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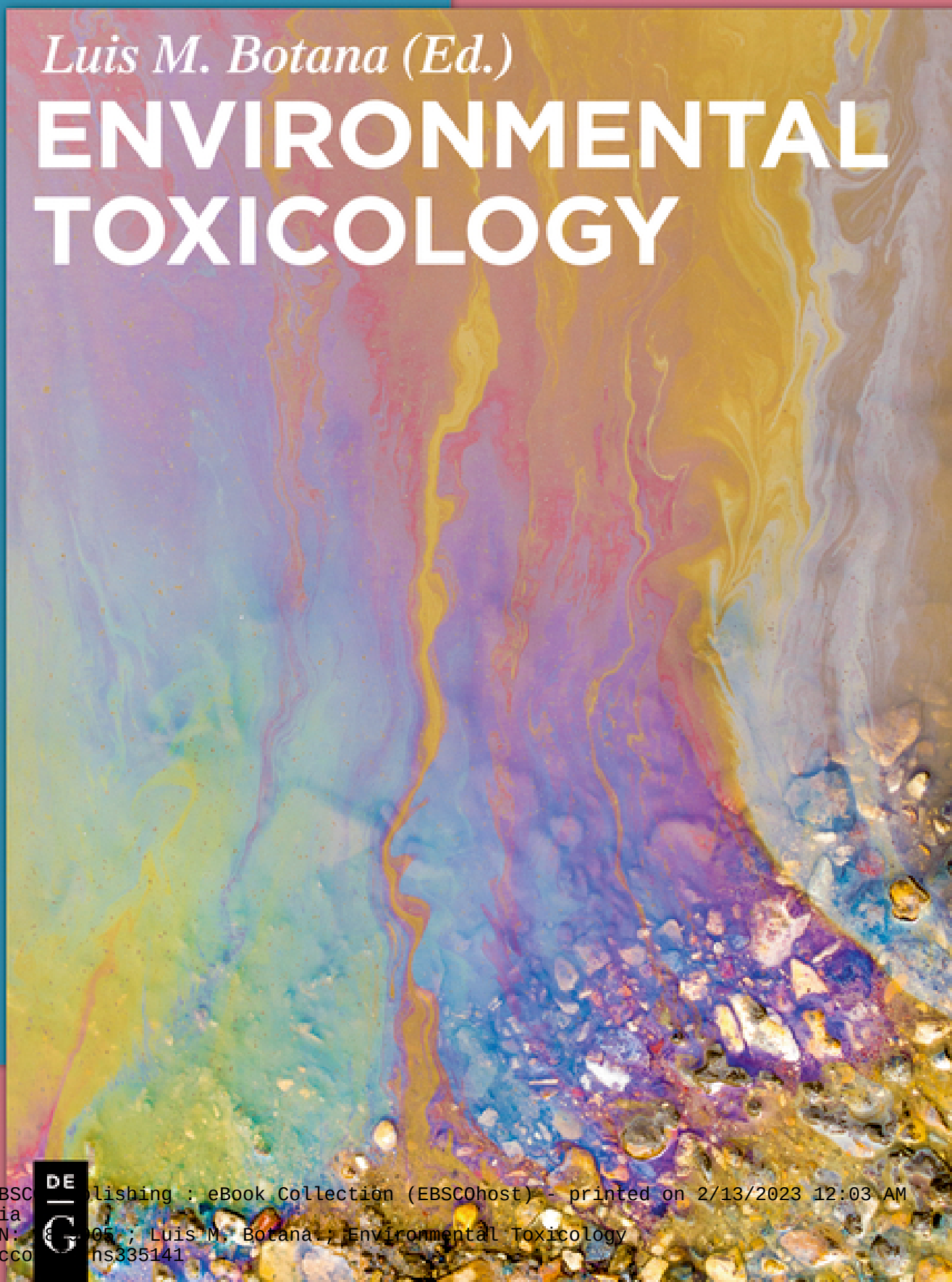

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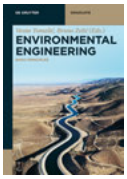

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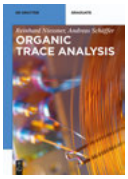

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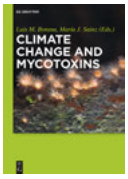

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Environmental Toxicology

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Preface

This book about environmental toxicants describes the risk of natural toxins. The topic of environmental risks is far too wide to be covered in a single book for graduate students. From a general standpoint, toxic compounds can be identified as natural or man-made. This book covers a part of the natural compounds, leaving aside those of bacterial origin, as food toxins are one of the main concerns in food safety today. The book has been divided into two parts: the first part describes the nature and risks of marine toxins, freshwater toxins and mycotoxins, thereby covering environmental risks from seafood, freshwater and agriculture; the second part describes how to deal with the control of these toxins. This part describes analytical and functional assays for the monitoring of the different toxins, the principles behind each of the approaches and the methodology to perform toxicological studies required to set toxic threshold levels. All chapters were written by colleagues from my group and institution, and I sincerely acknowledge their efforts to provide a clear, concise and pedagogic piece of information for each of the topics. Moreover, their generosity to spend the time necessary for the writing is much appreciated – to all of them, my sincere thanks.

The idea behind this book was that anyone new to the field would have in a concise book all necessary information to start working. In other words, I edited the book I would like to have when I started my PhD thesis.

Given the nature of the topic, this book would be of interest to any graduate starting in the areas of food safety, toxicology, analysis or medical research. I hope they find it useful.

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Luis M. Botana

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1 High-throughput detection methods

1.1 Introduction

High-throughput detection methods are experimental techniques that involve automated tools and allow rapid acquisition of data related to the presence or absence of a certain molecule or group of molecules. They are often referred to as high-throughput technologies or high-throughput screening (HTS) in the literature. These methods provide a remarkable increase of productivity when compared to traditional detection methods. Currently, high-throughput terminology is applied to processing of hundreds of samples in a short period of time.

These methods often comprise several approaches to achieve high workflow rates, among them laboratory automation, miniaturization and parallelization of detection technologies, effective experiment design, continuous processing, and computerized data interpretation. Although many of them have been developed for pharmaceutical screening of high numbers of products, they are also suitable for the detection of environmental contaminants.

This chapter will provide an overview of laboratory automation, detection technologies, and experimental design compatible with high-throughput detection using nonanalytical methods. Analytical methods will be presented in Chapter 2.

1.2 Laboratory automation

Laboratory robots are essential tools for handling high numbers of samples. Simultaneous assay of hundreds of samples is often performed in 96-, 384-, or 1536-well plates (Figure 1.1). Manual handling of 384- or 1536-well plates or several 96-well plates in parallel is impractical if not impossible (Figure 1.1) [1, 2]. Therefore, automated liquid handling is necessary for actual high throughput. Adequate programing will allow one to perform an experimental protocol simultaneously in a high number of samples. Currently, automated liquid handlers, dispensers, and workstations can effectively dispense liquids with high precision and good reproducibility in 96-, 384-, or 1536-well plates (for a review of liquid-handling technologies and principles see [2]). Automated workstations also perform sample transfer, tip replacement, incubations or mixing and shaking, depending on the device design and programing (Figure 1.2A). These instruments have pipetting arms with many-channel heads that allow parallel transfer of samples or reagents (Figures 1.2B and 2C). They are often endowed also with gripper arms to perform different tasks such as moving labware (tubes or plates) to different points of the station or removing plate lids. The pipetting and gripper arms can move along X-, Y-, and Z-axes across a stationary deck. In some instruments

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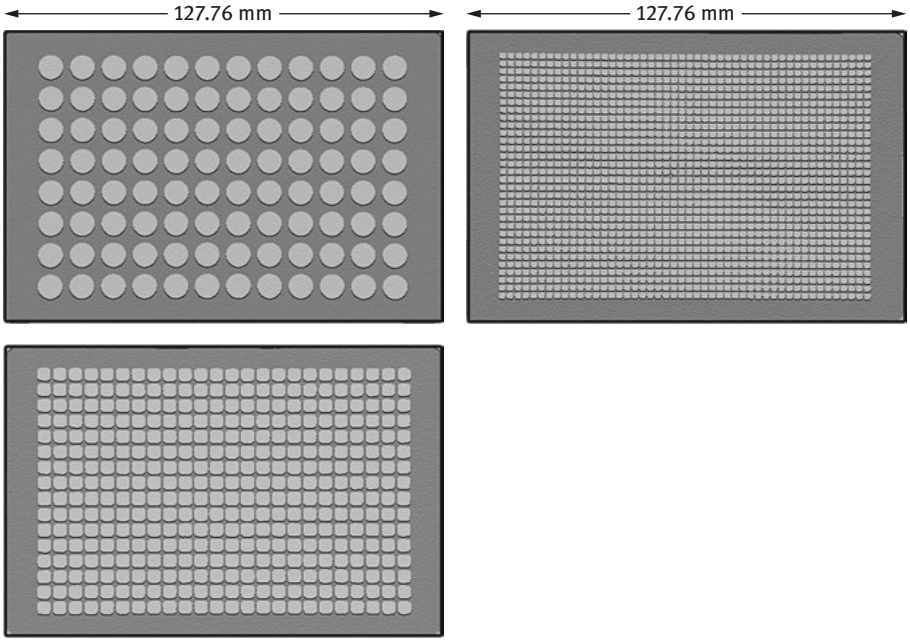

Figure 1.1: Distribution of wells in 96-, 384-, and 1586-well plates.

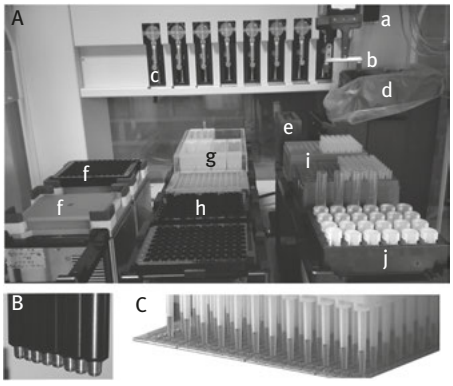

Figure 1.2: Automated liquid handling. (A) Automated liquid-handling workstation. Moving pipetting head (a), moving gripper hand (b), syringe pumps and valves (c), disposal station (d), tip washing station (e), shakers and temperature control blocks (f) and static positions for liquid containers (g), plates (h), tips (i), and tube racks (j). (B) 8-channel pipetting head. (C) 96-channel pipetting head.

volume measurement systems have been incorporated as feedback quality controls for precision and accuracy of pipetting or dispensing [2].

Automated laboratory workstations have been used mainly to execute detection assays in high numbers of samples before taking them to a reader. However, sample preparation previous to assay or analysis is often a bottleneck in most laboratories that slows down workflow [3]. Sample preparation automation is

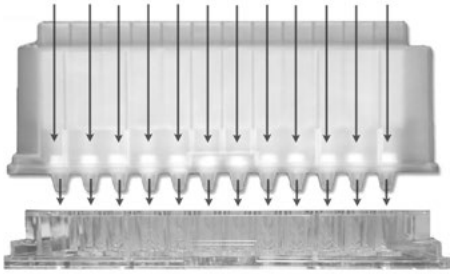

Figure 1.3: Schematic representation of simultaneous SPE cleaning in 96-well SPE plates.

critical to ensure high-throughput turnover of detection methods, including quantitative analytical techniques. Automated solid-phase extraction (SPE) procedures for sample cleanup have been developed for online and offline processing. Robots may be used for exchange of disposable online SPE columns. Alternatively, versatile liquid-handling SPE workstations allow simultaneous offline sample processing through 96- or 384-well SPE plates (Figure 1.3) using robot-operated vacuum manifolds [1]. Both online and offline SPE provide similar limits of detection, ranges, accuracy, and reproducibility; however online methods allow lower amounts of sample [3].

Many workstations include bar code readers which are critical for automated sample identification and tracking. The generation of log files related to protocols and sample tracking are also useful for quality control and auditing.

Laboratory robots and workstations are required to warrant high productivity rates in high-throughput detection methods, but they also reduce contamination and eliminate human error [1]. Moreover, these techniques help to optimize laboratory personnel hands-on time and save costs by scaling down volumes. However, programing of complex experimental procedures is not trivial, in spite of user-friendly platforms, and miniaturization of plate wells should be makes them highly sensitive to dust, air bubbles, and evaporation [2]. In addition, liquid-handling conditions must be adjusted to avoid dripping and cross-contamination, and optimized sometimes for application-specific solutions, such as aqueous buffers containing different detergent concentrations or viscous material [1, 2].

1.3 Miniaturization and parallelization of detection technologies

Miniaturized assay formats for high throughput require readers capable of simultaneous detection of miniaturized-well plates. There are many instruments in the market that provide data for 96-, 384-, and 1536-well plates in minutes. Many plate readers are endowed with different detection technologies. Multimode readers may include several of the following: absorbance, fluorescence, luminescence, fluorescence

polarization, time-resolved fluorescence (TRF), time-resolved fluorescence resonance energy transfer (TR-FRET), and image-based cytometry.

Spectrophotometric absorbance measures how much light a chemical substance in solution absorbs (Figure 1.4A). The absorption spectrum (wavelength range of the light absorbed) is characteristic of each molecule.

Fluorescence occurs as a result of absorption of the energy of an excitation photon by a fluorophore (molecule with fluorescence properties, F in Figure 1.4B), which creates an excited electronic state S_1' (Figures 1.4B, C). Some energy is dissipated coming to a S_1 energy state, and after a few nanoseconds the fluorophore returns to its ground state S_0 by emitting a photon that has lower energy and, therefore, higher wavelength, than the excitation one (Figure 1.4B, C). When fluorescence intensity is measured, excitation occurs simultaneously with emission detection. TRF consists of emission measurement a few milliseconds after excitation. Although TRF reduces background signal significantly, it requires specific fluorophores with prolonged emission properties such as lanthanides, and the instrumentation and reagents needed for TRF are more expensive.

Fluorescence polarization is based on the fact that if a fluorophore is excited by polarized light, the emitted light will be depolarized if the molecule is rapidly rotating (Figure 1.4D). Small molecules rotate rapidly, while large molecules rotate slowly. Therefore, the interaction of small fluorophore-labeled molecules with large molecules can be detected by a reduction of emitted light depolarization (Figure 1.4D,E).

Fluorescence resonance energy transfer (FRET) consists in energy transfer from an excited fluorophore (donor, D in Figure 1.4F–G) to another molecule (acceptor, A in Figure 1.4F–G) without emission of a photon when they are in close proximity. The transference of energy occurs at small distances comparable to macromolecule size (Figure 1.4F, G), and when the acceptor fluorophore returns to its ground energy state, it emits a photon of different wavelength than the donor. Therefore, the interaction of one molecule labeled with the donor fluorophore with another molecule labeled with the acceptor fluorophore will result in quenching of donor fluorescent signal and appearance of acceptor fluorescence (Figure 1.4G). The same principle used for TRF applies to TR-FRET, which uses lanthanides as donors. TR-FRET provides additional information about conformation, flexibility, and equilibrium populations of interacting molecules [4].

Luminescence is the emission of light by a molecule. It really comprises any emission of light from an excited molecule when it returns to its ground energy state independently of the process that leads to the high-energy state. Frequently, the emission is triggered by the generation of a high-energy intermediate as a result of a chemical reaction. This process is called chemiluminescence (Figure 1.4H). The chemical reaction may be catalyzed by an enzyme. The detector is configured to measure the intensity of emitted light. From a physicochemical point of view, fluorescence is a type of luminescence; however, it is usually considered separately when describing reader capabilities.

Some readers also incorporate label-free detection. This technology is based on the changes in light refraction caused by mass modifications in close proximity (150 nm) to the refractive surface. The changes in the refraction index are detected by a change

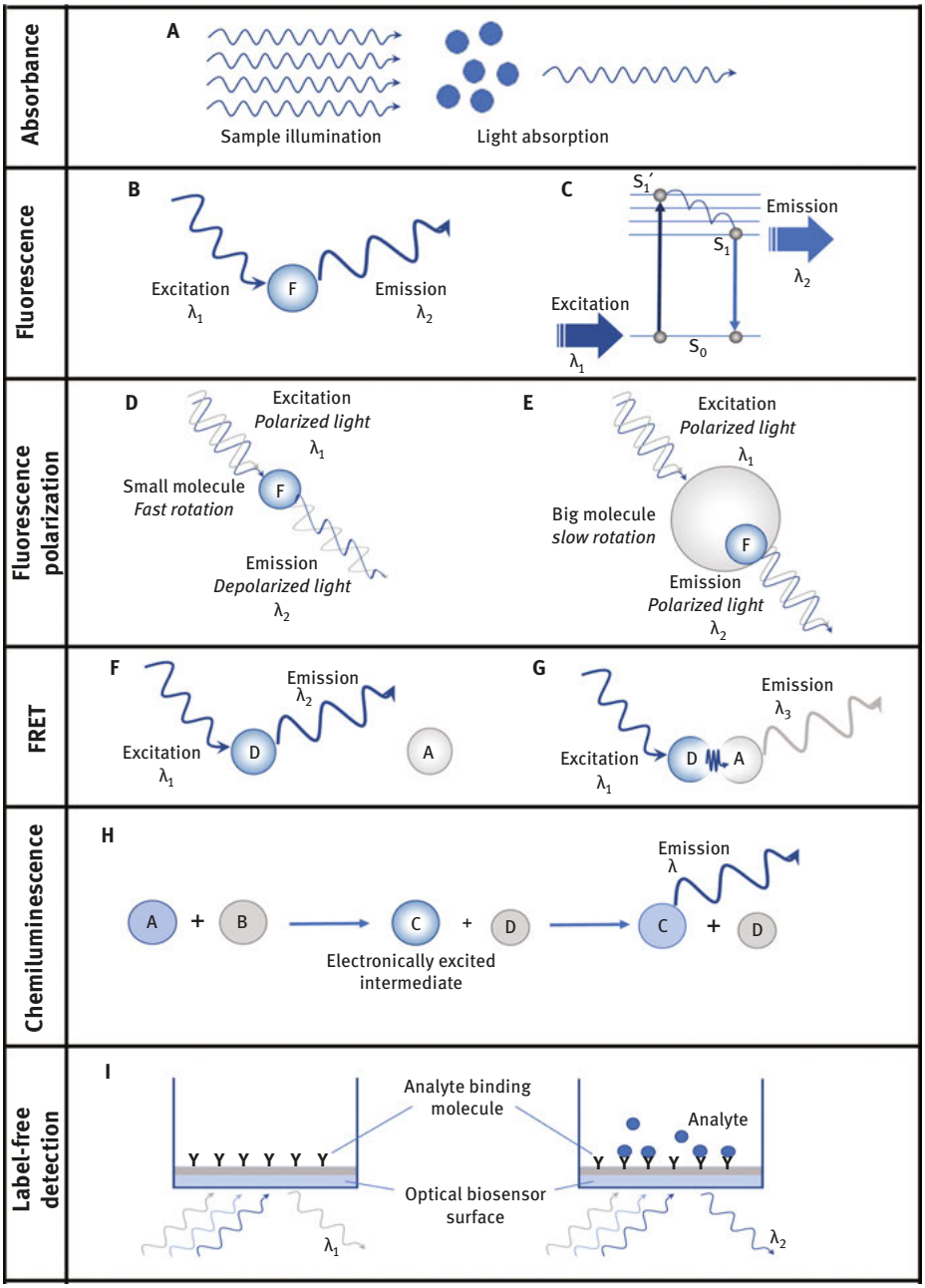

Figure 1.4: Schematic representation of (A) absorption; (B, C) fluorescence; (D, E) fluorescence polarization; (F, G) fluorescence resonance energy transfer; (H) chemiluminescence; and (I) label-free optical detection. See main text for detailed description.

of reflected light wavelength (Figure 1.4I) [5]. The advantage of these techniques is the capability of avoiding labeling with fluorescent dyes or other molecules, which may alter the results in some instances. However, label-free technology requires the use of special plates with an optical biosensor surface integrated in the clear bottom of black microplates. Electric impedance biosensing can also be used for HTS in cell-based assays using special microplates to evaluate cell viability and growth [6].

Another technology available in multimode readers is imaging of the plate bottom, including fluorescence intensity, bright field and digital phase contrast imaging. Three color images can be obtained for a 384-well plate in a few minutes. Imaging is mainly used for cell-based applications.

These readers can be incorporated into robots or automated workstations, or be upgraded with plate stackers to provide a continuous workflow suitable for high-throughput requirements.

A different strategy for incrementing laboratory throughput is multiplexing. Multiplexing consists of simultaneous detection of several analytes in the same sample. The most extended multiplexing technology is based on the use of microspheres with different characteristics of fluorescence and size that can be identified using flow cytometers or similar detectors. Each microsphere class is functionalized for the specific detection of a target analyte. Quantification of the analyte is also performed by fluorescence, using a fluorophore with excitation and emission wavelengths that do not overlap with the microsphere signal (Figure 1.5). Multiplexing is achieved by addition of a mixture of several classes of microspheres to the same sample, and posterior separation of the microspheres in the reader by a cytometer-like fluidics system (Figure 1.5). The number of analytes could be higher than 50. Recently, plate-based designs have been adapted to multiplexed assays for the detection of environmental contaminants, such as marine toxins, cyanotoxins, and mycotoxins [7–9]. Planar arrays for multiplexing have also been reported [10].

