

# ADVANCED PHARMACY

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**Bentham Books**

# **Advanced Pharmacy**

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## **Advanced Pharmacy**

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## **DEDICATION**

*Dedicates this book to God, to my wife Maria Gabriela and my daughter Gabriela Daiana. A special recognition to the National University of San Luis and INFAP-CONICET.*

**Martin Masuelli**

*My parents Mirta and Victor. My wife, Evangelina, and my children Mile, Ari and Valent. My teachers.*

**Mauricio Filippa**

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*A collection of thoughts should be a pharmacy where you can find a remedy for all ills.*

**Voltaire**

## PREFACE

Advanced Pharmacy is a book entirely dedicated to the latest knowledge regarding pharmaceutical science, its scope, and news. This book is oriented and directed to the professionals of the pharmaceutical industry, academics, researchers and students. It consists of thirteen chapters that cover different current topics, including the following chapters: Chapter 1 - Physical Pharmacy by M. Filippa; Chapter 2 – Preformulation: Active Pharmaceutical Ingredient - Excipient Compatibility Studies by Adriana Segall; Chapter 3 – On Some Medicinal Chemistry Applications of the QSAR/QSPR Theory by P. Duchowicz & J. Garro-Martínez; Chapter 4 – Computer-assisted study of garlic organosulfur as an antioxidant agent by M. Díaz *et al.*; Chapter 5 – Enzymes in Biocatalysis: characteristics, kinetic approach, production and uses by L. Chaillou *et al.*; Chapter 6 – Antifungal agents by E. Butassi *et al.*; Chapter 7 – Naturally and chemically sulfated polysaccharides in drug delivery systems by H. Prado *et al.*; Chapter 8 – Immunomodulatory plant extracts and their compounds. Evaluation of your safety by R. Davicino & C. Anesini; Chapter 9 – Development of biofilms and persistent cells, causes and consequences by M. G. Paraje. Chapter 10 –Development of Analytical Methods for Analysis of Drugs of Abuse in Biological Fluids using Design of Experiments and Response Surface Methodology by C. dos Santos, *et al.*; Chapter 11 – Steric exclusion chromatography. (including the chromatography of polymers in aqueous solution) by Marguerite Rinaudo; Chapter 12 – Intrinsic Viscosity Methods in Natural Polymer as Pharmaceutical Excipients. L. Lazo Delgado *et al.*; and Chapter 13. Extraction techniques in green analytical chemistry. A. Pighinet. Each chapter of this book fully expresses the latest advances in pharmaceutical science and raises the wishes of each author to make their research known and the pride of being able to develop this knowledge didactically and educationally.

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## Physical Pharmacy

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**Abstract:** In this chapter, we focus on solutions. In the introduction, general and descriptive aspects are defined, such as the classification of solutions and the addition of solids. Considering the properties of these systems, we focus on definitions related to colligative properties, and on the use of this property for the adjustment of isotonic solutions considering the selective capacity of the membranes, differentiation of tonicity and osmolarity. We also introduce the calculations necessary for the preparation of isotonic solutions with blood plasma, using the mass and volume adjustment method. The solutions require different methods of expressing their concentration, and in order to develop this point, we present the different forms of expression, with extensive detail on one of the variables, *i.e.*, normality, very important in the formulation of parenteral solutions. Since the preparation of solutions is an important aspect, we detail the existing interactions between the solute and the solvent, specify the thermodynamic aspects that condition the solubility of the solute in the solvent, and develop variables such as polar interactions, capacities to accept or give Hydrogen bridge junctions and the energy requirement to generate space within the solvent, giving a rational look at the process of improving the capacity of the solvent to contain the solute. The dissolution rate is another variable developed through simple equations, which make an analysis of the factors that modify it, such as agitation, temperature, particle size, and diffusion coefficient. We also describe variables such as the pH and the dielectric constant of the solvent to modify solubility.

**Keywords:** Cohesion force, Colligative properties, Concentration gradient, Co-solvent, Descriptive terms, Dissolution rate, Hildebrand coefficient, Hydrogen bridge, Intrinsic factors, Isosmotic, Isotonic, Melting point, pH, Polarizability, Solubility, Solute-solvent interaction, Solvatochromic properties, Staverman coefficient, Stagnant film, Tonicity.

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## INTRODUCTION

Numerous physical variables affect a pharmaceutical product and its pharmacological action. In the API (Active Principle Ingredient) development process, which also includes the configuration of the finished product, along with its manufacture and dispensing, many physical variables, such as temperature, transport, process fluidity, inter and intra-molecular interactions, pore size, permeation speed, dissolution speed, light effects, thicknesses and characteristics of the packaging materials in contact with the product, pressures and torque forces, and particle sizes play a transcendent role in the results.

Analyzing all these variables in this chapter is not feasible, for this requires a special space. However, there are responsibilities that for pharmacists—a professional included within health systems—are specific to their profession. One of them is the vehicle of APIs, *i.e.*, any process that requires conforming a mixture of API with other ingredients, commonly called excipients, and which may result in a product in solid, liquid, or gaseous form, which facilitates access and dispensing for the patient or the health team.

The formation of mixtures between two or more substances may lead to two very different results. On the one hand, mixing can generate what we commonly know as a *solution*. On the other, simply mixing may result in a spread of one material over another. Depending on the materials that are mixed, the formation of a system that is the sum of the two options described above may also occur. Such is the case of suspensions of a substance that has low solubility in the extensive medium.

Simply put, a *solution* can be defined as the portion of the system in which when two components are mixed, a single phase is observed. Normally, one of the components is in a lower proportion than the other. This is called a *solute*. The other component that is in a higher proportion is called a solvent. By definition, a *phase* is that portion of a system that has the same chemical and physical properties in all its parts, or, in other words, it is chemically and physically homogeneous. For this, it is necessary that the minority component, that is, the solute, is evenly distributed within the solvent. The best way to ensure this is for the solute to distribute each of its molecules within the solvent, each of them interacting independently. These solutions can be classified according to the physical state of the materials that give rise to them, as shown in Table 1.

**Table 1. Classification of solutions considering the state of their components.**

Solute	Solvent	Example
Gas	Gas	Gas mixture used in a respirator
Gas	Liquid	Carbon dioxide in water
Gas	Solid	Hydrogen in metals
Liquid	Gas	Water in a gaseous state in the air
Liquid	Liquid	Ethanol in water
Solid	Liquid	Glucose in water
Solid	Gas	I <sub>2</sub> vapors in the air
Solid	Solid	Metal alloys, eutectic mixtures

The independence of the behavior of the solute molecules is conditioned not only by the concentration of the solute but also by its molecular size. When the size of the solute molecule has significant values, the solvent—which normally has a small molecular size—presents difficulties in ensuring this independence and the necessary stability of the solution. This problem is observed for solute molecules whose sizes are greater than 1 nm, as is the case with some milk proteins. Table 2 shows the classification of the systems according to the particle size of the solute.

**Table 2. Characteristics of the solutions according to the molecular size of the solute.**

According to the size of the solute	Particle size	Example
True Solution	Up to 1 nm	Aqueous solutions of salt, alcohol, ketone, ester, acid, amine, and amide. All of the simple structures.
Colloidal Dispersion	Range from 1 to 100 nm	Aqueous dispersions of simple proteins.
Suspension	Over 100 nm	Silica particles in water.

When the molecule has a higher range, they are called *dispersions*, and we may find the *colloidal* ones where the size of the particle or solute molecule is 1-100 nm, and the *suspensions*, when their size exceeds 100 nm.

The process of mixing two substances for the transport of APIs is one of the main physicochemical topics that a pharmacist must know. The correct application of this knowledge significantly shortens the development and manufacturing process of the final product formulation. That is why in this chapter, we will focus on the propriety of these mixtures so widely used by the pharmaceutical professional.

## Preformulation: Active Pharmaceutical Ingredient-Excipient Compatibility Studies

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**Abstract:** A relevant area of research in the preformulation phase for the development of new dosages is active pharmaceutical ingredient (API)-excipient compatibility. The possibilities of chemical and physical interaction of API and the excipients may affect how efficient and effective it is, while displaying an impact on the nature, stability and availability of API. The most common signs of deterioration of an API are changes in the color, taste, odor, polymorphic form, or crystallization (pharmaceutical incompatibility). These changes arise from chemical reactions with the excipient, leading to degradation of the API. The active components are usually more stable than solid dosage forms, and although testing the compatibility of API-excipients is essential, no protocol has yet been accepted to evaluate their interactions. Fourier Transform Infrared Spectroscopy (FT-IR), Differential Scanning Calorimetry (DSC), Isothermal Stress Testing-High Performance Liquid Chromatography (IST-HPLC), Hot Stage Microscopy (HSM), Scanning Electron Microscopy (SEM), Solid state Nuclear Magnetic Resonance Spectroscopy (ssNMR) and Power X-ray Diffraction (PXRD) are commonly used as screening techniques for assessing the compatibility of an active pharmaceutical ingredient (API) with some currently employed excipients. The potential physical and chemical interactions between drugs and excipients can affect the chemical nature, the stability and bioavailability of drugs and, consequently, their therapeutic efficacy and safety. Once the solid-state reactions of a pharmaceutical system are understood, the necessary steps can be taken to avoid reactivity and improve the stability of drug substances and products. In this chapter, we summarize the techniques to investigate the compatibility between APIs and excipients.

**Keywords:** Compatibility, Differential scanning calorimetry, Fourier transform-infrared spectroscopy, Isothermal stress testing-high performance liquid chromatography, Solid state nuclear magnetic resonance spectroscopy, Scanning electron microscopy, Power x-ray diffraction.

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## INTRODUCTION

The way to determine the shelf life of a drug product is to perform stability studies according to the suggestions of ICH Q1A. Stability studies are very expensive to carry out for the laboratories since they demand the use of large amounts of drug products, standards, reagents, in addition to many hours of personal work and use of equipment. In addition, it takes three months to have the first result.

With this background, active pharmaceutical ingredient (API)-excipient compatibility studies are becoming increasingly relevant.

Compatibility studies are never going to replace stability studies, but they serve in the selection of excipients at an early stage as they increase the possibility of developing a more stable formulation. API-excipient incompatibility can alter drug stability, thereby affecting drug safety and efficacy. Estimation of API-excipient interactions is a crucial step in the preformulation studies of drug development to achieve consistent stability, bioavailability and manufacturability of solid dosage forms [1].

