v). β-Carotene, ellagic acid, (−)-epicatechin, and quercetin were dissolved in ethanol. The working solutions were prepared daily in a range between 3.1 and 766 μM by rigorous dilution of the respective stock solutions in water.

For the studies concerning different strategies for mixing sample and reagent, the following solutions were prepared: NaOH, 0.25 M; HCl, 0.10 M; and working standard solutions of gallic acid (2.5−100.0 mg L^{-1}). FCR was diluted 1:20 (v/v) with water.

For the automatic determination of FCR reducing capacity of compounds and food products, FCR was diluted 1:40 (v/v) (experiments using pure compounds) and 1:10 (v/v) (experiments using samples) with water. Sodium hydroxide solution (0.25 M) and working standard solutions containing gallic acid (2.5−40.0 mg L^{-1}) were also prepared.

For the batch method, FCR was diluted 1:5 (v/v) in water. Sodium carbonate (60 g L^{-1}) and working standard solutions of gallic acid (2.5 and 25.0 mg L^{-1}) were also prepared.

All food products were purchased at local markets. The tea extracts were prepared by pouring 200 mL of deionized water at 90 °C into a glass with tea bag (1.49−1.66 g of leaves) and by brewing for 5 min. No sample treatment other than dilution using water was applied before determination. The dilutions performed for the flow system and for the batch method varied from 1:25 to 1:200.

For determination of dispersion coefficient of Ruzicka (*21*), a bromothymol blue (BTB) solution was prepared from a stock solution (0.20 g L^{-1}) by dilution in 0.1 mol L^{-1} NaOH solution in order to provide an absorbance value of about 0.646 at 620 nm.

Apparatus. Solutions were propelled through the flow system by means of a multi-syringe piston pump (Crison Instruments, Allela, Spain) equipped with syringes of 5 mL (Hamilton, Switzerland). Each syringe is connected to a three-way solenoid valve (N-Research, Caldwell, NJ) that allows the access to two different channels (solutions flask or flow network). The multi-syringe module also comprises extra commutation valves. For all valves, the exchange options were classified in on/off lines. The "off" line was assigned to the solution flasks, and the "on" line was reserved for the flow network in the valves placed at the multi-syringe. For the other valves, the positions are assigned in order to maintain the valves turned "off" most of the time to avoid over-heating problems. All tubing connecting the different components of MSFIA was made of PTFE (Omnifit, Cambridge, U.K.) of 0.8 mm i.d. with Gilson (Villiers-le-Bel, France) end-fittings and connectors.

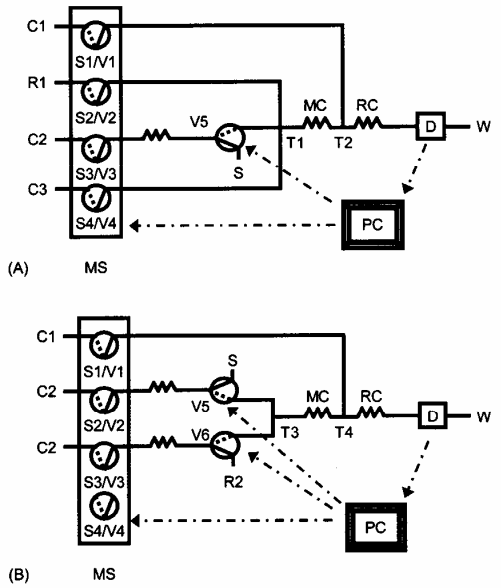

Figure 1. MSFIA manifolds for evaluation of different mixing strategies (**A**) and determination of FCR reducing capacity (**B**): MS, multi-syringe; S*i*, syringe; V*i*, commutation valves (solid and dotted lines represent the position on and off, respectively); MC, mixing coil; RC, reaction coil (100 cm); D, detector; T*i*, confluences; C1, NaOH 0.25 M; C2, water; C3, HCl 0.10 M; R1 and R2, Folin–Ciocalteu reagent diluted at 1:20 and 1:10 (v/v), respectively; S, standard solution or sample; PC, personal computer; W, waste.

A personal computer, running homemade software written in QuickBasic 4.5, controlled the multi-syringe operation (number of steps and direction of piston displacement) and the position of all commutation valves.

As detection system, a Jenway 6100 (Essex, U.K.) UV−vis spectrophotometer equipped with a flow-through cell from Hellma (internal volume = 80 μL, ref 178.710-QS, Mullheim/Baden, Germany) was used, and the wavelength was set at 750 nm. The data acquisition was performed through a PCL-711 B interface card at 3 Hz, using the same software developed for controlling the flow system. Furthermore, the analytical signals were also recorded in a Kipp & Zonen (Delft, The Netherlands) BD 111 strip chart recorder.

MSFIA Manifold and Procedure for Evaluation of Different Mixing Strategies. The system components were arranged as shown schematically in **Figure 1A**. The connection between the multi-syringe and the valve V5 was 200 cm long. The tubing length between valve V5 and confluence T1 was 20 cm long while the mixing coil (MC) was 100 cm long. The reaction coil (RC) had the same length.

These components constituted a flow network, where the management of solutions was defined through software control. This aspect allowed the implementation of different strategies for mixing sample and reagent (gallic acid−FCR) without physical reconfiguration of the manifold. Therefore, five different strategies were implemented based on continuous flow of FCR or on merging or intercalation of segments of sample and reagent. The protocol sequence was similar for each strategy adopted. Initially, the syringes were filled with solutions from the respective reservoirs (1650 μL) with all valves at off position. Then, 100 μL of gallic acid standard solution was aspirated by activating valves V3 and V5. After a dummy step (250 μL), applied to change the flow direction (*22*), the different mixing strategies were applied as described below in detail. Thereafter, at confluence T2 an alkaline solution was added, and the mixture was further propelled toward the detection system (total flow rate = 3 mL min^{-1}).

The first approach tested was similar to a conventional FIA procedure (*23*), involving the continuous flow of FCR. In this case, the sample

Table 1. Protocol Sequence for the Determination of FCR Reducing Capacity

step	description	position of the commutation valves[a] 1	2	3	4	5	6	volume[b] (μL)	time (s)
1	syringes are filled with the respective solutions	F	F	F	F	F	F	2300	6.90
2	sample and FCR are aspirated	F	N	N	F	N	N	200	12.00
3	dummy step to change the flow direction	F	F	F	F	F	F	500	1.50
4	sample, FCR, and NaOH are sent toward detection system	N	N	N	F	F	F	600	18.00
5	flow stop	F	F	F	F	F	F		240.00
6	carrier and NaOH are sent to wash the system	N	N	N	F	F	F	1400	21.00

[a] N and F represent the positions on and off, respectively. [b] The indicated values for volume refer to syringe 10 mL.

segment was merged at confluence T1 with reagent stream (valves V1, V2, and V3 were in position on).

The merging zones strategy (*24*) was also implemented, using equal (experiment merging zones I) or different volumes (experiments merging zones II and III) of FCR and sample. In the experiment merging zones I (100 μL of sample + 100 μL of FCR), the sample was pushed by carrier until confluence T1 (valves V1, V3, and V4 were in position on). After that, the sample and reagent segments were simultaneously sent to MC by activating valves V1, V2, and V3. In the experiment merging zones II, 100 μL of sample was merged with 300 μL of FCR. For this, sample and reagent zones were sent into the MC during a single forward displacement of the piston driver bar by activating valves V1, V2, and V3. The experiment merging zones III was performed using 100 μL of FCR and 300 μL of sample. After the sampling step, sample was pushed by carrier into the MC creating a front zone of sample of 100 μL (valves V1, V3, and V4 were in position on). After that, by activating valves V1, V2, and V3, 100 μL of sample and 100 μL of FCR were merged at confluence T1. Finally, in all these experiments, the final mixture was further propelled to the detector by activating valves V1, V3, and V4.

The last approach tested was implemented by sequential introduction of reagent and sample segments into MC channel, creating a plug of intercalated zones. The sample was placed between two segments of reagent and the mixture took place at the boundaries of each segment, as occurs in sequential injection analysis (SIA) (*25*). In this case, each segment had 100 μL of volume. The flow protocol was applied by sequentially activating valves V1, V2, and V3 (insertion of reagent) or valves V1, V3, and V4 (insertion of sample). For each experiment, the dispersion coefficient of the sample was calculated as recommended by Ruzicka and Hansen (*21*).

MSFIA Manifold and Procedure for Determination of FCR Reducing Capacity. The system components were arranged as shown schematically in **Figure 1B**. The connections between the multi-syringe and the valves V5 and V6 were 200 cm long. The connections between these valves and confluence T3 were 5 cm long. The mixing coil (MC) had the same length. The reaction coil (RC) was 100 cm long.

The following modifications were performed in the manifold presented in **Figure 1A**. The Folin–Ciocalteu reagent was introduced into the flow system by aspiration through one extra commutation valve (V6) instead of direct introduction through syringe 2. Moreover, the mixing coil was reduced from 100 to 5 cm in order to minimize the dispersion of standard/sample and FCR.

The protocol sequence for the determination of FCR reducing capacity is summarized in **Table 1**. Before starting the analytical cycle, syringe 1 was filled with NaOH solution while the other two syringes were filled with water. After flow reversal, these carriers were propelled toward the detection system. Thus, the flow-through cell was filled, and the absorbance signal was adjusted to zero.

In the first step of the analytical cycle, syringes were filled with the respective solutions. Then, 100 μL of standard/sample and 100 μL of FCR were aspirated. After a dummy step applied to change the flow direction (*22*), the standard/sample and FCR plugs were propelled through confluence T3 and MC up to confluence T4, where NaOH solution was added. After passing through RC, this mixture was propelled until it reached the flow-through cell. Then, the flow was stopped, and the absorbance at 750 nm was monitored during 240 s at room temperature. After the last step, in which carrier and NaOH were sent to wash the flow-through cell, the flow system was ready for a new analytical cycle. All experiments were performed in triplicate.

For the analysis of pure compounds, the same flow procedure was performed without flow stop. The reactivity of each compound was estimated through the establishment of linear calibration curves by plotting the absorbance as a function of concentration of testing compound (μM). Under these conditions, the slope of the calibration curve for testing compound was compared to the slope of the calibration curve for the standard compound (gallic acid). This ratio (%) reflected the FCR reducing capacity of the testing compound.

Folin–Ciocalteu Batch Method. The Folin–Ciocalteu method described by Singleton and co-workers (*9*, *10*) was adapted to a microplate reader (Synergy HT, Bio-Tek, Winooski, VT). Hence, 50 μL of gallic acid standard solution or food sample and 50 μL of FCR were placed in each well. After that, 100 μL of sodium carbonate solution was added. The absorbance of the blue complex formed was monitored at 760 nm every 60 s during 2 h. All experiments were performed in quadruplicate, and the temperature was kept at 25.0 ± 0.1 °C.

RESULTS AND DISCUSSION

Evaluation of Different Mixing Strategies. The chemistry behind the FC assay relies on the transfer of electrons in alkaline medium from phenolic compounds to phosphomolybdic/phosphotungstic acid complexes to form blue complexes that can be detected spectrophotometrically (*9*). In this case, the sequence of mixture is of utmost importance, especially in order to avoid premature alkaline destruction of the FCR (*10*). This aspect was considered when developing the automatic flow system. Therefore, the reagent solution (FCR) was placed in syringe 2, and it was mixed with gallic acid standard solution in mixing coil (MC) after confluence T1. This mixture was further merged at confluence T2 with NaOH solution propelled by syringe 1. As the FC reagent contains acid, HCl solution was placed in syringe 4 as the carrier in order to maintain the pH value through flow system.

As MSFIA systems are based on a flow network relying on computer-controlled solenoid valves, different strategies for mixing sample and reagent after confluence T1 were evaluated. This was performed through software control without manifold reconfiguration.

The analytical features of different mixing strategies are summarized in **Table 2**. The sensitivity estimated through the slope of calibration curve was inversely related to the dispersion coefficient of sample. Thus, for the larger sample volume tested (300 μL, experiment merging zones III), the sensitivity is about twice that obtained for other experiments. However, the linear range decreased, and the determination frequency also decreased from 27 to 21 determinations h^{-1}. For experiments using 100 μL of sample, the sensitivity was similar (in the range 8.88–9.28 mAU mg^{-1} L). However, as the reaction on the intercalated zones approach took place at the boundaries of each segment, the linear range decreased from 5 to 100 to 5–40 mg L^{-1}. On

Table 2. Analytical Features of Different Strategies for Mixing Sample and Reagent in MSFIA

mixing strategy	slope[a] (mAU mg^{-1} L)	linear range (mg L^{-1})	D_{sample}	determination frequency (h^{-1})	FCR consumption[b] (μL/determination)	NaOH consumption (mg/determination)
continuous flow of FCR (100 μL of sample)	8.88	5–100	7.83	27	75	15
merging zones I (100 μL of sample + 100 μL of FCR)	9.21	5–80	7.60	25	5	15
merging zones II (100 μL of sample + 300 μL of FCR)	8.90	5–100	7.83	26	15	15
merging zones III (300 μL of sample + 100 μL of FCR)	17.43	2.5–40	3.96	21	5	15
intercalated zones (100 μL of FCR/100 μL of sample/100 μL of FCR)	9.28	5–40	7.60	24	10	15

[a] For all calibration curves $R \geq 0.9997$, $n \geq 5$. [b] Values refer to the commercial solution.

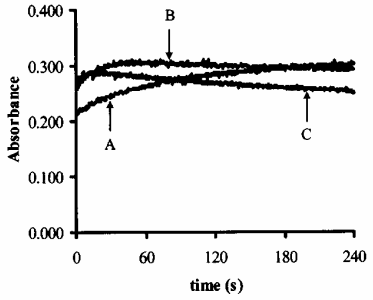

Figure 2. Reaction monitoring during 4 min after flow halting for different NaOH concentrations (M): A, 0.10; B, 0.25; C, 0.50. Other conditions: gallic acid concentration, 25 mg L^{-1}; FCR concentration, 1:20 (v/v).

the other hand, in the experiments with continuous flow of FCR, merging zones II, and intercalated zones approaches, the consumption of FCR was 15, 3 and 2 times of that verified on the experiment merging zones I. Therefore, this approach (100 μL of standard/sample and 100 μL of FCR) was chosen as it provided good linear range (5−80 mg L^{-1}), low reagent consumption (5 μL of commercial FCR per determination), and determination frequency similar to that obtained in the other experiments.

Study of Chemical Aspects. Studies concerning the reaction time, Folin−Ciocalteu reagent, and NaOH concentration were carried out using a univariate approach. A preliminary study on the effect of reaction time was carried out using FCR diluted 1:20 (v/v) and NaOH 0.25 M. The flow was stopped in the reactor (RC) during 0, 30, 60, and 90 s before the detection step; the slope of calibration curves obtained was 10.5, 11.2, 11.4, and 11.4 mAU mg^{-1} L, respectively. The sensitivity increased with time of flow stop up to 60 s; this value was chosen for the next experiment.

The Folin−Ciocalteu reagent concentration was evaluated at 1:40, 1:20, 1:10, and 1:5 (v/v) using the experimental conditions described above. The sensitivity obtained was 10.6, 11.2, 13.1, and 10.7 mAU mg^{-1} L, respectively. Thus, the FCR concentration 1:10 (v/v) was chosen as it provided the highest sensitivity.