In the last decades, flow cytometers have also been optimized to provide high-throughput flow rates [11, 12]. Although they do not reach the speed of the multimode spectrophotometers discussed above, there are flow cytometers that can process 384-well plates in less than 1 hour. This technology allows classification of events according to their characteristics of size, light dispersion, and fluorescence intensity at different excitation and emission wavelengths. Therefore, flow cytometers can combine high-throughput speed and multiplexing for simultaneous detection of several analytes in the same sample.

Nowadays, electrophysiology methods have also been automated. Electrophysiology is used to measure plasma membrane voltage or currents to detect ion fluxes through plasma membrane ion channels. Although some automated electrophysiology instruments would perform parallel recordings from 96- or 384-well plates, they have not been able to allow workflow rates as high as the other technologies yet; moreover, they are fairly expensive and require highly qualified personnel [13, 14]. Therefore, electrophysiology is not the best option for routine high-throughput contaminant detection at the moment.

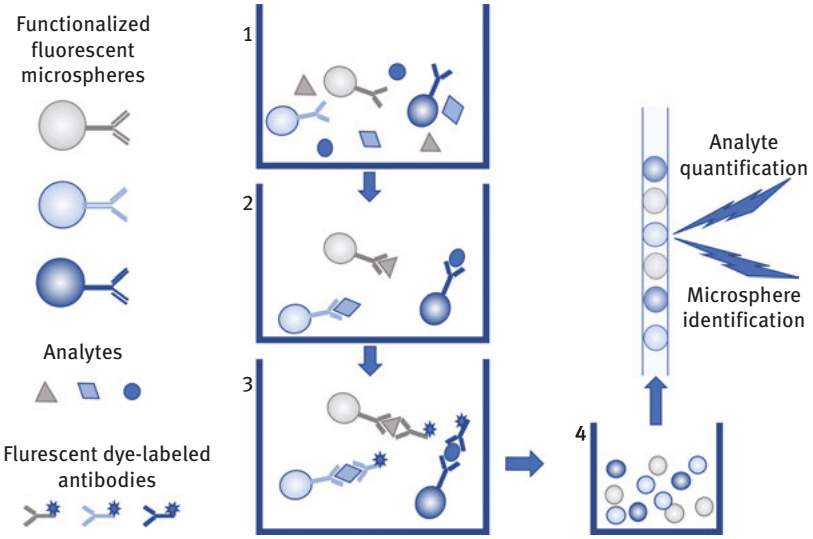

Figure 1.5: Diagram of a multiplexed immunoassay with a sandwich format. 1 Sample with different target analytes is combined with a mixture of several classes of microspheres, each class functionalized for the specific detection of an analyte. 2 Each microsphere class binds the specific analyte. 3 The amount of analyte bound to the microsphere surface is quantified using a specific fluorophore-labeled antibody. 4 The microspheres present in the sample are separated by the fluidics system of the reader. The internal fluorescence of the microsphere and the labeled-antibody signal on the microsphere surface, are recorded for analyte class identification and quantification, respectively.

1.4 Assay design

Assay design is critical for efficient, robust, high-throughput detection. Several detection strategies can be miniaturized and adapted to achieve a high-throughput workflow. Most of these techniques rely on the specific binding of the analyte to a detecting molecule. The nature of this detecting molecule will greatly determine the characteristics of the detection method. None of these techniques should be considered an analytical method, because they would not allow the unequivocal identification and quantification of the analyte. However, all these assay designs are suitable for HTS.

Methods based on the interaction of the analyte with antibodies, receptors, enzymes, aptamers, and cells will be discussed.

1.4.1 Immunodetection

Immunodetection is based on binding of the analyte to a specific antibody. It offers multiple possible designs that can be implemented in high-throughput assays. Enzyme-linked immunosorbent assay (ELISA) has been the most widely used immunoassay. There are several detection strategies, such as direct, indirect,

sandwich, or competition ELISA (Figure 1.6), depending on the characteristics of the antigen and the available antibodies. Independent of the assay format, in all ELISAs the reporter antibody is covalently bound to an enzyme and the read-out is a molecule generated by the enzymatic reaction that can be quantified by absorbance, luminescence, or fluorescence (Figure 1.6). Commonly used enzymes are horseradish peroxidase, alkaline phosphatase, and ß-D-galactosidase. The substrate depends on the final read-out. The enzymatic reaction provides significant signal amplification with a remarkable increase of sensitivity. Chemiluminescence is considered to provide the highest sensitivity and the widest detection range. ELISAs are robust, inexpensive, sensitive assays performed in plates, and they can be easily miniaturized for high throughput.

Other immunodetection possibilities amenable to high-throughput detection are labeling of the reporting antibody directly with a fluorescent dye or substitution of the flat bottom of a microplate well by microspheres as solid phase for multiplexing. Besides fluorescence intensity, TRF, FRET and RT-FRET have also been used for immunodetection.

Immunoassays are specific, easy to perform, reliable, and robust. However, antibody cross-reactivity with other molecules might be a problem. Frequently, antibodies

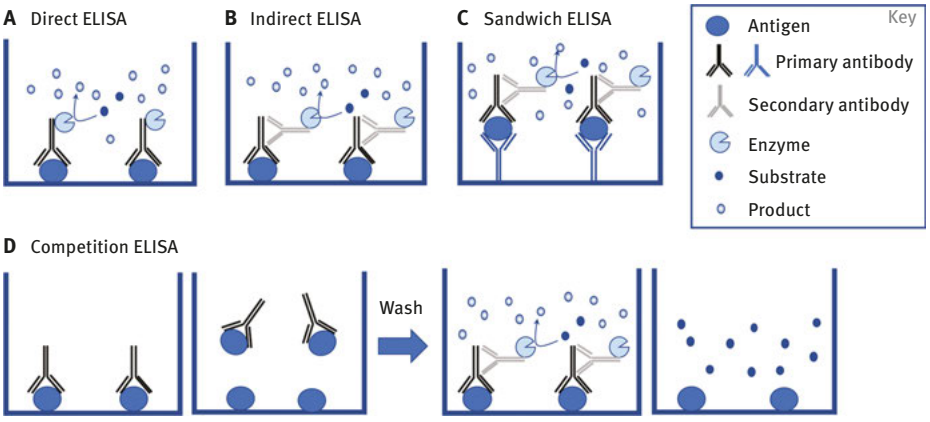

Figure 1.6: ELISA assay formats. (A) Direct ELISA: The analyte in the sample is immobilized onto the well surface and its presence is detected by an enzyme-labeled, primary antibody. A chemical reaction catalyzed by the enzyme produces a colored, fluorescent, or luminescent product. (B) Indirect ELISA: Similar to direct ELISA, except that in this case the enzyme is bound to a secondary antibody that interacts with the primary antibody. (C) Sandwich ELISA: An analyte-specific antibody is immobilized on the well surface. The analyte in the sample binds to this antibody and, then, it is detected by the addition of another primary antibody. Direct or indirect detection is possible as in (A) and (B) if the enzyme is bound to a primary or secondary antibody, respectively. (D) Competition ELISA. The analyte present in the sample competes for binding to a specific antibody with the analyte previously immobilized on the surface. Positive samples will cause a reduction of the amount of antibody attached to the surface and, therefore, a reduction of the signal. Direct and indirect designs are also possible.

bind not only to the molecules that have served as immunogen, but also to other molecules with similar structure. This characteristic might be a disadvantage if specific detection of a single molecule is required and the presence of similar compounds in the sample is possible. In other cases, it might be advantageous if there are several toxic analogs in the same sample that should be detected. Marine toxin groups, for instance, have multiple analogs with varying toxicity. Ideally, a good antibody for a marine toxin detection immunoassay should detect efficiently the highly toxic analogs and should have lower binding affinity for the less toxic molecules. Unfortunately, this perfect cross-reactivity balance is very difficult to achieve [15, 16].

Therefore, immunoassays can be considered quantitative if the sample cannot contain any other molecule that binds the antibody except for the target analyte. On the contrary, if the sample may contain several analogs of the target group, that bind the antibody, the immunosassay cannot be used to accurately quantify the contaminant levels and should be considered a qualitative screening tool.

Another limitation of immunoassays is the difficulty of producing antibodies against small molecules, which do not behave as antigens, and therefore have to be linked to bigger carriers to trigger the immune response. Many contaminants are quite small, and consequently, good-quality antibodies may not be available for assay development. In addition, the toxic potency of some compounds hinders the appearance of an immune response before death occurs [15, 17].

Although not strictly considered high throughput, rapid screening immunoassays for in situ detection of contaminants should be mentioned in this section. Parallel or simultaneous testing of many samples in short periods of time is possible with these techniques and they can also be used in the field. The most commonly used methods are lateral flow immunoassays (LFIAs, Figure 1.7A). The system consists of a combination of absorbent materials and nitrocellulose membrane that form a path for continuous flow of liquid sample driven by capillary forces (Figure 1.7B) [18, 19]. They usually follow a sandwich or competition assay design (Figure 1.6), depending on the size of the antigen and the antibodies [19]. A “deposit” of a specific antibody (antibody conjugate pad) usually labeled with colored nanoparticles is located in the path of the flow (Figure 1.7B). The sample enters in contact with the labeled, analyte-specific antibody and flows toward a test zone where another analyte-specific antibody is immobilized (sandwich assay design, Figure 1.7C). If the sample contains the analyte, the first antibody–analyte complex will bind to the immobilized antibody, causing a concentration of colored nanoparticles and revealing a color band (Figure 1.7A, C). Sample flow moves forward and reaches the control zone where an anti-immunoglobulin binds the excess nanoparticle-labeled antibody, which serves as a positive control of assay performance. For inhibition assays the analyte is immobilized in the test zone and the absence of the color band is the positive test result. These assays were initially developed for visual score, but colorimetric quantification or fluorescent read-out using fluorescent labeling of the antibody is also possible using adequate readers [20].

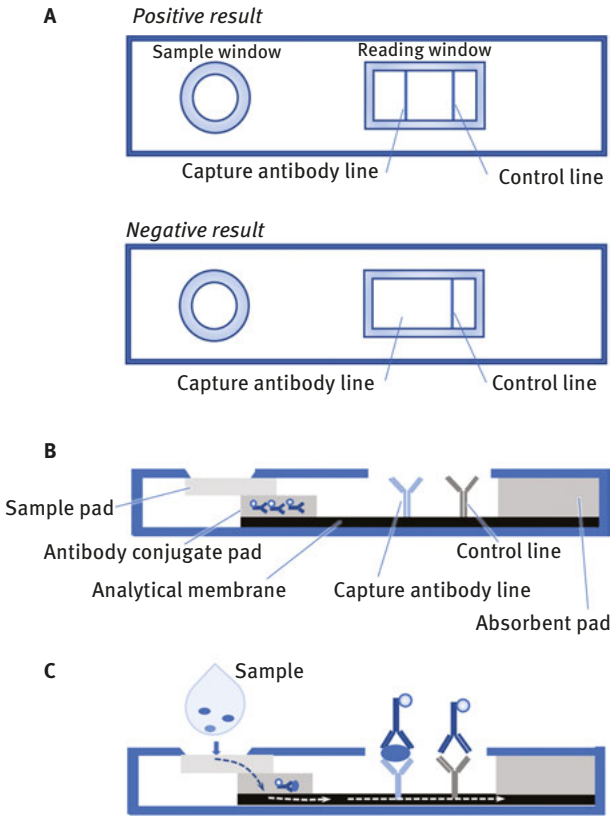

Figure 1.7: Lateral flow immunoassay (sandwich format). (A) Schematic representation of positive and negative results by visual score. (B) Internal design of the lateral flow device. (C) Diagram of sample application, flow (dashed lines) and capture antibody–analyte–nanoparticle-labeled antibody complex for a positive sample, and control line.

In spite of the limitations, there are immunoassays available for the detection of multiple contaminants, such as marine toxins, mycotoxins, or pesticides, using different approaches that include ELISA, multiplexing, or LFIA [10, 21, 22].

1.4.2 Receptor-based methods

Some techniques use binding of the analyte to a biological target as detection strategy. These techniques are not so different from immunodetection in design and may use similar formats to quantify binding to the receptor, although the competition assay is probably the most common.

An important advantage of receptor-based assays versus immunoassays is that the affinity of the receptor for the different analogs of a toxin group correlates better with toxic activity than antibody cross-reactivity [23]. On the other hand, receptor-based assays also have some limitations. Membrane receptors or ion channels are often the natural targets of toxicants. These proteins lose their activity and binding properties if they are extracted from the plasma membrane. Therefore, working with purified receptors is not always possible, and membrane fragments or vesicles containing the

receptors should be used. Although there are some receptor-based methods available for toxin detection, working with plasma membrane adds a degree of complexity and they are not so robust and commonly used as immunoassays. Another important disadvantage is that many of the receptor-based methods use radioactive labels, which are not well accepted for routine detection.

An example of target-based methods is the nicotinic receptor-based assay for cyclic imines that has been developed for detection in microplates with colorimetric, fluorescence, fluorescence polarization, or chemiluminescence read-outs and in multiplexed platforms using microspheres [24–27]. This method consists in a competition format, where cyclic imines compete for binding to the nicotinic receptor with fluorescent bungarotoxin. This assay also performed adequately after miniaturization in 384-well plates [25]. Receptor-based assays usually quantify the interaction of the analyte with the receptor. A better evaluation of toxicity would be provided by quantification of receptor functionality, which is usually performed with cells, instead of isolated receptors.

1.4.3 Enzymatic activity

Modulation of enzymatic activity can also be used to measure or detect contaminants. Frequently, the enzyme used for detection is a biological target of the analyte (or analyte class), providing as a result an evaluation of sample toxicity in relation with the contaminant class. Inhibition of enzymatic activity is most commonly used. In order to quantify the enzyme activity, the amount of a substrate transformed into a reporter product is measured by colorimetric, fluorescent, or luminescent detection. An enzymatic activity-based assay is the protein phosphatase inhibition assay for the detection of okadaic acid and its analogs. The inhibition of protein phosphatase activity is quantified by the reduction of a dephosphorylated product that can be measured by colorimetric or fluorescent techniques [28, 29]. Microcystins can also be detected with a similar assay [30]. Enzymatic assays are easily transferred to high-throughput platforms.

Optimization of enzymatic assays for sample analysis is complicated due to the complexity of biological matrixes. Many enzymatic reactions require specific conditions to take place, and therefore they are easily affected by modifications of the composition of the reaction medium after sample addition [31].

1.4.4 Aptamer-based detection

Aptamers are short sequences of single-stranded DNA or RNA that bind the analyte [17]. They are selected from synthetic libraries of oligonucleotides for high affinity binding to the analyte to increase selectivity and specificity. Aptamers offer advantages of high stability and chemical flexibility for labeling and addition of functional groups, when

compared to antibodies. Several designs are also possible, including sandwich and competitive formats. However, most aptamer-based detection methods are sensors, often called aptasensors, oriented to fast, low-cost, portable detection. Aptamer-based techniques are relatively recent and not as widely extended as immunoassays, although they have received a lot of attention and undergone remarkable development in the last decades. Therefore, interaction of analytes and aptamers might be used to optimize high-throughput methods in the future. Currently, aptamers for detection of mycotoxins, aquatic toxins, antibiotics, and pesticides have been reported [17].

1.4.5 Cell-based assays

Cell-based assays use the response of the cell to a substance to detect its presence in a sample. The nature of the measured response may be diverse. Probably, the most commonly used cell-based assay is quantification of cell viability, which serves as an indicator of cytotoxicity, reflecting a reduction of cell proliferation or cell death. There are multiple techniques to quantify cell viability with colorimetric, fluorescent, or luminescent read-outs (see Table 1.1), and many have been used in HTS of cytotoxic compounds [32].

Besides cell viability assays, other cellular events can also be used as markers of toxicity for the detection of hazardous substances, such as intracellular calcium concentration, membrane potential, or intracellular levels of cAMP. Plasma membrane proteins, mainly receptors and ion channels, are often targets of toxic compounds, and as mentioned above, they must be inserted in the membrane to preserve their binding properties and activity. At the moment, testing of an effect on the activity of these biomolecules is performed in the whole cell. Measurements of intracellular ion concentration, ion flux through the plasma membrane, or membrane potential have been optimized for the detection of molecules with activity on G-protein-coupled receptors or ion channels. HTS has been reported for these assay designs using ion- or voltage-sensitive fluorescent dyes and fluorescence plate readers with fluorescence intensity, FRET or fluorescence imaging read-outs [13, 33]. Automated electrophysiology is also available, although it is considered by some authors medium throughput [13]. Most electrophysiological screening methods measure current under voltage clamp conditions, providing an indication of ion fluxes through a specific channel type [13].

One of the main features of cell-based assays is the lack of specificity. The further down in the signaling cascade, the less specific the assay. For example, for a toxin that alters the function of an ion channel, a cell viability assay would be less specific than membrane voltage detection. However, specificity can be improved with an adequate assay design. A strategy used for specific detection of palytoxin in hemolysis and cytotoxicity assays consists of adding the drug ouabain that inhibits palytoxin effect on the Na^+/K^+ ATPase pump [34, 35]. Within the same assay, palytoxin is tested alone

Table 1.1: Techniques to measure cell viability in well plates [32].

Technique	Reagent	Measured product	Cell function	Read-out	Advantages/ disadvantages
Tetrazolium reduction assay	Tetrazolium compounds (MTT[a], MTS, XTT, WST)	Formazan	Viable cell metabolism	Colorimetric	Lower sensitivity Cytotoxic Endpoint assay[e] 1–4 h for signal Less expensive
Resazurin reduction assay	Resazurin	Resorufin	Viable cell metabolism	Fluorescent	More sensitive than tetrazolium assays Cytotoxic in the long term 1–4 h for signal Continuous assay[e] Less expensive
Protease viability marker assay	GF-AFC[b]	AFC	Constitutive protease activity	Fluorescent	Nontoxic 30 min–1 h for signal
ATP assay	Luciferin Firefly luciferase	Light[c]	Cellular ATP	Luminescent	Very sensitive Fast (10 min) Endpoint assay[e] Less handling required Expensive
Real-time luciferase assay	Shrimp luciferase Pro-substrate	Light[d]	Viable cell metabolism	Luminescent	Continuous assay[e] 10 min for read-out Real-time: rapid decay of signal after death Expensive

[a] 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.
[b] Glycylphenylalanyl-aminofluorocoumarin.
[c] Luciferin + ATP + O_2 → Oxyluciferin + PP_i + AMP + CO_2 + light. Reaction catalyzed by luciferase.
[d] Pro-substrate enters the cell and undergoes intracellular reduction to the luciferase substrate. The substrate exits the cell and luciferase catalyzes the enzymatic reaction with the production of light.
[e] Endpoint assay indicates that it requires cell lysis. Continuous assay allows monitoring of cell viability for hours without the need of cell lysis, and therefore may also be amenable to multiplexing.

to screen the sample for its presence, and in combination with ouabain as a positive control of the effect being caused through alteration of the Na^+/K^+ ATPase pump. Similarly, the presence of ciguatoxins is confirmed by potentiation of the response by veratridine in membrane voltage or cytotoxicity cell-based assays [36, 37].

In spite of the great potential of cell-based assays for contaminant detection, they have some important disadvantages that preclude their extended use for routine lab work. The maintenance of cell cultures requires significant resources in terms of equipment, material, and personnel. In addition, cell-based assays entail special challenges for method validation due to variability of cell culture conditions and genotypic and phenotytic instability of cell lines [13, 38], among others.

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Keywords: automation, screening, fluorescence, luminescence, absorbance, laboratory robot, immunoassay, ELISA, multiplexing, lateral flow immunoassay, cytotoxicity

Abbreviations: ELISA: enzyme-linked immunosorbent assay; FRET: fluorescence resonance energy transfer; HTS: high-throughput screening; LFIA: lateral flow immunoassay; SPE: solid phase extraction; TRF: time resolved fluorescence; TR-FRET: time-resolved fluorescence resonance energy transfer

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Inés Rodríguez and Amparo Alfonso

2 Analytical instrumentation and principles

2.1 Introduction

Analytical instrumentation provides information about chemical and physical properties of substances and about individual components of samples. Data obtained in this way can be used either for quantitative or qualitative purposes. Nowadays advanced analytical instruments are being developed, increasing not only their accuracy, but also their versatility and ease of use. An instrument for analysis converts the information about chemical or physical properties of some analyte to information that can be interpreted and manipulated. In this context several definitions should be considered:

- Analytical technique: chemical or physical principle used to study an analyte.
- Analytical method: application of a technique or a set of techniques to analyze a specific analyte in a specific matrix or used to know qualitatively and / or quantitatively the composition of a sample and the chemical state of the analyte.
- Analytical process: instructions for use an analytical method
- Analytical protocol: specific guidelines with all steps necessary to develop a specific analytical method

Analytical instrumentation includes traditional titrimetric and volumetric techniques and many other different procedures useful for the determination of active ingredients in a sample and for quantifying related compounds and impurities associated in order to know the composition of complex matrices. These techniques have the advantage of using small amounts of sample, reagents, and time.