Solid dosage forms are generally less stable than their API components. Despite the importance of API-excipient compatibility testing, there is no universally accepted protocol to assess such interactions [2]. The implementation of the ICH Q8 Guideline of Quality by Design makes it essential to carry out compatibility studies before the final formulation of the medicinal product is defined. Fortunately, there are many analytical techniques in the literature, mainly thermo analytical and spectroscopic techniques such as Differential Scanning Calorimetry (DSC), Hot Stage Microscopy (HSM), Scanning Electron Microscopy (SEM), Fourier Transform-Infrared Spectroscopy (FT-IR), Isothermal Stress Testing (IST) coupled with Liquid Chromatography (LC), Solid state Nuclear Magnetic Resonance Spectroscopy (ssNMR) and Power X-Ray Diffraction (PXRD).

In a pharmaceutical form, excipients are in direct contact with the API. Although the excipients are assumed to be inert, they may interact, physically affecting its organoleptic characteristics, decreasing the dissolution profile, or causing API degradation. Within the process of development of a pharmaceutical product, the concept of Quality by Design involves the complete knowledge of the possible physical-chemical interactions of API and excipients.

## Methodology

Solid pharmaceutical forms are generally less stable than API. The excipients or impurities present in the excipients can interact with the API and cause its degradation. Changes may be chemical, physical, or therapeutic. First, chemical interactions involve a chemical reaction between the API and the excipient. This may lead to the development of toxic degradation products or loss of potency (with a commitment to clinical efficacy). Second, physical interactions cause changes in the solubility, taste, odor, dissolution rate, and finally, bioavailability. These interactions may arise from changes in the color, odor, taste, polymorphic form, or API crystallization. In general, compatibility studies are conducted by studying the behaviour of the API and the excipient separately and in 50% physical mixture. This maximizes the contact between the API and the excipient. Ternary API mixtures can also be made with two excipients.

Both the presence of moisture in the excipient and/or the addition of water to the mixture may alter the properties of the API. Temperature also accelerates API degradation speed.

### *Thermo-analytical Techniques*

Thermo-analytical techniques have been developed to predict the suitability of the excipients to be employed in dosage forms in order to minimize undesired reactions (stability issues) between the API and the excipient [1]. Within the thermo-analytical technique, Differential scanning calorimetry (DSC) is the leading technique that has been used for more than 50 years.

DSC curve provides essential information of the API, such as its melting point, melting enthalpy, glass transition, as well as temperatures of crystallization and decomposition. Moreover, it is possible to assess interactions considering endothermic or exothermic peaks and variations as they appear, change, or disappear in drug-excipient mixture thermal curves [3 - 5]. It is worth mentioning that thermal data needs to be considered carefully since interactions at room temperature may not be observed in the same manner as they happen under high temperatures.

DSC has the advantage that it is a fast method, requires small amounts of sample, and is very useful for detecting a physical interaction, a change in the polymorphic form, or a transformation from a crystalline to an amorphous form.

DSC presents some disadvantages as well. It is a destructive method, and it is not useful when thermal changes are small, or when the material presents properties that hinder interpretation. DSC cannot solve overlapping thermal events.



## CHAPTER 3

## Some Medicinal Chemistry Applications of the QSAR/QSPR Theory

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**Abstract:** The application of QSAR/QSPR techniques and computer-aided modelling are considered valuable tools to initiate the search for new drugs, and nowadays, these are being intensively used for this purpose. Trustworthy models can provide insight into the structural characteristics that may influence the drug inhibitory activity, drastically improving the success and the pace of the development of more effective drugs with weaker secondary effects. The present book chapter revises and comments on different recent QSAR/QSPR applications conducted in medicinal chemistry field in the last five years (2016-2020), developed on various interesting biological activities and physicochemical properties of drug compounds.

**Keywords:** QSAR/QSPR theory, Medicinal chemistry, Molecular descriptors, Drug compounds, Multivariable linear regression, CORAL software, Monte carlo method, Pharmaceutical, Validation, *In silico* design, Biological activity, CoMFA and CoMSIA, Molecular docking, Consensus model, Machine learning, Drug-drug interaction, OECD guidelines.

### INTRODUCTION

The development of computer science has been of great help in the drug discovery area, while the Quantitative Structure-Activity/Property Relationships (QSAR/QSPR) theory [1 - 4] has found broad application over the years as a useful computational semi-empirical strategy for predicting the biological activities and physicochemical properties of chemical compounds. The theory becomes especially useful for predicting compounds with unknown experimental

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data and thus for filling data gaps. It is also vital for the design of novel drugs, since their biological activities may be predicted in the synthesis and, consequently, the design may be lead by the results of the QSAR. Therefore, the QSAR/QSPR technique constitutes a fast and cost-effective choice for *in vivo* or *in vitro* experimental measurements.

The fundamental hypothesis of the QSAR/QSPR studies is that the bioactivity/property exhibited by a compound is the direct result of its molecular structure. Therefore, a quantitative relationship is established between the molecular structure and the final effect, such as the analyzed biological activity or the physicochemical property, without the need of knowing specific details of the molecular mechanism of action involved.

The success of a QSAR/QSPR analysis is generally based on its modelling methodology. The research framework involved includes different steps that are not trivial: molecular structure representation, molecular descriptors calculation, model development, model evaluation, and applicability domain definition.

The chemical structure is represented by molecular descriptors. These are numerical variables giving specific and important information about the constitutional, topological, geometrical, hydrophobic, and/or electronic characteristics of the investigated compounds [5 - 8]. There exist thousands of molecular descriptors available for characterizing the chemical structure and establishing predictive models. These variables are calculated with mathematical formulae obtained from the Chemical Graph Theory, Information Theory, Quantum Mechanics, Markov Chains Theory, *etc* [6, 7, 9 - 11].

Appropriate molecular descriptors must be employed during QSAR/QSPR analyses. There exists an inclination for employing open-access descriptor software, while the number of descriptors analyzed in a given QSAR/QSPR study has greatly increased. Some studies tend to use non-conformational molecular descriptors instead of conformational ones, avoiding conformational analysis and geometry optimization, thus lowering the complexity of computations. Table 1 includes a brief comparison between descriptor types.

In addition, different data reduction techniques are available for quantifying the structure/activity relationship and finding out useful parallelisms. The most usual approach is the multidisciplinary Multivariable Linear Regression (MLR) technique, which is a simpler mathematical strategy when compared to a non-linear method. That is so, particularly when many of the molecular descriptors is available for distinguishing the chemical structures. MLR models clearly show the effect of including/excluding descriptors in the linear equation; then, it is possible to propose the cause/effect relationship through such types of parallelisms.

Whenever the results obtained by a linear model are acceptable, then this would probably be the preferred option in QSAR/QSPR modelling.

**Table 1. Comparison and information about molecular descriptor types.**

Details	Non-conformational Descriptors	Conformational (3D) Descriptors
<b>Complexity</b>	Require a simple descriptor calculation.	Require a geometrical optimization before the descriptor is calculated.
<b>Time</b>	Fast computation time.	Demanding computation time.
<b>Requirement</b>	Do not demand molecular conformation knowledge.	Demand molecular conformation knowledge.
<b>Structural information content</b>	Structural data based on atomic composition and connectivity.	Information related to the atomic composition, connectivity and conformational parameters.
<b>Isomerism</b>	Isomers are represented by defining the presence/absence of geometric elements.	Simple calculation of the isomer descriptor can be performed.
<b>Predictive capability</b>	Improve the predictive QSPR capability avoiding 3D-ambiguities.	If the molecular conformation is known, the predictive QSPR capability could be improved.

More advanced and sophisticated techniques are the so-called Machine Learning (ML) methods, which can also be applied to develop predictive QSAR/QSPR models through molecular descriptors. Some examples are: Partial Least Squares Discriminant Analysis (PLS-DA) [12], Artificial Neural Networks (ANN) [13], Random Forest (RF) [14], Support Vector Machines (SVM) [15], Gradient Boosting Machines (GBM) [16], or Radial Basis Functions with Self-Consistent Regression (RBF-SCR) [17].

The Parsimony Principle (Occam's Razor Principle) [18] says that the models must contain all that is necessary for modelling but nothing more. Thus, given a number of models with about the same predictive error, fewer parameters should be preferred because of its simplicity.

A training set of molecules with known experimental bioactivity/property data is used to calibrate the QSAR/QSPR model and to obtain its parameters, while a test set of molecules "never seen" during the calibration step but also with known experimental data, allows demonstrating the real predictive capability of the model by comparing it to the predicted results.

Finally, QSAR/QSPR models have limitations associated with different types of chemical structures and bioactivities/properties for which the models can produce good predictions. Thus, it is necessary to define the model's applicability domain (AD) [19, 20]. The AD is a theoretically defined area such that only the molecules

## Computer-assisted Study of Garlic Organosulfur as Antioxidant Agents

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**Abstract:** Although many sulfur-containing garlic compounds exhibit antioxidant activity, little is known about the molecular mechanisms through which these compounds react with reactive oxygen species. For this reason, in this chapter, we present a summary of various papers in which, the scavenging of hydrogen peroxide and hydroxyl radical by garlic compounds allyl methyl disulfide, allyl methyl sulfide, and diallyl sulfide is analyzed from a theoretical-quantum outlook.

Different computational methods and methodologies were analyzed. The DFT functional B3LYP, CAM-B3LYP, BKM, M05-2X, and M06-2X and even other methods such as Gaussian-n (G3MP2B3) were also evaluated. A broad series of basis sets were used from the simple 6-31G(d) to the extended triple-zeta 6-311++G(3df,2p).

The thermodynamic and kinetic aspects of different proposed reactions were explored. Epoxidation, sulfonation, and hydrogenation were some of the processes raised as possible reaction pathways. Reaction mechanisms were proposed for each pathway, and different methods used to obtain the TS structure (TS Berny, QST2, and QST3) were compared. The kinetic and the rate constants were obtained through the Intrinsic Reaction Coordinate calculations. Gas and aqueous phases were mostly utilized in our papers; however, we included and studied the behavior of the systems in non-polar environments in our last publication.

In addition, we analyzed several published papers by other authors with important relevance to this topic. The results of some of them were of great usefulness to our work. The main objective is to provide information for the design of new and more powerful antioxidant agents.