To evaluate the influence of NaOH concentration, the kinetic of the reaction was also considered. For this, the flow was stopped when the sample segment reached the flow-through cell (18 s after the solutions were sent toward the detector) and the absorbance was monitored during 240 s (**Figure 2**). For 25 mg L^{-1} gallic acid, it was observed that higher alkali levels accelerated the color development and its fading. For that reason, it is important to have enough but not excessive alkalinity because it affects the kinetic of the reaction and also the stability of the complex formed (*10*).

Therefore, the influence on the sensitivity and on the time necessary to attain the maximum value of absorbance was assessed. For NaOH concentrations of 0.10, 0.20, 0.25, 0.30, and 0.40 M, the sensitivity obtained was 10.0, 14.1, 13.7, 13.1, and 12.6 mAU mg^{-1} L, respectively. Moreover, the time necessary to reach the maximum absorbance value was 2.5, 5, 4, 2.5, and 1.5 min, respectively. Although the highest sensitivity was obtained with 0.20 M NaOH, the time required to reach a stable absorbance value increased to 5 min. Thus, the concentration chosen was 0.25 M since the sensitivity was similar and the time of stopped flow was reduced to 4 min.

Application to Pure Compounds. Several phenolic and nonphenolic compounds were tested, including phenolic antioxidants as propyl gallate and BHA that are frequently used as additive in foods. Moreover, nonphenolic compounds with known antioxidant properties (ascorbic acid, β-carotene, sodium sulfite) and other compounds which are known to react with FCR but are not effective as antioxidant (citric acid, ferrous sulfate, D-glucose) were also evaluated. Cinnamic acid was chosen as negative control.

The FCR reducing capacity, expressed as the ratio between the slopes of the calibration curves determined for pure compounds and for gallic acid, are presented in **Table 3**. The values obtained for the MSFIA system were in agreement with those obtained using the conventional batch procedure; they are also similar to those described by other authors (*9*, *10*).

Some exceptions were observed, as occurred for resorcinol that originated a lower ratio value for MSFIA when compared to the batch procedure employing carbonate buffer solution for pH adjustment. When performing the batch procedure using NaOH solution, results similar to MSFIA were attained. For (−)-epicatechin, a lower ratio value was also found for the MSFIA procedure when compared to the batch method. Nevertheless, when the reaction conditions in the MSFIA system were changed (flow stop during 4 min and FCR 1:10 (v/v)), similar results were observed (RD = +2.3%).

Some nonphenolic substances, such as ascorbic acid and ferrous ion, also reacted with FCR. On the other hand, β-carotene, cinnamic acid, citric acid, D-glucose, and sodium sulfite did not react with FCR (the upper limits of concentration tested were 0.005, 1.00, 5.01, 11.2, and 16.0 mM, respectively). Therefore, the present method is not suitable for determination of total phenolic content unless interfering substances are considered or removed. Moreover, the application of this method for determination of antioxidant capacity in food samples is

Table 3. Relative FCR Reducing Capacity Obtained for Phenolic and Nonphenolic Compounds Determined by the Proposed MSFIA and Batch Method[a]

compounds	linear range[b] (μM)	MSFIA	batch	RD[c] (%)
	Phenolic and Polyphenolic			
BHA	25–126	88	37	+138
caffeic acid	15–150	103	104	−1.0
catechol	22–93	107	108	−0.9
ellagic acid	10–50	202	196	+3.1
(−)-epicatechin	13–64	169	214	−21
ferulic acid	31–123	79	80	−1.3
propyl gallate	15–150	98	94	+4.3
pyrogallol	15–150	109	108	+0.9
quercetin	14–58	191	195	−2.1
resorcinol	22–354	58	83	−30
sinapic acid	29–116	88	137	−36
tannic acid	3.1–11	762	789	−3.4
trolox	29–353	58	39	+49
	Nonphenolic			
ascorbic acid	28–224	63	64	−1.6
ferrous sulfate	153–766	8.4	16.9	−50

[a] The results are expressed as the ratio (%) between the slope of the calibration curves obtained for the testing compound and for gallic acid. [b] For all calibration curves $R \geq 0.9995$, $n \geq 4$. [c] RD = relative deviation.

proposed for evaluation of the contribution from phenolic and other reducing substances (as ascorbic acid, for instance). The contribution from other antioxidant compounds with different mechanism of action (such as β-carotene) may not be considered.

Application to Food Samples. The assessment of FCR reducing capacity of several food products including wines, beers, teas, soft drinks, and juices was performed using the proposed MSFIA system. The absorbance value obtained for samples was interpolated in the following calibration curve: $A = 0.0132\ (\pm 0.0002) \times C + 0.001\ (\pm 0.001)$ and $R^2 = 0.9998$, where A is the absorbance and C is the concentration of gallic acid (mg L^{-1}). Thus, the FCR reducing capacity was expressed as gallic acid equivalents (mg L^{-1}). This result was multiplied by the respective dilution factor.

The results obtained by the proposed methodology (C_{MSFIA}) and by the conventional batch method (C_{batch}) for the analysis of the samples are presented in **Table 4**. The FCR reducing capacity values obtained for wines were in agreement with those reported by other authors (*26*, *27*) that have also found values about 10 times higher for red wines in comparison to white wines.

For comparison purposes, a linear relationship ($C_{MSFIA} = C_0 + S \times C_{batch}$) was established ($n = 15$), and the values for intercept (C_0), slope (S), and correlation coefficient were 13.5 (± 18.4), 0.994 (± 0.015), and 0.9997, respectively. Considering the limits of the 95% confidence intervals presented (values in parentheses), the calculated slope and intercept do not differ significantly from the values 1 and 0, respectively. Therefore, there is no evidence for systematic differences between the two sets of results (*28*) obtained by the proposed methodology and by the conventional batch method. Furthermore, when a paired t-test was performed on the data obtained for all samples, a t value of 1.416 was calculated. The comparison between this value and the t ($P = 0.05$; df = 14) = 2.145 indicates no significant difference for the mean concentrations obtained by the two methods (*28*).

The repeatability of the developed method was assessed by calculating the relative standard deviation from 10 consecutive determinations of three gallic acid standard solutions (2.5, 10.0, and 40.0 mg L^{-1}) providing values of 1.33, 0.53, and 0.34%, respectively.

Table 4. Results (mg L^{-1}) Obtained for Analysis of Different Samples by MSFIA Methodology (C_{MSFIA}) and Batch Method (C_{batch}) for the Determination of FCR Reducing Capacity[a]

sample	C_{MSFIA}[b]		C_{batch}[c]		RD[d] (%)
red wine A	2422 ± 11	(1:200)	2419 ± 21	(1:100)	+0.1
red wine B	2526 ± 11	(1:200)	2490 ± 20	(1:100)	+1.4
red wine C	2278 ± 15	(1:200)	2329 ± 9	(1:100)	−2.2
red wine D	1890 ± 35	(1:200)	1889 ± 26	(1:100)	+0.1
white wine A	294 ± 8	(1:100)	305 ± 3	(1:50)	−3.6
white wine B	280 ± 2	(1:50)	282 ± 2	(1:25)	−0.7
dark beer	1073 ± 4	(1:50)	1052 ± 39	(1:100)	+2.0
lager beer	469 ± 3	(1:25)	467 ± 28	(1:100)	+0.4
green tea	768 ± 9	(1:100)	773 ± 4	(1:50)	−0.6
melissa tea[e]	623 ± 2	(1:50)	605 ± 25	(1:200)	+3.0
soft drink A	420 ± 1	(1:25)	393 ± 8	(1:100)	+6.9
soft drink B	121 ± 1	(1:50)	118 ± 4	(1:25)	+2.5
fruit juice A	312 ± 4	(1:100)	290 ± 6	(1:50)	+7.6
fruit juice B	455 ± 7	(1:25)	426 ± 12	(1:100)	+6.8
orange juice	526 ± 3	(1:25)	503 ± 9	(1:100)	+4.6

[a] Each value corresponds to the mean ± standard deviation. The values in parentheses correspond to the dilution performed prior to analysis. [b] $n = 3$. [c] $n = 4$. [d] RD = relative deviation between the two methods. [e] Honey flavor.

The detection limit was calculated as the concentration corresponding to the intercept value plus three times the statistic $s_{y/x}$ (*28*). For four different calibration curves, the calculated detection limit was about 0.6 mg L^{-1}. A complete analytical cycle (**Table 1**) took 335 s, considering the time taken for each step and also the time necessary for data transference between the computer and the multi-syringe. Therefore, the determination frequency was approximately 12 h^{-1}.

In conclusion, the present automatic methodology for the determination of FCR reducing capacity represents a suitable tool for routine determinations. It was successfully applied to food samples of diverse origin, providing results that were in agreement with those obtained by the time-consuming batch method proposed for standardization. Moreover, the strict control of reaction conditions (mixing of reagent/sample, reaction time) and the reduced intervention of operator contributed to achieving reliable results, with good repeatability.

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CHAPTER 6

Automatic flow system for sequential determination of $ABTS^{\bullet+}$ scavenging capacity and Folin-Ciocalteu index: a comparative study in food products

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Automatic flow system for sequential determination of ABTS$^{\bullet+}$ scavenging capacity and Folin-Ciocalteu index: A comparative study in food products

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Abstract

In the present work, an automatic flow procedure for the sequential spectrophotometric determination of Folin-Ciocalteu reducing capacity (FC assay) and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation (ABTS$^{\bullet+}$) scavenging capacity expressed as the trolox equivalent (TEAC assay) is proposed for a comparative study of antioxidant properties in food products. Exploiting the flexibility of flow management associated to the computer control offered by multisyringe flow injection analysis, both methodologies were carried out in the same manifold using gallic acid and trolox as standard compounds. The proposed system configuration allowed the performance of each method separately or in tandem, providing 24 determinations per hour, which accounts for its application in routine analysis.

The present methodology was applied to a large number of beverages ($n = 72$), namely red and white wines, herbal and tea infusions, juices and beers. The results obtained showed that FC reducing capacity and TEAC values of red wines were significantly different from those obtained for the other beverages, while tea infusions provided significantly higher TEAC values when compared to white wines, herbal infusions, juices and beers. A good correlation was found between TEAC and FC reducing capacity ($R > 0.9$) for red wines, herbal and tea infusions, and beers. For these beverages, similar slope values were found (106–140 mg L^{-1} of gallic acid per mM of Trolox), except for beers that showed a higher response for FC assay. These results provided evidence that the correlation between these assays vary according to the type of sample, reinforcing the idea that more than one method should be used for evaluation of antioxidant capacity.

Keywords: Multisyringe flow injection analysis; Folin-Ciocalteu assay; 2,2′-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation; Beverages

1. Introduction

Nowadays, the development and validation of analytical methods for assessment of antioxidant capacity in food products are an increasing area of research [1]. These methods are of great interest not only to the food industry but also to medical and nutritional researchers as active dietary constituents including phenolic compounds, vitamins C and E, and carotenoids, are capable of preventing free radical-induced reactions [2,3]. These reactions are implicated in the oxidative rancidity of food products and also in the development of several human pathologies such as cardiovascular diseases, diabetes, neurological degeneration, and certain types of cancer [4,5]. However, the methods currently used to assess this property differ from each other in terms of substrates, probes, reaction conditions, and in the form that results are expressed. Even when only one of these assays is considered, different antioxidant standard compounds, solvents, reaction time and pH value are frequently applied [6], which makes the comparison of results from different studies difficult. This situation stresses the importance to standardize analytical methods for application in routine assessment of antioxidant capacity.

In this context, the Folin-Ciocalteu reducing capacity (FC assay) and Trolox equivalent antioxidant capacity (TEAC assay) have been recently proposed as standardized methods for measurement of antioxidant capacity of food products and dietary supplements [7]. These methods are based on electron-transfer donation from the antioxidant to the oxidant probe. The degree of color change of the probe is proportional to the antioxidant concentration. For the FC assay, the oxidant is a

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molybdotungstophosphoric heteropolyanion and the absorbance increase is measured at 750 nm, whilst for the TEAC assay the oxidant is the 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{\bullet+}$) and the absorbance decrease at 734 nm is measured [1,6]. Nevertheless, for the implementation of standardized methods exhaustive studies are required for reliable comparison of data generated by different assays. Moreover, the application of batch methodologies for research tasks and/or routine analysis is time-consuming, tedious and laborious.

The drawbacks outlined above could be circumvented by the implementation of automatic analytical methodologies based on flow analysis, which are characterised by high throughput, reduced intervention of operator, reagent and sample saving, reduced production of residues and improved reproducibility [8,9]. Moreover, owing to its higher versatility, recent computer-controlled automatic methods are capable to accommodate a wide variety of assays without the need for system reconfiguration, allowing simultaneous and/or sequential determinations, which makes them especially suitable to establish comparisons between methods [10–12].

Therefore, the objective of the present work was the development of an automatic flow procedure for the sequential determination of FC reducing capacity and TEAC intended for a comparative study of the antioxidant capacities of several food products. The automation of these two methodologies was based on multisyringe flow injection analysis (MSFIA) [13,14], which combines the multichannel operation of flow injection analysis and the flexibility of flow management offered by the multicommutation technique. The proposed method was applied to a large number of beverages with recognised antioxidant properties and the results were compared within and between methods.

2. Experimental

2.1. Reagents, standards and samples

For the preparation of all solutions, water from Milli-Q system (resistivity > 18 MΩ cm) and ethanol absolute pro analysis were applied. All chemicals used were of analytical-reagent grade with no further purification.

Folin-Ciocalteu reagent (FCR), gallic acid and horseradish peroxidase (HRP) from type VI-A (EC 1.11.1.7, 1280 units mg^{-1}) were purchased from Sigma (St. Louis, MO, USA). ABTS [2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)] in the crystallized diammonium form and trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) were obtained from Fluka (Buchs, Switzerland). H_2O_2 (30%, v/v) was obtained from Riedel-de Haën (Seelze, Germany). Sodium hydroxide and sodium acetate were purchased from Merck (Darmstadt, Germany).

For the determination of FC reducing capacity, FCR was diluted 1:10 (v/v) with water. Sodium hydroxide solution (0.25 mol L^{-1}) was also prepared. For the determination of Trolox equivalent antioxidant capacity (TEAC), ABTS, H_2O_2, and HRP stock solutions of 18 mM, 20 mM, and 3.2×10^6 units L^{-1}, respectively, were prepared in 0.060 mol L^{-1} acetate buffer pH 4.6. These stock solutions were stable for over a month when stored at 4 °C. $ABTS^{\bullet+}$ working solution in 0.060 mol L^{-1} acetate buffer pH 4.6 was obtained by mixing appropriate volumes of the following solutions at the indicated final concentrations: ABTS (0.80 mM), H_2O_2 (0.080 mM), and HRP (3 units mL^{-1}). This solution was prepared 12 h before analysis and protected from light. It was stable for at least 2 days at room temperature.

For the determination of FC reducing capacity and TEAC, gallic acid and trolox were used as antioxidant standard compounds, respectively. Gallic acid (500 mg L^{-1}) and trolox (2.0 mM) stock solutions were prepared by dissolving the appropriate amount of the respective solid in water and in ethanol solution 50% (v/v), respectively. These stock solutions were found to be stable for at least 1 week when stored at 4 °C. The working solutions containing either gallic acid (5.0–75 mg L^{-1}) or trolox (0.020–0.20 mM) were prepared daily by dilution in water.