The classification of methods included in analytical instrumentation can be done in two ways:

1. According to the information obtained:
   (a) Qualitative methods
   (b) Quantitative methods
2. According to the property measured:
   (a) Physicochemical methods: spectroscopic methods, including colorimetry, spectrophotometry, fluorimetry, nephelometry, turbidimetry, nuclear magnetic resonance (NMR), and mass spectrometry (MS).
   (b) Electro-analytical methods: potentiometry, amperometry, voltammetry, electrophoresis, and polarography.
   (c) Separation-based methods: used to separate individual components from a mixture. This includes methods such as extraction, filtration, distillation, chromatography, centrifugation, and crystallization. Electrophoretic techniques are also included in separation methods.

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In this way, a wide range of old and new techniques, tools, and instruments can be included in analytical instrumentation. The present chapter will focus on separation systems, particularly liquid chromatography (LC) coupled to different devices to identify and quantify environmental toxicants. A summary of separation and chromatographic techniques is provided in Figure 2.1. To select a separation technique it is important to consider physical and chemical properties of substances that need to be separated, the size of the sample, the concentration of analyte, the composition of matrix, and the number of samples.

Environmental toxicants such as marine toxins, cyanotoxins, or mycotoxins are small molecules with different chemical characteristics, Table 2.1. (see Chapters 6, 7 and 8). These compounds are present in complex matrices; therefore, for their analysis it is necessary to use a combination of several separation methods, mainly extraction procedures and chromatographic techniques for final identification (Figure 2.2).

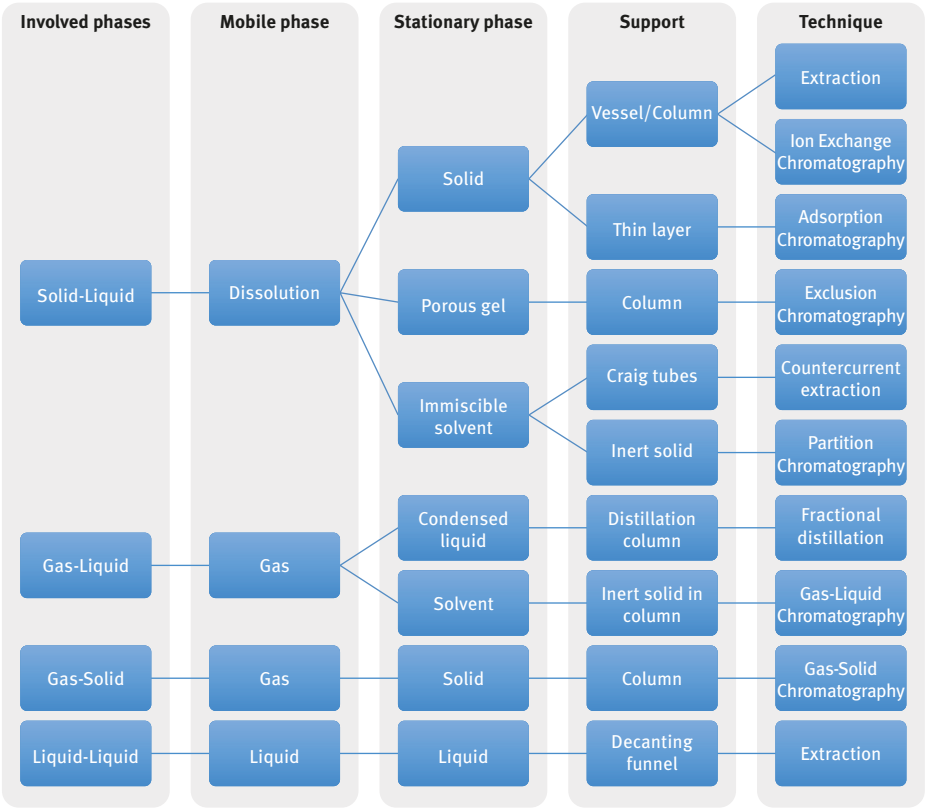


Figure 2.1: Separation and chromatographic techniques.

Table 2.1: Solubility and polarity characteristic of main marine toxins, cyanotoxins and mycotoxins groups.

TOXICANT	LIPOPHILIC/APOLAR	HYDROSOLUBLE/POLAR
Marine toxins	Okadaic acid, Dinophysistoxin I and Dinophysistoxin II (Diarrheic Shellfish Toxins, DSP) Azaspiracids (AZAs) Yesotoxins (YTXs) Pectenotoxins (PTXs) Cyclic Imines (CIs) Ciguatoxins (CTXs) Brevetoxins (PbTXs)	Saxitoxin group (Paralytic Shellfish Toxins, PSPs) Domoic acid (DA) Tetrodotoxins (TTXs)
Cyanotoxins	Microcystins (MCs) Nodularins (NOD)	Anatoxins (ATXs) Cylindrospermopsin (CYN) Saxitoxin group
Mycotoxins	Fumonisins Zearalenone Aflatoxins	Deoxinivalenol Patulin

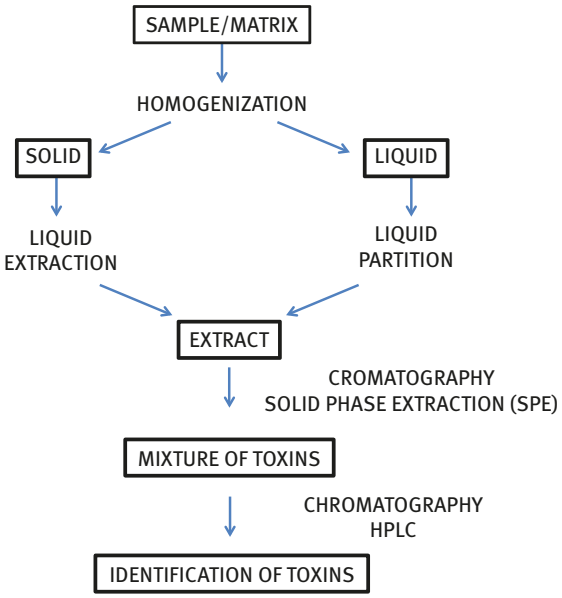


Figure 2.2: Scheme of separation methods used to analyze toxins.

2.2 Sample/matrix extraction

The first essential step in the analysis of toxic compounds is the adequate extraction of the compound of interest from the matrix. This step includes sample preparation and extraction procedures.

2.2.1 Sample preparation

The sample or matrix should be homogeneous in order to achieve good extraction efficiency. To homogenize the sample, if solid (e.g.,: shellfish, fish, or grains), a blender or grinder is often used. In the case of official analysis, the sample should be representative of the lot to be analyzed, therefore the sampling procedure should be carried out according to specific rules [1–3]. In any case the sample should as representative as possible. Table 2.2 shows the amount of sample that should be processed and homogenized and the amount used from this homogenate for the extraction of each group of toxins following official standard procedures.

2.2.2 Extraction procedure

The extraction procedure for compounds is different depending on the matrix state (solid of liquid):

2.2.2.1 Extraction procedures from solid matrices (solid–liquid extraction)

In this case the matrix is mixed with a solvent. The polarity of the solvent used should be according to the nature of the compound to be extracted. Organic solvents such as acetone, methanol, or acetonitrile are used to extract nonpolar/lipophilic compounds as in the case of lipophilic marine toxins, while aqueous solutions are used to extract polar/hydrophilic

Table 2.2: Amount of sample processed for representative sampling and amount necessary for the extraction following official standard operating procedures to identify toxins. AOAC: Association of Official Analytical Chemists.

Toxicant	Sample processed	Amount used for extraction	Official method
Lipophilic marine toxins: DSP, AZAs, YTXs, PTXs, CIs	100–150 g shellfish tissue	2.00 ± 0.05 g	[4]
PSPs	100–150 g shellfish tissue	5.00 ± 0.1 g	[5, 6]
DA	100–150 g shellfish tissue	4.00 ± 0.1 g	[7]
	Scallops: at least 10 specimens		[8]

Table 2.3: Solvents used to extract toxins from solid matrices.

Toxicant	Extraction solvent
Lipophilic marine toxins: DSP, AZAs, YTXs, PTXs, CIs	Methanol
CTXs	Acetone or Methanol
PbTXs	Acetone
PSPs	HCl 0.1N or Acetic acid 1%
Domoic acid	Methanol (50%)
TTXs	Acetic acid 1%
Mycotoxins	Water: Acetic Acid (49:1) – Acetonitrile (50)

compounds such as PSPs, domoic acid, or tetrodotoxin (TTX). Sometimes a mixture of solvents in different proportions is used to extract compounds of different nature from the same sample; this is the case of mycotoxins. The solvent (pure or combined) is mixed with the matrix and the separation of both phases is achieved by gravity, filtration, or centrifugation. The compound of interest will be in the liquid phase. A summary of solvents used to extract toxins from different solid matrices is given in Table 2.3.

2.2.2.2 Extraction procedures from liquid matrices (liquid–liquid extraction)

In this case the extraction of toxins from the liquid is based on the toxin distribution between both phases and on its ability to migrate from one to the other. In a simple liquid–liquid extraction two immiscible phases are used, one aqueous and the other an organic solvent such as diethyl ether, chloroform, or hexane. These phases are immiscible and form two layers, with the denser phase at the bottom. The extraction efficiency is the percentage of toxin moving from one phase to the other, and it is determined by the partition coefficient of the toxin between both phases. In some cases, such as mycotoxin extracts, the partition between phases is forced using a mixture of salts to saturate the aqueous phase and to force mycotoxins to migrate to the organic phase, this is called dispersive liquid–liquid extraction [9]. In addition, to extract some toxins from liquid matrices, resins or polymers to attach these compounds are also used [10]. The binding toxin-resin is specific and saturable. The resin is introduced in mesh bags to be easily extracted from liquids. In the case of cyanotoxins coming from water sampling, the sample should be filtrated and concentrated and some step to lysate cells should be incorporated [11].

2.3 Chromatography

Chromatography is a commonly used technique to analyze and identify the extract obtained from matrix. In addition, chromatography is also used to clean and prepare the sample before analysis.

Chromatography is defined as an analytical technique of separation in which a chemical mixture (in solution or suspension) carried by a liquid or gas is separate into components by passing it through a stationary phase. Several methods are included under this term, this is the case of LC, thin-layer chromatography (TLC), gas chromatography (GC), and supercritical-fluid chromatography (SFC) among others. The method is based on the distribution of components between a mobile phase and a stationary phase. Sample components interact with the stationary phase with different affinity and are dragged by the mobile phase. Their ability and rate of migration are based in their interaction with the stationary phase, higher interaction gives rise to lower migration.

2.3.1 Classification

The classification of chromatographic methods can be done according to different criteria:

2.3.1.1 Based on phase combination

- Stationary phase: liquid or solid
- Mobile phase: liquid, supercritical-fluid or gas

Stationary phase	Mobile phase		
	Gas	**Supercritical-fluid**	**Liquid**
Liquid	Gas–Liquid Chromatography (GLC)	Supercritical-fluid Chromatography (SFC)	Liquid–Liquid Chromatography (LLC)
Solid	Gas–Solid Chromatography (GSC)	Supercritical-fluid Chromatography (SFC)	Liquid–Solid Chromatography (LSC or LC)

2.3.1.2 Based on the mechanism of separation

- Partition chromatography
- Adsorption chromatography
- Ion-exchange chromatography
- Molecular exclusion, size exclusion, or gel filtration chromatography
- Affinity chromatography
- Electrophoresis
- Chiral chromatography

2.3.1.3 Based on phase polarity

- Normal-phase chromatography: mobile phase nonpolar and stationary phase polar
- Reverse-phase chromatography: mobile phase polar and stationary phase nonpolar

2.3.1.4 Based on the shape of chromatography bed

- Planar chromatography: Paper chromatography
  TLC
- Column chromatography: Packed chromatography
  Open tubular column chromatography

2.3.1.5 Based on the development procedure

- Frontal chromatography
- Displacement chromatography
- Elution chromatography

To separate toxins and to obtain qualitative and quantitative information, column chromatography, with different solid stationary phases and different mobile phases (liquid) depending on the analyte, is frequently used. The terms commonly used in the context of column chromatography are summarized in Table 2.4. In general, the mobile phase is a liquid and the stationary phase is a solid held in a column.

2.4 Column chromatography – Solid phase extraction (SPE) and sample preparation

In the classic liquid column chromatography the stationary phase is placed in a vertical glass or plastic column. The mobile phase, a liquid, is added to the top of the column and flows down (through the stationary phase) by either gravity (Figure 2.3) or an external low-pressure, flash chromatography (Figure 2.4). The sample, with compounds to be separate, is added on the top and separation of components is

Table 2.4: Terms used in column chromatography.

Term	Definition
Mobile phase or carrier	Solvent moving through the column
Stationary phase or adsorbent	Substance that stays fixed inside the column (solid or liquid)
Eluent	Fluid entering the column
Eluate	Fluid exiting the column (collected in fractions)
Elution	The process of washing out a compound through a column using a suitable solvent
Analyte	Mixture whose individual components (toxins) have to be separated

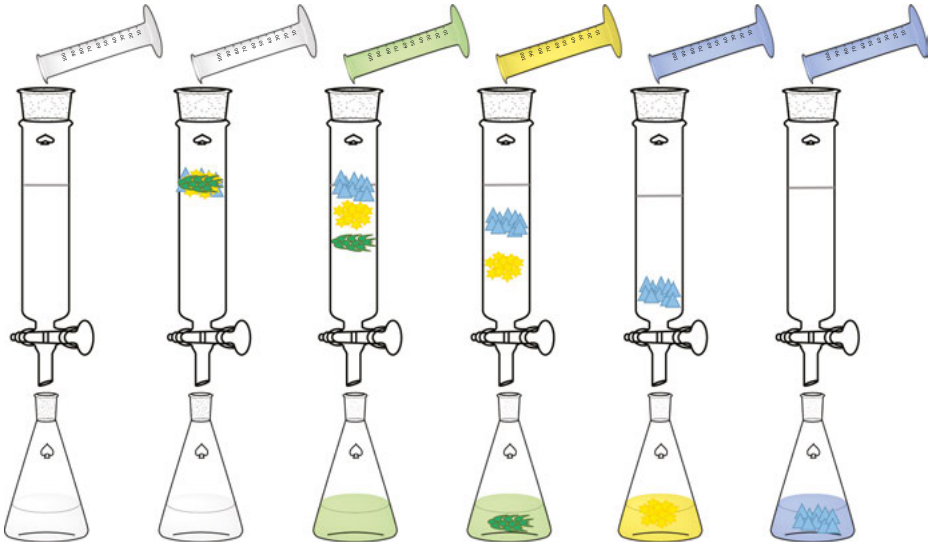


Figure 2.3: Separation of analytes by gravity column chromatography (solid stationary phase-liquid mobile phase).

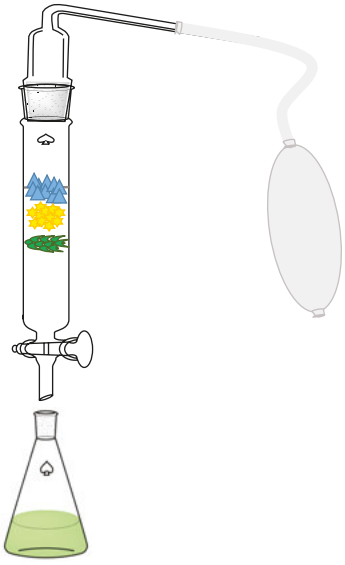


Figure 2.4: Separation of analytes by flash chromatography (solid stationary phase-liquid mobile phase).

achieved through the varying interaction between phases. Fractions with components are separated by time units (minutes or seconds) or volume (drops or mL) and collected at the end of the column. The quality of the separation depends on several factors, particularly the absence of air bubbles in the stationary phase. To avoid air bubbles correct packing of stationary phase is important.

This type of chromatography currently applied to prepare and clean samples to avoid interferences in detection is also called SPE. The stationary phase is a solid adsorbent such as silica gel, alumina, dextran or agarose polymers, porous graphitic carbon, or synthetic resins like Biogel®, Diaion®, or Florisil®. The size of particles is usually given by the mesh value, which refers to the number of holes in the mesh used to sieve the adsorbent. Therefore, higher mesh value means more holes per unit area and correspondingly smaller particles. Around 70–230 mesh particles are used for gravity columns and 230–400 mesh particles for flash columns.

Silica gel is usually modified with long hydrocarbon chains (8 or 18 carbon atoms) and the columns are called C18 ODS (octadecyl silice), or C8. Alumina is available in three types: I, II and III (from lower to higher water content). The basis of separation in this chromatography is the polarity of molecules, and silica gel and alumina are both polar adsorbents. In this way chromatography techniques can be classified into two types.

Normal-phase chromatography: when the stationary phase is more polar than the mobile phase. In this case, the more polar components in a sample will be retained strongly on the stationary phase while nonpolar compounds will be not fixed by these adsorbents. Therefore, nonpolar compounds will be eluted faster than polar compounds. Thus, the components of a mixture can be adequately separated by increasing the polarity.

Reverse-phase chromatography: when the mobile phase is more polar than the stationary phase. Common polar solvent mixtures are water, methanol, and acetonitrile. Main solid adsorbents used as stationary phases are polar by nature. Nonpolar stationary phases are prepared for example by coating silanized silica gel with nonpolar liquids (with silicone or hydrocarbons). With reverse phase, the most polar compounds will be eluted first and the components following will have decreasing polarities.

When the stationary phase is a resin, depending on its nature, ion-exchange, size-exclusion, partition, or affinity chromatography can be used.

Flash chromatography is a modification of classic column chromatography in which the mobile phase moves faster through the column with the help of either vacuum or pressurized air. For this reason, it is considered as medium-pressure chromatography while use of gravity is considered low-pressure chromatography. The air used should be inert in order to not interact with the mobile or stationary phases or the sample.

These SPE protocols are currently used for preparative and cleanup purposes, to clean samples with toxins before analysis. As mentioned, the polarity of toxins is important to choose the stationary and mobile phases. C8, C18, porous graphitic carbon, or Biogel® is used to clean and prepare samples with PSPs [6, 12, 13]. C8, C18, and porous graphitic carbon cartridges are used to process samples with TTXs, while C18, silica gel, and Florisil® are used with ciguatoxin (CTX) samples [14–17]. The mobile phase and the protocol of elution should be different in each case.

2.5 High-Performance Liquid Chromatography (HPLC) analysis

HPLC is the most common analytical technique used worldwide to analyze a sample because it allows the separation, identification, and quantification of components in a mixture. HPLC is a modification of column chromatography. In this case, the stationary phase, a resin, is tigthly packed into a column. The resin is a granular material made of small solid particles such as silica or polymers. A pump is necessary to force the elution of mobile phase through the column under high pressures of up to 400 atmospheres (404 bars). The pressure makes the technique much faster compared to column chromatography, the efficiency is higher and this method provides continuous quantitative and qualitative information. Smaller particles in stationary phase have a much greater surface area for interactions with molecules passing through. This results in a much better separation of the components of a mixture. The components of a liquid chromatograph are presented in Figure 2.5:

- Mobile phases: A and B reservoirs with solvents or mixtures of solvents such as water, acetonitrile, and or methanol
- Degasser: to eliminate bubbles solved in the mobile phases to avoid irregular fluxes and fluctuations in detector response
- Pump: to force the mobile phase through the column
- Injector: to introduce the sample without modification in mobile phase pressure
- Column oven: to keep constant column temperature (T)
- Chromatographic column: stationary phase
- Detector: to detect the components eluted from the column

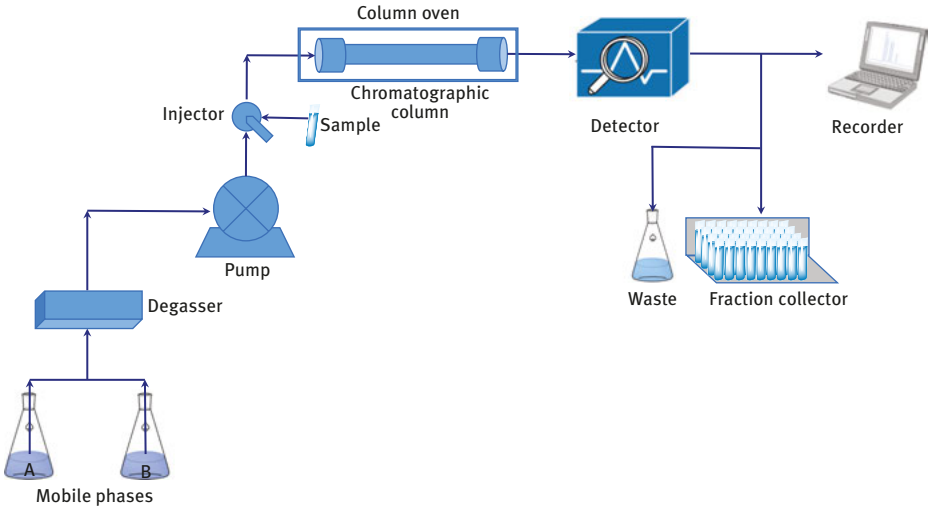


Figure 2.5: Liquid chromatographer.