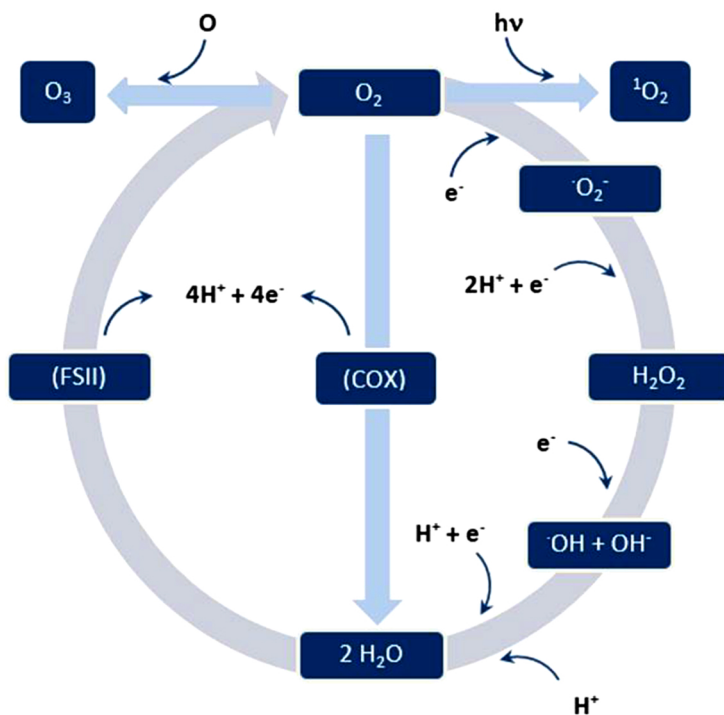
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**Keywords:** Allyl methyl disulfide, Allyl methyl sulfide, Antioxidant activity, Diallyl sulfide, Density functional theory, Epoxidation, Garlic compounds, Hydrogenation, Hydroxyl radical, Hydrogen peroxide, IRC, Reactive oxygen species, Sulfoxidation.

## INTRODUCTION

Oxygen ( $O_2$ ) is considered synonymous with life because living beings use it for their respiratory function. However, there is much scientific evidence that this molecule is responsible for many diseases in organisms, even at low concentrations [1]. One explanation for the toxicity of  $O_2$  is the formation of chemically reactive oxygen species (ROS) which, are natural products of the normal aerobic metabolism and are derived from the partial and incomplete reduction of oxygen [2] (Fig. 1).



**Fig. (1).** Some ROS generation cycle.  $^1O_2$ , singlet oxygen;  $O_2^-$ , superoxide;  $H_2O_2$ , hydrogen peroxide;  $OH^\cdot$ , hydroxyl radical; and  $OH$ , hydroxyl ion.

The principal ROS can be classified as:

1. Those derived from the product of oxygen breakdown or excitation, such as atomic oxygen, ozone ( $O_3$ ), and singlet oxygen;

2. Oxygen species partially reduced, such as superoxide anion, hydrogen peroxide, and hydroxyl radical.

In addition to free radicals ROS, other highly reactive species such as peroxides and species derived from nitrogen, sulfur, and carbon are also considered in this group [3 - 5].

Some ROS play a physiological role by acting as chemical messengers that help maintain optimal physiological functions [6, 7]. However, these species have inherent chemical properties that provide them extensive reactivity against various molecules of biological relevance. The latter makes them responsible for oxidative stress and suggests their participation in pathologies that involve lipid, protein, and DNA damage [2].

In this sense, the free radicals that can initiate a reaction with lipids are the hydroxyl radical ( $\bullet\text{OH}$ ), the peroxide radical ( $\text{ROO}\bullet$ ), the alkoxide radical ( $\text{RO}\bullet$ ), and the alkyl radical ( $\text{R}\bullet$ ). These cause a chain reaction, which leads to the oxidation of a large number of substances, especially short-chain hydrocarbons [3]. On the other hand, the proteins have amino acid residues susceptible to free radical attacks, mainly by the hydroxyl radical. Among the physiological amino acids that are most prone to oxidation are: tyrosine, phenylalanine, tryptophan, histidine, methionine, and cysteine [8]. This oxidation can cause a conformational change of the protein and, therefore, a loss or modification of its biological function. Besides, DNA is also susceptible to oxidative damage, which should not be considered independently of the oxidative damage associated with proteins [9]. Thus, the harmful or beneficial effects on human health will largely depend on the type of ROS, the quantity and temporal and spatial distribution within the organism [7].

Meanwhile, to protect from damage caused by ROS, the cells contain a defense system that involves endogenous and exogenous antioxidants. Both types of agents cooperate and act as scavengers of ROS [10]. The endogenous antioxidants are products present in each organism, either enzymatic or non-enzymatic such as Glutathione, Alpha-lipoic acid, Coenzyme Q, Ferritin, Uric acid, Bilirubin, Metallothionein, L-carnitine, and Melatonin. On the other hand, exogenous antioxidants are compounds incorporated with the diet as vitamin C, vitamin E, and carotenoids, among others [11 - 15].

In addition to those listed above, garlic (*Allium sativum*) has antioxidant properties attributed to the organosulfur compounds present in this vegetable. Several studies show the current interest of these compounds in the field of antioxidant agents [16 - 19]. Fig. (2) shows the main organosulfur compounds present in garlic with possible antioxidant activity.

**CHAPTER 5****Enzymes in Biocatalysis: Characteristics, Kinetic Approach, Production, and Uses****Lucrecia L. Chaillou<sup>1</sup>, Valeria Boeris<sup>2,3,4</sup>, Darío Spelzini<sup>2,3,4</sup> and Mónica A. Nazareno<sup>1,3,\*</sup>**

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**Abstract:** Enzymes are proteins that efficiently catalyze chemical reactions of specific substrates; they are highly specific for one reaction or a class of reactions, based on the structure of their active sites. This chapter presents the classification according to the nature of the reactions where enzymes are involved as biocatalysts and shows examples of biocatalyzed chemical processes. Kinetic aspects are discussed, and the relevance of the kinetic parameters is highlighted. Inhibitors of enzyme-mediated reactions are also described and classified; their kinetic implications are revealed; besides, examples of enzyme inhibition, examples of pharmacological drug-inhibition are presented. The roles of enzyme cofactors and cosubstrates are described taking examples of biological systems. Enzymes are also used in bioremediation processes and examples are mentioned. Enzyme production strategies developed to enable industrial application are presented, taking lactase as a model example; enzyme preparation, purification, recovery, and stabilization are the key steps in their utilization. Nowadays, with the development of genomics and proteomics, it is possible to access new enzyme activities as well as manipulate, design and improve new and traditional enzyme activities. Biocatalysis is a multidisciplinary area of science that is gaining increasing interest both from a scientific point of view and for its growing industrial applications due to its high specificity in the conversion of substrates into specific products, the reduced volume of waste generated and the non-aggressive operating conditions. Specifically, the enzymes' use in pharmacological drugs synthesis is remarkably interesting, since they allow to improve both the performance and the stereoselectivity of the active principles.

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**Keywords:** Active-site, Biocatalysis, Chromatography, Classification, Cofactor, Competitive inhibitors, Downstream processing, Enzymes, Immobilization, Inhibitors, Intermolecular Union, Irreversible Union, Isolation, Kinetic aspects, Michaelis- Menten, Non-competitive inhibitors, Purification, Recovery, Reversible Union.

## INTRODUCTION

Enzymes are biomolecules that have the ability to increase the rate of a chemical reaction of a specific reagent called substrate without undergoing, in general, any chemical modification. To fulfill this role as a biological catalyst, it may or may not require the presence of a non-proteic molecule called a cofactor.

Many enzymatic proteins lack catalytic activity in the absence of their cofactor, which can be a certain specific organic molecule or a certain metal ion. Thus, the inactive protein component of an enzyme is called the apoenzyme, and the active enzyme, including the cofactor, is the holoenzyme. Each enzyme is characterized by being quite specific, acting on a certain substrate to produce a particular product or products. Selectivity stands out among the most relevant aspects of enzymes as biocatalysts; chemoselectivity [1], stereoselectivity: diastereo- and enantioselectivity [2] and regioselectivity [3] are the most remarkable traits among enzyme catalytic properties. Enzymes can be obtained from readily available renewable resources, are biodegradable, and are essentially nonhazardous and nontoxic [4]. Advantages given from such properties have been utilized in various industrial applications, such as in food processing, biomass conversion, bioremediation, polymer synthesis, organic synthesis and particularly for the production of pharmaceutical intermediates. Biocatalysis is a multidisciplinary subject that is gaining increasing interest both from a scientific point of view and for its growing industrial uses.

### Enzyme Classification

The nomenclature of enzymes was empirically carried out using the substrate that enzyme recognizes, as the name root, and the suffix -ase is added, *e.g.*, lipase is an enzyme that catalyzes the hydrolysis of lipids; the name also gives some indication of the reaction catalyzed by the enzyme, *e.g.*, glucose oxidase. In the 1950s, due to the increase in the number of enzymes being discovered, attempts began to address a systematic classification. Malcolm Dixon and Edwin Webb made a list of all known enzymes and they observed that the types of reaction involved were quite small, despite the large number of enzymes (659 enzymes) compiled. Enzymes were classified into three groups according to the type of reaction catalyzed: the hydrolyzing enzymes, the transferring enzymes, and other enzymes. These main groups were subdivided according to the type of the substrates or kind of reaction catalyzed [5]. The 659 enzymes published were



listed sequentially: hydrolyzing enzymes (1–221); transferring enzymes (222–579); and other enzymes (580–659).

Enzymes are now classified by the Enzyme Commission (EC) of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB) number with four digits. The first digit classifies enzymes into major classes based on the substrates transformed and products formed by an enzyme, which is shown in Table 1. The seventh class was added in 2018 [6]. The second digit corresponds to the subclass, and it indicates the groups involved in the reaction catalyzed. For class 3 hydrolases, for example, each subclass corresponds to the group that is hydrolyzed, *e.g.*, subclass EC 3.1 acts on ester bonds: subclass EC 3.2 acts on glycosidic bonds. The third digit specifies in greater detail the groups involved in the reaction, *e.g.*, sub-subclass EC 3.2.1 contains enzymes that hydrolyze O- and S-glycosyl compounds, and sub-subclass EC 3.2.2 denotes enzymes that hydrolyze N-glycosyl compounds. The fourth digit identifies the specific enzyme within a sub-subclass.