All samples were purchased at local markets. Herbal and tea infusions were prepared by pouring 200 mL of deionized water at 90 °C into a glass with herbal or tea bag and by brewing for 5 min. Carbon dioxide from white wines and beers was completely removed by magnetic stirring. All beverages were diluted with water just before measurement. The dilutions performed for red wines, herbal infusions, and tea infusions (green and black) were 1:200, 1:4 to 1:20, and 1:40, respectively. White wines, juices and beers (blond and dark) were diluted 1:20. Samples were analysed in a randomised way.

2.2. Apparatus

Solutions were propelled through the flow network by means of a multisyringe piston pump (Crison Instruments, Allela, Spain) equipped with syringes of 5 mL (Hamilton, Switzerland). Each syringe is connected to a three-way commutation valve (N-Research, Caldwell, NJ, USA) that allows the access to two different channels (solution flasks or flow network). Two extra commutation valves were included for introduction of standard/sample (V5) and FCR (V6). The flow assembly also includes two 8-port multiposition selection valves, disposed in the same module (Crison Instruments, Allela, Spain) that accommodated six reaction coils (RC*i*). The three and four-way connectors (T1 and T2, respectively) were used as confluences. All tubing connecting the different components of MSFIA was made from polytetrafluoroethylene (Omnift, Cambridge, U.K.) of 0.8 mm i.d., except for tubes between flasks and syringes, which were of 1.5 mm i.d. in order to avoid back pressure or vacuum at high flow rates. End-fittings and connectors from Gilson (Villiers-le-Bel, France) were also used.

To perform the FC and TEAC assays, the analytical absorbance measurements should be carried out at 750 and 734 nm, respectively [7]. For this, an Ocean Optics PC 2000-ISA (Winter Park, FL) spectrophotometer connected to 200 μm fiber optic cable and a DH-2000 deuterium-halogen light source (Top Sensor Systems, Eerbeek, The Netherlands) were used. Facing the fiber optic, a Hellma (Müllheim/Baden, Germany) 178.710-QS flow-through cell (10 mm light path, 80 μL inner volume) was placed in an Ocean Optics CUV cell support.

A personal computer equipped with an Advantec PCL-711 B interface card, running homemade software written in Quick-Basic 4.5 (Microsoft), controlled the multisyringe and valves operation. The data acquisition at 4 Hz, corresponding to an integration time of 0.023 s and an average scan of 11, was performed by SpectraWin (version 4.2) through an external trigger signal from the interface card.

2.3. Statistical analysis

For each sample, data are reported as mean ± standard deviation ($n = 3$) (see supplementary data, Tables S1–S6). One-way analysis of variance (ANOVA) was performed on these values to determine whether they differed significantly at a 95% level. Since the Levene test showed that there was no homogeneity of variances ($p < 0.05$), the Welch and Brown-Forsythe statistics were estimated instead of the usual F test. A post hoc comparison test (Tamhane's T2) was also applied to determine which group(s) differ from each other. Linear regression was applied for studying the possible correlation between the studied parameters. All statistical analyses were carried out using SPSS version 14.0 for Windows.

2.4. MSFIA manifold and procedure for FC reducing capacity and TEAC assays

The system components were arranged as shown schematically in Fig. 1. The connections between the multisyringe and the valves V5 and V6 were 200 cm long. The tubing length between these valves and confluence T1 were 5 cm long. The mixing coil (MC) had the same length. The connection between confluence T2 and the central channel of selection valve VA was 20 cm long, while the connection between the central channel of selection valve VB and the flow-through cell was 25 cm long. The reaction coils, where the FC reducing reaction (RC2, RC3, RC4) or the ABTS$^{\bullet+}$ scavenging reaction (RC6, RC7, RC8) took place, were 250 cm long. The washing coil L1 had the same length.

These components constituted the flow network for the sequential spectrophotometric determination of FC reducing capacity and TEAC of several food products. Nevertheless, as the FC and TEAC assays are standardized for different antioxidant compounds [7], namely gallic acid and trolox respectively, the calibration procedures were performed separately.

The protocol sequence adopted for the FC and TEAC calibration is described in Table 1. Before starting the calibration procedures, all syringes were filled with the respective solutions. For the FC procedure, syringes S1, S2 and S3 were simultaneously activated and depending on the position of selection valves, the NaOH solution and carrier were propelled through reaction coils RC2, RC3 or RC4 towards the detector and the absorbance signal was adjusted to zero. For the TEAC procedure, carrier and ABTS$^{\bullet+}$ solution (delivered by syringes S2, S3, and S4) were simultaneously propelled through reaction coils RC6, RC7 or RC8. The absorbance value measured (0.760 ± 0.010) was due to the ABTS$^{\bullet+}$ radical solution from syringe S4 after dilution inside the flow system.

Briefly, the calibration protocol can be divided in the following stages: antioxidant standard solution uptake and delivery to the reaction coil (steps 1–4); flow stop for reaction development (steps 5, 6); and spectrophotometric measurement (steps 7, 8).

In particular, for FC calibration 100 μL of gallic acid standard solution and 100 μL of FCR were aspirated into the flow system through commutation valves V5 and V6, respectively. After that syringes S1, S2, and S3 were simultaneously activated and the antioxidant plug pushed by carrier was sequentially merged with FCR plug and NaOH stream at confluence T1 and T2, respectively. This mixture was directed to reaction coil RC2. These operations were repeated twice more, in order to fill the other two reaction coils (RC3 and RC4). After a waiting period of 250 s, the reaction product stored in each RC was successively

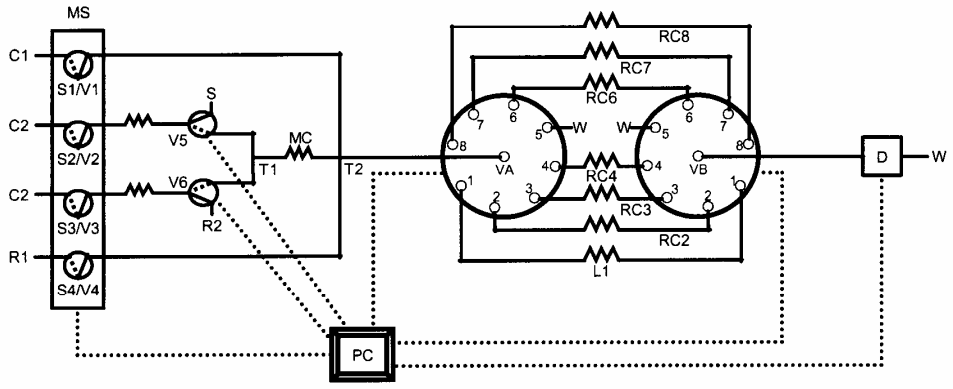

Fig. 1. MSFIA manifold used for the sequential determination of FC reducing capacity and TEAC in food products: MS, multisyringe; Si, syringes; Vi, commutation valves (solid and dotted lines represent the position on and off, respectively); T1 and T2, confluences; MC, mixing coil (5 cm); VA and VB, 8-port multiposition selection valves; L1, washing coil (250 cm); RCi, reaction coils (250 cm); D, detector; C1, NaOH 0.25 mol L^{-1}; C2, water; R1, ABTS$^{\bullet+}$ in 0.060 mol L^{-1} acetate buffer pH 4.6; R2, Folin-Ciocalteu reagent diluted at 1:10 (v/v); S, standard solution or sample; PC, personal computer; W, waste. The exchange options of the commutation valves were classified in on/off lines. The "off" line was assigned to the solution flasks and the "on" line was reserved for the flow network in the valves placed at the multisyringe, while for the other valves the positions are assigned in order to maintain the valves turned off most of the time to avoid over-heating problems.

Table 1
Protocol sequence for the Folin-Ciocalteu and TEAC calibration using the MSFIA method

Step	Instrumentation	Protocol[a]	Description
Folin-Ciocalteu calibration			
		start loop: standard injection	
1	Multiposition valve	Valve VA and VB move to position X	X (FC calibration) = position 2 to 4
2	Multisyringe Piston	Pick up 1362 μL at 10.0 mL min^{-1} (V1 off, V2 off, V3 off, V4 off, V5 off, and V6 off)	Loading syringes with carrier/reagents
3	Multisyringe piston	Pick up 100 μL at 1.0 mL min^{-1} (V1 off, V2 on, V3 on, V4 off, V5 on, and V6 on)	Aspiration of gallic acid standard solution and FCR
4	Multisyringe piston	Dispense 362 μL at 2.0 mL min^{-1} (V1 on, V2 on, V3 on, V4 off, V5 off, and V6 off)	Merging gallic acid with FCR and then with NaOH stream towards RC X
		End loop: standard injection	Repeat 3 times
5	Multiposition valve	Valve VA and VB move to position 1	
6	Stopped-flow	Wait 250 s	Development of FC reducing reaction
		Start loop: standard measurement	
7	multiposition valve	Valve VA and VB move to position X	X (FC calibration) = position 2 to 4
8	Multisyringe piston	Dispense 1100 μL at 2.0 mL min^{-1} (V1 on, V2 on, V3 on, V4 off, V5 off, and V6 off)	Propulsion of RC X content to detector (λ = 750 nm); signal acquisition; system clean-up
		End loop: standard measurement	Repeat 3 times
TEAC calibration			
		start loop: standard injection	
1	Multiposition valve	Valve VA and VB move to position X	X (TEAC calibration) = position 6 to 8
2	Multisyringe piston	Pick up 1362 μL at 10.0 mL min^{-1} (V1 off, V2 off, V3 off, V4 off, V5 off, and V6 off)	Loading syringes with carrier/reagents
3	Multisyringe piston	Pick up 100 μL at 1.0 mL min^{-1} (V1 off, V2 on, V3 off, V4 off, V5 on, and V6 off)	Aspiration of trolox standard solution
4	Multisyringe piston	Dispense 362 μL at 2.0 mL min^{-1} (V1 off, V2 on, V3 on, V4 on, V5 off, and V6 off)	Merging trolox with carrier and then with ABTS$^{\bullet+}$ stream towards to RC X
		end loop: standard injection	Repeat 3 times
5	Multiposition valve	Valve VA and VB move to position 1	
6	Stopped-flow	Wait 295 s	Development of ABTS$^{\bullet+}$ scavenging reaction
		Start loop: standard measurement	
7	Multiposition valve	Valve VA and VB move to position X	X (TEAC calibration) = position 6 to 8
8	Multisyringe piston	Dispense 1100 μL at 2.0 mL min^{-1} (V1 off, V2 on, V3 on, V4 on, V5 off, and V6 off)	Propulsion of RC X content to detector (λ = 734 nm); signal acquisition; system clean-up
		End loop: standard measurement	Repeat 3 times

[a] The indicated values of volume and flow rate are referred to syringe 1 (5 mL).

propelled towards the detector and the absorbance increase at 750 nm was measured.

For the TEAC calibration, after loading the trolox standard solution (100 μL), the antioxidant plug was sequentially merged with carrier and ABTS$^{\bullet+}$ stream at confluence T1 and T2, respectively, and propelled further up to RC6. These steps were repeated twice more and, depending on the position of selection valves, the mixture was directed to reaction coils RC7 or RC8. After a flow stop period of 295 s, the content of each reaction coil was directed to the detector and the absorbance decrease due to radical scavenging was measured at 734 nm.

Noteworthy, both determinations started with the measurement of a blank signal through aspiration of water as sample. The obtained signals correspond to the absorbance values in the absence of reducing or scavenging compounds.

The MSFIA procedure for the sequential determination of FC reducing capacity and TEAC of food products is summarized in Table 2. In the first commands (steps 1–4), sample and FCR were aspirated and further propelled to reaction coils RC2–RC4, similar to FC calibration procedure. Then, carrier and ABTS$^{\bullet+}$ radical solution were dispensed in order to clean the manifold line between confluence T2 and selection valve VA (steps 5, 6). After loading syringes with carrier/reagent, sample was aspirated and further sent with carrier and ABTS$^{\bullet+}$ solution to reaction coils RC6–RC8, similar to TEAC calibration procedure (steps 7–10). Next, NaOH solution and carrier were dispensed through L1 towards the detector in order to establish the absorbance baseline for the FC assay (steps 11, 12). Following the piston bar adjustment (step 13), the reaction product of the FC assay stored in RC2–RC4 was propelled through the detector (steps 14, 15), providing three replicate measurements of FC capacity. Afterwards, with selection valves in position 1, the ABTS$^{\bullet+}$ radical solution and carrier were sent to the detector in order to establish the absorbance baseline for TEAC assay (steps 16, 17). Thereafter, the contents of RC6–RC8 were sent to the detector and the ABTS$^{\bullet+}$ scavenging capacity was measured ($n = 3$, steps 19, 20). Finally, the NaOH solution and carrier were propelled through L1 toward the detector in order to re-establish the initial conditions, rendering the system for analysis of the next sample.

For the determination of intrinsic absorption of sample, the same flow procedure was applied (Table 2). However, in these experiments acetate buffer solution (0.060 mol L^{-1}) at pH 4.6 was placed in syringe S4 instead of ABTS$^{\bullet+}$ radical solution,

Table 2
Protocol sequence for the automatic sequential determination of FC reducing capacity and TEAC using the MSFIA method

Step	Instrumentation	Protocol[a]	Description
		Start loop: sample injection for FC assay	
1	Multiposition valve	Valve VA and VB move to position X	X (FC assay) = position 2 to 4
2	Multisyringe piston	Pick up 1150 μL at 10.0 mL min^{-1} (V1 off, V2 off, V3 off, V4 off, V5 off, and V6 off)	Loading syringes with carrier/reagents
3	Multisyringe piston	Pick up 100 μL at 1.0 mL min^{-1} (V1 off, V2 on, V3 on, V4 off, V5 on, and V6 on)	Aspiration of sample and FCR
4	Multisyringe piston	Dispense 362 μL at 2.0 mL min^{-1} (V1 on, V2 on, V3 on, V4 off, V5 off, and V6 off)	Merging sample with FCR and then with NaOH stream towards RC X
		End loop: sample injection for FC assay	Repeat 3 times
5	Multiposition valve	Valve VA and VB move to position 5	
6	Multisyringe piston	dispense 1000 μL at 2.0 mL min^{-1} (V1 off, V2 on, V3 on, V4 on, V5 off, and V6 off)	Dispense ABTS$^{\bullet+}$ solution and carrier to clean the manifold lines
		start loop: sample injection for TEAC assay	
7	Multiposition valve	valve VA and VB move to position X	X (TEAC assay) = position 6 to 8
8	Multisyringe piston	Pickup 1150 μL at 10.0 mL min^{-1} (V1 off, V2 off, V3 off, V4 off, V5 off, and V6 off)	Loading syringes with carrier/reagents
9	Multisyringe piston	Pick up 100 μL at 1.0 mL min^{-1} (V1 off, V2 on, V3 off, V4 off, V5 on, and V6 off)	Aspiration of sample
10	Multisyringe piston	Dispense 362 μL at 2.0 mL min^{-1} (V1 off, V2 on, V3 on, V4 on, V5 off, and V6 off)	merging sample with carrier and then with ABTS$^{\bullet+}$ stream towards RC X
		End loop: sample injection for TEAC assay	Repeat 3 times
11	Multiposition valve	Valve VA and VB move to position 1	
12	Multisyringe piston	Dispense 1525 μL at 2.0 mL min^{-1} (V1 on, V2 on, V3 on, V4 off, V5 off, and V6 off)	Dispense NaOH solution and carrier to clean the manifold lines
13	Multisyringe piston	Pick up 2000 μL at 10.0 mL min^{-1} (V1 off, V2 off, V3 off, V4 off, V5 off, and V6 off)	Piston bar adjustment
		Start loop: sample measurement for FC assay	
14	Multiposition valve	valve VA and VB move to position X	X (FC assay) = position 2 to 4
15	Multisyringe piston	Dispense 1100 μL at 2.0 mL min^{-1} (V1 on, V2 on, V3 on, V4 off, V5 off, and V6 off)	Propulsion of RC X content to detector (λ = 750 nm); signal acquisition; system clean-up
		End loop: sample measurement for FC assay	Repeat 3 times
16	Multiposition valve	Valve VA and VB move to position 1	
17	Multisyringe piston	Dispense 1500 μL at 2.0 mL min^{-1} (V1 off, V2 on, V3 on, V4 on, V5 off, and V6 off)	Dispense ABTS$^{\bullet+}$ solution and carrier to clean the manifold lines
18	Multisyringe piston	Pickup 4800 μL at 10.0 mL min^{-1} (V1 off, V2 off, V3 off, V4 off, V5 off, and V6 off)	Piston bar adjustment
		Start loop: sample measurement for TEAC assay	
19	Multiposition valve	Valve VA and VB move to position X	X (TEAC assay) = position 6 to 8
20	Multisyringe piston	Dispense 1100 μL at 2.0 mL min^{-1} (V1 off, V2 on, V3 on, V4 on, V5 off, and V6 off)	propulsion of RC X content to detector (λ = 734 nm); signal acquisition; system clean-up
		End loop: sample measurement for TEAC assay	repeat 3 times
21	Multiposition valve	Valve VA and VB move to position 1	
22	Multisyringe piston	Dispense 1500 μL at 2.0 mL min^{-1} (V1 on, V2 on, V3 on, V4 off, V5 off, and V6 off)	Dispense NaOH solution and carrier to re-establish the initial conditions

[a] The indicated values of volume and flow rate are referred to syringe 1 (5 mL).

while HCl 0.20 mol L^{-1} was aspirated through valve V6 instead of FCR. All measurements were carried out at room temperature (25 ± 2 °C).