- Fraction collector: to collect fractions after detection
- Recorder: to collect data that can be interpreted and manipulated.

The response obtained after a chromatography is called chromatogram where the signal obtained from the detector is represented *versus* the time. Each component is represented as a peak and the time when the peak appears is called retention time. The components of a sample are separated according to the time retained by the stationary phase, that is according to the affinity for the stationary/mobile phases. These differences can be due to different chemical or physical properties including:

- Ionization state
- Polarity and polarizabilty
- Binding (hydrogen bonding, Van del Waals forces)
- Hydrophobicity
- Hydrophilicity

2.5.1 Components of liquid chromatograph

2.5.1.1 Mobile phases and elution

Mobile phases should be free of solids or particles in suspension, therefore should be filtrated or of high purity. Reservoirs are glass containers (clear or amber). In general, a 2–5-µm filter is placed in the inlet of suction tube to avoid any particles in the suspension. In addition to eliminating air bubbles solved in the mobile phases, a degasser is connected before the pump. Depending on the mobile phase composition the elution can be:

- Isocratic elution: the composition of the mobile phase is constant though the separation time.
- Gradient elution: the composition of mobile phase changes with time during separation in order to change the polarity to increase efficiency. If the chromatography is normal phase, the polarity of solvents in gradient must be increasing (from less to high). If the chromatography is in reverse phase, the polarity of solvents must be decreasing.

Methanol, water, and acetonitrile are the most common solvents used as mobile phase. When the sample contains ionizable compounds, the mobile phase pH should be controlled, for this reason buffers are used. In addition, the stability of the columns is also affected by mobile phase pH. Depending on the pH, different buffers should be selected taking into account that a buffer is most effective at ±1 pH units of its pKa. Besides the buffer is also important in terms of detection, since it can be appropriated or not, depending on the detector. For example, with citrate buffers ultraviolet detection bellow 220 nm is not possible and MS cannot be used either.

2.5.1.2 Pumps

Pumping systems force flow of mobile phases through the column. To properly perform this function, some operating requisites are necessary:

- Wide range of pressure (0–1200 bars)
- Variable fluxes (0.01–10 mL/min)
- Controlled and reproducible fluxes
- To be mechanically reliable
- Pulsation-free fluxes
- Inert materials in contact with fluids

Reciprocating piston pumps with two pistons are the pumping systems most frequently used. The basic elements in these pumps are a cylindrical pump chamber that holds a piston with a motor that operates a driving cam (this is called plunger because the motor rotates and the piston is moved in and out of the pumper chamber) a pump seal and a pair of check valves. Pistons are usually made in sapphire, stainless steel or graphite. Check valves control the direction of flow through the pump. The pump seal keeps the mobile phase from leaking around the piston. The systems with two plungers provide more uniform fluxes. In addition, a pulse damper is included to avoid signal fluctuations in detectors and to improve baseline noise. Nowadays binary and quaternary pumps are commonly chosen in HPLC systems. Binary pumps have two channels. Each channel has a system with two plungers and inlet and outlet valves. Both channels are connected through a low-volume mixer chamber. Quaternary pumps are based in a system of one channel with two plungers and a valve to select the solvent line from four.

2.5.1.3 Injection

The injection system, autosampler, is located between the pumping system and the column. This device introduces the sample (0.1–100 µL for analytical purposes or up to 5.000 µL for preparative purposes) at atmospheric pressure into the high-pressure system. This is a critical step in the chromatographic process. In this way, the requirement of stopping the mobile phase flow to inject the sample is eliminated. To allow this, sample injection valves (switching valves or rotor seal valves) are used. This system introduces reproducible amounts of sample in the mobile phase stream without causing changes in pressure or flow. In addition, any disturbance in the chromatographic baseline is minimized. Autosamplers are the first contact of sample with HPLC instrumentation and therefore should be manufactured from inert materials. But, contamination problems can still appear in the form of sample carryover due to sample adsorption in the injection system (rotor seal contamination). To avoid this, autosamplers are provided with washing systems able to eliminate any sample rest. Washing solutions should be selected according to sample solubility to clean residual sample.

2.5.1.4 Columns

Columns are the essential part of HPLC system that will provide narrow peaks. Column efficiency and performance are measured by the number of theoretical plates (TPN), the bigger the TPN, better the column. A typical column has an internal diameter of 4.6 mm or smaller and a variable length from 50 to 250 mm. Small internal diameter columns increase sensitivity, but they may decrease efficiency and resolution. In semi-preparative columns and in preparative columns internal diameter is increased to 10mm and 20 mm, respectively. These columns require flow rates of 10–20 mL/min while in analytical columns the optimum flow rate is 1–0.2 mL/min. Long columns increase efficiency and resolution over short columns, but also increase retention times and backpressure. Short columns can provide adequate resolution and high speed of analysis. The particle size of resin inside the column is also important. A particle size of 5 µm is the most common for speed and resolution in analytical columns. Smaller particles can increase resolution and decrease analysis time. When the size is lower than 3 µm the pressure is increased, this is the case of ultra-high performance liquid chromatography (UPLC) columns, with 1.5–3-µm particles. The pore size of internal particles is also important, the smaller the pore size the larger surface area. For small molecules like environmental toxicants the pore size should be 80–150 Å. Silica is the most popular resin used for pH between 1.0 and 7.5. Other polymers are used for extreme pH. The resin is usually packed in stainless steel cartridges to support high pressures. Depending on the molecules to be separated, different chromatographic techniques will be chosen. Normal-phase columns are packed with polar resins like silicia, amide, or hydrophilic interaction liquid chromatography (HILIC) to separate small polar compounds by hydrophilic interactions [18]. Reverse-phase columns are the most widely used for samples with polar, nonpolar, ionic, or ionizable analytes. This phase is compatible with mobile phases containing methanol, water, or acetonitrile. C6, phenyl, C18, and C8 resins are often selected for reverse phase, while for polar analytes others like porous graphitic carbon are used. To separate toxins, either normal or reverse-phase columns are selected [16, 19, 20]. Figure 2.6 shows different stationary-phase resins for HPLC. Other chromatography resins for ion chromatography, ion-exchange chromatography, ligand exchange, or size exclusion, among others, can be selected depending on the analyte, inorganic compounds, amino acids, pesticides, carbohydrates, etc.

2.5.1.5 Oven

In addition to the appropriate column, the temperature of separation is also crucial. This should be as constant as possible; therefore the oven is essential to keep constant column temperature.

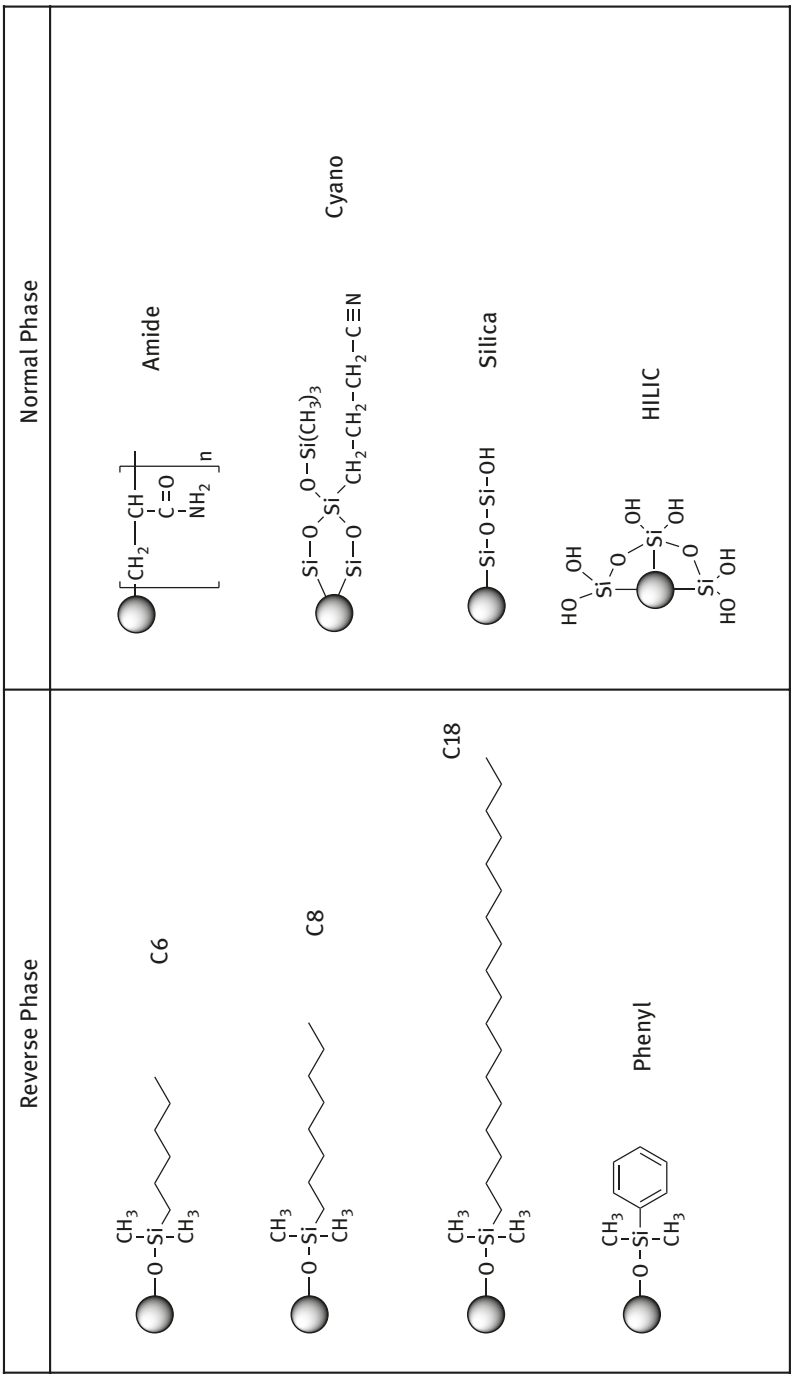


Figure 2.6: Resins for stationary phases (normal and reverse).

2.5.1.6 Detectors

Detectors are devices to convert a physical or chemical change into a measurable signal and in this way to recognize an analyte. In LC detection, these devices are used to identify and determine the concentration of eluting compounds in the mobile phase coming from the column. Detectors should have several characteristics such as high sensitivity toward solute over mobile phase, reproducible responses, either specific or general response to compounds in a mixture, wide dynamic range, unaffected by changes in temperature or mobile phases, and fast response, among others [21]. In addition, detectors should have a low volume in detection cell and low noise detection and limits. The detectors often used in LC can measure specific or bulk properties and in this way can be classified into two groups

2.5.1.6.1 Bulk property detectors

Electrochemical detectors

These are used for compounds that can be oxidized or reduced and require the use of electrically conductive mobile phase. The system has three electrodes (a counter, a working, and a reference electrode) and the reaction takes place in the working electrode surface after the application of a fixed potential difference between working and reference electrodes [21]. These detectors are sensible and selective but aqueous mobile phases or polar solvents are required with electrolytes (oxygen free). In addition, flux and mobile phase conditions are important.

Refractive index detectors

These are the oldest LC detectors. In this case, the property measured is the difference in optical reactive index between mobile phase and sample. This is a differential detector, therefore the higher optical reactive index the bigger signal.

Conductivity Detection detectors

In this case, the conductivity of the mobile phase is measured. This property changes when analytes are passing through the detection cell.

Light Scattering detectors

The property measured is the amount of scattered light, and these detectors are useful to measure large-molecular weight compounds present in the mobile phase.

2.5.1.6.2 Specific property detectors

Ultraviolet (UV)-Visible detectors

The property measured is the absorbance of eluate. This is a common LC detector since many compounds of interest absorb in the UV or visible region. Sample concentration

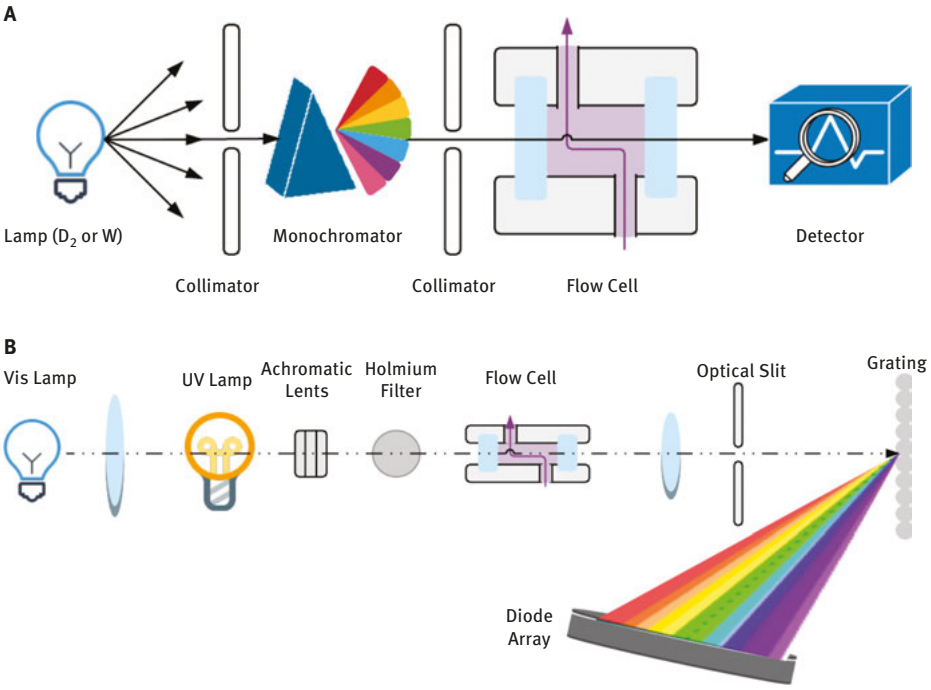


Figure 2.7: A.-Scheme of UV detector. B.-Scheme of photodiode array detector.

is a function of the fraction of light transmitted through the detection cell. Mobile phase composition is important for optimum sensitivity and linearity in detection. These detectors can have fixed or variable wavelength (Figure 2.7A), or photodiode array that rely one or more wavelengths generated by a broad-spectrum lamp. As Figure 2.7B shows, in this case the light passes through the flow cell prior to hitting the grating, allowing it to spread the spectrum across an array of photodiodes. UV detection is used in the official method of DA detection after HPLC separation and also to detect palytoxins, PbTX, and some mycotoxins [22, 23].

Fluorescence detectors (FLDs)

The property measured is the optical emission of light by analytes after they have been excited to a higher wavelength. These detectors are more sensitive, specific, and selective than UV-visible detectors. Analytes should have native fluorescence or should be converted into derivatives with fluorescent properties. The excitation light source is a lamp with broad spectrum (deuterium or xenon) and excitation wavelength is selected by a filter or a monochromator (Figure 2.8). In some cases a laser can be used instead of the lamp. After sample excitation, the FLD should have a filter system or monochromator to select the most appropiated emission wavelength of analyte. The dynamic range is high (change in fluorescence when concentration of analyte changes); however the

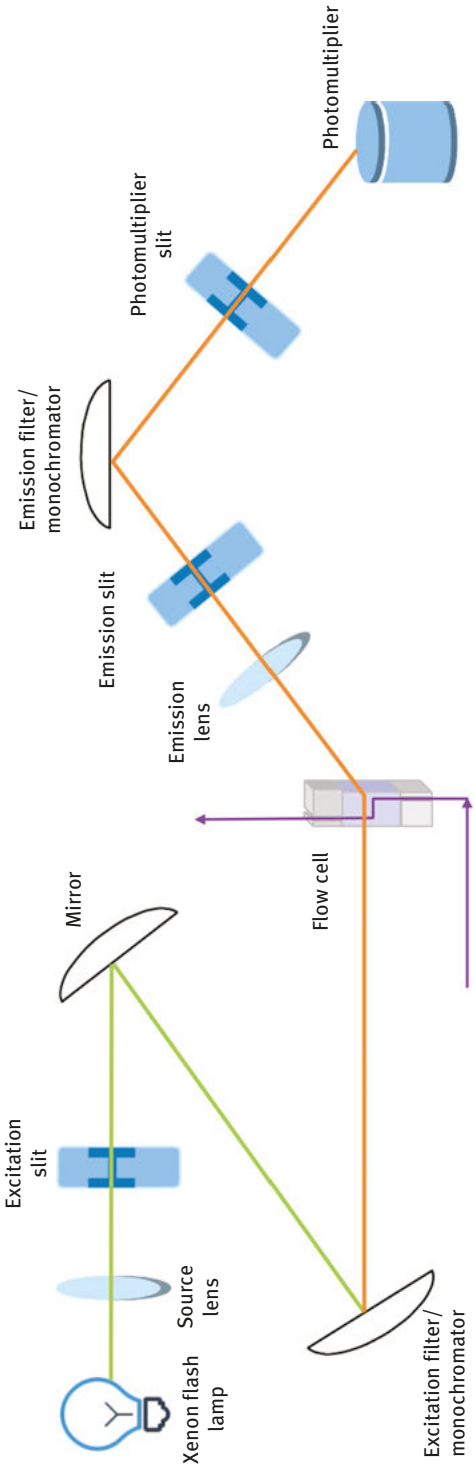


Figure 2.8: Scheme of FLD detector.

linear range is small. In addition, the composition of mobile phase and interferences of matrix should be taken into account. The FLD is often used to detect environmental toxicants after HPLC separation [23]. Several cyanotoxins and marine toxins can be detected by fluorescence after derivatization [23]. In the case of PSPs, the conversion into fluorescent molecules by oxidation can be done before or after HPLC separation [6, 24]. In the case of palytoxins a pre-column derivatization method was also developed. FLDs are also used to detect microcystins (MCs) and anatoxins (ATXs) [11, 25].

Mass spectroscopy (MS) detectors

The property measured is the difference in mass-to-charge ratio (m/z) of ionized molecules to separate them. In addition, molecules can be fragmented by electrical fields. In this way MS allows the quantification of analytes and provides structural information by identification of distinctive fragmentation pathways. This is currently the detection with higher applications because of advantages such as small sample size, high sensitivity, specificity, and resolution of time. However, using MS samples are destroyed; therefore for preparative purposes a splitter should be included. In addition, this is a very costly technique, in terms of technical and human resources.

MS is an analytical technique by which chemical substances are identified by the sorting of gaseous ions in electrical and magnetic fields. MS consists of four basic parts: first a handling system to introduce the sample in the equipment, second a gas phase where ions should be created, then the analyzer where ions will be separated in space or time based on their m/z and finally a detector where detection and measure of the quantity of ions of each m/z will be done (Figure 2.9).

MS operates under vacuum conditions to have a collision-free path for ions. The sample inlet system is designed for minimal loss of vacuum when the sample coming from LC is introduced. From the inlet system, the sample is introduced into

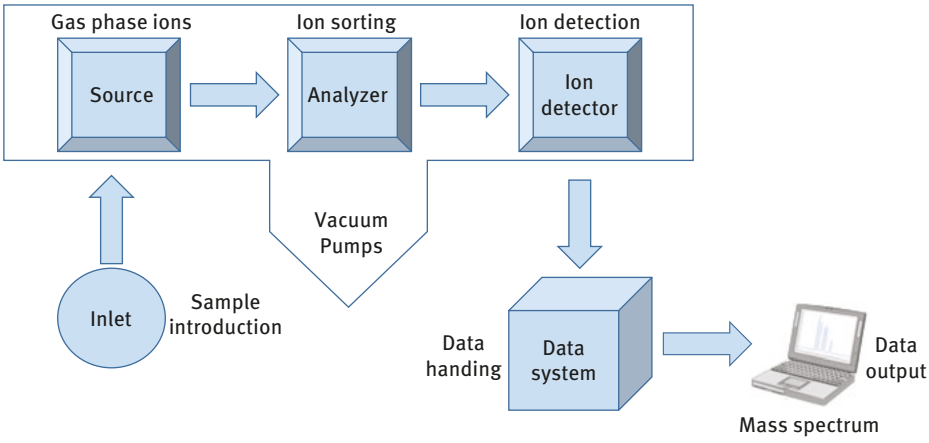


Figure 2.9: Scheme of mass spectrometer detector.

the ionization chamber. Ion fragments can be created in two different ways, gas phase (volatile substances and vaporized samples) or desorption techniques (samples in condensed phase inside the ionization chamber):

Gas phase: This ionization includes:
- chemical ionization (ionization is produced by collision of sample molecules with a reagent of gas)
- electro-impact ionization (ionization is produced by an electron beam generated from a filament; ions are formed during collision of the electron beam and sample molecules)
- field ionization (ionization is produced because molecules can lose an electron when placed in a very high electric field created by applying high voltage)

Desorption: This ionization includes:
- field desorption, useful for nonvolatile, thermally unstable molecules and large lipophilic or polar molecules (ionization is produced by high potential applied in an electrode)
- fast atom bombardment, useful for large biological molecules difficult to get into gas phase (ionization is produced when a high-energy beam of neutral atoms, xenon, or argon, strikes a solid sample)
- electrospray ionization, in this case ionization is produced at atmospheric pressure and it is useful for polar molecules (the sample is dispersed by an electrospray into a fine aerosol and it is highly charged due to high voltage employed to disperse the liquid following by solvent evaporation)
- matrix-assisted laser desorption ionization: sample and matrix are co-crystallized (ionization is produced after crystallization application of short pulses of laser in a high vacuum, which causes the absorption of energy by the matrix, that it is converted into excitation energy and used to sample ionization)

In mass analyzers, the ions produced in the ion source are separated according to their *m/z* ratios. The most common analyzers are magnetic sector, quadrupole, and time of flight. Magnetic sector uses a magnetic field that causes ions to travel in a circular path. In quadrupole analyzers, the field is formed by four electrically conducing parallel rods (adjacent rods have opposite polarity) where ion circulation is dependent on applied voltage (for given voltages only ions with certain *m/z* ratios are allowed to pass through while others, such as uncharged molecules, are carried away). Time-of-flight analyzers use the differences in transit time though a drift region to separate ions of different masses previously accelerated by an electrical pulse. In addition, ion-trap analyzers use electrical fields to trap ions in a small space and to separate them by certain *m/z* values. Others like ion cyclotron resonance and electrostatic mass analyzers can also be included.