**Table 1. Examples of the different enzyme classes according to NC-IUBMB and a typical reaction that enzymes catalyze.**

Enzyme Class	Name	Example of Reactions Catalyzed by the Mentioned Enzyme
1	Oxidoreductases	EC 1.3.5.1 (succinate dehydrogenase) succinate + quinone → fumarate + quinol
2	Transferases	EC 2.7.7.49 (RNA-directed DNA polymerase) 2'-deoxyribonucleoside 5'-triphosphate + DNA <sub>n</sub> → diphosphate + DNA <sub>n+1</sub>
3	Hydrolases	EC 3.2.1.108 (lactase) lactose + H <sub>2</sub> O → D-galactose + D-glucose
4	Lyases	EC 4.1.3.32 (2,3-Dimethylmalate lyase) propanoate + pyruvate → (2 <i>R</i> ,3 <i>S</i> )-2,3-dimethylmalate
5	Isomerases	EC 5.1.2.1 (lactate racemase) ( <i>S</i> )-Lactate → ( <i>R</i> )-lactate
6	Ligases	EC 6.2.1.1 (acetate-CoA ligase) ATP + acetate + CoA → AMP + diphosphate + acetyl-CoA
7	Translocases	EC 7.2.1.1 [NADH: ubiquinone reductase (Na <sup>+</sup> -transporting)] NADH + H <sup>+</sup> + ubiquinone + n Na <sup>+</sup> [side 1] → NAD <sup>+</sup> + ubiquinol + n Na <sup>+</sup> [side 2]

Fig. (1) shows the partial expansion of 3.2.1 sub-subclass, for example, in EC 3.2.1.108, numbers correspond specifically to lactase, a hydrolase that hydrolyzes O- glycosyl compounds such as lactose. The ability of metabolic pathways to adapt to environmental and chemical conditions, during evolution, is related to

## Antifungal Agents

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**Abstract:** Fungal infections represent an increasing threat to a growing number of immune- and medically compromised patients. Fungi, like humans, are eukaryotic organisms and there are a limited number of selective targets that can be exploited for antifungal drug development. This has also resulted in a very restricted number of antifungal drugs that are clinically available for the treatment of superficial and invasive fungal infections at the present time. Moreover, the utility of available antifungals is limited by toxicity, drug interactions and the emergence of resistance, which contribute to high morbidity and mortality rates. These limitations have created a demand for the development of new antifungals, particularly those with novel mechanisms of action.

The 1990s can be considered the “golden era” of antifungal drug development with multiple big pharmaceutical companies actively engaged in the discovery and development of novel antifungals. However, this has largely become stagnant since then, and it has been two decades since the newest class of antifungal agents (the echinocandins) reached the market. Overall, there are currently few classes of FDA-approved antifungal agents clinically used in the treatment of fungal infections. In this chapter, we reviewed antifungal drugs and summarized their mechanisms of action, pharmacological profiles, and susceptibility to specific fungi. Approved antimycotics inhibit nucleic acid and microtubule synthesis, membrane ergosterol synthesis and cell wall polymers’ synthesis, or sequester ergosterol. The experimental antifungal drugs in clinical trials are also reviewed. We report sphingolipids and protein biosynthesis inhibitors, which represent the most promising emerging antifungal therapies.

**Keywords:** Allylamines, Aureobasidin a, Azoles, Benzylamines, Cell wall, Echinocandins, Ergosterol, Flucytosine, Manogepix, Morpholines, Nucleoside-peptides, Olorofim, Polyenes, Sordarin, Tavaborole, Thiocarbamates, Triterpenes.

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## INTRODUCTION

Medically important fungi can be unicellular (yeasts), multicellular or filamentous (molds) microorganisms or can exist in each of these morphological forms (polymorphic fungi) [1]. Although there are millions of species of fungi described, only fewer than 150 are known to be etiologic agents of disease in humans, which can cause superficial infections to invasive diseases.

Superficial fungal infections are caused by pathogens that are restricted to the stratum corneum with little or no tissue reaction, and include dermatophytes, *Candida*, *Malassezia*, *Trichosporon* and *Hortaea* [2]. These pathogens are rarely dangerous or life-threatening but are important because of their worldwide distribution, frequency, person-to-person transmission, and morbidity [3]. Dermatophytes (genera *Microsporum*, *Epidermophyton* and *Trichophyton*) infect keratinized tissues, human hair, nails, and epidermis, leading to conditions such as athlete's foot, ringworm of the scalp, and infection of the nails. *Candida* requires a warm and humid environment and is responsible for skinfold and mucosal infections such as vaginitis in women and oral thrush common in babies, denture wearers, and immunocompromised patients [4]. *Malassezia* needs a moist microenvironment and lipids to grow and is associated with pityriasis versicolor, folliculitis, seborrheic dermatitis, and dandruff. Piedra is characterized by nodules on the hair shaft that can be caused by *Trichosporon* (white piedra) and *Hortaea* (black piedra).

Subcutaneous mycoses (chromoblastomycosis, eumycetoma and sporotrichosis), also classified as implantation mycoses, are the most common mycosis in rural areas of underdeveloped countries caused by saprophytic fungi that enter by traumatic implantation and affect the skin and subcutaneous tissue [5]. Chromoblastomycosis is caused by melanized fungi belonging to the genus *Fonsecaea*, *Cladosporium*, *Rinocladiella* and *Exophiala*. *Madurella mycetomatis* is the most common cause of eumycetoma worldwide. Sporotrichosis is a subacute or chronic infection caused by the saprophytic dimorphic fungus *Sporothrix schenckii*.

Endemic mycoses are caused by true pathogenic dimorphic fungi of restricted geographic distribution that enter the organism through the respiratory tract. Although they can cause asymptomatic or self-limited infections in immunocompetent individuals, they produce a more severe course in immunosuppressed patients [5]. Histoplasmosis is caused by varieties of *Histoplasma capsulatum* endemic to temperate zones worldwide; paracoccidioidomycosis is caused by *Paracoccidioides brasiliensis* and *Paracoccidioides lutzii* endemics of regions of Central and South America;

blastomycosis is caused by *Blastomyces dermatitidis* complex and distributed in North America; and coccidioidomycosis (Valley Fever) is caused by *Coccidioides immitis* and *Coccidioides posadasii* endemics of arid and semi-arid regions of North and South America, respectively.

The immune system prevents fungal infections in healthy people, but the prevalence of invasive fungal infection is increasing in immunocompromised hosts with solid organ and blood or marrow transplantations, treated with aggressive chemotherapy and immunosuppressive drugs, and more patients live with underlying immunosuppressive infections, such as human immunodeficiency virus (HIV)/ acquired immunodeficiency syndrome (AIDS). These infections have lower incidence than superficial ones, but have a major impact on morbidity and mortality rates in humans and are caused by opportunistic fungi belonging to the genera: *Candida*, *Cryptococcus*, *Aspergillus*, and *Pneumocystis* [4].

There are currently five main classes of antifungal agents used in the treatment of mycoses: polyenes, azoles, allylamines, echinocandins and antimetabolites. In practice, these agents are often constrained by dose-limiting toxicities, drug-drug interactions, intolerance, and the routes of administration. The increase of both invasive and superficial mycoses coupled with the emergence of resistance, innate or acquired, and the practical limitations of existing agents, has created a demand for the development of new antifungals, particularly those with novel mechanisms of action. New agents and classes are a welcome addition to the antifungal armamentarium and results of ongoing clinical trials are eagerly awaited [6, 7].

This chapter summarizes the most relevant facts regarding the chemical structure, mechanisms of action, pharmacological profiles, and susceptibility to specific fungi of the established systemic and topical antifungal agents. The experimental antifungal drugs in clinical trials are also reviewed.

## **HISTORY OF ANTIFUNGALS**

Fig. (1) shows a historical timeline of antifungal agents that are used clinically. Benzimidazole was the first antifungal described in 1944. Polyenes (*e.g.* nystatin and amphotericin B) were developed in the 1950s, with amphotericin B deoxycholate being the only option for the treatment of invasive mycosis for many years, despite having significant side effects, including renal failure, electrolyte abnormalities, and infusion reactions [8, 9]. Griseofulvin is a systemic antifungal agent introduced in 1959 used to treat skin and nail infections. Flucytosine, a pyrimidine analogue, was developed in the 1970s. Then, in late 1960s and 1970s, the development of topical imidazoles, clotrimazole, miconazole and econazole broadened the spectrum of antifungal therapy [8, 10].

## Naturally and Chemically Sulfated Polysaccharides in Drug Delivery Systems

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**Abstract:** Sulfated polysaccharides have always attracted much attention in food, cosmetic and pharmaceutical industries. These polysaccharides can be obtained from natural sources such as seaweeds (agarans, carrageenans, fucoidans, mannans and ulvans), or animal tissues (glucosaminoglycans). In the last few years, several neutral or cationic polysaccharides have been sulfated by chemical methods and anionic or amphoteric derivatives were obtained, respectively, for drug delivery and other biomedical applications. An important characteristic of sulfated polysaccharides in this field is that they can associate with cationic drugs generating polyelectrolyte-drug complexes, or with cationic polymers to form interpolyelectrolyte complexes, with hydrogel properties that expand even more their applications. The aims of this chapter are to present the structural characteristics of these polysaccharides, to describe the methods of sulfation applied and to review extensively and discuss developments in their use or their role in interpolyelectrolyte complexes in drug delivery platforms. A variety of pharmaceutical dosage forms which were developed and administered by multiple routes (oral, transdermal, ophthalmic, and pulmonary, among others) to treat diverse pathologies were considered. Different IPECs were formed employing these

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sulfated polysaccharides as the anionic component. The most widely investigated is  $\kappa$ -carrageenan. Chitosan is usually employed as a cationic polyelectrolyte, with a variety of sulfated polysaccharides, besides the applications of chemically sulfated chitosan. Although chemical sulfation is often carried out in neutral polysaccharides and, to a less extent, in cationic ones, examples of oversulfation of naturally sulfated fucoidan have been found which improve its drug binding capacity and biological properties.

**Keywords:** Sulfated polysaccharides, drug delivery, polyelectrolyte drug complexes, interpolyelectrolyte complexes, nanotechnology.