3. Results and discussion

3.1. Development of MSFIA system for the sequential determination of FC reducing capacity and TEAC

The objectives of the present work were (i) the development of an automatic flow method for the sequential determination of FC reducing capacity and TEAC; (ii) the study of reducing and antioxidant capacities of food products, and (iii) the evaluation of correlation between these two methods. Therefore, taking into account the first purpose, the MSFIA system recently reported for the determination of FC reducing capacity [15] was improved to accommodate in the same manifold the FC reduction reaction and the ABTS$^{\bullet+}$ scavenging reaction (TEAC assay). For this, the flexibility of flow management offered by MSFIA was exploited by merging antioxidant standard solution/sample with FCR (FC assay) or with water (TEAC assay). Afterwards, NaOH solution (FC assay) or ABTS$^{\bullet+}$ radical solution (TEAC assay) was added to the prior mixture and directed to the reaction coil. Taking into consideration that both methodologies require time for reaction development [15,16], a stopped-flow approach was chosen and implemented before the determination. Furthermore, in order to enhance the sample throughput, the two assays were carried out in tandem. For this, sampling and reagent mixing for TEAC

assay were performed during the waiting period of FC assay, whilst the spectrophotometric measurement of FC assay was attained during the flow stop period of TEAC assay.

For this analytical procedure, four syringes were necessary: one of them containing the alkaline solution for FC assay, two syringes containing water to propel the standard/sample and the FCR, and the last one containing ABTS$^{\bullet+}$ radical solution (Fig. 1). Confluence T2 was connected to the central channel of selection valve VA, whereas the central channel of valve VB was connected to the flow cell. This arrangement allowed the establishment of six coiled reactors, three for FC assay and the other three for TEAC assay, by connecting the side ports of the selection valves. The washing coil L1 allowed the access of flow cell in order to establish the baseline, without disturbing the content of the reaction coils. The port 5 of selection valve VA was directed to waste to permit the exchange of standard/sample and also the cleaning of the tubing between confluence T2 and valve VA (steps 5 and 6, Table 2), without passing through the flow cell. Otherwise, the time of exchange and reagent consumption would be increased.

The mixing coil (MC) and the line between confluence T2 and valve VA were made as short as possible to prevent high sample dispersion. The length of the reaction coil (150–250 cm) and the volume of solution sent to the reaction coil (200–450 μL) prior to flow stop were studied in order to guarantee that all sample segments were inserted in the reaction coil. These experiments were carried out using gallic acid (40 mg L^{-1}) as sample and a procedure similar to "FC calibration". Thus, after loading the gallic acid standard solution and FCR, these segments were sent to RC2 by activating syringes S1, S2 and S3. After flow stop, the selection valves were in position 1 and the standard solution that would be outside the RC2 (before the valve VA and/or after the valve VB) was propelled towards the detector. Finally, the content of RC2 was sent to the detector. Using this procedure, it was observed that the reaction coil length of 250 cm and a volume of 362 μL allowed the accommodation of all sample within the reaction coil. Moreover, the time of stopped-flow applied in the FC and TEAC calibration procedures (step 6, Table 1) was optimized in order to guarantee that the standards were subjected to the same reaction time as samples. For the FC and TEAC calibration, the time values were 250 and 295 s, respectively.

The chemical conditions for the determination of FC reducing capacity were similar to those reported in previous work [15]. For the TEAC assay, the concentration of ABTS$^{\bullet+}$ solution was studied in order to provide an absorbance value near 0.8, after dilution inside the flow system. This evaluation was performed taking into account that the stoichiometry of ABTS oxidation by H_2O_2 is 2 ABTS:1 H_2O_2 and that the ABTS concentration should be in about five-fold excess. This excess, along with a 12-h preincubation period before use, guaranteed that hydrogen peroxide was exhausted, preventing the possible reaction between the antioxidant compounds present in the sample and ABTS/H_2O_2/HRP system [17]. Therefore, the HRP concentration was fixed at 3 units mL^{-1} and the following solutions of ABTS/H_2O_2 concentrations were prepared: 1.28/0.128, 0.80/0.08 and 0.64/0.064 mM, providing absorbance values of 1.190, 0.760 and 0.594, respectively. Thus, the ABTS$^{\bullet+}$ radical solution was obtained from 0.80 and 0.08 mM of ABTS and H_2O_2, respectively. The radical solution was stable during continuous operation for 8 h (R.S.D. <1.3%).

Using the described analytical procedures for FC and TEAC calibration (Table 1) and sequential determination for samples (Table 2), the analytical signals were obtained from different reaction coils ($n = 3$, per assay). Therefore, in order to evaluate if there was significant difference between them, 10 consecutive calibration procedures for FC and TEAC assays were performed using 25 mg L^{-1} of gallic acid and 0.10 mM of trolox, respectively. For each method 30 determinations were obtained, corresponding to 10 analytical signals per RC. An ANOVA test was performed and the results obtained ($p = 0.375$ for FC assay and $p = 0.363$ for TEAC assay) indicated that the analytical signals obtained by the different reaction coils were not significantly different [18].

3.2. Analytical features of the developed MSFIA system

Under the optimal conditions described above, linear calibration plots for gallic acid (5.0–75.0 mg L^{-1}) and for trolox (0.020–0.20 mM) were obtained for the FC and TEAC assay, respectively. Fig. 2 displays a typical signal output, including the FC and TEAC calibration and also the analysis of some samples. The absorbance values obtained for samples were interpolated in the following calibration curves: $A_{750} = 0.0078\ (\pm 0.0001) \times C_{\text{gallic acid}} + 0.026\ (\pm 0.005)$ ($n = 5$, $R \geq 0.9989$); $A_{734} = -3.05\ (\pm 0.05) \times C_{\text{trolox}} + 0.742\ (\pm 0.015)$ ($n = 4$, $R \leq -0.9988$); where A is the absorbance and C is the concentration of gallic acid (mg L^{-1}) or trolox (mM); values between parenthesis are the standard deviation of the parameters corresponding to twelve calibration curves performed on different days. The interpolation values were multiplied by the respective dilution factor. Furthermore, the sample blank for both methods was measured and absorbance values <0.006 were found for all samples tested. As these values represent <5% of analytical signal of samples for FC assay and <1% of initial ABTS$^{\bullet+}$ absorbance, their contribution to the absorbance measured was not significant.

The detection limit was calculated as the concentration corresponding to the intercept value plus three times the statistic $s_{y/x}$ [18]. For 12 different calibration curves, the calculated detection limit for FC and TEAC assays was about 3 mg L^{-1} and 0.008 mM, respectively. The precision of the developed method was estimated by calculating the relative standard deviation (R.S.D.) from 12 consecutive determinations of samples A11, B12, and D12 (see supplementary data). The values obtained for FC assay were 3.6, 4.0 and 3.1%, while for TEAC assay they were 3.1, 3.1, and 2.4%, respectively. The reproducibility of the methodology was assessed by the R.S.D. of calibration slopes performed in different days ($n = 12$). The results obtained were 1.3 and 1.6% for the FC and TEAC assay, respectively.

The sequential analysis of samples by the two methods allowed 24 determinations to be carried out per hour. Considering that the sample was analysed for both methodologies ($n = 3 + 3$), the present method provided FC reducing capacity and TEAC values for four samples per hour. The calibration pro-

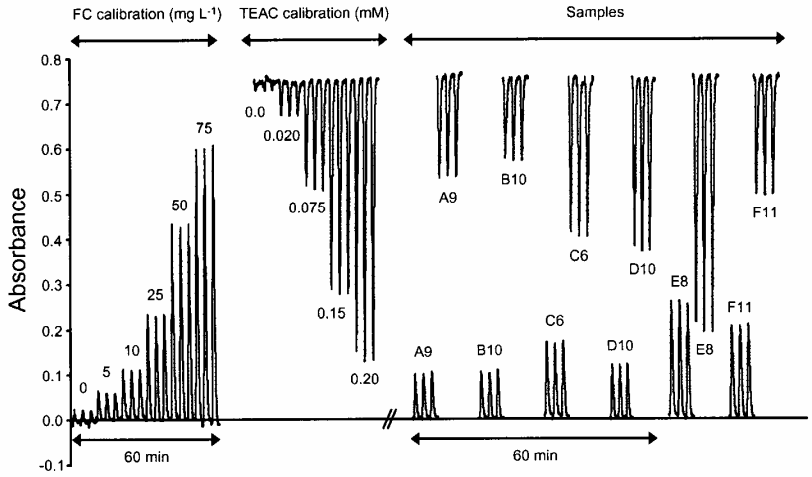

Fig. 2. Signal profile obtained for FC and TEAC assays using gallic acid (mg L^{-1}) and trolox (mM) as standard compounds, respectively. The sequential determination of FC reducing capacity and TEAC for some food samples is also presented.

cedures allowed 18 determinations per hour, corresponding to 6 standards ($n = 3$). Furthermore, it should be stressed that each method can be carried out separately, even for sample analysis. In fact, using the calibration procedure, the FC reducing capacity or the TEAC of samples can be easily assessed.

3.3. Analysis of food products and comparison of results within and between methods

The proposed automatic method was applied to a large number of beverages ($n = 72$) with recognised antioxidant capacity [19–22], namely red and white wines, herbal infusions, tea infusions (green and black), juices and beers (blond and dark). Sample details and analysis values for twelve products of each group are provided as supplementary data (Tables S1–S6). The statistical treatment of the data obtained by beverage type and assay is shown in Table 3.

The TEAC and FC reducing capacity of red wines were higher than the other beverages, ranging from 9.1 to 22.6 mM and from 1284 to 3274 mg L^{-1}, respectively. Actually, red wines showed TEAC and FC reducing capacity values approximately 10 times higher than white wines, ranging from 1.14 to 2.83 mM and from 193 to 327 mg L^{-1}, respectively. These differences between red and white wines are in agreement with results found by other authors [20,21,23]. The error plot of means with 95% confidence intervals for TEAC and FC reducing capacity of each group of beverage is presented in Fig. 3. The TEAC and FC reducing capacity of red wines were statistically different ($p < 0.05$) from the other beverages. These results are expected owing to the high content of anthocyanins and other phenolic compounds extracted from the skins and seeds during the fermentation of red wine [24]. High TEAC values were observed in tea infusions ($p < 0.05$), whereas white wines, herbal infusions, juices and beers showed no significant difference ($p > 0.05$) between them. In relation to FC reducing capacity, white wines were statistically different ($p < 0.05$) from the other beverages, with exception to herbal infusions ($p = 0.945$).

Noteworthy, the TEAC and FC reducing capacity of dark beers ($n = 6$) tested in this investigation were 2.18 ± 0.48 mM and 782 ± 212 mg L^{-1}, respectively, whilst the average values of blond beers ($n = 6$) were 1.00 ± 0.14 mM and 390 ± 69 mg L^{-1}, respectively. They were statistically different

Table 3
Statistical summary from TEAC (mM) and FC reducing capacity (mg L^{-1}) assays by beverage type

	Red wines	White wines	Herbal infusions	Tea infusions	Juices	Beers
TEAC (mM)						
Mean ± S.D.[a]	15.4 ± 3.3	1.81 ± 0.45	1.89 ± 1.16	5.49 ± 2.10	2.23 ± 0.63	1.59 ± 0.70
Max.	22.6	2.83	3.63	10.9	3.58	2.58
Min.	9.1	1.14	0.28	2.72	1.35	0.85
Interval	13.5	1.69	3.35	8.18	2.23	1.73
FCR capacity (mg L^{-1})						
Mean ± S.D.[a]	2047 ± 487	252 ± 43	325 ± 171	558 ± 228	448 ± 124	586 ± 254
Max.	3274	327	543	1081	659	962
Min.	1284	193	46	220	304	321
Interval	1990	134	497	861	355	641

[a] Results are the mean ± standard deviation (S.D.) of 12 samples.

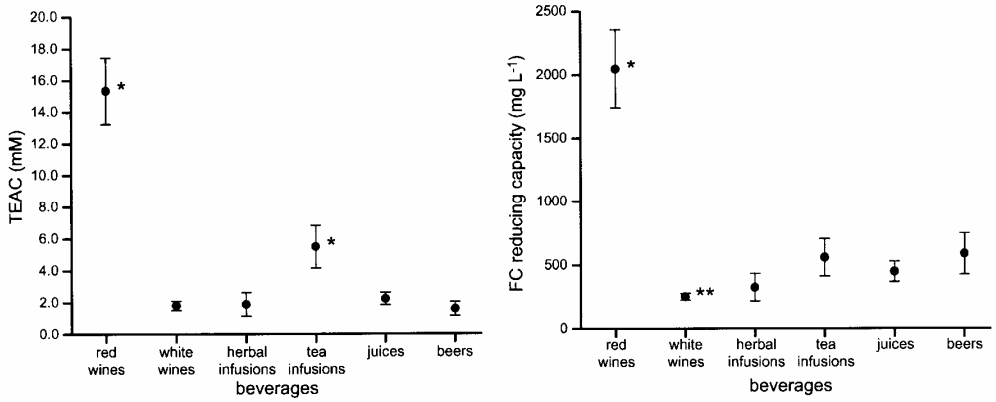

Fig. 3. Error plot of means with 95% confidence intervals for TEAC and FC reducing capacity of each group of beverage. *Significantly different from the other means ($p < 0.05$). **Significantly different from the other means ($p < 0.05$), except for herbal infusions ($p = 0.945$).