Tandem mass spectrometry (MS/MS) refers to the use of a second stage of analysis, that is two mass analyzers in the same experiment; in this way selected ions from a

complex mixture can be studied. The first analyzer (Q1) is used to select the ion of interest, which is driven into a pressurized collision cell with an inert gas. In this collision cell, selected ions are dissociated after collision with the gas (collision-induced dissociation). Ions produced after dissociation, fragment ions, are analyzed in the second analyzer (Q3). In this way, product ions from the precursor ion are analyzed. This is called MS^n, where n is the number of mass analysis. An example of tandem equipment is formed by tandem quadrupoles. Originally, three tandem quadrupoles were used but nowadays devices are composed by two quadrupoles separated by the collision cell (Figure 2.10). In these devices dissociation and fragmentation of precursor ion is produced in the collision cell where fragments are retained and then liberated to the second analysis stage. Different experiments can be done with tandem MS detection:

- Full scan: Q1 analyzer is working in scan mode covering a specific mass range, in this way a mass spectrum is registered for each analyte. The chromatogram obtained shows a line (total ion chromatogram) where the mass spectrum of each time interval can be observed.
- Single-Ion Monitoring (SIM): Q1 analyzer only detects selected ions. The chromatogram obtained shows peaks of selected ions.
- Multiple Reaction Monitoring (MRM): Q1 and Q3 working in SIM mode. Q1 analyzer selects precursor ions that are fragmented in the collision cell and Q3 monitors the corresponding product ions of each precursor ion. The chromatogram obtained shows a peak with all product ions (*m/z* values with different intensity) for each precursor.
- Product Ion Scan: In Q1 analyzer a precursor ion is selected, then this ion is fragmented in the collision cell and finally the product ions are detected in the Q3 analyzer. The chromatogram obtained shows peak of precursor, and the mass spectrum of peak shows *m/z* values of product ions.
- Precursor Ion Scan: Q1 analyzer works in full scan mode and Q3 analyzer works in SIM mode. In this way, Q1 is scanning across a specific mass range. Ions in this mass range are passed into the collision cell where they are fragmented, thus giving the product ions of interest. The chromatogram obtained shows the peak of product ions, and the mass spectrum of peak shows the *m/z* value of precursor ion.
- Neutral Loss Scan: Q1 and Q3 both work in full scan mode. In this way, Q1 is scanning across a specific mass range. The selected ions are passed into the collision cell where they are fragmented and Q3 is scanned over a similar mass range, offset by the neutral mass of the diagnostic fragment.

Examples of these chromatograms for TTX are shown in Figure 2.11

Finally, ion beams after passing through mass analyzer strike the detector, where ions separated according to their *m/z* values in the mass analyzer can be electrically detected. In this way, a mass spectrum is obtained. The spectrum represents the relative abundance of ions of different *m/z* values produced in an ion source and it contains molecular weight, structural, and quantitative information.

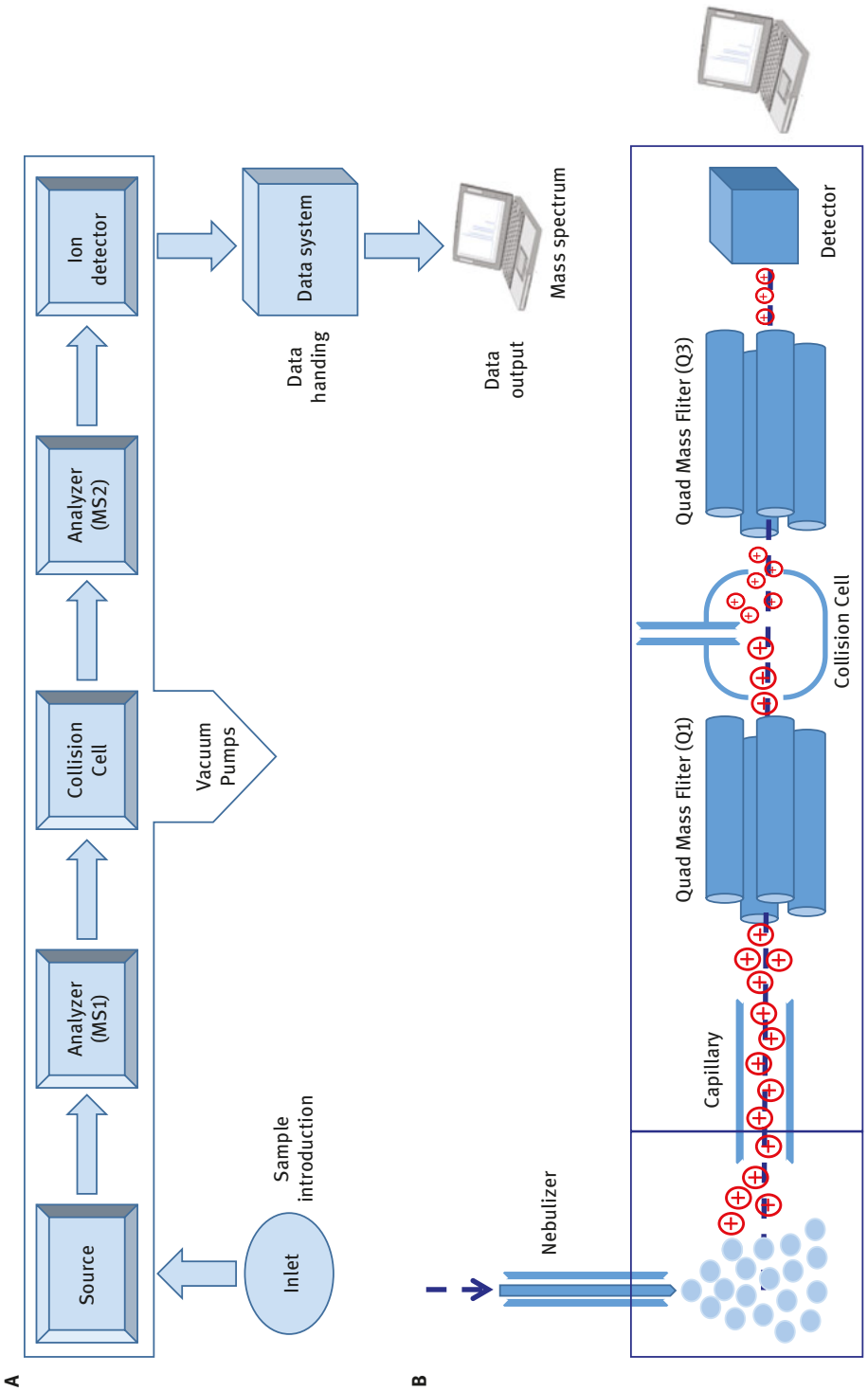


Figure 2.10: (A) Tandem mass spectrometer. (B). Detail of ion fluxes and fragmentations.

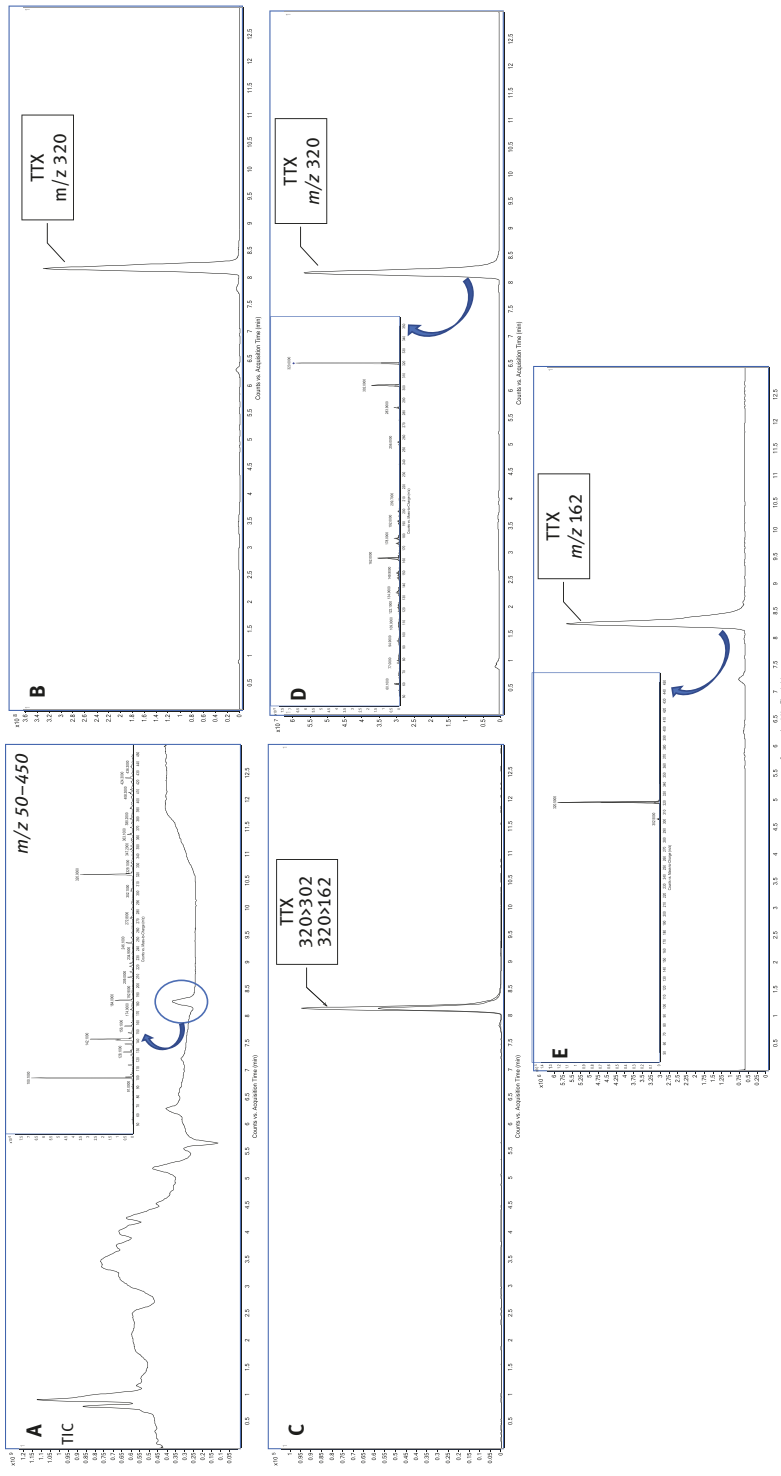


Figure 2.11: Chromatograms and mass spectrum of different experiments with TTX obtained by tandem Mass spectrometry. (A).-Full Scan. (B).-Selected Ion Monitoring (SIM) mode. (C).-Multiple reaction Monitoring (MRM) mode (D).-Product Ion Scan. (E).-Precursor Ion Scan mode.

When a sample is analyzed, all MS parameters such as source temperature, voltage, or collision energy, should be previously optimized with compound standards in order to achieve the maximum level of sensitivity since these parameters are dependent both on equipment and toxin.

MS/MS tandem detectors coupled to LC are widely used to identify and quantify all kinds of environmental toxicants. Calibration curves of signal versus concentration can be constructed (see below) and any toxin can be quantified. The calibration curve of one toxin standard is sometimes used to quantify other toxins from the same group, assuming an ionization-conversion factor of 1:1. However, it is incorrect to assume that analogs from the same group provide an equimolar response by MS/MS tandem detection.

2.6 Liquid chromatography for toxin identification

The analysis of environmental toxicants is a challenge due to their wide range of structures and chemical properties of these molecules. The largest effort to detect these compounds is being done in HPLC with fluorescent detection (HPLC-FLD), ultraviolet detection (FPLC-UV), and MS detection (LC-MS) [26]. As discussed, these methods must be able to deal with compounds extracted from complex matrices and be capable of differentiating toxins of interest from other compounds. In addition, in all cases the use of certified toxin standards is necessary for method validation and quality control purposes. Although fluorescence and ultraviolet detection have been used for years to detect these compounds and other methods can be used for detection purposes, MS detection has proved to be a better technique [23, 26–28]. In all cases, after matrix extraction and cleanup, samples are injected in LC-MS/MS systems and chromatograms obtained are compared with standards. LC-MS detection methods can be used for monitoring the presence of toxicants in samples with official purposes, in that case should be validated (see chapter 3) or to detect and describe new compounds or unknown toxin metabolites. Below, several applications of UPLC-MS to identify each group of toxins in samples as well as the use of high-resolution MS to identify new compounds are shown. In MS, it is always important to bear in mind the matrix effect, that is the deviation in MS signal when the toxin is solved in matrix. For this reason, matrix effect and matrix corrections should be always determined using spiked matrices with a known toxin concentration. In addition, the recovery of the extraction procedure should also be evaluated, that is the amount of toxin extracted from a reference material with certified toxin amount.

2.6.1 Lipophilic toxins

LC-MS/MS is the official method to identify lipophilic toxins in shellfish for consumption according European Union (EU) regulations (EU 15/2011) since 2014 [29]. LC-MS/MS methodology is the reference method for the following lipophilic compounds: DSP group (OA, DTX1, DTX2 and DTX3), PTX group (PTX1 and PTX2), YTX

group (YTX, 45 OH YTX, homo YTX, and 45 OH homo YTX), and AZA group (AZA1, AZA2 and AZA3), although the spirolides (from the CI family) can also be detected. To develop this analysis, a standard protocol is currently available harmonized between several laboratories in Europe. Samples are first extracted with methanol, 2.00 ± 0.05 g of homogenate tissue / 20 mL. The methanolic extract is filtered through a methanol-compatible 0.45-mm filter and injected in the LC-MS system to detect free toxins. To convert esterified forms of the DSP group in free acids, an aliquot of extract is hydrolyzed in alkaline conditions. Toxins can be detected using acidic or basic chromatographic conditions, in both cases employing two mobile phases in elution gradient and reversed-phase columns, C8 or C18 silica columns. For basic chromatographic conditions, mobile phases are composed by water (A) and acetonitrile-water (90:10) (B), both containing 0.05% ammonia. For acidic conditions, mobile phases are water (A) and acetonitrile-water (95:5) both containing 50mM formic acid and 2mM ammonium formate. Table 2.5 summarizes LC parameters for acidic conditions in a reverse-phase column and 6.5 min per injection. Gradient is done between 0 to 3 min starting in 30% of mobile phase B (organic) until 70%, then initial conditions are recovery and the column is equilibrated for 2 min. MS/MS detection is performed in MRM mode using two transitions per toxin. The transition with higher intensity is used for quantification, while the lower transition is used for confirmatory purposes. PTX, AZA, and SPX groups are ionized in positive ion mode, while DSP and YTX groups are ionized in the negative mode (Table 2.5). MS parameters were previously optimized with toxin standards injected in mobile phases in order to achieve the maximum level of sensitivity. Figure 2.12 shows the chromatogram obtained (two peaks for toxin) when a mixture of lipophilic marine toxins solved in methanol was injected. The retention time order is a function of polarity, the higher polarity the first elution. That is Spirolide 20 (SPX20), the most polar of lipophilic toxins and the first eluted. A calibration curve with at least five concentrations of each toxin should be injected. In this way a calibration curve representing the transition with higher intensity versus concentration is constructed, obtaining a linear equation for each toxin with a regression line. When a sample is injected, the chromatogram can be compared with the standard one, and any peak (with the same transitions and retention time) can be identified as a toxin and quantified using the calibration curve.

2.6.2 Hydrosoluble marine toxins: *PSPs, TTXs, DA*

To officially detect and quantify the amount of PSPs and DA in shellfish for consumption, different HPLC methods are used. In the case of TTXs, there is no official method for detection although LC-MS is being used. The STX group does not have natural ultraviolet adsorption or fluorescence. Therefore to detect these toxins, before (pre-column oxidation, Pre-COX) or after (post-column oxidation, P-COX) HPLC this group of compounds should be converted into purine derivatives with fluorescent properties [6, 24].

Table 2.5: Main characteristics of UPLC and MS/MS methods for detection of Lipophilic Marine Toxins (Fig.2.12).

LC conditions						
Column	Waters Acquity UPLC BEH C18, 100 mm x 2.1 mm, 1.7 µm particle size					
Flow	0.4 mL/min					
Injection volume	5 µL					
Column T	40°C					
Mobile phase A	H_2O (2mM ammonium formate and 50mM formic acid)					
Mobile phase B	CH_3CN H_2O (95:5) (2mM ammonium formate and 50mM formic acid)					
	Time (min)	**Mobile phase A (%)**	**Mobile phase B (%)**			
Gradient	0	70	30			
	3	30	70			
	4.5	30	70			
	4.51	70	30			
	6.5	70	30			
MRM conditions						
Compound	**Precursor Ion** ***m/z***	**Product Ion** ***m/z***	**CE**	**Fragmentor**	**CAV**	**Polarity**
45-OH-homo-YTX	1171.5	1091.5	40	250	4	Negative
		869.5	88			
45-OH-YTX	1157.5	1077.5	38	240	4	Negative
		871.5	86			
homo-YTX	1155.5	1075.5	40	250	4	Negative
		869.5	88			
YTX	1141.2	1061.3	38	240	4	Negative
		855.3	86			
PTX1	892.5	821.5	28	270	2	Positive
		213.2	44			
PTX2	876.5	823.5	28	270	2	Positive
		213.2	44			
AZA2	856.5	838.5	36	213	2	Positive
		820.5	40			
AZA1	842.5	824.5	32	206	4	Positive
		806.5	44			
AZA3	828.5	810.5	32	216	4	Positive
		792.5	44			
DTX1	817.5	255.2	52	320	7	Negative
		113.2	70			
OA/DTX2	803.5	255.2	50	320	7	Negative
		113.2	66			
SPX20G	706.5	688.3	32	233	4	Positive
		164.2	56			
SPX13	692.5	674.3	36	75	4	Positive
		164.2	60			
SPX13,19	678.5	660.3	36	135	4	Positive
		164.2	60			

CE: collision energies; CAV: cell accelerator voltage

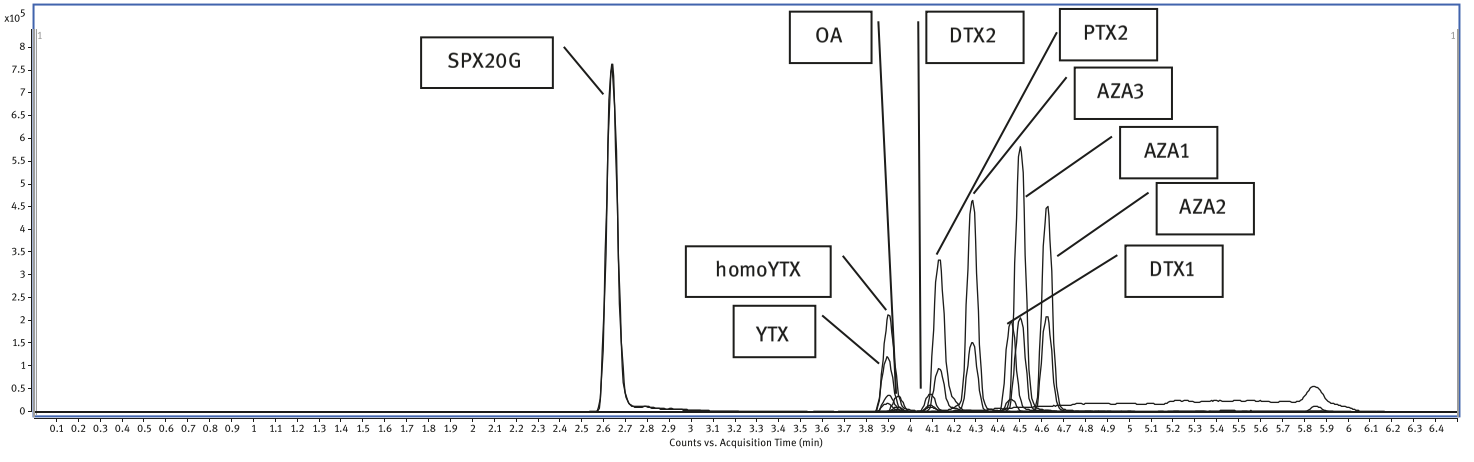


Figure 2.12: Characteristic chromatogram of lipohilic marine toxins. Chromatographic separation was carried out using a 1290 Infinity ultra-high-performance liquid chromatography system coupled to an Agilent G6460C Triple Quadrupole mass spectrometer equipped with an Agilent Jet Stream ESI source (Agilent Technologies, Waldbronn, Germany). The triple quadrupole was operated with the following optimized parameters: drying gas temperature of 350°C and a flow of 8 L/min; nebulizer gas pressure of 45 psi (Nitrocraft NCLC/MS from Air Liquid); sheath gas temperature of 400°C and a flow of 11 L/min and the capillary voltage was set to 4000 V in a negative mode with a nozzle voltage of 0 and 3500 V in a positive mode with a nozzle voltage of 500 V. The collision energy (CE), cell accelerator voltage (CAV), and fragmentor were optimized using MassHunter Optimizer software.