## INTRODUCTION

Sulfated polysaccharides have always attracted much attention in food, cosmetic and pharmaceutical industries. These polysaccharides can be obtained from natural sources such as seaweeds (carrageenans, agarans, fucoidans, ulvans), or animal tissues (glucosaminoglycans). Also, several neutral or cationic polysaccharides have been sulfated by chemical methods and anionic or amphoteric derivatives were obtained, respectively, for biomedical applications. An important characteristic of sulfated polysaccharides in this field is that they can associate ionically with cationic drugs, generating polyelectrolyte-drug complexes, or with cationic polymers to form interpolyelectrolyte complexes (IPECs) with hydrogel properties that expand even more their applications [1]. In Fig. (1), a general IPEC preparation scheme is presented. Experimental and simulation evidence indicates that salt and polyelectrolyte concentration, pH, mixing ratio and order of addition, polyelectrolyte molecular weight and topology are useful parameters for the regulation of the size, internal structure, and drug release properties of IPECs [2, 3].

The aims of this chapter are to present the structural characteristics of these polysaccharides, to describe the methods of sulfation applied and to review the development in their use alone or their role in IPECs in drug delivery platforms as excipients [4, 5].

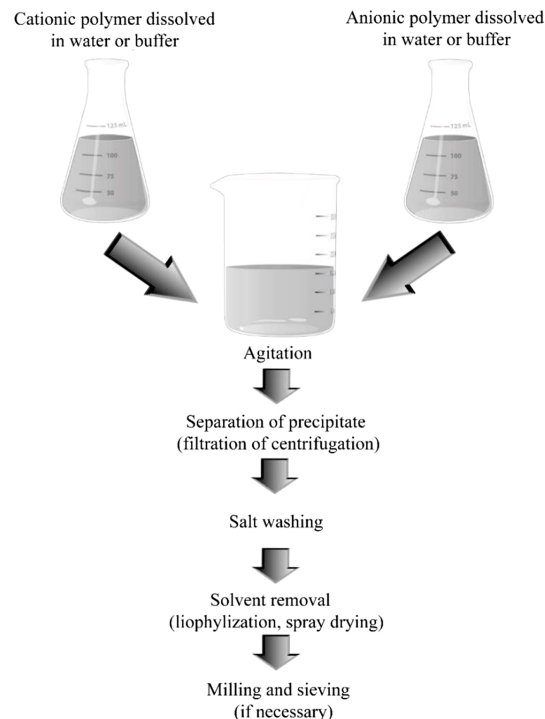
## STRUCTURAL CHARACTERISTICS

### Naturally Sulfated Polysaccharides

#### *Carrageenans and Agarans*

The major matrix polysaccharide components in most of the red seaweeds are sulfated galactans. They are constituted by linear chains of alternating 3-linked  $\beta$ -galactopyranosyl residues (A-units) and 4-linked  $\alpha$ -galactopyranosyl residues (B-units). The A units always belong to the D-series, whereas the B units can belong

to the D- or L-series, many times occurring as 3,6-anhydrogalactopyranosyl residues. These galactans are divided into two different groups, carrageenans and agarans, according to whether the B-units belong to the D- or L-series [6 - 8].



**Fig. (1).** IPEC general preparation scheme.

In agarans, the 4-linked units can be partially or wholly converted to 3,6-anhydro forms by enzymatic elimination of sulfate from the 6-position. According to the initial substitution pattern, this conversion may afford the neutral polysaccharide agarose or substituted agaroses. Agarose is the major component of the products industrially obtained mainly from seaweeds of the orders Gelidiales, Gracilariales, and Ahnfeltiales [9], and known as “agar” or “agar-agar”. The term “agarose” has a strict chemical sense (Fig. 2), whereas for other polysaccharides of the group, a classification considering the sulfation pattern of the A-units has been proposed [8]. Although sulfated agarans appear in all the agarophytes, they are the predominant galactans in seaweeds that do not have at present important industrial applications, they usually give complex mixtures, not only due to their sulfation pattern, but also by the presence of pyruvate acetals and methoxyl groups, as well as non-reducing monosaccharides as side chains. These mixtures are difficult to purify, and they have not been widely used in drug delivery systems, so in this chapter, we will focus on galactans of the carrageenan group.

## CHAPTER 8

# Immunomodulatory Plant Extracts and their Compounds. Evaluation of your Safety

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**Abstract:** Medicinal herbs have been in use for the management of human health, for prevention, as well as for the cure of human diseases since ancient civilizations. In recent times, the use of herbal drugs has increased in both developed and developing countries, because of the large chemical, pharmacological, and clinical knowledge of plant drugs and their derivatives, the development of new analytical methods for quality control, the development of new forms of preparation and administration of plant drugs and their derivatives and finally the relatively wide therapeutic margins with less frequent adverse effects. However, naturals are not a synonym for innocuous as many adverse effects can occur. In this regard, there are different levels of perceptions about the safety of medicinal herbs, varying from “completely safe” to “completely harmful”, although there is also a clear idea about its side effects depending on factors such as dosage, characteristics of the plant material and consumer-related factors. Because of this, medicinal plants need to be studied and effective and innocuous doses must be established.

Nowadays, immunomodulatory drugs have gained a main role principally as a consequence of COVID-19 produced by the SARS-CoV-2 virus.

Some South American plants frequently used in Argentine folk medicine such as *Larrea divaricata* and *Ilex paraguariensis* and others used all over the world like *Tilia* spp. and *Coffea Arabica* are known to exert immune-enhancing effects.

In this review, we discussed some reports about the immunological effect of the mentioned plants and their majority compounds, focusing on their efficacy and safety.

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**Keywords:** Adverse effects, Immunomodulatory effects, Plants isolated compounds, Popular uses, Medicinal plant.

## INTRODUCTION

Medicinal herbs have been in use for the management of human health and for the prevention, as well as the cure of human diseases since ancient civilizations. For example, the Greek physician Hippocrates (459–370 BC) wrote a book on 300 medicinal plants classified by their physiological actions. Dioscorides, “the father of pharmacognosy, wrote the work “De Materia Medica” that offers plenty of data on medicinal plants constituting the basic materia medica until the late Middle Ages and the Renaissance.

Recently, in accordance with OMS, 80% of people in the world attend their primary health care with medicinal plants [1] by using them as herbal medicines, dietary supplements, and nutraceuticals in the global market.

The reason why people in both developed and developing countries prefer medicinal herbs rather than synthetic ones is that most medicinal plants contain dozens of different compounds, which can synergize themselves and mitigate adverse effects, exerting a plethora of effects that help the body to fight a disease. Moreover, scientific studies have shown that the effects of whole plants are higher than the effects of the isolated principle [2]. In addition, other factors are taken into consideration, such as the high adverse effects of synthetic products, the fact that there is large chemical, pharmacological, and clinical knowledge of plant drugs and their derivatives, the development of new analytical methods for quality control, the development of new forms of preparation and administration of plant drugs and their derivatives and finally the relatively wide therapeutic margins with less frequent adverse effects. Frequently, a well-standardized crude extract exerts more beneficial effects and less toxic side effects than isolated compounds [3].

However, naturals are not a synonym for innocuous, as many adverse effects can occur. These side effects may occur through different mechanisms, including direct toxic effects of the herbs, by the effects of contaminants, and by interactions with drugs or other herbs [4]. Some compounds present in medicinal plants are known to be associated with adverse effects, for example, saponins are related to gastric complications, essential oils with convulsions and dermic irritation, anthraquinones and pyrrolizidine alkaloids with hepatic alterations, furanocoumarins with photosensitive and so on [5]. Because of this, medicinal plants need to be studied and effective and innocuous doses must be established.

Nowadays, immunomodulatory drugs have gained a main role principally as a

consequence of COVID-19 disease, the pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The inhibition of cellular components of host immunity has also been reported for SARS-CoV-2 and MERS-CoV infections. During these infections, a downregulation of gene expression related to antigen presentation, and increased level of exhausted CD8+ T cells and loss of CD4+ T cell function were found in the peripheral blood of patients infected with SARS-CoV-2 [6]. This immune insufficiency or misdirection may increase viral replication and cause tissue damage. On the one hand, uncontrolled immunity may result in pulmonary tissue damage, functional impairment, and reduced lung capacity. Therefore, under this context, the induction of a balanced host immune response against pathogens in general, and SARS-CoV-2, is crucial to control and eliminate infection, employing adaptive and innate immune responses. The role of immunomodulatory drugs is crucial.

Some South American plants frequently used in Argentine folk medicine such as *Larrea divaricata* and *Ilex paraguariensis* and others used all over the world like *Tilia* spp. and *Coffea Arabica* are known to exert immune-enhancing effects.

In this review, we will discuss some reports about the effect of the mentioned plants and their majority compounds focusing on their efficacy and safety.

This study was done because these plants are used by people either as medicines or functional foods. It is intended through this review to answer the following question: Are medicinal plants really safe?

### ***Larrea divaricata* Cav. and NDGA**

*Larrea divaricata* Cav. (Zygophyllaceae), whose common names “jarilla hembra”, “jarilla”, “jarilla del cerro”, and “jarilla de la sierra” is an autochthonous South American plant widely distributed in the north-western, center, and south-eastern regions of Argentina [7]. It is an evergreen shrub 3 to 6 feet (0.9 to 1.8 m) tall. In folk medicine, *L. divaricata* is used for healing sores and wounds, rheumatism, inflammation of the respiratory and intestinal tracts, gastric disorders, venereal diseases, and as a tonic, corrective, antiseptic, expectorant, and emetic [8]. The plant is also used for the treatment of arthritis, cancer [9], tuberculosis, common cold [10, 11] and rubefaction [12].

Also in folk medicine, *L. divaricata* is widely used as an anti-inflammatory [13]; scientific studies have demonstrated a dual pro-inflammatory and anti-inflammatory behavior that depends on the type and extract concentration. Pedernera *et al.* (2006) studied the anti-inflammatory effect by two methods: the cotton pellet-induced granuloma and the carrageenan-induced arthritis models. The authors also demonstrated the anti-ulcerogenic activity of a methanol extract.

## Biofilms: the Achilles' Heel of Antimicrobial Resistance

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**Abstract:** Microbial biofilms are communities of sessile cells with a three-dimensional (3D) extracellular polymeric substance (EPS). The EPS consists of exopolysaccharides, nucleic acids (eDNA and eRNA), proteins, lipids, and other biomolecules, that they produce and are irreversibly attached to living or non-living surfaces. This is the most frequent growth mode of microorganisms in nature. The biofilm formation consists of several steps, starting with attachment to a surface and the formation of microcolonies. Subsequently, in the maturation step, three-dimensional structures are formed and end the life cycle of biofilms with the dispersal or detachment of the cells. This type of growth has been reported to be more resistant to antimicrobial treatment and immune response than its planktonic (free-living) counterparts. Several intrinsic resistance factors including the interaction between antimicrobial and biofilm matrix components, reduced growth rates, persister cells presence, increased production of oxidative stress, and antagonist and degradation mechanisms may be active in some parts of the biofilms have been described. Extrinsic factors such as increased horizontal genes transmission conferring antimicrobial resistance have been described contributing to the biofilm antimicrobial resistance.