Table 4
Slope and correlation coefficients between FC reducing capacity (mg L^{-1}) vs. TEAC (mM) for the beverages analysed

	Red wines	White wines	Herbal infusions	Tea infusions	Juices	Beers
Slope (mg L^{-1} mM^{-1})	140 ± 33	57 ± 55	135 ± 41	106 ± 18	136 ± 101	349 ± 69
Correlation (R)[a]	0.947	0.590	0.918	0.972	0.690	0.962

[a] Correlation coefficients were determined by least-squares method ($n = 12$); correlation is significant at the 0.01 level (2-tailed).

for both parameters ($p < 0.05$). This higher antioxidant capacity of dark beers compared to blond beers was reported before and it may be related to their higher content in phenolic compounds and melanoidins that are formed during malting and brewing processes and that could act synergistically [22].

In the present study, the average values of TEAC and FC reducing capacity of green tea infusions ($n = 6$) were 6.78 ± 2.17 mM and 692 ± 228 mg L^{-1} respectively, while for black tea infusions ($n = 6$) the results obtained were 4.19 ± 0.96 mM and 424 ± 137 mg L^{-1}, respectively. The higher in vitro antioxidant capacity of green tea ($p < 0.05$) observed was also reported by Ivanova et al. [25] using the TEAC assay. Moreover, Serafini et al. [26] evaluated in vitro the length of the peroxyl radical induced lag-phase and observed that green tea was six-fold more potent than black tea. These differences may be attributed to the fermentation step from the black tea processing, where phenolic compounds are oxidized and polymerized enzymatically to theaflavins and thearubigens. The degradation of the major green tea catechin, epigallocatechin gallate, which is a powerful antioxidant, also takes place [27]. Nevertheless, for a better comparative assessment of the antioxidant efficiency of green and black tea infusions, other factors should be taken into consideration, such as raw material provenience, the brewing time, and the chopping grade of the tea leaves, for instance.

A good correlation was found between TEAC and FC reducing capacity when the whole set of samples was considered (FC capacity = 121.8 ± 8.6 TEAC + 127.3 ± 60.5, $R = 0.959$, $n = 72$). For each group of samples, the regression data including the slopes and the correlation coefficients established between the two methods are given in Table 4. Taking into account that these assays are based on electron transfer mechanisms [6], it is expected a significant correlation between them. The results obtained in the present study demonstrated a good correlation for red wines, herbal and tea infusions and beers ($R \geq 0.918$). Nevertheless, a lower correlation was established for white wines ($R = 0.590$) and juices ($R = 0.690$). In the case of white wines this can be an artifact caused by the low dispersion of FC capacity and TEAC values (Fig. 3). On the other hand, as juices were obtained from a variety of fruits (see supplementary data, Table S5) there was a high heterogeneity in the composition of these samples, which could explain the lower correlation obtained. Comparing the slope values obtained when a good correlation was attained (Table 4, $R > 0.9$), similar values were obtained for red wines, herbal and tea infusions, while the slope for beers was higher (about 2.5 times). This indicated a higher response for FC assay, probably due to the presence of dextrins, melanoidins, and proteins in dark beers [28].

4. Conclusions

The automatic method developed in the present work allowed the consecutive determination of the FC reducing capacity and TEAC, representing a useful tool for routine analysis as also for comparison purposes between these parameters. The fact of performing these determinations in parallel has also the advantage of processing the sample at the same time, avoiding errors that might arise with sample modification over time. The flexibility introduced by the proposed configuration associated to the computer control also allowed the performance of each assay separately. The present flow system was successfully applied to a large number of beverages providing reliable information about

the antioxidant properties in a simple, rapid and automatic way. The results showed that the correlation between these assays may vary according to type of sample. This observation reinforces the idea that more than one method should be used for evaluation of antioxidant capacity, especially in complex samples as food matrices.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.aca.2007.04.028.

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Supplementary Data (Tables S1–S6)

Table S1. Trolox equivalent antioxidant capacity (mM) and FC reducing capacity (mg L^{-1}) for red wines[a]

A – Red wines, origin	Dilution[b]	TEAC assay (mM)	FC assay (mg L^{-1})
A1, Terras do Sado	1:200	22.6 ± 0.7	3274 ± 44
A2, Terras do Sado	1:200	14.8 ± 0.6	1910 ± 62
A3, Table wine	1:200	13.2 ± 0.1	1617 ± 65
A4, Douro	1:200	16.2 ± 0.3	1912 ± 26
A5, Alentejo	1:200	15.5 ± 0.5	2063 ± 74
A6, Beiras	1:200	13.4 ± 0.5	1973 ± 77
A7, Alentejo	1:200	16.7 ± 0.7	2134 ± 38
A8, Dão	1:200	13.5 ± 0.5	1763 ± 60
A9, Dão	1:200	14.2 ± 0.1	2107 ± 90
A10, Douro	1:100	9.1 ± 0.4	1284 ± 52
A11, Alentejo	1:200	16.4 ± 0.2	2031 ± 64
A12, Dão	1:200	18.6 ± 0.8	2496 ± 99

[a] Each value corresponds to the mean ± standard deviation (n = 3). [b] Dilution performed prior to analysis.

Table S2. Trolox equivalent antioxidant capacity (mM) and FC reducing capacity (mg L^{-1}) for white wines[a]

B – White wines, origin	Dilution[b]	TEAC assay (mM)	FC assay (mg L^{-1})
B1, Dão	1:20	1.45 ± 0.08	248 ± 9
B2, Alentejo	1:20	1.91 ± 0.07	225 ± 3
B3, Dão	1:20	1.88 ± 0.01	252 ± 5
B4, Alentejo	1:20	1.25 ± 0.01	193 ± 4
B5, Beiras	1:20	1.55 ± 0.03	243 ± 5
B6, Minho	1:20	2.24 ± 0.04	327 ± 12
B7, Minho	1:20	1.84 ± 0.02	254 ± 3
B8, Beiras	1:20	1.82 ± 0.05	198 ± 1
B9, Algarve	1:20	1.79 ± 0.06	323 ± 3
B10, Alentejo	1:20	1.14 ± 0.05	219 ± 7
B11, Minho	1:20	1.96 ± 0.07	254 ± 4
B12, Minho	1:20	2.83 ± 0.03	290 ± 5

[a] Each value corresponds to the mean ± standard deviation (n = 3). [b] Dilution performed prior to analysis.

Table S3. Trolox equivalent antioxidant capacity (mM) and FC reducing capacity (mg L^{-1}) for herbal infusions[a]

C – Herbal infusions, description	**Dilution**[b]	**TEAC assay (mM)**	**FC assay (mg L^{-1})**
C1, *Tilia cordata* L.	1:4	0.28 ± 0.01	46 ± 2
C2, *Melissa officinalis* L.	1:20	2.69 ± 0.09	536 ± 13
C3, *Mentha piperita* L.	1:10	1.35 ± 0.01	257 ± 4
C4, *Aloysia citriodora* Palau	1:20	1.42 ± 0.04	372 ± 13
C5, *Melissa officinalis* L.	1:20	3.22 ± 0.05	543 ± 4
C6, *Hypericum perforatum* L.	1:20	2.21 ± 0.04	359 ± 8
C7, *Equisetum arvense* L.	1:20	1.05 ± 0.03	175 ± 7
C8, *Thea Sinensis sims* (Pu-Erh)	1:20	3.30 ± 0.08	408 ± 4
C9, *Matricaria chamomilla* L.	1:10	0.40 ± 0.01	73 ± 1
C10, mixture of plants	1:20	3.63 ± 0.11	498 ± 11
C11, mixture of plants	1:20	2.29 ± 0.10	416 ± 7
C12, mixture of plants	1:20	0.83 ± 0.02	219 ± 7

[a] Each value corresponds to the mean ± standard deviation (n = 3). [b] Dilution performed prior to analysis.

Table S4. Trolox equivalent antioxidant capacity (mM) and FC reducing capacity (mg L^{-1}) for tea infusions[a]

D – Tea infusions, description	**Dilution**[b]	**TEAC assay (mM)**	**FC assay (mg L^{-1})**
D1, *Camellia sinensis*	1:40	5.95 ± 0.12	619 ± 18
D2, *Camellia sinensis*	1:40	4.92 ± 0.17	429 ± 19
D3, *Camellia sinensis*	1:40	6.99 ± 0.11	709 ± 19
D4, *Camellia sinensis*	1:80	10.9 ± 0.2	1081 ± 15
D5, *Camellia sinensis*	1:40	6.69 ± 0.16	784 ± 16
D6, *Camellia sinensis*	1:40	5.25 ± 0.18	531 ± 9
D7, *Camellia sinensis*	1:40	4.54 ± 0.02	442 ± 16
D8, *Camellia sinensis*	1:40	4.17 ± 0.08	510 ± 6
D9, *Camellia sinensis*	1:40	3.52 ± 0.10	304 ± 5
D10, *Camellia sinensis*	1:40	4.72 ± 0.10	483 ± 5
D11, *Camellia sinensis*	1:40	2.72 ± 0.11	220 ± 8
D12, *Camellia sinensis*	1:40	5.46 ± 0.18	587 ± 9

[a] Each value corresponds to the mean ± standard deviation (n = 3). [b] Dilution performed prior to analysis. D1–D6, green tea; D7–D12, black tea.

Table S5. Trolox equivalent antioxidant capacity (mM) and FC reducing capacity (mg L^{-1}) for juices[a]

E – Juices, description	Dilution[b]	TEAC assay (mM)	FC assay (mg L^{-1})
E1, orange	1:20	1.98 ± 0.07	463 ± 5
E2, tropical fruits	1:20	2.13 ± 0.03	397 ± 14
E3, tomato, tropical fruits, carrot	1:20	1.70 ± 0.02	304 ± 3
E4, orange, carrot	1:20	1.51 ± 0.06	305 ± 7
E5, tropical fruits, carrot	1:20	2.46 ± 0.07	384 ± 12
E6, orange	1:20	2.66 ± 0.08	659 ± 16
E7, apple	1:20	2.51 ± 0.05	636 ± 9
E8, orange, passion fruit	1:20	3.58 ± 0.08	587 ± 9
E9, orange	1:20	1.35 ± 0.05	435 ± 11
E10, pineapple, coconut	1:20	2.75 ± 0.07	478 ± 9
E11, orange	1:20	2.45 ± 0.03	428 ± 4
E12, tropical fruits	1:20	1.72 ± 0.07	304 ± 11

[a] Each value corresponds to the mean ± standard deviation (n = 3). [b] Dilution performed prior to analysis.

Table S6. Trolox equivalent antioxidant capacity (mM) and FC reducing capacity (mg L^{-1}) for beers[a]

F – Beers, description	Dilution[b]	TEAC assay (mM)	FC assay (mg L^{-1})
F1, blond beer	1:20	0.92 ± 0.04	465 ± 13
F2, blond beer	1:20	0.96 ± 0.05	328 ± 8
F3, blond beer	1:20	1.03 ± 0.03	482 ± 5
F4, blond beer	1:20	1.26 ± 0.05	379 ± 7
F5, blond beer	1:20	0.85 ± 0.03	321 ± 10
F6, blond beer	1:20	1.00 ± 0.02	362 ± 6
F7, dark beer	1:20	2.58 ± 0.08	962 ± 14
F8, dark beer	1:20	2.50 ± 0.04	898 ± 10
F9, dark beer	1:20	2.43 ± 0.09	925 ± 23
F10, dark beer	1:20	1.52 ± 0.04	573 ± 13
F11, dark beer	1:20	1.60 ± 0.01	456 ± 4
F12, dark beer	1:20	2.43 ± 0.12	875 ± 9

[a] Each value corresponds to the mean ± standard deviation (n = 3). [b] Dilution performed prior to analysis.

CHAPTER 7

Automatic in vitro determination of hypochlorous acid scavenging capacity exploiting multisyringe flow injection analysis and chemiluminescence

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Automatic in Vitro Determination of Hypochlorous Acid Scavenging Capacity Exploiting Multisyringe Flow Injection Analysis and Chemiluminescence

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In the present work, a chemiluminometric automatic flow methodology for the in vitro determination of hypochlorous acid scavenging capacity, under pH and concentration conditions similar to those found in vivo, is proposed. As the pH found in physiological conditions (7.4) and the pH required for the chemiluminescence detection reaction (>10) are different, the multisyringe flow injection analysis features were exploited to perform the in-line reaction of HOCl and the scavenger molecule at physiological pH prior to reaction of the remaining HOCl with luminol at alkaline conditions. These two reactions were carried out in about 3 s, allowing the determination of fast reacting antioxidants, in a time frame closer to in vivo generation of HOCl when compared to previously described methods. The developed method was applied to nonsteroidal anti-inflammatory drugs of different chemical families, and positive controls (cysteine, gallic acid, lipoic acid). The HOCl scavenging capacity was evaluated at pH 7.4 and 10.0; different results were found for oxicam derivatives, providing evidence that the pH of in vitro methods should be carefully selected to allow assumptions about putative in vivo effects.

The formation of reactive oxygen species (ROS) and metabolites in biological systems plays an important role in the mechanism of defense against microbes.[1–3] However, the continuous overproduction of these reactive species and/or the decrease in antioxidant defenses may cause damage by destroying the surrounding tissue and contributing to the development of several human pathologies including atherosclerosis, neurological degeneration, and several chronic inflammatory processes.[4–6] In the latter pathological condition, among the various ROS that may be produced during the inflammatory response, a relevant role is played by the potent bactericidal hypochlorous acid (HOCl).[1] This nonspecific oxidizing and chlorinating agent which is produced in vivo by the myeloperoxidase/H_2O_2/Cl^- system of the stimulated polymorphonuclear leukocytes rapidly reacts with amines and sulfhydryl groups, causing damage to several biomolecules or cell structures.[6,7] This makes HOCl as a potential target for the chemotherapy of inflammation.

For this reason, several analytical procedures have been developed for studying in vitro the scavenging activity of therapeutic agents against HOCl using electron spin resonance (ESR),[8] UV-visible spectrophotometry,[9–12] fluorescence,[13] and chemiluminescence[8,14] as detection systems. However, these methods, which are not suitable for fast screening, presented some limitations. For instance, ESR-based methods need expensive instruments that are not available in routine laboratories. Furthermore, in the enzymatic-based assay developed by Haenen and Bast,[9] employing α_1-antiproteinase (α_1-AP) and elastase, compounds which have inhibitory effects on the activities of either enzyme could falsely be interpreted to be HOCl scavengers. For the 5-thio-2-nitrobenzoic acid (TNB) oxidation assay,[10] developed later by the same group, it was observed that compounds containing free thiol groups, such as dihydrolipoic acid, cysteine, and glutathione, interfered in this method, yielding an excess of TNB. Nève et al.[11] verified that tenoxicam and piroxicam, two nonsteroidal anti-inflammatory drugs (NSAIDs), scavenged HOCl, but it was not

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possible to quantify their action as both compounds absorbed at the wavelength of detection. To circumvent this type of interference, a liquid chromatographic system has been applied to carry out the separation of the drugs and their oxidation products before the determination.[12,13] However, this makes these methodologies time-consuming, expensive, and often not suitable for screening determinations.