This happens by reaction in alkaline media at high temperatures [31]. In this way, PSPs can be detected by fluorescence after acidic extraction. In the case of DA, HPLC with UV detection after an aqueous/methanol extraction of shellfish is the official method. In the case of TTXs, there is no official method, although some LC-MS validated method was published [31]. In any case to know the amount of all hydrophilic toxins in a sample at least three different analyses, with different equipment, should be done. For this reason, the unification of the three groups into one method is very interesting. This is only possible by using LC-MS technology. However, the conventional reversed-phase columns used in LC are not useful to separate all analogs. In this regard, the use of normal-phase resins like ion-pair reagent for HILIC will help to solve this problem. With an amide column (normal-phase) and a gradient starting in a high percentage of organic phase, the three groups can be analyzed in one injection. Each toxin group should be extracted in previous conditions, that is for PSPs or TTXs acidic conditions, 5.00 ± 0.01 g of homogenate tissue / 5 mL AcOH 1%, and cleanup by SPE with carbon resins while DA is extracted with methanol:water (4.00 ± 0.01 g of homogenate tissue /16 mL AcOH 1%, methanol 50%). Then, extracts obtained are injected in the LC-MS system. Conditions for this analysis are summarized in Table 2.6. In this case, normal-phase column, the gradient is done between 0 and 11 min starting with 95 % of mobile phase B (organic) until 5% is reached, then initial conditions are recovered and the column is equilibrated for 2 min. MS/MS detection is performed in the MRM mode using two transitions per toxin (Table 2.6). In these conditions, the three groups of toxins can be detected and identified, Figure 2.13. In this case, DA, with the lower polarity, is eluted

Table 2.6: Main characteristics of UPLC and MS/MS methods to detect hydrophilic marine toxins (Fig.2.13).

	LC conditions					
Column	Waters Acquity UPLC BEH Amide, 100 mm x 2.1 mm, 1.7 µm particle size					
Flow	0.4 mL/min					
Injection volume	5 µL					
Column T	40°C					
Mobile phase A	H_2O (10mM ammonium acetate and 0.1% formic acid)					
Mobile phase B	98% CH_3CN + 2%H_2O (100mM ammonium acetate and 0.1% formic acid)					
	Time (min)	**Mobile phase A (%)**	**Mobile phase B (%)**			
Gradient	0	5	95			
	11	95	5			
	12	95	5			
	13	5	95			
	15	5	95			
	MRM conditions					
Toxins	**Precursor Ion** ***m/z***	**Product Ion** ***m/z***	**CE**	**Frag**	**CAV**	**Polarity**

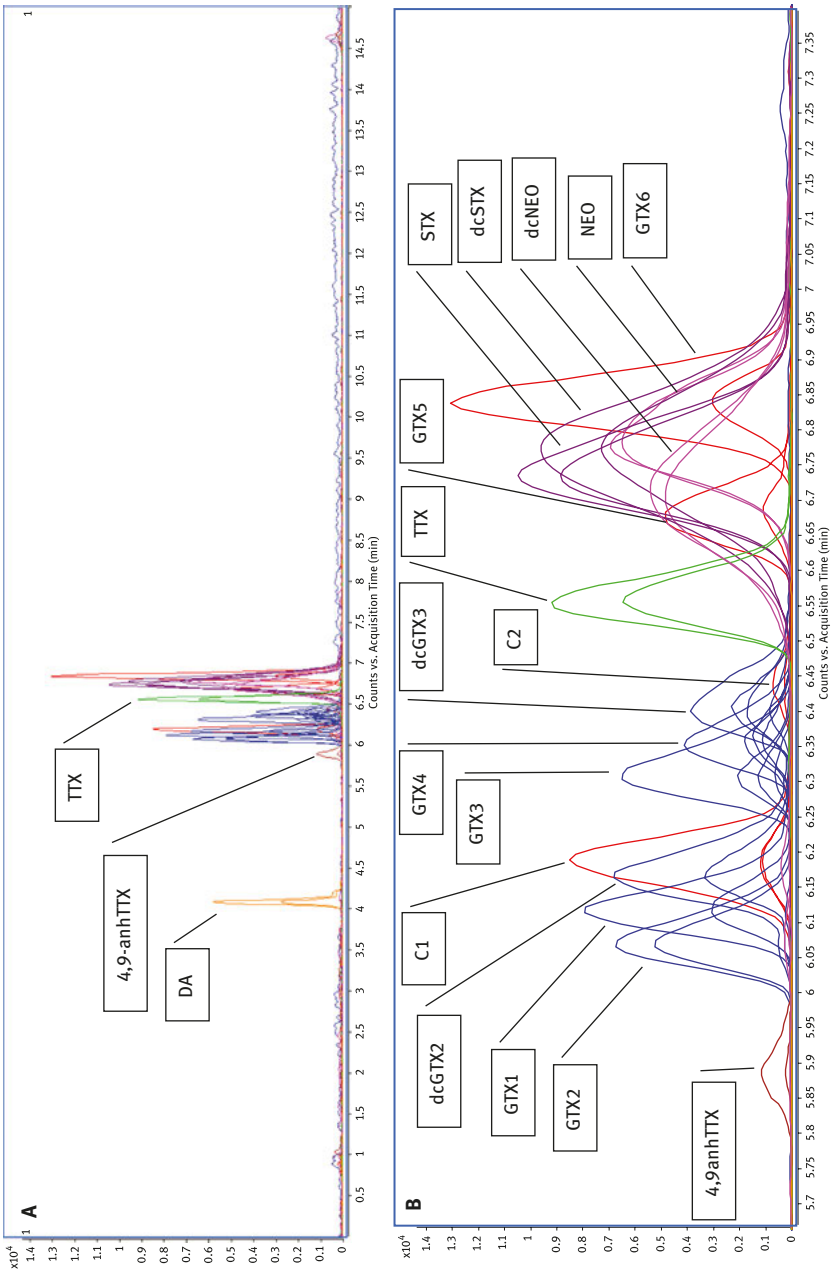
(continued)

Table 2.6: (continued)

	MRM conditions					
Toxins	**Precursor Ion** *m/z*	**Product Ion** *m/z*	**CE**	**Frag**	**CAV**	**Polarity**
DA	312.1	74.2	24	116	4	Positive
		266	12			
C1&C2	474	121.9	40	147	4	Negative
		351	24			
dcGTX2	351	164	32	156	2	Negative
		333	16			
dcGTX3	353	255	16	89	2	Positive
		335	2			
dcSTX	257	84.1	32	128	2	Positive
		126	20			
GTX1	410	349	20	142	2	Negative
		367	12			
GTX2	394	333	20	151	2	Negative
		351	12			
GTX3	396	110	56	108	2	Positive
		298	16			
GTX4	412	314	16	93	4	Positive
		332	12			
GTX5	378	121.9	24	147	2	Negative
		360	12			
GTX6	396.1	316.1	8	68	4	Positive
	394.2	122	20	129	1	
NEO	316	110.1	52	112	2	Positive
		298.1	20			
dcNEO	273.1	110.1	44	111	2	Positive
		126	20			
STX	300	60.2	40	108	2	Positive
		204	24			
4,9-anhydroTTX	302	161.9	40	152	4	Positive
		256	28			
TTX	320	161.9	36	160	2	Positive
		302	24			

CE: collision energies; CAV: cell accelerator voltage

▸ Figure 2.13: (A).-Characteristic chromatogram of hydrophilic marine toxins. (B).-Zoom-in of A chromatogram from 5.5 to 7.5 min. Chromatographic separation was carried out using a 1290 Infinity ultra-high-performance liquid chromatography system coupled to an Agilent G6460C Triple Quadrupole mass spectrometer equipped with an Agilent Jet Stream ESI source (Agilent Technologies, Waldbronn, Germany). The triple quadrupole was operated with the following optimized parameters: drying gas temperature of 250°C and a flow of 11 L/min; nebulizer gas pressure of 55 psi (Nitrocraft NCLC/MS from Air Liquid); sheath gas temperature of 400°C and a flow of 12 L/min and the capillary voltage was set to 3000 V in a negative mode with a nozzle voltage of 0 and 3000 V in a positive mode with a nozzle voltage of 0 V. The collision energy (CE), cell accelerator voltage (CAV), and fragmentor were optimized using MassHunter Optimizer software.



first while PSPs and TTX with higher polarity are eluted together at the end of the gradient. Similar to the previous procedure, the calibration curve with different standard concentrations can be constructed and any sample be quantified.

2.6.3 Cyanotoxins

Several LC-MS/MS methods had been developed to quantify cyanotoxins, however a longer time, 70 min, was necessary to separate all toxins [32]. By using UPLC the time of analysis is significantly reduced. In this case instead of extraction from matrix, the sample should be in some way concentrated because toxins are solved in water [33]. Figure 2.14 shows a chromatogram of a mixture of six cyanotoxins eluted in LC and MS/MS conditions summarized in Table 2.7. To separate these toxins a reverse-phase column is used with a gradient starting in 0% of mobile phase B at minute 4 until 70% at minute 8, then initial conditions are recovered and the column is equilibrated for 2 min. The analysis is improved by adding an isocratic period (0–4 min) with 0% of organic phase [34]. With this period, phenylalanine can be easily separated from anatoxin. These two compounds have the same molecular weight and transitions and therefore can produce misidentifications. MS/MS detection is performed in the MRM mode in positive mode using two transitions (three for ATX-a) per toxin (Table 2.7). As it can be expected, the most polar compound is the first to be eluted, that is, CYN and ATXs are eluted first and then MCs and NOD.

2.6.4 Mycotoxins

Mycotoxins are accumulated in solid matrices like cereals, figs, nuts, grapes, or coffee beans or liquid like wine or bear. After toxin extraction from these matrices, additional cleanup processes developed for pesticide analysis, such as QuEChERS methodology (from quick, easy, cheap, effective, rugged, and safe) and dispersive liquid–liquid extractions should be done before analysis to avoid interferences. UPLC-MS/MS is the technique more used, although HPLC-UV or HPLC-FLD have been traditionally used for mycotoxin analysis [35]. UPLC-MS/MS allows the simultaneous detection of many mycotoxins with good sensibility and specificity. Mycotoxin separation was done with conditions of LC and MS/MS summarized in Table 2.8. A 13-min gradient with a reverse-phase column was used. The gradient was done in several steps starting in 0% of organic phase (methanol) up to 100%. Acid mobile phase allows the simultaneous detection of fumonisins and trichothecenes. MS is used in dynamic multiple reaction monitoring mode, selecting transitions during a specified period of time, hence providing higher sensitivity and reproducibility due to the dwell times for each transition that are maximized [36]. In this way, as Figure 2.15 shows, 22 mycotoxins can be easily identified in 13 min.

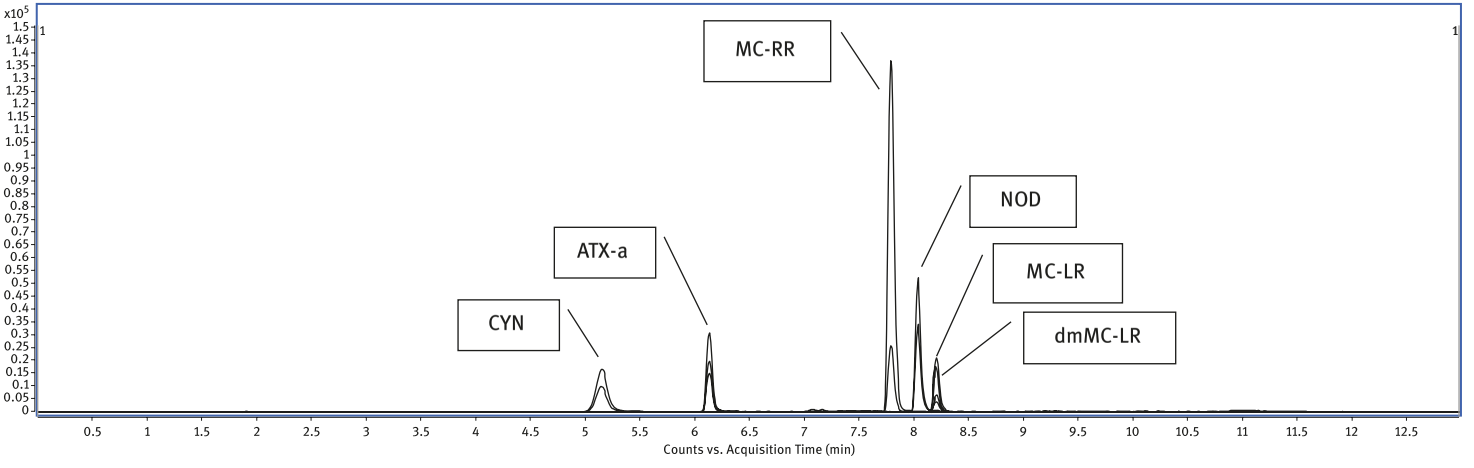


Figure 2.14: Characteristic chromatogram of a mixture of six cyanotoxins. Chromatographic separation was carried out using a 1290 Infinity ultra-high-performance liquid chromatography system coupled to an Agilent G6460C Triple Quadrupole mass spectrometer equipped with an Agilent Jet Stream ESI source (Agilent Technologies, Waldbronn, Germany). The triple quadrupole was operated with the following optimized parameters: drying gas temperature of 350°C and a flow of 5 L/min; nebulizer gas pressure of 30 psi (Nitrocraft NCLC/MS from Air Liquid); sheath gas temperature of 400°C and a flow of 12 L/min and the capillary voltage was set to 3500 V in a positive mode with a nozzle voltage of 1500 V. The collision energy (CE), cell accelerator voltage (CAV), and fragmentor were optimized using MassHunter Optimizer software.

Table 2.7: Main characteristics of UPLC and MS/MS methods to detect cyanotoxins (Fig.2.14).

LC conditions			
Column	Waters Acquity UPLC HSS T3, 100 mm x 2.1 mm, 1.8 µm particle size		
Flow	0.4 mL/min		
Injection volume	5 µL		
Column T	35°C		
Mobile phase A	H_2O (0.05% formic acid)		
Mobile phase B	CH_3CN (0.05% formic acid)		
	Time (min)	**Mobile phase A (%)**	**Mobile phase B (%)**
Gradient	0	100	0
	4	100	0
	8	30	70
	10	30	70
	10.5	100	0
	13	100	0

MRM conditions						
Compound	**Precursor Ion** *m/z*	**Product Ion** *m/z*	**CE**	**Fragmentor**	**CAV**	**Polarity**
Epoxy-homoATX-a	196	138	16	93	1	Positive
		178	12			
H2-homoATX-a	182	147	16	93	1	Positive
		164	12			
Epoxy-ATX-a	182	138	16	93	1	Positive
		164	12			
homoATX-a	180	145	16	93	1	Positive
		163	12			
H2-ATX-a	168	133	16	93	1	Positive
		150	12			
ATX-a	166.13	43.2	24	93	1	Positive
		131	16			
		149	12			
CYN	416.13	194	40	146	1	Positive
		336.1	20			
MC-YR	1046	103	120	151	1	Positive
		135	80			
MC-FR	1029	103	120	151	1	Positive
		135	80			
MC-LW	1026	103	120	151	1	Positive
		135	80			
MC-YM	1020	103	120	151	1	Positive
		135	80			
MC-LY	1003	103	120	151	1	Positive
		135	80			
MC-LR	995.56	135	76	185	1	Positive
	498.28	861.4	8	105		

Table 2.7: (continued)

MRM conditions						
Compound	**Precursor Ion** *m/z*	**Product Ion** *m/z*	**CE**	**Fragmentor**	**CAV**	**Polarity**
MC-LF	987	103	120	151	1	Positive
		135	80			
MC-AW	983	103	80	151	1	Positive
		135	120			
dmMC-LR	981.54	103	120	285	1	Positive
		135	80	215		
MC-VF	973	103	120	151	1	Positive
		135	80			
MC-YA	960	103	120	151	1	Positive
		135	80			
MC-AR	953	103	120	151	1	Positive
		135	80			
MC-LL	952	103	120	151	1	Positive
		135	80			
MC-LA	911	103	120	151	1	Positive
		135	80			
MC-RR	520.11	103.1	68	151	1	Positive
		135	32			

CE: collision energies; CAV: cell accelerator voltage

Table 2.8: Main characteristics of UPLC and MS/MS methods to detect mycotoxins (Fig.2.15).

LC conditions			
Column	Waters ACQUITY HSS T3, 100 mm x 2.1 mm, 1.8 µm particle size		
Flow	0.3 mL/min		
Injection volume	5 µL		
Column T	40 °C		
Mobile phase A	H_2O (5 mM ammonium formate and 0.1% formic acid)		
Mobile phase B	Methanol		
	Time (min)	**Mobile phase A (%)**	**Mobile phase B (%)**
Gradient	0	100	0
	0.5	86	14
	2	86	14
	3	40	60
	3.5	40	60
	6.5	0	100

(continued)

Table 2.8: (continued)

	Time (min)	Mobile phase A (%)	Mobile phase B (%)
	10	0	100
	10.5	100	0
	13	100	0

MRM conditions

Compound	Precursor Ion *m/z*	Product Ion *m/z*	CE	Fragmentor	CAV	RT	Polarity
Aflatoxin B_1	313.07	285	24	142	2	6.45	Positive
		241	44				
Aflatoxin B_2	313.07	287	28	147	2	6.25	Positive
		259	32				
Aflatoxin G_1	329.07	243	28	132	2	6.05	Positive
		200	48				
Aflatoxin G_2	331.1	245	32	75	2	5.9	Positive
		217	40				
Beauvericin	806.4	402.1	56	280	2	10.05	Positive
		302.1	68				
Deoxynivalenol	297.15	249	8	74	2	5	Positive
		203	12				
3+15-Acetyl-deoxynivalenol	339.15	137	8	65	2	5.72	Positive
		261	8				
Deoxynivalenol 3-glucoside	503.18	457.1	12	125	2	4.9	Negative
		427.0	20				
Enniatin A	704.4	557.2	52	255	2	10.4	Positive
		210	64				
Enniatin A_1	690.4	520.1	60	280	2	10.25	Positive
		232.1	64				
Enniatin B	662.4	467.2	52	240	2	9.95	Positive
		449.1	48				
Enniatin B_1	676.4	168.1	70	260	2	10.1	Positive
		236	66				
Fumonisin B_1	722.4	352.2	40	170	2	7.08	Positive
		334.3	40				
Fumonisin B_2	706.5	336.2	40	165	2	8	Positive
		318.2	48				
HT-2 toxin	447.1	345	16	108	2	7.14	Positive
		285	20				
Moliniformin	96.99	96.99	0	51	2	1.1	Negative
		41.1	12				
Neosolaniol	400.19	215	16	74	2	5.38	Positive
		169	28				
Ochratoxin A	404.09	239	22	84	2	8.0	Positive
		102	75				

Table 2.8: (continued)

	MRM conditions						
Compound	**Precursor Ion** *m/z*	**Product Ion** *m/z*	**CE**	**Fragmentor**	**CAV**	**RT**	**Polarity**
T-2 toxin	484.25	245	12	84	2	7.64	Positive
		197.1	16				
Zearalenone	317.14	273	20	123	2	8.08	Negative
		131	28				
α-zearalenol	319.1	275.1	20	155	2	7.9	Negative
		130	36				
β-zearalenol	319.1	275.1	20	155	2	7.4	Negative
		130	36				

CE: collision energies; CAV: cell accelerator voltage

2.6.5 Ciguatoxins

The presence of CTX in fish is not regularly monitored due to the high variability of toxins, the variability of fish species and due to large inter-species variations [27]. In addition, the lack of standards hinders correct identifications. In any case UPLC-MS is a methodology regularly used for these toxins. The extraction from fish matrices is done with acetone or methanol and then several SPEs and liquid–liquid extractions to avoid matrix interferences, followed by LC-MS analysis [15, 17, 20]. Table 2.9 summarizes LC and MS conditions to separate and identify several CTX and other analogs like gambierol and gambierone. To separate these toxins a reverse-phase column is used with a 4.5-min gradient starting in 50% of mobile phase B at minute 2.5 until 100% at minute 7. Then the column is stabilized for 5 min. MS identification is done in two steps. First, MS is used in SIM mode, positive mode, selecting *m/z* values of compounds described in bibliography. Then, each peak is confirmed using scan mode and checking the typical fragmentation pathway of CTX, that is ammonium, sodium, and potassium adducts and several water losses. In this way as Figure 2.16 shows CTX can be identified even when no standards are available. In this case, the calibration curve of one toxin standard will be used to quantify other toxins, assuming an ionization-conversion factor of 1:1, which is not exact.