Due to the heterogeneous nature of biofilms, it is likely that multiple mechanisms of biofilm antimicrobial resistance are useful in order to explain biofilm survival in a number of cases, being the result of an intricate mixture of intrinsic and extrinsic factors. The understanding of the nature of biofilm development and drug tolerance are great challenges for the use of conventional antimicrobial agents and indicate the need for multi-targeted or combinatorial therapies.

**Keywords:** Biofilms, Intrinsic resistance factors, Extrinsic resistance factors antimicrobial agents, Biofilm antimicrobial resistance.

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## INTRODUCTION

### Understanding Biofilms: What are Biofilms?

In 1684, in a report to the Royal Society of London, Antoine Von Leeuwenhoek was the first to display “animalcules” found on scrapings of plaque on his own teeth. But just 260 years later, Zobell (1943) reported an “effect” in seawater and described many of the fundamental characteristics of attached microbial communities [1]. However, it was not until 1970s that a Canadian microbiologist, J. William Costerton, used the name “biofilms” as a more generic term for microorganisms adhering to wet surfaces in freshwater ecosystems [2]; and with Rodney M. Donlan (2002) made a description of biofilms as “a microbial microbial-derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or each other, embedded in a matrix of extracellular polymeric substances that they have produced, and exhibit an altered phenotype concerning growth rate and gene transcription” [3].

Microorganisms exist in two principal forms, as free-floating planktonic cells and, as sessile cells in biofilms. However, it is now generally acknowledged that the majority of microbial cells (Gram-positive and Gram-negative, motile and non-motile, aerobic, anaerobic and facultative bacteria, unicellular and multicellular fungi) on earth are living in three-dimensional (3D) structures, referred to as biofilms. According to recent global estimates, 40–80% of all prokaryotes live in biofilms [4]. Biofilms are a microbial multicellular lifestyle as different mixing communities, being attached to an inert or living surface [5]. The extracellular matrix (ECM) is composed of multiple types of extracellular polymeric substances (EPS) typically consisting of a protein in a majority (>2%), polysaccharides between 1 to 2%, as well as extracellular DNA and RNA (<1%), ions, peptides, lipids, and finally 97% of water [6, 7]. Biofilms are permeated at all levels by a network of channels through which water, microbial garbage, nutrients, enzymes, metabolites, and oxygen move to and fro. Oxygen may be depleted within only 30–40  $\mu\text{m}$  of the interface, although depending on the oxygen content in the bulk water, temperature, and flow [8]. This ensemble of chemically and functionally diverse biomolecules is termed as the ‘matrixome’ [9].

The ecological advantages of forming biofilms include protection from achieving a favorable habitat, retaining nutrients, and ensuring survival. In cases of adverse conditions, such as desiccation, changes in pH, osmolarity, or exposure to toxic compounds, UV radiation, lack of nutrients, and various external mechanical forces or predators, the microbial community can provide protection [10]. They also help in providing resistance by blocking the access of antimicrobial (ATM) agents and immune cells of the host [11, 12].

Some biofilms contain only a single species, while some others contain a multitude of species living in a sociomicrobiological way [13]. Some of the examples of biofilm-forming bacteria and eukaryotic microorganisms are shown in Fig. (1). Microbial multicellular communities have a variety of sizes and shapes that allow that sessile cells to form long-term relationships, interact with each other, and establish metabolic cooperation [14, 15]. Therefore, the fact that, in biofilms, the participating microbial cells are situated in close proximity seems to be advantageous, since metabolites can easily be transferred and metabolized further. Moreover, there occurs the acquisition of new genetic traits because the genetic material is easily exchanged because of the proximity of the cells, thus maintaining a large gene pool [16 - 18].

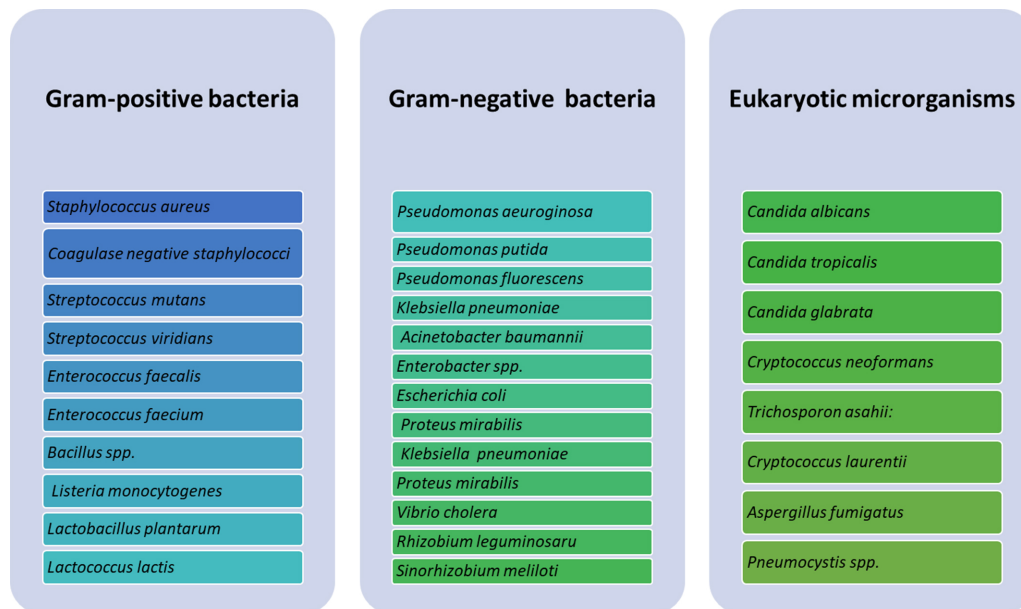


Fig. (1). Predominant biofilm-forming microorganisms.

Human microbial communities natively populate mucous membranes, for example, skin, oral mucosa, digestive, and female reproductive tract. Despite our bodies being colonized with a mixed microbial community of characteristic compositions and they are important and beneficial to us as they can degrade nutrients and thereby make these accessible [19 - 21]. In addition, communities play key roles in the development of our immune systems and in the anatomy of the mucosal surfaces [22, 23]. The relationship between the host and its microbial communities is delicately balanced, but under certain conditions, it can break down and result in infectious diseases. The National Institutes of Health reported that among all microbial (65%) and chronic (80%) infections are associated with

## Development of Analytical Methods for Analysis of Drugs of Abuse in Biological Fluids using Design of Experiments and Response Surface Methodology

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**Abstract:** New Psychoactive Substances (NPS), also known as design drugs, are developed by modification of the chemical structure of the initially prohibited substances. The idea behind this strategy is to create alternatives for consumption and to evade national and international control measures applied to controlled substances, bypassing the legislative prohibition. In this context, the emergence of NPS has raised questions about the analytical methods that can be applied to identify and to characterize these substances in different scenarios, including biological fluids (serum/plasma, whole blood, oral fluid, and urine). Because biological fluids are complex matrixes, a sample preparation step is required to remove undesired endogenous matrix components and to isolate and pre-concentrate the analytes before chromatographic analysis. Different extraction or sample preparation techniques such as liquid-liquid extraction, solid phase extraction, dispersive liquid-liquid microextraction, and microextraction by packed sorbent can be used prior to chromatographic analysis (gas chromatography, mass spectrometry, or liquid chromatography mass spectrometry). All these techniques involve many factors that must be optimized so that the analytical method can detect NPS in biological samples. Tools like design of experiments (DoE) and Response Surface Methodology (RSM) can contribute to the study and optimization of the variables involved in these analytical techniques. This book chapter shows how experimental design tools (full factorial design, fractional factorial design, Plackett-Burman design, Box-Behnken design, central composite design) and response surface methodology can aid the development of analytical methods for the analysis of drugs of abuse in biological fluids.

**Keywords:** New psychoactive substances, Sample preparation, Chromatographic analysis, Design of experiments, Optimization.

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## INTRODUCTION

The most recent World Drug Report presented by the United Nations Office on Drugs and Crime (UNODC) [1] on June 26<sup>th</sup>, 2019, in Vienna, estimated that in 2017, about 271 million people aged 15–64 worldwide (which correspond of 5.5% of the global population) had used drugs at least once in the previous year (Fig. 1a). To circumvent prohibitions imposed by the legislation of different countries, new drugs have been developed by chemical modification of the originally prohibited structures, to give the so-called NPS (New Psychoactive Substances), also known as design drugs, legal highs, and bath salts, among other names. According to the UNODC, 106 countries and territories reported the emergence of 739 NPS between 2009 and 2016 (Fig. 1b), making identification of these substances difficult and their inspection ineffective.

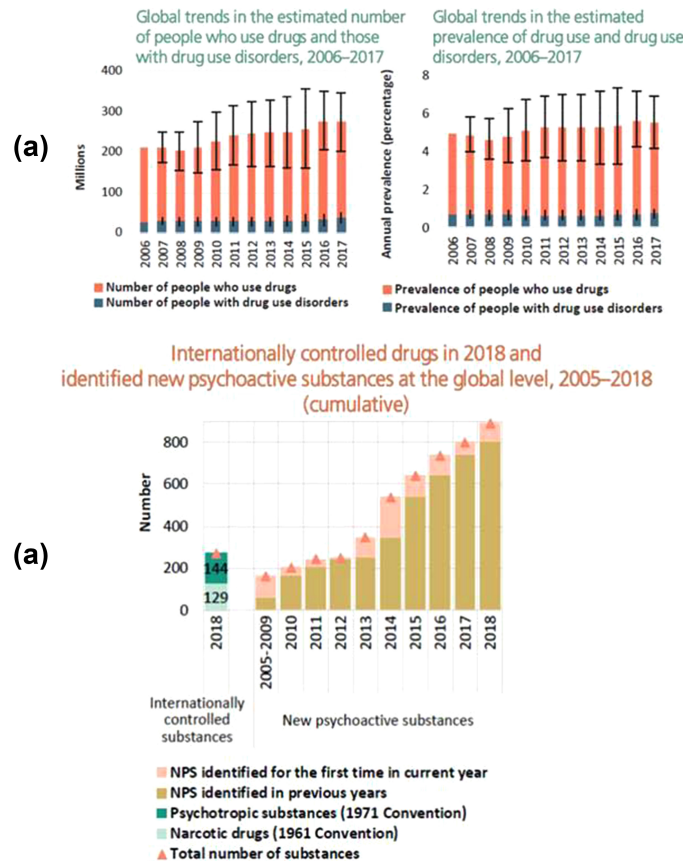


Fig. (1). a) Global trends in drugs of abuse and NPS identified at a global level, b) Identified NPS at a global level. Source UNODC [1].