Chemiluminescence (CL) detection offers great analytical advantages over the other techniques; it is selective, sensitive, simple, inexpensive, and rapid.[15] Nevertheless, the implementation of fast CL reactions (as HOCl–luminol)[16] based on batch methods is laborious, as rapid and reproducible sample/reagent mixing and immediate measurement are necessary. The automation of these reactions, based on flow techniques, meets these requirements, improving the precision and accuracy of the methodology.[17] In fact, a flow injection analysis (FIA)[18] and a sequential injection analysis (SIA)[19] using CL as detection system for the evaluation of scavenging of hypochlorite ion have been developed, but its application was restricted to standard compounds (ascorbic acid, α-tocopherol, and trolox). Moreover, as the CL reaction is favored under alkaline conditions, the scavenging reaction was carried out at pH > 9.5, under conditions far different from those found in physiological milieu.

Therefore, the main purpose of the present work was the development of a chemiluminometric automatic flow methodology for the in vitro determination of hypochlorous acid scavenging capacity in conditions closer to those found in vivo. For this, luminol was chosen as chemiluminometric reagent because of its specificity for detecting HOCl among other reactive species produced by human neutrophils after activation of the respiratory burst response.[20] Furthermore, multisyringe flow injection analysis (MSFIA)[21–23] was chosen, as it offers versatility of flow management and multichannel operation in a wide range of flow rates. These characteristics allow the accommodation in a single protocol of different reaction conditions, concerning both reaction time and pH value. Taking into consideration the differences between the pH found in physiological conditions and the pH required for the CL detection reaction, these MSFIA features were exploited to perform the in-line reaction of HOCl and the scavenger molecule at physiological pH (or at the pH of CL detection, for comparison purposes) prior to oxidation of luminol by the remaining HOCl at alkaline conditions. The developed method was applied to several NSAIDs of different chemical families, and the HOCl scavenging capacity at different pH values (7.4 and 10.0) was evaluated.

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EXPERIMENTAL SECTION

Reagents and Solutions. All chemicals used were of analytical-reagent grade with no further purification. Aminopyrine was obtained from Aldrich (Milwaukee, WI). Lornoxicam was kindly provided by Euro-Labor Pharmaceutics. Lipoic acid and luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) were purchased from Fluka (Buchs, Switzerland). Ethylenediaminetetraacetic acid (EDTA) disodium salt and potassium dihydrogen phosphate were obtained from Probus, S.A. (Barcelona, Spain). All other chemicals were purchased from Sigma (St. Louis, MO), including the sodium hypochlorite solution (4% available chlorine, ref 239305).

Water from Milli-Q system (resistivity >18 MΩ cm) and ethanol absolute pro analysis were used for the preparation of all solutions. All solutions were protected from light. The stock solutions of NSAIDs were prepared by dissolving aminopyrine, cysteine, dipyrone, gallic acid, ibuprofen, and meloxicam in water. Lornoxicam, piroxicam, sulindac, and tenoxicam were dissolved in water with 2.0 mM NaOH. Acemetacin, indomethacin, and lipoic acid were dissolved in ethanol. The working solutions (2.5–10000 μM) were prepared daily by dilution in water.

Hypochlorous acid (HOCl) solutions were prepared daily, immediately before use. First, a 1% (v/v) solution of NaOCl was prepared from the commercial solution. The pH was adjusted to 6.2 with diluted sulfuric acid and the concentration of HOCl was further determined spectrophotometrically (Model 8453, Hewlett-Packard, Waldgrohn Germany) at 235 nm using the molar absorption coefficient of 100 M^{-1} cm^{-1}.[24] Next, the working solution containing 50 μM of HOCl was prepared by dilution of the previous solution in 20 mM potassium phosphate buffer pH 7.4 or in 20 mM carbonate buffer pH 10.0. Due to the pK_a (7.5) of hypochlorous acid, the solution contains approximately 1:1 HOCl and OCl^- at pH 7.4, while at pH 10.0 OCl^- are the main species. However, it is usually referred as "HOCl" independently the pH value of the medium.

The chemiluminogenic reagent was prepared by dissolving 22.2 mg of luminol in 250 mL of 0.10 M carbonate buffer (pH 10.0) containing 1.0 mM EDTA. This solution should be prepared at least 24 h before analysis and kept in dark at room temperature.

Flow Manifold and Instrumentation. The automated multisyringe flow system designed for the chemiluminometric determination of hypochlorous acid scavenging capacity is depicted in Figure 1A. It comprises a multisyringe piston pump (MicroBu 2030, Crison, Alella, Spain) equipped with syringes of 5 and 10 mL (Hamilton, Switzerland) at positions S1, S3 and S2, S4, respectively. Each syringe is connected to a three-way commutation valve (N-Research, Caldwell, NJ) that allows the access to two different channels (solutions flask or flow network). The flow assembly also includes a 10-port multiposition selection valve (Crison, Alella, Spain) used as sampling device.

All tubing connecting the different components of MSFIA was made from polytetrafluoroethylene (PTFE – Omnifit, Cambridge, U.K.) of 0.8 mm i.d., except for tubes between the flasks and syringes, which were of 1.5 mm i.d. in order to avoid back pressure or vacuum at high flow rates. The four and three-way connectors (T1 and T2, respectively) used as confluences were built from polymethylmethacrylate (PMMA).

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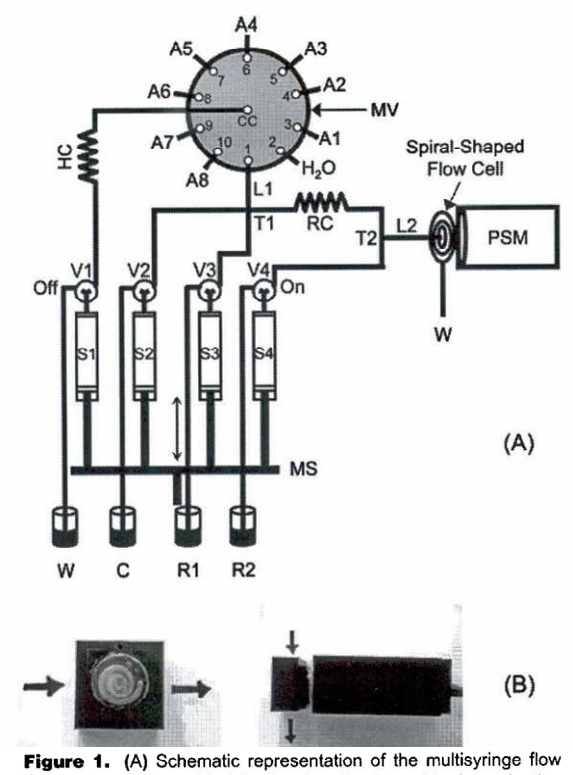

Figure 1. (A) Schematic representation of the multisyringe flow injection setup assembled for the chemiluminometric determination of hypochlorous acid scavenging capacity. MS, multisyringe; MV, multiposition valve; PSM, photosensor module; S1 and S3, syringes 5 mL; S2 and S4, syringes 10 mL; V*i*, three-way commutation valves; T*i*, confluences; CC, central communication channel; HC, holding coil (350 cm); RC, reaction coil (100 cm); L1 and L2, connecting tubing (10 cm); C, carrier (10 mM phosphate buffer pH 7.4 or 10 mM carbonate buffer pH 10.0); R1, HOCl 50 μM in 20 mM phosphate buffer pH 7.4 or in 20 mM carbonate buffer pH 10.0; R2, luminol 500 μM in 0.10 M carbonate buffer pH 10.0 and 1.0 mM EDTA; A*i*, sample; and W, waste. The exchange options of the commutation valves were classified in on/off lines. The "off" line was assigned to the solution flasks, and the "on" line was reserved for the flow network. (B) Frontal view of the custom-built spiral-shaped flow cell and upper view of the flow cell positioned over the photosensor module, located inside a light-tight box. The arrows indicate the flow direction.

The control of multisyringe and valve operation, and also CL data acquisition and treatment, were attained by a personal computer running the software package Autoanalysis written in Delphi (version 5.0) and Visual C++ (version 6.0) (Sciware, Balearic Islands, Spain).

As detection system, a lab-made CL detector adapted to flow assemblies was applied. The CL detector that was previously described for utilization in MSFIA manifolds[23] was improved by engraving the flow path on an Acetal-Delrin block, providing a spiral-shaped flow-through cell with an inner volume of 80 μL and an effective emitting surface of approximately 0.85 cm^2. This flow cell was placed and fixed over a photosensor module (Hamamatsu HS5784, Bridgewater, NJ) window, producing a compact light-tight box (Figure 1B).

Analytical Procedure of the MSFIA-CL Method for the Determination of HOCl Scavenging Capacity. The experimental MSFIA-CL procedure for the determination of hypochlorous acid scavenging capacity including the scavenging reaction of HOCl at a fixed pH value and subsequent CL luminol oxidation in alkaline medium is summarized in Table 1. For this, the buffer solution used as carrier, HOCl prepared in the respective buffer, and luminol were placed in syringes S2, S3, and S4, respectively. Syringe S1 and the multiposition valve were used for sample loading into the holding coil and for dispensing a fixed aliquot of sample into the reaction coil. Two confluences were incorporated into the manifold: the first one to promote the merging of sample with HOCl solution, and the second one allowed the merging of this mixture with luminol just before light collection.

Briefly, the first commands of MSFIA-CL procedure (steps 2−8, Table 1) involved the sample selection and loading into the holding coil. After this, the sample injection protocol begins by dispensing simultaneously and at the same flow rate the sample (150 μL) and HOCl solution (150 μL) into the reaction coil, where the scavenging reaction took place (step 10, Table 1). Next, the detector was activated, and the measurement of light intensity at 4 Hz within the wavelength interval ranging from 185 to 850 nm was initiated (step 11, Table 1). Subsequently, the mixture HOCl/sample was further propelled, mixed with luminol and driven to the spirally shaped flow cell at a total flow rate of 10 mL min^{-1} for CL measurements at room temperature (step 12, Table 1). Thereafter, the sample injection protocol (step 9, Table 1) was again started. This analytical cycle was performed in quadruplicate for each sample. Finally, the sample selection protocol (step 2, Table 1) was restarted, rendering the system for analysis of the next sample. The automated procedure devised allows the determination of scavenging capacity of up to eight samples (n = 4) within the same analytical cycle.

The measurement of CL-blank signal was performed through aspiration of water as sample, providing the maximum CL emission. The HOCl scavenging capacity of the NSAIDs was evaluated through the decrease in CL emission. The sample concentration providing 50% inhibition of CL-blank emission (IC_{50}) was then estimated.

RESULTS AND DISCUSSION

Design of MSFIA-CL Method. The objective of the present work was the development of an in vitro methodology for determination of HOCl scavenging capacity in conditions close to those found in vivo concerning pH and concentration of both HOCl and antioxidant compounds. Therefore, a manifold based on MSFIA was devised to accommodate the reaction between HOCl and antioxidant compound at a different pH from the reaction of remaining HOCl with the chemiluminogenic reagent at alkaline pH (Figure 1). For this, the flexible channel operation offered by MSFIA was exploited by merging HOCl solution (syringe 3) to the antioxidant compound (syringe 1), both propelled by buffer solution at pH 7.4 through the reaction coil. Afterward, by activation of a third syringe, the chemiluminogenic reagent at alkaline pH was added to the prior mixture and directed at a higher flow rate toward the detector.

Taking into account that low concentrations of HOCl and NSAIDs, in a range compatible to the respective physiological[25] and therapeutical[26] levels would be tested, a high sensitivity was

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Table 1. Analytical Procedure for Determination of Hypochlorous Acid Scavenging Capacity Using the MSFIA-CL Assembly

step	instrumentation	protocol[a]	description
1	multisyringe piston pump	dispense 5000 μL at 15.0 mL min^{-1} with heads V1 off, V2 off, V3 off, and V4 off	piston bar adjustment
2		start loop: sample selection	
3	multisyringe piston pump	pick up 1350 μL at 15.0 mL min^{-1} with heads V1 off, V2 off, V3 off, and V4 off	syringes are filled with the respective solutions
4	multiposition valve	valve moves to position X	X = valve position 2 to 10
5	multisyringe piston pump	pick up 2000 μL at 4.0 mL min^{-1} with heads V1 on, V2 off, V3 off, and V4 off	sample is aspirated into HC
6	multiposition valve	valve move to position 1	
7	multisyringe piston pump	dispense 250 μL at 1.0 mL min^{-1} with heads V1 on, V2 off, V3 off, and V4 off	cleanup sample tubing L1
8	multisyringe piston pump	dispense 500 μL at 2.5 mL min^{-1} with heads V1 off, V2 on, V3 off, and V4 off	dispense carrier to clean manifold lines
9		start loop: sample injection	
10	multisyringe piston pump	dispense 150 μL at 1.0 mL min^{-1} with heads V1 on, V2 off, V3 on, and V4 off	merging sample plug with oxidant reagent plug into RC
11	CL detector	light collection with a 900-fold photomultiplier gain	measure every 0.25 s, 2 points to average
12	multisyringe piston pump	dispense 500 μL at a total flow rate of 10.0 mL min^{-1} with heads V1 off, V2 on, V3 off, and V4 on	merging oxidant-sample plug with luminol; taking place the CL reaction
13	CL detector	stop chemiluminescence measurement	
14		repeat n times from loop: sample injection	n = 4 (number of injections)
15		repeat n times from loop: sample selection	n = number of samples
16	multisyringe piston pump	pick up 5000 μL at 15.0 mL min^{-1} with heads V1 off, V2 off, V3 off, and V4 off	piston bar adjustment

[a] The indicated values of flow rate and volume are referred to syringe 1 (5 mL)

needed. Therefore, the main goal of the optimization studies was the improvement of the fast CL reaction between HOCl and luminol. Since the proposed procedure involved CL inhibition, the analytical signal corresponding to the blank (absence of antioxidant compound) should be maximized in order to ensure the best performance in terms of sensitivity. For this, the flow rate applied during the detection step (Table 1, step 12), the volume of HOCl introduced into the reaction coil, and the concentration of luminol were studied. The initial concentration of HOCl was fixed at 50 μM as concentration values up to 100 μM can be achieved in vivo at sites of inflammation,[25] but epithelial cell death was reported for concentrations between 25 and 50 μM within 4 h of exposure.[27] The length of reaction line for the fast CL luminol oxidation (L2) was also fixed at 10 cm.

The flow rate is a relevant variable especially when the oxidant and chemiluminogenic reagent are mixed just before light collection, because it determines the time interval available for the development of the CL reaction and measurement, which are also dependent on the length of the CL reaction coil (L2). In view of the manifold configuration and the analytical procedure devised, the dependence of the flow rate on the light collection by the photomultiplier tube was performed by activating simultaneously syringe/valve S2/V2 and S4/V4. This study was carried out using luminol 250 μM and 100 μL of HOCl. Total flow rates from 1.5 to 30 mL min^{-1} were investigated (Figure 2A). It was observed that the CL intensity increased with flow rate up to 10 mL min^{-1} and then decreased. The decrease in the analytical signal was due to the maximum CL emission taking place after the detector flow cell. Therefore, the total flow rate 10 mL min^{-1} was selected for further studies.