2.6.6 Identification of unknown and novel toxins

MS/MS analysis is based on a targeted methodology of finding predetermined compounds while missing other non-predetermined compounds that could be present in the sample. When no standards are available, but the compounds to be identified are described in the bibliography and the fragmentation pathway is known, they can be identified in the same way as was described for CTXs. However, when unknown compounds should be identified this cannot be applied. The term SWATH from "sequential

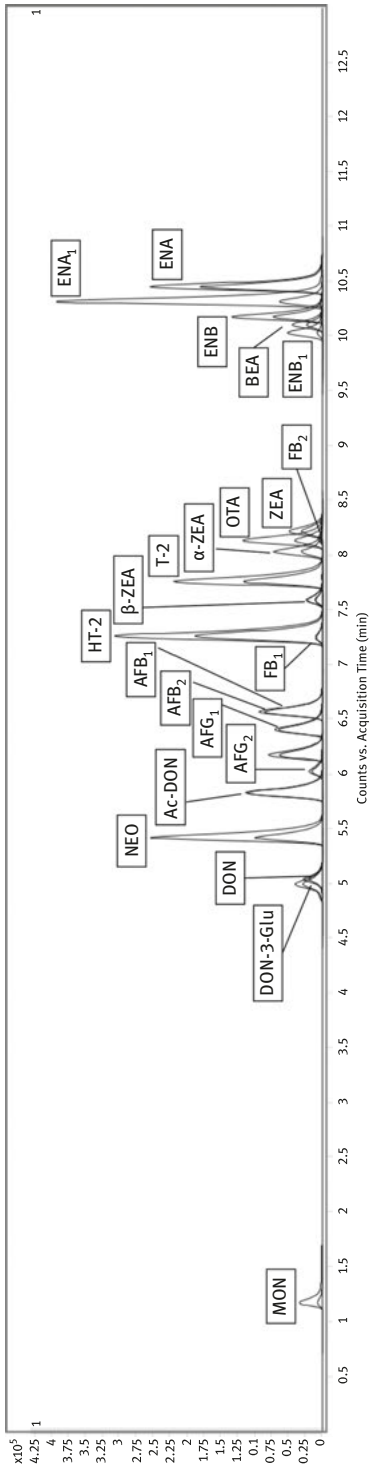


Figure 2.15: Characteristic chromatogram of a mixture of mycotoxins: deoxynivalenol (DON), deoxynivalenol 3-glucoside (DON-3-Glu), 3+15-acetyl-deoxynivalenol (Ac-DON), neosolaniol (NEO), aflatoxin G_2 (AFG_2), aflatoxin G_1 (AFG_1), aflatoxin B_2(AFB_2), aflatoxin B_1(AFB_1), fumonisin B_1(FB_1), HT-2 toxin (HT-2), T-2 toxin (T-2), fumonisin B_2 (FB_2) ochratoxin A(OTA), enniatin B_1 (ENB_1), beauvericin (BEA), enniatin B (ENB), enniatin A_1 (ENA_1) and enniatin A (ENA), monilifor-min (MON), zearalenone (ZEA), α-zearalenol (α-ZEA), β-zearalenol (β-ZEA). Chromatographic separation was carried out using a 1290 Infinity ultra-high-performance liquid chromatography system coupled to an Agilent G6460C Triple Quadrupole mass spectrometer equipped with an Agilent Jet Stream ESI source (Agilent Technologies, Waldbronn, Germany). The triple quadrupole was operated with the following optimized parameters: sheath gas temperature, 400 °C; sheath gas flow, 12 L/min, gas temperature, 350 °C; gas flow, 8 L/min; nebulizer, 45 psi; capillary voltage, 4000 V; and nozzle voltage 0 V. Cell voltage accelerator (CAV) employed for all analytes was 2 volts. The collision energy (CE) and fragmentor were optimized using MassHunter Optimizer software.

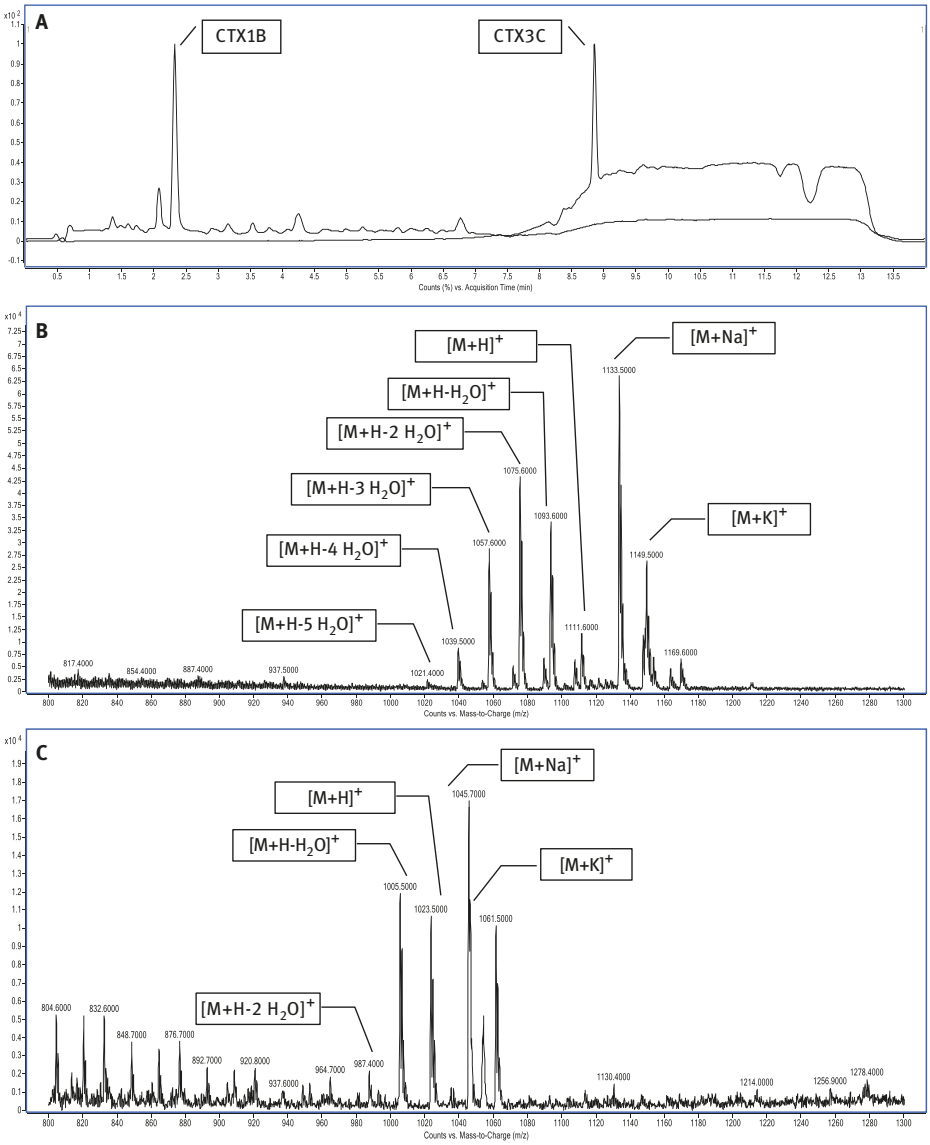


Figure 2.16: (A).-Characteristic chromatogram of a mixture of two ciguatoxins obtained in SIM mode. (B).-Mass spectrum of CTX1B peak obtained in scan mode. (C).-Mass spectrum of CTX3C peak obtained in scan mode. Chromatographic separation was carried out using a 1290 Infinity ultra-high-performance liquid chromatography system coupled to an Agilent G6460C Triple Quadrupole mass spectrometer equipped with an Agilent Jet Stream ESI source (Agilent Technologies, Waldbronn, Germany). The triple quadrupole was operated with the following optimized parameters: drying gas temperature of 350°C and a flow of 8 L/min; nebulizer gas pressure of 45 psi (Nitrocraft NCLC/MS from Air Liquid); sheath gas temperature of 400°C and a flow of 11 L/min: and the capillary voltage was set to 4000 V in a positive mode with a nozzle voltage of 0 V. The collision energy (CE), cell accelerator voltage (CAV), and fragmentor were optimized using MassHunter Optimizer software.

Table 2.9: Main characteristics of UPLC and MS/MS methods for detection of Ciguatoxins (Fig.2.16).

LC conditions			
Column	Acquity UPLC BEH C18, 100 mm x 2.1 mm, 1.7 µm particle size		
Flow	0.4 mL/min		
Injection volume	5 µL		
Column T	35 °C		
Mobile phase A	H_2O (2mM ammonium formate and 50mM formic acid)		
Mobile phase B	CH_3CN H_2O (95:5) (2mM ammonium formate and 50mM formic acid)		
	Time (min)	**Mobile phase A (%)**	**Mobile phase B (%)**
Gradient	0	50	50
	2.5	50	50
	7	0	100
	11.5	0	100
	11.6	50	50
	14	50	50

SIM conditions				
Compound	**Precursor Ion *m/z***	**Fragmentor**	**CAV**	**Polarity**
I-CTX3 and I-CTX4	1157.6	240	7	Positive
CTX-1143	1143.6	240	7	Positive
Caribean-CTX	1141.7	240	7	Positive
CTX-1127	1127.6	240	7	Positive
CTX-1123	1123.6	240	7	Positive
CTX1B	1111.6	240	7	Positive
CTX-1109	1109.5	240	7	Positive
54-deoxy-CTX/1B and 52-epi-CTX1B	1095.6	240	7	Positive
M-CTX-4A/4B	1079.6	240	7	Positive
CTX-4A/4B	1061.6	240	7	Positive
2,3-OH-CTX3C and M-CTX3C	1055.6	240	7	Positive
2-OH-CTX3C and M-CTX3C	1041.6	240	7	Positive
CTX-1040	1040.6	240	7	Positive
51-OH-CTX3C	1039.6	240	7	Positive
Gambierone	1025.5	180	7	Positive
49-epo-CTX3C and CTX3C	1023.6	240	7	Positive
Gambierol	757.9	240	7	Positive
CTX-1159	1159.6	240	7	Positive

CAV: cell accelerator voltage

windowed acquisition of all theoretical fragment ion mass spectra" is sometimes applied for the total search of compounds in a sample, eliminating the targeted nature of MS/MS analysis. However this is not easy when the number of compounds to look for is high, with a wide molecular weight range and complex structures, in addition to the complexity of many different matrices [37]. On the other hand the use of a high-resolution MS (Time of Flight or Orbitrap MS) can be a solution to identify unknown compounds. With this technology the accurate mass data as well as the fragmentation pathway can be obtained and the formula of any molecule predicted [19, 38–40].

Keywords: Analytical instrumentation, chromatography, mass spectrometry, fluorescence, ultraviolet-visible

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Carmen Alfonso and Álvaro Antelo

3 Quantitative and qualitative methods, primary methods

3.1 Quantitative methods: Beyond the numerical data

Laboratories perform thousands of analytical measurements at every moment. One main advantage of analytical methods is their reliability and fitness for purpose of results. The aim of an analytical method is to deliver a result with an acceptable uncertainty level. Therefore, analytical measurements should have well-defined quality control and quality assurance procedures. Quality in analytical laboratory can be understood as depending on the method's ability to produce reliable results with a defined degree of confidence.

The six principles of good analytical practice [1]:

Principle 1: Analytical measurements should be made to satisfy an agreed upon requirement.

Principle 2: Analytical measurements should be made using methods and equipment which have been tested to ensure they are fit for purpose.

Principle 3: Staff making analytical measurements should be both qualified and competent to undertake the task.

Principle 4: There should be a regular independent assessment of the technical performance of a laboratory.

Principle 5: Analytical measurements made in one location should be consistent with those made elsewhere.

Principle 6: Organizations making analytical measurements should have well-defined quality control and quality assurance procedures.

Without a proper laboratory quality system, the analysis development is very costly since too many mistakes can take place or wrong decisions implemented which could cause damages due to loss of reputation. Analysts with adequate understanding of the principles underlying a method are more likely to achieve reliable results. Analysts with the right knowledge and understanding will appreciate the risks involved and act appropriately; but, without a quality system, the cost of the analysis comes largely from:

- Repetition of analysis in order to ensure the results.
- Investigation of problems.
- Review of procedures.

The quality system should prevent mistakes by means of:

- Quality assurance measures.
- Quality control of analytical results.
- Thorough documentation of the system.

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- Efficient maintenance of records.
- Regular audits of all aspects of the system.

The most frequent activity of an analytical laboratory is the performance of quantitative chemical analyses, which provides information on the amount of the analyte in a sample, that is numerical data. Less frequently: qualitative analyses (positive/negative, detected/not detected answer, and substance identity, *vide infra*) and semiquantitative (similar to qualitative assays in detecting the presence or absence of an analyte but it goes on to provide a numerical representation of the amount of the analyte) analyses are also carried out. The quantitative analytical techniques basically fulfill three main objectives:

1. They provide information about its magnitude and its uncertainty. The obtainment of as high metrological quality as possible [2].
2. They solve analytical problems derived from a great variety of activities (health, general and food industries, the environment, etc.).
3. They allow to define a decision-making process in compliance with legislation. They can be used as a preventive measure.

Analytical laboratories use methods that must be evaluated and tested to ensure that they produce valid results suitable for their intended purpose. Therefore the methods must be validated, thus validation is defined as "Confirmation, through the provision of objective evidence, that the specified requirements for a specific intended use or application have been fulfilled" [3]. The use of validated methods is an important aspect of quality assurance in the laboratory.

In practice, the objective of analytical method validation is to ensure that every future measurement in routine analysis will be close enough to the unknown true value for the content of the analyte in the sample through a series of method-performance characteristics, such as precision, trueness, selectivity/specificity, linearity, operating range, recovery, limit of detection (LOD), limit of quantification, sensitivity, ruggedness/robustness, and applicability.

Metrology includes all theoretical and practical aspects of measurement, whatever the measurement uncertainty and field of application. The combined effect of unknown factors and inaccurate knowledge on the known factors is described quantitatively with the measurement of uncertainty.

Therefore a reliable result is supported on: calibration, method validation, uncertainty measurement, and metrological traceability, see Figure 3.1 [4]. Accordingly, the objectives of validation are not simply to obtain estimates of trueness or bias and precision but also to evaluate those risks that can be expressed by the measurement uncertainty associated with the result [5]. Two fundamental requirements of ISO 17025 standard, i.e. validation of the methods and estimation of the uncertainty of measurements, can give a way to check whether an analytical method is correctly fit for the purpose [6].

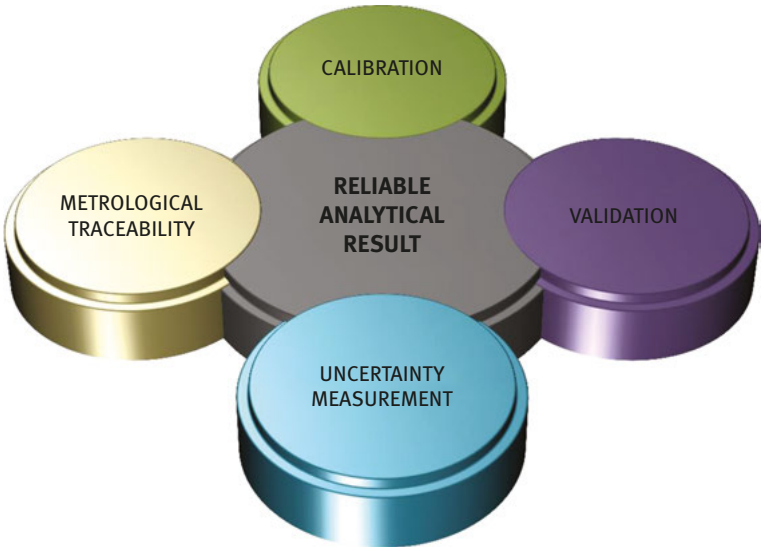

Figure 3.1: Relationship between metrological traceability, calibration, validation, and measurement uncertainty of results to get a reliable analytical result.

The uncertainty of a measurement is a parameter, associated with its result, which characterizes the dispersion of the values that could reasonably be attributed to the measurand, namely its quality [7]. Measurement uncertainty is an issue that is sometimes misunderstood, by beginners and experts. The authors only point out the crucial difference between error and uncertainty:

- Error is the difference between the measured value and the "true value" of the thing being measured.
- Uncertainty is a quantification of the doubt about the measurement result.

Uncertainty of measurement can arise from many sources. Calculating uncertainty in measurement becomes quite a challenge and it is necessary to develop a step-by-step process to achieve the best conformance with a specification. Figure 3.2 shows the basic steps of the evaluation of the measurement uncertainty. In the overall process, the most time-consuming step is identifying the factors that influence uncertainty in measurement results. There are several sources of uncertainty which contribute to estimate final method uncertainty.

1. incomplete definition of the measurand;
2. imperfect realization of the definition of the measurand;
3. nonrepresentative sampling – the sample measured may not represent the defined measurand;
4. inadequate knowledge of the effects of environmental conditions on the measurement, or imperfect measurement of environmental conditions;
5. personal bias in reading analog instruments or data management;

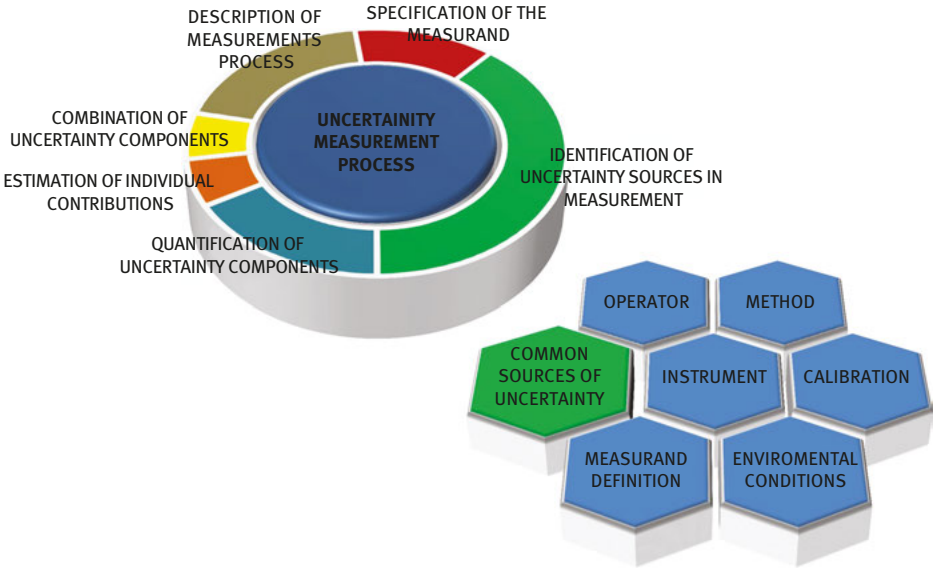

Figure 3.2: Usually the contribution of the uncertainties carried by the references to the total uncertainty is small relative the contributions that originate from the measurement process.

6. finite instrument resolution or discrimination threshold;
7. inexact values of measurement standards and reference materials (RMs);
8. inexact values of constants and other parameters obtained from external sources and used in the data-reduction algorithm;
9. approximations and assumptions incorporated in the measurement method and procedure;
10. variations in repeated observations of the measurand under apparently identical conditions.

Some sources of uncertainty found in all traceable measurements are: the uncertainty of the reference standard used for calibration, the repeatability of the calibration process, and the repeatability of the actual measurement. Fluctuations in environmental factors such as the temperature will be significant sources of uncertainty for many measurements. Once the uncertainty has been determined for a measurement process, it can then be used to establish whether a measurement is in conformance or nonconformance with a specification.

Uncertainty in measurement process is relevant to decisions on compliance with regulatory or manufacturing limits where a decision is made on the basis of a measurement result accompanied by information on the uncertainty associated with the result. In order to utilize a result to decide whether it indicates compliance or noncompliance with a specification, it is necessary to take into account the measurement uncertainty, see Figure 3.3.