NPS identification methods require a lot of resources and time to determine the chemical structure and the underlying properties of the substances. In many countries, such compounds are rarely included in the drug control legislation and may even fall outside generic legislative systems because they are mainly synthetic derivatives and analogues of existing controlled drugs and pharmaceutical products, or even natural derivatives. Therefore, it is important to identify the NPS chemical structure so that it can be monitored through the dissemination of national and international records.

The main difficulty in identifying drugs by forensic science is related to finding appropriate methodologies to extract parameters or characteristics that are crucial to treat and to contextualize the definition of the drug data profile [2]. The analytical methods that are used to identify an unknown drug usually entail determining at least two uncorrelated parameters and considering the available resources as well as any information about sample constitution. Different jurisdictions may dictate the actual practices followed by a particular laboratory. Recommendations of the Scientific Working Group for the Analysis of Seized Drugs (SWGDRUG) establish three categories of methods grouped according to their highest potential level of selectivity, as follows [3]:

- Category A: Selectivity through structural information. Methods: infrared spectroscopy, mass spectrometry, nuclear magnetic resonance, Raman spectroscopy, and X-ray diffractometry.
- Category B: Selectivity through chemical and physical characteristics. Methods: capillary electrophoresis, gas chromatography, ion mobility spectrometry, liquid chromatography, microcrystalline tests, supercritical fluid chromatography, ultraviolet/visible spectroscopy, and macro/microscopic examination (cannabis only).
- Category C: Selectivity through General or Class Information. Methods: color tests, fluorescence spectroscopy, immunoassay, melting point, and pharmaceutical identifiers.

The SWGDRUG recommendations state that:

- When a Category A method is incorporated into an analytical scheme, at least one other method (from either Category A, B, or C) exploiting different chemical or physical properties of the analyte shall be used to support identification.
- When a Category A method is not used, at least three separate methods shall be employed; two shall be from Category B, the combination of which provides high degree of selectivity. The third method (Category B or C) is required to support identification.
- For the results of the method within the analytical scheme to be considered of



**CHAPTER 11****Steric Exclusion Chromatography (including the Chromatography of Polymers in an Aqueous Solution)****Marguerite Rinaudo<sup>1,\*</sup>**<sup>1</sup> *Biomaterials Applications, 6 Rue Lesdiguières, 38000 Grenoble, France*

**Abstract:** This chapter describes the different difficulties encountered when studying a new polymer by GPC or SEC. This technique is known as liquid chromatography in which a soluble polymer is eluted through a porous gel filling a column. The different molecular weights are separated following their hydrodynamic volumes compared with the pore diameters. It appeared in the sixties firstly in an aqueous medium. The main factors playing a role in the elution through the porous support are examined. Especially, the SEC behaviour of water-soluble polymers is discussed introducing the behaviour in aqueous medium where H bonds and hydrophobic interactions are important. Examples of dextrans and neutral oligosaccharides, rich in -OH groups are discussed showing that weak adsorption increases the elution volumes when eluted in water. Other important interactions concern the electrostatic interactions causing exclusion from the gels and changes in the polyelectrolyte conformation. Elution with monovalent electrolytes (NaNO<sub>3</sub> or NaOAc) around 0.1M is recommended. SEC of charged oligosaccharides, hyaluronan, pectins and chitosan are briefly described.

Fortunately, new equipment appeared progressively and especially in 1983 the multiangle laser light scattering (MALLS) was introduced, which is probably the most useful detector to associate with the differential refractometer. In that case, Mw is obtained independently of the elution volume as soon as there are no aggregates and good solubility of the polymer tested in the solvent selected. To conclude, it is necessary to insist on the quality of the polymeric solution avoiding the presence of aggregates which may be identified by dynamic light scattering (DLS). In their presence, even after filtration on 0.2 µm pore membrane, the SLS overestimates the Mw.

**Keywords:** Aqueous medium, Chromatography, GPC, Molar mass, Persistence length, Polyelectrolyte, SEC.

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## INTRODUCTION

The steric exclusion chromatography (SEC) is a very important technique for characterisation of linear or branched polymers. It is a liquid chromatography allowing fractionation in the solution of molecules in dependence of their molecular weights (Mw) or most precisely of their hydrodynamic volume (Vh). Experimentally, columns are filled with porous beads with different porosities and granulometries through which the solute is eluted at a constant rate or pressure at a given temperature in a given solvent. Our objective is to present the evolution of the SEC technique used for characterising soluble polymers whatever the polymer and the solvent used. Especially, this chapter covers the experiments on the aqueous solution and on natural polymers based on our long experience with this technique.

This technique was firstly developed on hydrophilic gels in an aqueous medium to separate salts from proteins and fractionate proteins with different Mw. The first supports were crosslinked dextrans produced under the trade name Sephadex and launched by Pharmacia in Sweden (in 1959). At that time, the method was named gel permeation chromatography (GPC) or gel filtration based on the work of Porath and Flodin [1]. Those gels are used under low elution pressure and need a calibration relating the elution volume  $V_e$  and the Mw in given thermodynamic conditions (temperature, and salt concentration playing a role in the swelling degree of the gels). The first calibrations were performed using well-defined Mw standards (dextrans or proteins) which are valid on the same set of columns, in the same solvent and the same type of polymers as the unknown polymeric systems being studied. This point is very important as the influence of the conformation must be taken into consideration for chromatographic analysis as it will be discussed later.

Other polymers were also crosslinked as Bio-Gel P produced by Bio-Rad which consists of a polyacrylamide (crosslinked with bisacrylamide) or Sepharose based on agarose from Pharmacia. After some time, Sephadex LH beads were produced from crosslinked dextran which has been hydroxypropylated to allow GPC experiments in aqueous and in organic solvents. In fact, all those porous supports were mainly used in aqueous solvents.

An important development of GPC occurs in the application of synthetic polymers when Moore in 1964, in States, prepared organic gel beads with controlled porosities by crosslinking of polystyrene organic gel beads with controlled porosities by crosslinking of polystyrene [2]. Using those porous gels, it was possible to get the molecular weight distribution for polymers in solvents compatible with the gels available. At that time, tetrahydrofuran (THF) was often

used. This GPC technique was considered a technical revolution to access the characteristics of polymers in the solution (especially their  $M_w$ ).

Nevertheless, it was considered that it is practically impossible to make a new calibration curve each time a new polymer is studied. The objective was to find a way to use the polystyrene calibration curve valid for all polymers. In 1965, the equipment produced by Waters company was introduced including a pump to get a stable flow of solvent, an injector and a differential refractometer. It allows to study many polymers in organic solvents at low pressure using long columns in series. During this period, it took many hours to get chromatograms which were analysed by hand.

At that time, Benoit *et al.* proposed the universal calibration curve when they established that the elution volume  $V_e$  was not related to the  $M_w$  but to the volume of the molecules taking into account the intrinsic viscosity. More precisely, the product  $[\eta]M$  corresponding to the hydrodynamic volume of the coil must be considered [3 - 5]. Using the universal calibration established with available polystyrene fractions, each elution volume of an unknown polymer gives  $[\eta]M$  from calibration and consequently  $M$  if the Mark-Houwink parameters are known ( $K$  and  $a$  are obtained from the relation  $[\eta] = KM^a$  established in the same solvent at the same temperature).

From that time, the rapid evolution of the experimental equipment occurs: 1) new rigid porous gels were produced (starting with silica gels especially for water-soluble polymers), 2) smaller porous particles and granulometries allowing to work at high pressure with smaller columns and smaller elution times, and 3) new detectors were adapted on line. The use of multidetectors like differential refractometers, viscosimeters, and multiangle laser light scattering detectors (MALLS) prevents the need of calibration since the result of a chromatography would give a complete characterization (radius of gyration ( $R_g$ ), molar mass distribution and average molar masses  $M_w$  and  $M_n$ ,  $K$  and  $a$  parameters) of new soluble polymers in a solvent compatible with the gels used.

With the evolution of the technique, and considering the universal calibration indicating that the molar hydrodynamic volume controls the diffusion in the porous support, the method is named steric exclusion chromatography (SEC).

In this paper, different examples of experiments realised from 1964 will be given to detect the main important difficulties involved in the elution of a given polymer on a given gel in a specific solvent.

## Intrinsic Viscosity Methods in Natural Polymer as Pharmaceutical Excipients

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**Abstract:** Intrinsic viscosity is the most economical and used measure in the determination of polymers and biopolymers used as excipients in the pharmaceutical industry. The most used methods in the measurement of intrinsic viscosity are Huggins, Kraemer, Schulze-Blashke and Martin, the first being used as a standard and reference for the others. There are also Simple Point methods such as Solomon Ciuta and others that help in this regard. In this chapter, we will focus on those methods best known and applied in intrinsic viscosity measurements. In the measurement of intrinsic viscosity in dilute solutions of polymers, experimental methods such as Huggins, Martin, Kraemer and Shulze-Blashke are particularly useful. In dilute concentrations, graphical methods such as those of Fuoss, Fidors, and Tanglertpaibul and Rao can also be used without major errors. Although there are many more methods these can be more difficult and impractical in their calculations and graphs. The methods furthest from experimental practicality are those that depend on other methods and constants, such as Budtov's and Baker's. As for the simple point methods, the simplest and most used is that of Solomon-Ciuta, the rest have similar or better results. As for the proposed methods, the most prominent and with the least error is Square, the rest being affordable but with a somewhat higher margin of error.

**Keywords:** Biopolymers, Intrinsic viscosity, Polymers.

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Martin Masuelli & Mauricio Filippa (Eds.)  
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## INTRODUCTION

### Plot Methods

In recent years, polymers have been increasingly used as a support and as a tool for the controlled release of drugs or active substances found in a certain formulation. There are many applications of polymers for drug delivery due to the unique characteristics of these materials. Some of these are: protection, support, and improvement of the stability of the formulation, good processability, hydrophilic or hydrophobic character according to the requirement, rapid or controlled release of the active substance, improvement of the bioavailability or acceptability of the drug by the patient, and finally, its safe use [1 - 3].