The volume of HOCl injected into reaction coil RC was evaluated between 25 and 250 μL (Figure 2B). The CL intensity increased with HOCl volume up to 150 μL. For higher volumes the CL peaks were larger but the maximum CL intensity was similar because the dilution of the HOCl solution due to dispersion was negligible. In fact, it was dependent on the ratio between the flow rates of syringes S1 and S3. For this reason, 150 μL of HOCl was adopted for the next experiments.

The effect of luminol concentration was estimated through the establishment of calibration curves by plotting the CL intensity as a function of HOCl concentration (μM). For this, three different luminol concentrations 100, 250, and 500 μM were evaluated; the slopes obtained were 17.0, 50.7, and 127.9 CL intensity counts/μM of HOCl, respectively. A linear response interval was found for HOCl concentrations ranging from 50–400, 10–100, and 10–50 μM, respectively. The larger concentration of luminol was selected because the range of HOCl concentration was compatible with those expected in vivo and the highest sensitivity was attained.

Some nonsteroidal anti-inflammatory drugs (NSAIDs) have low water solubility at neutral pH, but it increases when they are dissolved in ethanol or in alkaline medium. For this reason, it was decided to evaluate the influence of different ethanol concentrations (v/v) in the performance of the CL reaction. Hence, using the devised procedure and the optimized conditions stated above, the CL blank signal obtained using water (as sample) was related to those attained with different ethanol concentrations ranging from 0.5 to 100% (v/v). The CL inhibition was <2.2% for ethanol concentrations up to 2% (v/v), whereas 5, 10, 25, 50, and 100% (v/v) of ethanol originated 7, 11, 44, 65, and 76% of CL inhibition, respectively. Therefore, as a compromise between the solubility of tested compounds and the interference on the methodology, the maximum concentration of ethanol in the

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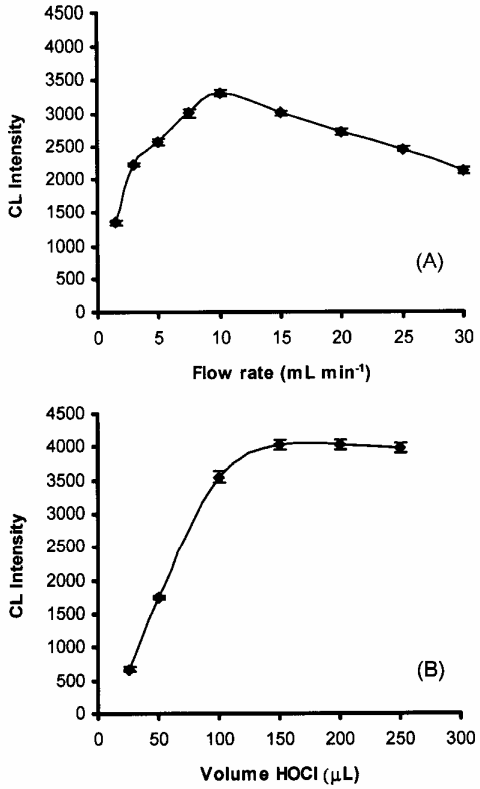

Figure 2. Optimization of the physical variables. (A) Flow rate. Experimental conditions: 250 μM luminol, 50 μM HOCl, 100 μL of HOCl. (B) Volume of HOCl. Experimental conditions: 250 μM luminol, 50 μM HOCl, 10 mL min^{-1} of total flow rate. For both graphs each point represents the values obtained from four determinations (mean ± standard deviation).

NSAIDs solutions allowed for determination using the flow system was 2% (v/v). Furthermore, under the present assay conditions the application of 2 mM of NaOH for preparation of some NSAIDs stock solutions had no effect on the pH value of the scavenging reaction.

The repeatability and reproducibility of the methodology were assessed for the determination of IC_{50} values of lipoic acid performed within and between-day ($n = 3$). The relative standard deviation obtained was 0.1 and 1.8%, respectively.

The precision of the MSFIA-CL system was also estimated by calculating the relative standard deviation from 12 consecutive determinations of three lipoic acid standards (10, 20, and 40 μM) providing values of 2.3, 1.7, and 0.5%, respectively. Therefore, the present MSFIA-CL method represents a noteworthy improvement in the repeatability and reproducibility when compared to CL-batch methodologies (RSD > 10%).[14] The determination throughput was 92 h^{-1}, corresponding to a sampling rate of 23 h^{-1} as each sample was analyzed in quadruplicate. Reagent consumption was 0.030 μmol of HOCl and 2.0 μmol of luminol per sample analyzed.

Application of the MSFIA-CL Method to Nonsteroidal Antiinflammatory Drugs (NSAIDs). The MSFIA-CL method was applied to NSAIDs representative of various families, namely arylpropionic acid (ibuprofen), pyrazolone (aminopyrine, dipyrone), indole (acemetacin, indomethacin, and sulindac), and oxicam derivatives (lornoxicam, meloxicam, piroxicam, and tenoxicam). Compounds reported as effective HOCl scavengers, such as cysteine, gallic acid, and lipoic acid,[28,29] were used as positive controls of the assay system.

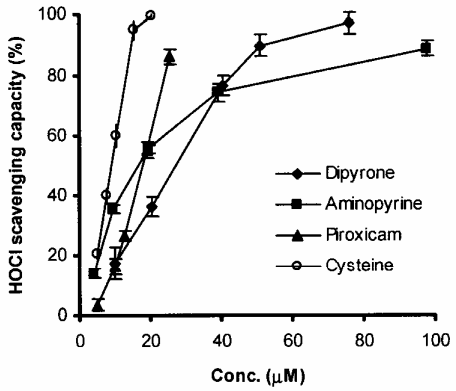

Figure 3. HOCl scavenging capacity of dipyrone, aminopyrine, piroxicam, and cysteine at pH 7.4.

In the present work, the CL-reaction is preceded by an oxidant−antioxidant reaction (HOCl-NSAIDs) at a fixed pH value. The extension of this reaction is determined mainly by its rate, which provides the ability of NSAIDs to scavenge HOCl. This feature is important in evaluating the putative in vivo effect of the drug, as its scavenging action against HOCl must be as fast as possible to minimize cellular damage. In opposition to that, the results provided by in vitro end-point methodologies, based on measurements after minutes of reaction, do not reflect the in vivo action of the drug as slow or fast reacting compounds are not distinguishable.

The scavenge effectiveness was expressed as the percentage of inhibition of luminol oxidation as a function of scavenger concentration and the results obtained for some of the compounds tested at pH 7.4 are presented in Figure 3. These compounds scavenge HOCl in a concentration-dependent manner. Moreover, the sample concentration that originates 50% inhibition of luminol-CL (IC_{50}) was also determined (Table 2). The values obtained by other methodologies are also included in Table 2, but it should be emphasized that a straightforward value comparison is not possible, as the reaction conditions (pH, HOCl concentration, probe species) are quite different. Nevertheless, a general comparison about relative potencies is feasible.

The results obtained in the present study demonstrate that HOCl is effectively scavenged by several NSAIDs and the potency of scavenging capacity was similar to that found for the positive controls. Cysteine (containing thiol group) exhibits greater HOCl scavenging capacity than that of lipoic acid (oxidized form of dihydrolipoic acid), since the IC_{50} values at pH 7.4 of cysteine and lipoic acid was 9.1 ± 0.4 and 26.3 ± 0.4 μM, respectively. Similar results were found when performing the scavenging reaction at pH 10.0. Similar results were found using the protein carbonyl assay at pH 7.4.[28]

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Table 2. HOCl Scavenging Capacity of Positive Controls and NSAIDs

	present method,[a] IC_{50} (μM)		
	pH 7.4	pH 10.0	other methods
	Positive Controls		
cysteine	9.1 ± 0.4	13.8 ± 0.7	52 ± 6[c]
gallic acid	9.7 ± 0.6	7.4 ± 0.2	
lipoic acid	26.3 ± 0.4	24.3 ± 1.3	99 ± 9[c]
	NSAIDs		
ibuprofen	NA[b]	NA[b]	NA[d]
aminopyrine	16.8 ± 0.4	20.9 ± 1.1	20.2 ± 2.5[e]
dipyrone	27.6 ± 1.3	21.5 ± 0.3	4.5 ± 0.7[e]
acemetacin	NA[b]	NA[b]	NA[d]
indomethacin	NA[b]	NA[b]	NA[d]
sulindac	NA[b]	NA[b]	NA[d]
lornoxicam	17.5 ± 0.1	1131 ± 1	4.3 ± 0.7[f]
meloxicam	9.9 ± 0.1	665 ± 4	17 ± 3[f]
piroxicam	17.7 ± 0.1	373 ± 37	3.6 ± 0.7[f]
tenoxicam	13.8 ± 0.8	1013 ± 16	4.0 ± 0.7[f]

[a] Results are expressed as the mean ± standard deviation of four determinations performed in duplicate. [b] NA, no activity was found within the tested concentrations ranging from 100 to 10000 μM. [c] IC_{50} (μM) values, using protein carbonyl assay (ref 28). [d] NA, no activity was found at concentrations ranging from 0 to 2000 μM, using taurine chlorination assay (ref 11). [e] IC_{50} (μM) values, using HOCl-induced luminol-CL assay (ref 14). [f] Second-order rate constants (10^3 M^{-1} s^{-1}) for the reaction of NSAIDs with HOCl, using a competitive fluorimetric assay based on *p*-aminobenzoic acid chlorination (ref 13).

For ibuprofen, acemetacin, indomethacin, and sulindac at concentrations up to 10000, 100, 100, and 1000 μM, respectively, no detectable activity against HOCl was observed for either pH 7.4 or 10.0. The lack of HOCl scavenging capacity agreed well with previous studies using taurine chlorination assay,[11] in which these compounds were unable to react directly with HOCl and to protect taurine against chlorination. Nevertheless, it should be noted that when acemetacin and indomethacin were dissolved in 2 mM NaOH, instead of ethanol, the IC_{50}s (μM) were 68 ± 2, 80 ± 4 at pH 7.4 and 138 ± 31, 130 ± 5 at pH 10.0, respectively. The reason for these results could be explained by the rapid alkaline hydrolysis of the amide bond of acemetacin and indomethacin, even at low concentrations of OH^-, yielding the formation of chloramines, the reaction products of HOCl with amines.[30] Thus, the scavenging effect observed in these conditions was due to the products of hydrolysis instead of the original molecule. For sulindac, which does not have the amide group, no scavenging capacity was obtained.

The pyrazolone NSAIDs displayed potent scavenging effects at both pH values, aminopyrine being the most potent when the reaction took place at pH 7.4, while at pH 10.0 the scavenging potency was similar for both compounds tested. In a previous study, using HOCl-induced luminol-CL at pH 12, it was clearly shown that both compounds were highly potent scavengers of HOCl.[14] Nevertheless, in these conditions dipyrone showed higher scavenging capacity than aminopyrine, and this may be attributed to the higher pH value applied.

Antwerpen et al.,[13] using a competitive fluorimetric assay based on *p*-aminobenzoic acid (PABA) chlorination for the determination of the rate constants for the reaction of NSAIDs with HOCl, reported that oxicam NSAIDs scavenge HOCl in physiologically relevant conditions. Our results at pH 7.4 are in agreement with those found, since the drugs seemed to have roughly the same order of potency for HOCl scavenging and further indicate that meloxicam is the most effective scavenger of this family. This is related to the methylthiazole ring in meloxicam, which has a clear influence on the increase of scavenging capacity of the drug toward HOCl.[13] Noteworthy, the observed IC_{50}s at pH 10.0 are much higher (about 100 times higher) and the order of scavenging potency was different. At pH 10.0, the thiophene ring of tenoxicam and lornoxicam and the benzene ring of piroxicam and meloxicam probably affect the oxidation on the C3-carbon of the enolic function of these molecules toward HOCl, since the potency of these drugs were in the same order. Therefore, these results show that the pH of HOCl scavenging reaction affects the ability of some compounds to react with HOCl, indicating that the conditions of in vitro testing should be as close as possible to those found in vivo.

In conclusion, the features of MSFIA, that allow the application of flow rates between 0.6 and 30 mL min^{-1}, enabled the precise delivery of low sample volumes at low flow rates and the fast reaction and detection by application of higher flow rates. When compared to predecessor techniques, the application of MSFIA to this particular application is clearly advantageous. First, the consumption of reagents is not continuous as that which occurs in FIA,[18] promoting the economy of luminol, which is propelled to the detector just before signal measurement, after the premixing of HOCl and putative antioxidant compound. In SIA systems, the reagent consumption is not continuous, but in the application proposed by Nakamura et al.,[19] the luminol solution is continuously added by an auxiliary pump to the main channel that connects the selection valve and the detector. Furthermore, it would be difficult to attain a homogeneous mixture between the HOCl/buffer solution and the putative antioxidant in a SIA system. In these systems, the mixing of solutions takes place at the boundaries of the segments of reagent stacked in a holding coil; thus, it would not be possible to guarantee a strict control of reaction pH.

In this particular application of MSFIA, the system configuration and the high flow rates applied allowed a high determination throughput. In fact, the time taken here between the contact of HOCl and the potential antioxidant compound plus chemiluminescence detection was about 3 s, which is well below what has been previously described in the literature. This is an advantage over other methods because the antioxidant compounds that react fast, closer to the time frame of generation of HOCl in vivo, are determined. Furthermore, in the proposed method, the reaction conditions (HOCl concentration and pH) were similar to those found in the physiological milieu, despite the fact that the detection of remaining HOCl took place at alkaline pH. This methodology would be cumbersome to perform in a batchwise manner, requiring manual pH adjustment before detection, but it was successfully implemented here using MSFIA. Finally, the MSFIA-CL methodology represents a suitable tool for the

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screening analysis of HOCl scavenging capacity, especially for pharmaceutical and biomedical research.

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CHAPTER 8

General conclusions

8.1. Analytical features of the developed flow-based antioxidant capacity assays

In the present dissertation, several automatic methodologies based on multisyringe flow injection analysis were developed for the routine/screening determination of antioxidant capacity of pure compounds and complex matrices as food products, including beers, isotonic and soft drinks, infusions, juices and wines. Their relevant analytical features are summarised in Table 8.1.

The automation of antioxidant capacity assays enabled strict control of reaction conditions including (i) reproducible mixture of antioxidant compounds/sample and oxidant species ($DPPH^{\bullet}$, FC reagent, $ABTS^{\bullet+}$, or HOCl), (ii) reproducible reaction time, and (iii) in-line pH adjustment. In these systems, the contact of oxidant and antioxidant species with oxygen and other substances that are present in the working environment was reduced. Moreover, the reduced intervention of the operator contributed to achieving reliable results with good repeatability (RSD < 4.0%). In fact, the automatic methods provided an improved repeatability when compared to batch procedures; for instance, the precision of the MSFIA-CL system developed for assessment of HOCl scavenging capacity was lower than 2.3%, compared to 10% obtained by CL-batch methodologies.

In batch methods used for the assessment of antioxidant capacity, the results obtained for samples are related to an antioxidant standard compound that shows different kinetic behaviour toward oxidant species. Thus, in order to attain results independent of time of analysis, these assays are usually based on end-point measurements. The reaction time for the $DPPH^{\bullet}$, the Folin-Ciocalteu, and the $ABTS^{\bullet+}$ assay vary between 20 min to 2 h, which decreases drastically their determination throughput. In this context, and with the purpose of attaining results statistically comparable with those provided by the end-point batch methods, a stopped-flow approach was implemented. This strategy allowed to follow the scavenging/reduction reaction development and to assess if total consumption of antioxidant is attained during reaction monitoring.

Table 8.1. Analytical features of the developed flow-based methods for the determination of antioxidant capacity.

Antioxidant assay	Standard compound (analytical range)	Time of stopped flow (s)	Det. rate (h^{-1})	RSD (%)	Expression of antioxidant capacity	Type of sample[d]
$DPPH^{\bullet}$ assay	–	180[a]	14	–	Number of $DPPH^{\bullet}$ molecules reduced by one molecule of antioxidant	Pure compounds
$DPPH^{\bullet}$ assay	Ascorbic acid (0.44 – 10.6 mg 100 mL^{-1})	180[a]	13	<1.0	Equivalents of ascorbic acid (VCEAC, mg 100 mL^{-1})	Food products
FC assay	Gallic acid (2.5 – 40 mg L^{-1})	240[a]	12	<1.3	Equivalents of gallic acid (mg L^{-1})	Food products
FC assay	Gallic acid (5.0 – 75 mg L^{-1})	250[b]	24 (12 + 12)	<4.0	Equivalents of gallic acid (mg L^{-1})	Food products
+						
$ABTS^{\bullet+}$ assay	Trolox (0.020 – 0.20 mM)	295[b]		<3.1	Equivalents of trolox (TEAC, mM)	
HOCl scavenging capacity assay	Lipoic acid (5.0 – 50 μM)	–[c]	92	<2.3	IC_{50} (μM)	NSAIDs

[a] the mixture of sample and reagent was stopped in the detector (Ch. 3–5); [b] the mixture of sample and reagent was stopped in the reaction coil before the detector (Ch. 6); [c] no stopped flow was applied, the contact of sample and HOCl plus chemiluminescence detection was about 3 s (Ch. 7); [d] food products include beers (blond and dark), isotonic and soft drinks, infusions (herbal and tea), juices, wines (red and white); IC_{50}, concentration of sample that inhibits 50% the analytical signal; NSAIDs, nonsteroidal anti-inflammatory drugs; TEAC, trolox equivalent antioxidant capacity; VCEAC, vitamin C equivalent antioxidant capacity.

For the $DPPH^{\bullet}$ and FC assay, the reaction mixture was stopped in the detector during 180 and 240 s, respectively, whilst for the sequential determination of FC and $ABTS^{\bullet+}$ assay, the mixture of sample and reagent was stopped in the reaction coil before the detector during 250 and 295 s, respectively. The reaction time was markedly reduced when compared to batch procedures; therefore the determination rate increased (varied between 12 and 24 h^{-1}). For the HOCl scavenging capacity assay, the stopped-flow approach was not applied because the objective was to attain a reaction time between the putative antioxidant compounds and HOCl close to the time frame of generation of HOCl in vivo. In fact, the contact between sample and HOCl plus chemiluminescence detection was about 3 s. This strategy allowed to obtain a high determination throughput (about 92 determinations per hour).

The standard compounds used for each assay were selected according to the corresponding batch methodology and results were expressed as equivalents of the respective compounds. For the study of reaction conditions of $DPPH^{\bullet}$ assay, the kinetic profile of radical consumption for the first 3 min of reaction was determined for several antioxidant compounds and the number of $DPPH^{\bullet}$ molecules reduced by one molecule of antioxidant was calculated. In the HOCl scavenging capacity assay, lipoic acid was used as the positive control and the results were expressed as the concentration of the sample that provided 50% inhibition of analytical signal (IC_{50}). For all automatic methods developed, the results obtained were in agreement with those reported in the literature and/or they were statistically comparable with those provided by the batch procedures.

Computer-controlled flow-based methodologies, as MSFIA, enable the aspiration/propulsion of precise volumes of sample, reagents and carrier, and represent powerful tools to develop greener analytical procedures by decreasing the reagent consumption and minimising the waste generated. The quantity of reagent(s) consumed and the total waste (volume) generated per determination for the automatic flow systems developed versus batch methods are presented in Table 8.2 and 8.3, respectively. All calculations were based on the information given in the previous chapters and on the information reported in the literature. For the determination of Folin-Ciocalteu reducing capacity, the consumption of FC reagent was reduced about 500 times, while 10 mg of

NaOH were used as alkaline reagent instead of 3 g of Na_2CO_3 used in the batch procedure. The higher consumption of alkaline solution, about 2.5 times, for the sequential assay was due to the increase in the number of operation steps involved in the analytical cycle (Chapter 6). Moreover, a considerable decrease in the volume of waste produced was obtained for the flow method of FC assay (3 mL per determination compared to 100 mL for batch assay). Taking into account that this method is widely used to estimate the reducing/antioxidant capacity and represents a routinely assay in the agrochemical field, the high reduction of FC reagent consumption and the volume of waste generated are valuable features. On the other hand, the reagent consumption and the volume of waste produced for $DPPH^{\bullet}$ assay and for the determination of HOCl scavenging capacity was higher using the flow-based methods. This took place because the manual discrete methods used for comparison purposes are not classical methods as Folin-Ciocalteu assay. In fact, they use a single spectrophotometric cell ($DPPH^{\bullet}$ assay) or a multiwell microplate (HOCl assay) whereby the sample/reagent(s) are added successively, and therefore the consumption of reagents as well as the volume of waste produced per determination are minimized (varying between 0.25 to 4 mL per determination). Furthermore, the automatic flow system developed for automation of $DPPH^{\bullet}$ assay replaced methanol for a less harmful reaction media, ethanolic solution 50% v/v.

Table 8.2. Quantities of reagents consumed per determination for the developed flow-based and for the batch antioxidant methods.

Antioxidant assay	**MSFIA method**		**Batch method**	
$DPPH^{\bullet}$ assay	$DPPH^{\bullet}$	0.34 μmol[a]	$DPPH^{\bullet}$	0.24 μmol[d]
	$DPPH^{\bullet}$	0.38 μmol[b]		
FC assay	FC reagent	10 μL[c]	FC reagent	5 mL[e]
	NaOH	10 mg	Na_2CO_3	3 g[e]
FC assay + $ABTS^{\bullet+}$ assay	FC reagent	10 μL[c]	FC reagent	5 mL[e]
	NaOH	24.7 mg	Na_2CO_3	3 g[e]
	ABTS	1.8 μmol	ABTS	3.0 μmol[f]
	H_2O_2	0.18 μmol	H_2O_2	0.03 μmol[f]
	HRP	0.12 nmol	HRP	0.5 nmol[f]
HOCl scavenging capacity assay	HOCl	7.5 nmol	HOCl	6.25 nmol[g]
	Luminol	500 nmol	Luminol	62.5 nmol[g]

[a] Flow method developed for studying $DPPH^{\bullet}$ scavenging reaction conditions (Ch. 3). [b] Flow method developed for determination of antioxidant capacity in food products (Ch. 4). [c] Values refer to the commercial solution. [d] Calculated from Brand-Williams *et al.* (1995). [e] Calculated from Singleton *et al.* (1999). [f] Calculated from Cano *et al.* (1998). [g] Calculated from Costa *et al.* (2006).

Table 8.3. Volume of effluent (mL) produced per determination for the developed flow-based and for the batch antioxidant methods.

Antioxidant assay	**MSFIA method**	**Batch method**
$DPPH^{\bullet}$ assay	4.2 [a] 5.2 [b] (50% v/v ethanolic solution)	4.0 [c] (methanol)
FC assay	3.0	100 [d]
FC assay + $ABTS^{\bullet+}$ assay	7.4 6.9	100 [d] 2.0 [e]
HOCl scavenging capacity assay	2.6	0.25 [f]

[a] Flow method developed for studying $DPPH^{\bullet}$ scavenging reaction conditions (Ch. 3). [b] Flow method developed for determination of antioxidant capacity in food products (Ch. 4). [c] Calculated from Brand-Williams *et al.* (1995). [d] Calculated from Singleton *et al.* (1999). [e] Calculated from Cano *et al.* (1998). [f] Calculated from Costa *et al.* (2006).

8.2. Contributions for the improvement of antioxidant capacity assays

The multisyringe flow systems presented in this dissertation contributed with new automatic flow methodologies for the assessment of antioxidant capacity. Besides the advantages attained with automation, the developed methods provided some improvements in the performance of the analytical methodologies.

Considering the DPPH$^{\bullet}$ assay, the evaluation of antioxidant capacity in batchwise manner is generally performed in methanol media and the results are expressed as the sample concentration that reduce 50% the initial DPPH$^{\bullet}$ concentration. Moreover, the scavenging reaction is time-consuming and it may take 20 min up to hours for a single analysis (Brand-Williams *et al.*, 1995). In the automatic method developed for studying the influence of pH and solvent in the DPPH$^{\bullet}$ scavenging reaction (Chapter 3), the number of radical molecules reduced per molecule of antioxidant obtained in methanol and ethanolic solution 50% (v/v) were similar, indicating that the replacement of methanol for a less harmful reaction media is a valid environmentally friendly alternative. This outcome was only possible due to the stopped-flow approach implemented in the present system and could not be achieved in the previously described flow-based methods based on single-point measurements (Ukeda *et al.*, 2002; Polasek *et al.*, 2004). Moreover, the MSFIA system proposed allowed the establishment of different reaction conditions (solvents, pH) only by changing the content of the syringes and it may be applied to other antioxidant assays based on bleaching of chromogenic radicals.

Using the automatic method developed for the determination of antioxidant capacity of food products through the DPPH$^{\bullet}$ reaction (Chapter 4), it was verified that the amount of radical consumed by antioxidant standards (ascorbic and caffeic acid) was independent of the initial concentration of radical, except for situations where DPPH$^{\bullet}$/antioxidant molar ratio was lower than the stoichiometric value. Since the percentage of radical consumed is calculated as the ratio between absorbance decrease and initial absorbance of radical, the values of percentage of DPPH$^{\bullet}$ consumed will be different for the same amount of antioxidant. Therefore, it was proposed that the antioxidant capacity should be expressed as the absorbance variation (or amount of radical consumed) rather than the percentage of

radical consumed as a function of antioxidant concentration. It was also concluded that the sample dilution factor plays an important role to attain exhaustion of the scavenging ability of the sample during the period of absorbance measurement, and consequently to achieve results comparable to those obtained from the batch method. For samples that did not exhaust its scavenging capacity within the time of reaction monitoring, a mathematical model was applied to estimate the total consumption of $DPPH^{\bullet}$. The combination of automation and mathematical modelling for predicting chemical equilibrium results was applied here for the first time and it offers a fast and reliable alternative to the time-consuming end-point batch method. Furthermore, the qualitative information about the kinetic behaviour of antioxidant compounds initially present in the samples or formed during the reaction with the radical was also provided.

Considering the automatic method developed for the determination of Folin-Ciocalteu reducing capacity (Chapter 5), the sodium hydroxide solution was employed as alkaline supporting medium instead of the carbonate buffer solution used in the batch procedure (Singleton *et al.*, 1999). This replacement allowed a considerable increase on the rate of the formation of coloured products. Nevertheless, the excessive alkalinity of the medium also originated an absorbance decrease along time due to the destruction of the colour complexes formed. In this context, the control of reaction conditions offered by MSFIA systems were exploited to implement these more drastic conditions, providing a reproducible balance between the two reactions. Actually, this methodology would be cumbersome to perform in a batchwise manner, as it requires a strict control of reaction time and mixing of reagent/sample. The results obtained by the proposed method after 4 min of reaction were statistically comparable with those attained by the time-consuming batch method (2 h) proposed for standardisation of determination of antioxidant capacity, with good repeatability (RSD < 1.3%).

The automatic method developed for the sequential determination of FC reducing capacity and $ABTS^{\bullet+}$ scavenging capacity (Chapter 6), represents a useful tool for routine analysis and also for real-time comparison between the two methods. A large number of samples were analysed and the results showed that the correlation between these assays may vary according to the type of sample, reinforcing the idea that more than one method should be

used for evaluation of antioxidant capacity. The fact of performing these determinations in parallel has also the advantage of processing the sample at the same time, avoiding errors that might arise with sample degradation over time. The possibility to perform each assay separately or in tandem through software control should also be highlighted as an advantage of the methodology proposed.

Regarding the automatic method developed for the determination of HOCl scavenging capacity (Chapter 7), the proposed multisyringe flow system provides the application of a large range of flow rates, enabling the precise delivery of low sample and HOCl volumes at a low flow rate and then the fast reaction and detection of remaining HOCl by application of a higher flow rate. This strategy was exploited to accommodate in a single protocol different reaction conditions, concerning both reaction time and pH value. In fact, the proposed flow method represent a valuable improvement over previously described methods, because the antioxidant compounds reacted with oxidant species in conditions closer to in vivo generation of HOCl (pH 7.4, HOCl concentration of 50 μM, and 3 s of reaction time). Moreover, the results obtained provide the evidence that the pH of in vitro methods should be carefully selected to allow assumptions about putative in vivo effects.

In conclusion, the automatic methods developed in this work showed to be a fast and reliable way for determination of antioxidant/radical scavenging capacity, so that they could be a suitable alternative to the currently existing methods and even a choice for implementation in routine/screening analysis, especially for food, pharmaceutical and biomedical research.

8.3. Perspectives and future trends

At the present moment, a high quantity and diversity of analytical methods for determination of antioxidant capacity are available. These assays differ from each other in terms of reaction mechanisms, oxidant and target/probe species, reaction conditions, and in the form that results are expressed. Even when only one of these assays is considered, different antioxidant standard compounds, solvents, reaction time and pH are frequently

applied. Moreover, as the *total antioxidant capacity* is dependent of a multitude of factors, a "battery" of assays measuring different aspects of the behaviour of antioxidants is strongly required to generate a complete antioxidant profile. In a broad spectrum of areas, including physiology, pharmacology, nutrition and agrochemical, this situation may difficult the selection of the most appropriate method(s), originating inappropriate applications and misinterpretation of the results. The comparison of data from different studies is also difficult. In this context, a primary factor to consider in selecting an antioxidant method is the mechanism of reaction and its relationship to what might occur in the target application. It is also advantageous to select methods that are commonly accepted, validated and standardised, with a large body of comparable data available in the literature. Therefore, the efforts that have been made during the last two years to standardize analytical methods and provide valid guidelines are of utmost importance to bring some order to this field, and they should be pursued by future researchers.

In the future, other in vitro analytical assays will be needed as more is learned about the oxidation sources (radical and non-radical) including their concentrations, the interactions with other oxidant species and biological targets, their significance to oxidative stress, and the surround environment. The design of novel methods relies definitely on the utilization of oxidant and target/probe species (proteins, triacylglycerols, and cell models) with relevant biological significance and in reaction conditions (concentrations, reaction time, pH) as close as possible to those found in vivo. The possibility to develop assays where more than one oxidant species is present in the reaction medium simultaneously should also be considered. Regarding the automation of antioxidant assays, an interesting field may be the development of automatic methodologies for the assessment of biomarkers of oxidative stress, such as F2-isoprostanes or malondialdehyde as indicators of oxidative damage to lipids, protein carbonyls or nitrotyrosine as indicators of oxidative damage to proteins, and 8-hydroxydeoxyguanosine as indicator of oxidative damage to nucleic acids. The miniaturisation of flow-based systems for measurement in-field and for obtaining real-time information may also be an actively pursued topic in future analytical chemistry research applied to this area.

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