The support polymer in pharmaceutical applications is an excipient, it is inactive in the formulation and serves as a vehicle to enable the preparation of the drug and to give it consistency and stability, among other functions. A medicine can contain one or more than one active ingredients. The latter is called an active ingredient and its activity depends on its nature but is related to the amount ingested or absorbed. This substance with pharmacological activity extracted from a living organism, once purified and / or modified, is called a drug or medicine. Morphologically, the polymer can be the matrix of the pharmaceutical compound, or it can be the shell or coating of the preparation [4 - 6].

In writing regarding pharmaceutical polymers and biopolymers, it is essential to be able to determine their physicochemical properties, and thus control their quality and be able to evaluate their raw materials before the final drug. For this reason, the measurement of intrinsic viscosity is essential since its determination is simple and inexpensive for its evaluation [7 - 9].

Viscosity is one of the most important properties of polymer solutions. The viscosity depends on the chemical structure of the polymer, interactions with the solvent and molecular weight. Normally, a high molecular weight molecule in a good solvent acquires a large hydrodynamic volume and the viscosity of the solution increases. In case of polyelectrolytes, the hydrodynamic volume depends, not only on the molecular weight, but also on the number and distribution of ionic groups in the polymer chain. Ionic groups can cause repulsion between chains, which results in an expansion of the molecule and, consequently, an increase in the viscosity of the solution. The viscosimetry of dilute solutions is related to the measurement of the intrinsic ability of a polymer to increase the viscosity of a solvent at a given temperature and is useful for obtaining information related to the size and shape of the polymer molecules in solution and the polymer-solvent interactions. In the dilute regime, the viscosity of a polymer solution (for excepti-

onally low polymer concentrations) is determined relative to the viscosity of the solvent [10, 11].

Viscosity is a physical phenomenon related to the transport of the momentum within a fluid that is not in equilibrium, but rather moves macroscopically in a certain direction. The more viscous a fluid is, the greater resistance it must flow. From a microscopic point of view, viscosity is understood as the friction between adjacent layers of the fluid that slide over each other: those that move at a slower speed tend to slow down the others, while those that move faster tend to accelerate to the slowest. The more viscous the fluid, the slower this transport of momentum occurs, perpendicular to the macroscopic displacement of the fluid, and the greater is the stress generated between adjacent layers. The viscosity of a polymer in a solution is an important property in determining its average molecular mass. In this type of solution, the viscosity is due to two contributions, that of the solvent and the polymer, so we will have to separate both effects [12].

The quantity that the viscometer measures is called the coefficient of viscosity, or simply, viscosity, and is symbolized by  $\eta$ . The SI units of  $\eta$  are  $\text{N} \cdot \text{s} \cdot \text{m}^{-2}$ , and in CGS the poise, P, which is  $\text{dyne} \cdot \text{s} \cdot \text{cm}^{-2}$ . Viscosities are usually expressed in centipoise. This magnitude is, in the case of a Newtonian fluid, independent of the speed at which it moves and is measured as the quotient between the friction stress exerted by some fluid layers on others and the velocity gradient that exists in them. In this practice, viscosity measurements are made using a rotational viscometer, which is based on measuring the resistance to rotation of a cylinder or disk immersed in the fluid [13, 14].

Viscosity measurements of dilute polymer solutions are important in the characterization of macromolecules. Each polymer chain in a solution contributes to the total viscosity of the solution. In a very dilute solution, the contribution of different chains is additive, and solution viscosity,  $\eta$ , increases, above the solvent viscosity, linearly with the concentration of the polymer. The virial expansion of viscosity at low concentrations is given by the equation below, which has the same form for osmotic pressure and light scattering:

$$\eta_s = \eta_0 (1 + [\eta] c + k_H [\eta]^2 c^2 + \dots) \quad (1)$$

The term that is linear with the concentration includes the intrinsic viscosity  $[\eta]$  and the quadratic term includes the Huggins coefficient  $k_H$ . Intrinsic viscosity is the initial slope on a graph of relative viscosity ( $\eta_s/\eta_0$ ) as a function of polymer concentration. As the relative viscosity is dimensionless, the unit of intrinsic viscosity is the reciprocal concentration [15].

## Extraction Techniques in Green Analytical Chemistry

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**Abstract:** Analytical chemistry determinations are not exempted from generating environmental contamination. A variety of strategies are now being proposed to reduce the impact on the environment caused by the different stages of the analytical process. These strategies can focus on the different stages of the analysis, ranging from sample collection and preparation to the acquisition and processing of analytical signals. Sample preparation constitutes a basic and crucial stage in the success of any analytical method and extraction is one of the most chosen techniques. Extractions often involve the use of a large amount of harmful solvents that may damage the health of the operator and the environment, into which these solvents are disposed of, often without treatment. Therefore, new techniques have been applied in order to reduce the impact of this procedure, also focusing on lowering the costs and complexity, always taking into account the quality of the procedures. Current trends in green analytical chemistry are directed towards simplification, miniaturization, and automation, also involving the use of solvent-free, environmentally friendly procedures and, at the same time, maintaining acceptable extraction efficiencies in a short time.

In this chapter, the fundamentals and technological advances in green extraction systems will be presented. Through representative examples of different compounds in different matrices, the advantages and limitations of different procedures will be presented, including ultrasound-assisted extraction, pressurized solvent extraction, microwave-assisted extraction, single drop liquid-liquid extraction, headspace extraction, dispersive liquid-liquid microextraction, hollow-fiber liquid-phase microextraction, micro-solid phase extraction, stir-bar sorptive extraction and stir-cake sorptive extraction.

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**Keywords:** Deep eutectic solvents, Dispersive liquid-liquid microextraction, Environmentally-friendly techniques, Green analytical chemistry, Green extractions, Headspace extraction, Hollow-fiber liquid-phase microextraction, Ionic liquids, low organic solvents consumption, Micro-solid phase extraction, Miniaturization, Microwave-assisted extraction, Pressurized solvent extraction, Sample pretreatment, Single drop liquid-liquid extraction, Stir-bar sorptive extraction, Solid-liquid miniaturized extraction, Stir-cake sorptive extraction, Sustainable, Ultrasound-assisted extraction.

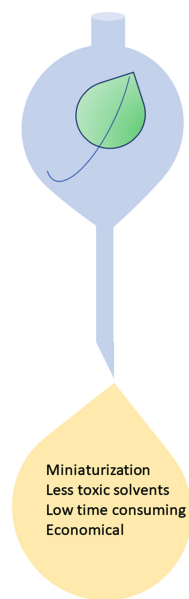
## INTRODUCTION

Over decades, tons of hazardous reagents from analytical determinations have been irresponsibly disposed of into the environment. In recent years, the awareness of the damage that this could be causing led to the emergence of new, more environmentally friendly analytical methods, focused on prevention more than on remediation of pollution which gave rise to the concept of Green Analytical Chemistry (GAC) [1 - 3]. The 12 principles of GAC [4], promote the use of analytical procedures that generate less hazardous waste and that are benign to the environment without affecting the generation of results quickly, efficiently, and effectively. This can be accomplished by developing new analytical methods or by modifying old ones to incorporate procedures such as automation, miniaturization, and in-situ treatment of analytical waste to achieve those goals. Besides, these greener techniques may offer other advantages such as lower cost, shorter analysis time and require less sample amount [2 - 4]. Since these other criteria also affect the quality and applicability of the green methods, a new concept has been proposed by Nowak *et al.* [5], the “White Analytical Chemistry”. In this new approach, analytical efficiency, environmental friendliness, safety, and practical and economic aspects are considered.

Remote sensing and direct measurement of unprocessed samples have always been the first choice [6]. However, in most analytical procedures, sample processing is essential and sampling, sample transportation, storage, and preparation are absolutely necessary before acquiring analytical determination [7]. Sample preparation usually involves total or partial digestion, mineralization, extraction, filtration, or distillation. Besides, sample dissolution and analyte extraction often involve the use of hazardous reagents and energy. Consequently, sample preparation is the step that most influences the total environmental impact of the method [6]. In this case, analytical green methods should avoid the use of toxic reagents, and energy and reagents consumption, waste generation, and time and operator effort must be minimal. Therefore, soft methods at room temperature such as assisted extractions and digestions, minimization, and automation have become the basic tools of green analytical methods providing fast and safe methodologies [1].



In analytical chemistry, extractions are performed in order to isolate quantitatively the target analyte from the matrix; and increase the concentration level of the target analyte in the final solution to be measured. Traditionally, large amounts of hydrophobic solvents and inorganic acids have been used both in solid-liquid and liquid-liquid extractions. Most of the used reagents are hazardous to laboratory workers and the environment. Consequently, new techniques emerged in which traditional water-immiscible solvents are replaced by others that are more environmentally friendly, and miniaturization, automation, and pressurized-assisted, ultrasound-assisted, and microwave-assisted extractions are used in many laboratories today. The main strategies for greening the sample treatment and analyte extraction are based on: i) miniaturization, which reduces reagent consumption and waste generated [8 - 11], ii) automation, which reduces environmental risk and exposition to dangerous substances and allows waste remediation steps [12 - 14], iii) energy saving, to improve energy efficiency in the non-spontaneous process [15, 16], iv) consumable saving, to avoid the use of single-use plastic [2], and v) on-line waste treatment to reduce costs and avoid toxic residues accumulation [17] (Fig. 1). The applications of these new techniques also provide an increase in the extraction yield and reproducibility of operations, and a reduction in the extraction time and solvent consumption, which implies a significant improvement in cost-saving and environmental benefits. In this chapter, an update of the newest green extraction techniques used in green analytical chemistry is reviewed.



**Fig. (1).** Schematic representation of the strategies for greening analyte extractions.

## SUBJECT INDEX

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*The book thoroughly displays state-of-the-art techniques of pharmacy regarding the potential of drugs and biopolymers considering their chemical structure, molecular level, and branching type, by using separation or spectroscopy techniques. A special attention has also been given to mesoscopic scale by using spectroscopic and microscopic methods in order to probe the structure of covering drugs, enzymatic kinetics, theoretical chemistry, biofilms etc. This book is very useful for students, academics, professionals, researchers and pharmacy industrialists, which will help in further development of this science, which is important for health.*

Martin A. Masuelli