In the simplest terms, measurements are made to achieve knowledge, or more commonly, they are used in commerce and industry to verify product conformance prior to acceptance. Figure 3.3 shows typical scenarios arising when measurement results, for example on the concentration of analyte, are used to assess compliance with a regulatory specification limit. The vertical small spheres represent uncertainty on each result, showing that there is a larger probability of the value of the measurand lying near the center of the expanded uncertainty interval than near the ends [8].

Cases of purple and green values are reasonably clear; the measurement results and their uncertainties provide good evidence that the value of the measurand is well above or well below the limit, respectively. In the orange case, however, there is a high probability that the value of the measurand is above the limit, but the limit is nonetheless within the expanded uncertainty interval. Depending on the circumstances, and particularly on the risks associated with making a wrong decision, the probability of an incorrect decision may be or may not be sufficiently small to justify a decision of noncompliance. Similarly, in the blue case the probability that the value of the measurand is below the limit may or may not be sufficient to take the result to justify compliance. Without further information, which has to be based on the risks associated with making a wrong decision, it is not possible to use these two results to make a decision on compliance.

Target measurement uncertainty is important in order to establish decision rules and explains how to use them to demonstrate whether a method is fit for purpose [9]. It must be assumed that the uncertainty has been evaluated by an appropriate method that takes all relevant contributions into account. The rule may also give the procedure for dealing with repeated measurements and outliers. The determination of the acceptance/rejection zone will normally be made by the laboratory based on the decision rule and the information available about the uncertainty in their own measurement result. For example: a laboratory of food analysis must analyze samples of different mussel batches from canning companies before the product can be released to the consumer chain, assuring that okadaic acid (marine toxin) is ≤ 160 μg okadaic acid/Kg. The decision rule states the mussel will be considered to be compliant with regulatory limits if the probability of the mass concentration of okadaic acid in mussel is lower than 160 μg okadaic acid/Kg. The rejection zone is set with a guard band of >150 μg/kg (value of t for 0.05 one-tailed test probability), see Figure 3.3 In this case, the guard band (calculated according to the method's uncertainty) has been chosen to provide a low risk of false acceptance, and therefore the blue mussel batch is also rejected.

The cost of a measurement, or of a method, is an important consideration when deciding on the required measurement uncertainty. In general, the lower the uncertainty value the more expensive the process will be. A laboratory could develop a state–of-the-art method with expensive, sophisticated instrumentation and highly qualified personnel and perform many replicates to reduce the

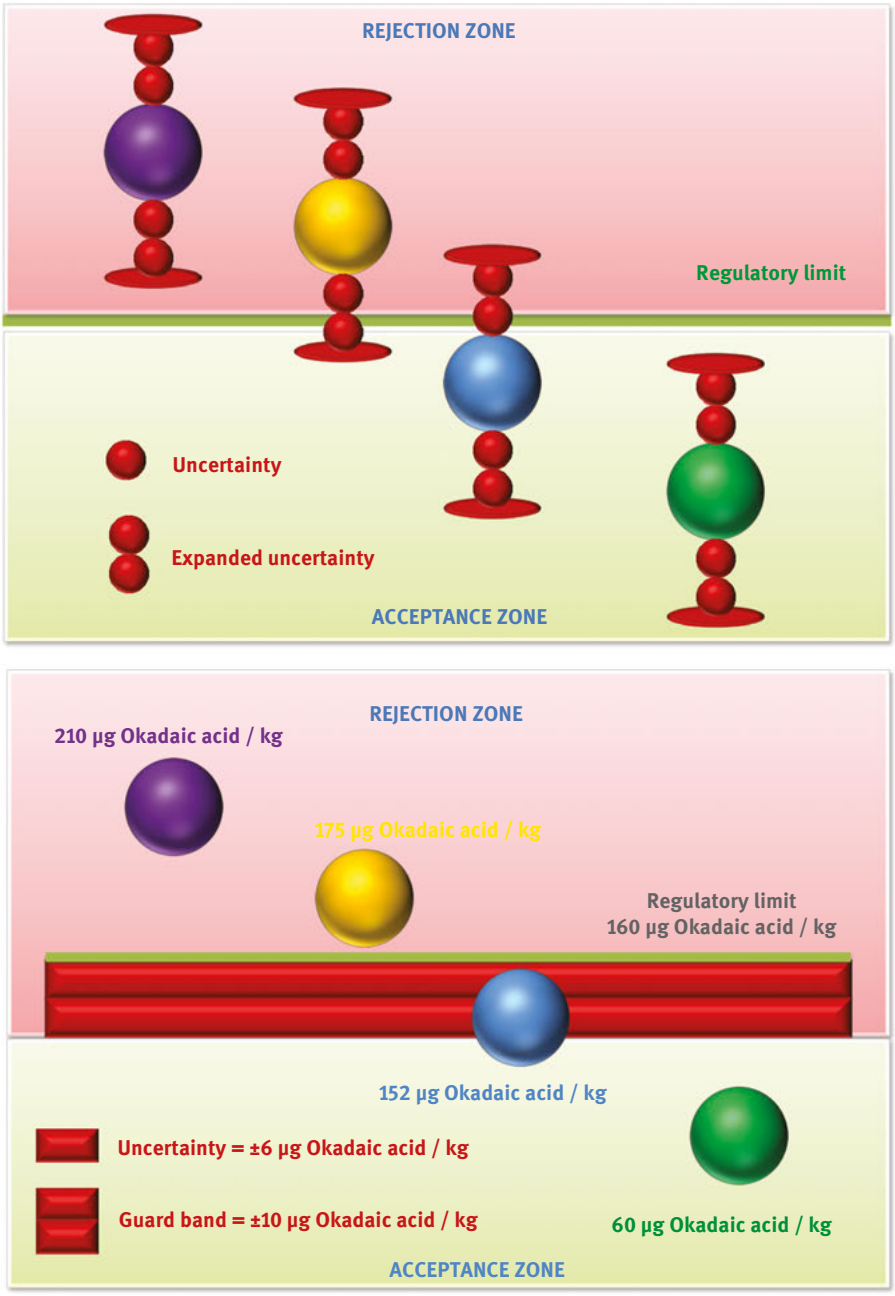

Figure 3.3: Implication of measurement uncertainty on mean values in relation to a criterion limit. Four scenarios showing mean counts. Upper figure shows uncertainty around mean count, lower figure takes into account a guard band around regulatory limit for okadaic acid marine toxin versus measurements.

measurement uncertainty to the absolute minimum value obtainable with the latest technology at great expense.

As the measurement uncertainty gets larger, the cost of making incorrect decisions based on the test result increases. As the examples above illustrate, the probability of making an incorrect decision can be determined. One (depict in Figure 3.3) is fast and of low cost, and gives results with an uncertainty of 6 μg/Kg. A better, but slower and more expensive, process could give results with an uncertainty of 3 μg/Kg. The optimum choice depends on the nature of the samples to be analyzed. If it is expected that the concentrations in the samples will always be close to the limit, the more expensive method must be used. However, if most of the concentrations are likely to be far from 160 μg/Kg, a more cost-effective solution is to use the cheaper method for screening since the expensive analytical method would be wasted if the result did not need that low measurement uncertainty.

Using this information, the potential cost of an incorrect decision (e.g., assuming mussel tons as non-conforming product when they are conforming.) can be determined. At some point the benefit of reducing the measurement's uncertainty does not warrant the expense involved.

3.2 Metrological traceability

The concept of traceability is an important element for metrological measurements, and its practical definition is: "property of a measurement result whereby the result can be related to a reference through a documented unbroken chain of calibrations, each contributing to the measurement uncertainty" [10]. A traceable result is comparable to that provided by the reference used and, therefore, it is accurate. The metrological traceability can be presented as a pyramid. Figure 3.4 shows an example of a metrological pyramid hierarchy for an analytical laboratory.

It should be noted that:

- The measurement uncertainties and bias are determined according to the metrological traceability measurement procedure pyramid and a closely related level of calibration standards pyramid. The measurement uncertainty and bias decrease from bottom to top. On the contrary, the level of accreditation laboratory and accuracy increase significantly from the top of the hierarchy.
- Metrological traceability, linked to the legitimate use of the SI unit, is the property of the result of a measurement which confirms the compatibility with the SI unit. Traceability gives the user confidence that the measurements agree with the national standards within the stated uncertainty.
- Metrological traceability is an attribute of the measurement result and, therefore, expressions such as "traceable equipment" or "traceable to the organization ..."

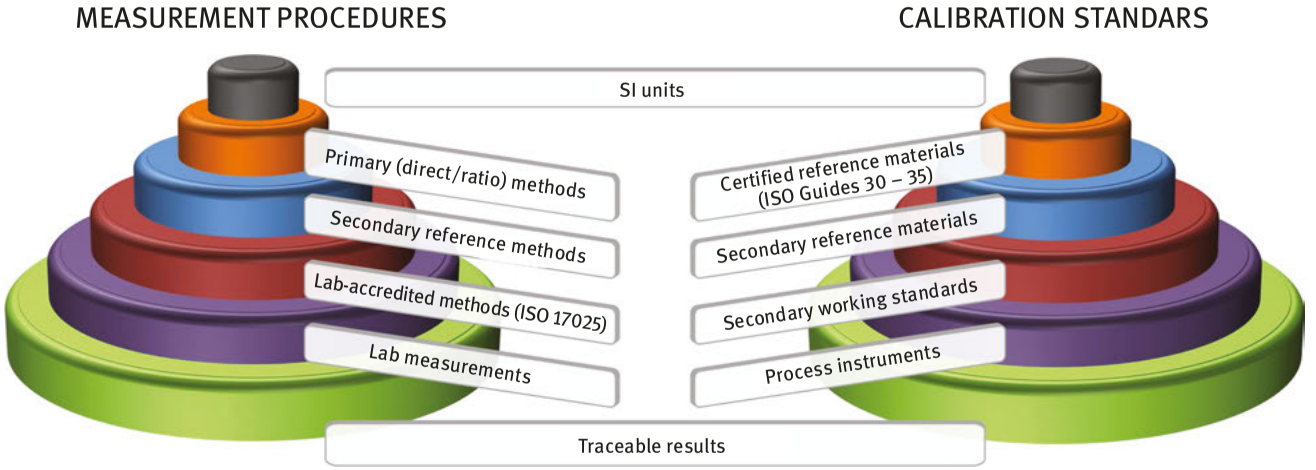

Figure 3.4: Traceability hierarchy from the realization of the SI Unit down to the measurements performed in the analytical laboratory through standards reference for equipment and method calibration. The pyramid illustrates how the different levels in the traceability are located. As lab process instruments are located in the lowest level, their traceability is dependent on all the levels above. The higher the pyramid step, the smaller the uncertainty is (smaller pyramid step), that is, the better the accuracy is.

are incorrect in metrology. Traceability must not refer to an instrument or calibration certificate, nor is it obtained by a specific calibration procedure or by using special equipment. Only the measurement results and the values defined by the measurement standards are traceable metrologically. Figure 3.4 shows how a process measurement that is implemented in a lab is traceable up to an international level through an unbroken chain of measurements.

– Metrological traceability is a fundamental property of a measurement result, but in isolation it cannot guarantee accuracy. Validated measurement procedures and estimates of measurement uncertainty are also essential if fit-for-purpose results are to be achieved [11].

Traceability through one of the primary methods is the best reference possible, provided that the application of the method in the laboratory takes place under conditions of very strict quality assurances. This basically involves the implementation of an instrument calibration plan and the knowledge and quantification of the uncertainty of all the parameters of the analytical procedure. Of course, in no case can the analytical method be compared with a definitive method, so other available references should be used, going down the pyramid ladder.

To achieve metrological traceability, no step of the pyramid should be missing since all measurements below that level have no traceability and are suspect to error. Analytical quality assurance procedures should be based on a system of traceability and feedback. Traceability, in this context, requires that all steps in a procedure can be checked, wherever possible, by reference to documented results, calibrations, standards, calculations, etc. To maintain this hierarchy the following actions are necessary:

– **Calibrations:** All the calibrations in the traceability chain have to be done routinely. An instrument is calibrated with a reference standard which should be calibrated to do recalibrations at regular intervals. The calibration of any measurement instrument only remains valid for a stated period of time. Therefore, the traceability expires when the calibration expires.
– **Documentation:** Every step needs to be documented according to a quality system in order to get reliable results. Every calibration, validation, and process data in the traceability chain needs to be documented. This means of course that the completion of calibration, validation, and measurement are documented in the calibration certificate, validation accreditation, and final measurement report. Without these documents calibration, uncertainty, and proper result values cannot be proven to be traceable.
– **Uncertainty:** Every step needs to include measurement uncertainty. It is logical that if uncertainty information is missing from any measurement step, traceability is lost. The main reason is that without knowing and documenting the uncertainty, instrument calibration could be verified by comparing the performance data with those claimed by less accurate method.

Analyzing a sample generates a chemical or physical signal whose magnitude is proportional to the amount of analyte in the sample. In the case of quantitative analysis, we distinguish between primary and secondary methods. A general scheme for establishing traceability of chemical measurements to the SI units needs the indispensable first step of primary methods. Primary methods of measurement have a central function in metrology. They are an essential component in the realization of the SI units and therefore are indispensable for establishing traceability of measurements of all kinds of physical quantities to the corresponding SI units [12]. In chemical measurements, metrological traceability refers to the amount of substance, the mole according to the International System of Units.

3.3 Method of analysis: Primary methods

Primary methods are the first methods used in quantitative analytical chemistry. VIM defines a primary method of measurement as a reference measurement procedure used to obtain a measurement result without relation to a measurement standard for a quantity of the same kind. Primary methods are also defined as methods having "the highest metrological qualities, whose operation can be completely described and understood, for which a complete uncertainty statement can be written down in terms of SI units, and whose results are, therefore, accepted without reference to a standard of the quantity being measured" [13].

Intrinsic to a primary method definition, this could be either:

- Primary direct method: defined as a method that measures the value of an unknown without reference to a standard of the same quantity.
- Primary ratio method: defined as a method that measures the value of a ratio of an unknown to a standard of the same quantity, its operation must be completely described by a measurement equation.

Primary direct methods measure extensive quantities. However, analytical chemistry is mainly concerned with the measurement of composition, which is represented by intensive quantities. Intensive quantities may be developed and maintained ratiometrically (i.e., without reference to any external standard) using primary ratio methods.

While a primary method allows to characterize many substances using one independent reference, the major criterion for the definition of a secondary analytical method is the specificity of the method, and needs the chemically identical compound as its reference. The primary ones, such as gravimetry, freezing point depression, tritimetry, coulometry, isotope dilution mass spectrometry, or quantitative NMR, are based on a fully understood physical or stoichiometric principle; therefore no RMs in the classical sense, identical with the analyte, is used (but for titration, isotope dilution mass spectrometry, and NMR, well-characterized and

stable chemicals for standardization are needed). On the other hand, widely used methods such as UV spectroscopy, colorimetry, atomic emission, or chromatography can only report a quantitative result by the comparison of a sample with a RM of the same identity.

The vast majority of routine measurements of amount of substance are not made using primary measurement methods; they are made with methods that have measurement equations that are not completely described from first principles. The operation of secondary methods is described completely by a measurement equation based on the signal comparison of sample and reference solutions, respectively: $C_{analyte} = I_{analyte} \times C_{reference}/I_{reference}$, where $C_{analyte}$ is the concentration of the analyte in the sample solution (the final measuring solution after sample preparation); $C_{reference}$ is the concentration of the analyte in the reference solution; $I_{analyte}$ and $I_{reference}$ are the signal of the sample and of the reference solutions, respectively, such as spectrometric absorbance or chromatographic peak area.

It is not necessary that the content of the reference is 100.0%, but it is crucial that its purity is known, together with the uncertainty of this value (e.g., 99.9 ± 0.05%). Any high-quality analytical result should include information about the associated measurement uncertainty, and the purity uncertainty of the reference is a parameter which always appears in the overall measurement uncertainty calculation of the measurand (such as the concentration or content of an analyte) [14].

The operation of a primary method is described completely by a measurement equation which leads to results that are unbiased and allows a rigorous calculation of the accompanying measurement uncertainty. The most general equation is $I = k \cdot \mathrm{N}$. In this expression, I is the signal intensity output, N is the amount of substance for this signal, and k is a proportionality constant. Primary ratio methods run as two steps of primary direct methods according to the previously equation and, finally the combination of the equations, see Table 3.1, of both steps give the ratio method [15]. They can generally be considered to be methods that operate in two parts, each of which is a primary "direct" method.

Like any analytical method, primary analytical methods have to be validated before routine applications on qualified instruments and its application has to take place under conditions of very strict quality assurances. Secondary methods are only

Table 3.1: Classification of primary methods.

Primary direct methods	Primary ratio methods
$\boldsymbol{I = k \cdot N}$	$I_1/I_2 = \cdot [(k_1/k_2)/N_2] \cdot N_1$
Gravimetry	Isotope dilution mass spectrometry (ID-MS)
Freezing point depression	Quantitative NMR
Tritimetry	
Coulometry	

able to provide full traceability when calibrated with standards that have "primary" status (those produced by a primary method).

An example of such a method is GC where the sensitivity of the GC instrument is not described in terms of a measurement equation, but it is known to be stable and linear over a specified range. Therefore, it cannot be a candidate primary ratio method, but it can be used to provide traceability in the manner described here. Other examples would include colorimetry, many MS methods, ion chromatography, and stripping voltammetry. As with other nonprimary methods, GC is an instrumental technique whose response to an unknown sample is indicative of the amount, or content of an analyte within the sample. The sensitivity of the measuring instrument is calibrated with respect to a known standard. Therefore, such a measurement method relies on standards underpinned by primary gravimetric preparation. The accuracy of the GC method will depend, among other parameters, on the accuracy of the standards used for calibration, and on the repeatability of the measuring instrument [15]. Secondary analytical method could use a reference nonidentical with the analyte where the concentration of analyte is achieved by conversion using the relative response factors, see Table 3.2 for comparison of performance of primary direct and ratio methods and secondary methods.

In the case of primary methods, "uncertainty statement can be written down in terms of SI units" and this specifies an essential property that is required to provide traceability to SI. If there are significant parts of the operation of the method, which when written as a measurement equation cannot be expressed in SI units, then the results will not be traceable to SI. This would indicate the presence of significant influence on the measurement from parameters or quantities that are not evaluated with respect to the stated reference of the SI [16].

Gravimetric methods are the shortest link to primary standards (weigh), setting SI-traceability via of mass unit. Gravimetric analysis does not require expensive equipment, Figure 3.5. The most common type of balance is an electronic balance in which the balance pan is placed over an electromagnet. The sample to be weighed is placed on the sample pan, displacing the pan downward, the balance detects this downward movement and generates a counterbalancing force using an electromagnet.

Gravimetric measurement has limitations, for example, where a balance is used in a laboratory, the accuracy of measurement must be regularly checked. The weights used for this purpose should either have a certificate demonstrating that they conform to a standard, or the balance must be regularly checked against such standards by the regular use of standard weights which are well documented and thus can be linked within the laboratory to the calibration standard. This principle also applies to the calibration of other equipment. But there exist some limitations: (i) one must have enough mass for accurate weighing, (ii) one must demonstrate that only the analyte is being measured, for example, moisture-sensitive samples and, (iii) the method is very laborious for complicated samples, for example volatile liquid samples.

Table 3.2: Comparison of a primary direct method, primary ratio method, and secondary method.

	Gravimetry	qNMR	HPLC, GC
Method	Primary direct	Primary ratio	Secondary
Cost	low	high	medium
LOD	Balance precision dependent.	Mass dependent. Sample size aprox. 1.0 mg	low
Traceability	direct, easy	direct, easy	low
Time	fast	15 min	Time-consuming
Reference	Calibrated weights	Any material, high purity	Same material, high purity
Impurities	Must demonstrate that the analyte only is being measured	Data available, possible overlap	Difficult
Mode	Nondestructive	Nondestructive	Destructive
Calibration	One	One	One for each compound
Enantiomers	No	Rather no	Yes
# of compounds	1 compound	Separate resonances from > 1 compound at a time	More than 1 calibration.
Combination with other analytical methods	Optional	Optional	Mandatory (mass balance method)

Quantitative NMR (qNMR) spectroscopy may be considered as a potential primary method as recommended by CCQM because of being free of empirical factors in the uncertainty budget [17]. In this case, qNMR needs expensive instrumentation and maintenance and highly qualified staff to perform the measurements, Figure 3.5.

qNMR is the state of the art procedure for routine certification from the point of view of a commercial producer of certified reference materials (CRMs) under ISO/IEC 17025 and ISO Guide 34 accreditation [18]. In addition, NMR has been used to determine concentrations of synthetic and biosynthetic products, fine chemicals, and pharmaceuticals, as well as metabolites, catabolites, and endogenous compounds in biological fluids. Quantitative qNMR has been shown to be particularly useful in metabolomics, drug discovery and analysis, and natural product analysis.

Quantification is made possible by the fact that the intensity of the NMR signal is directly proportional to the number of atomic nuclei represented by that signal. So, quantification is achieved by measuring the sample peak area of interest with respect to a signal from an appropriate internal or external standard, such as an internationally accepted primary CRM. Proton is the most used atomic nuclei for quantitative purposes.

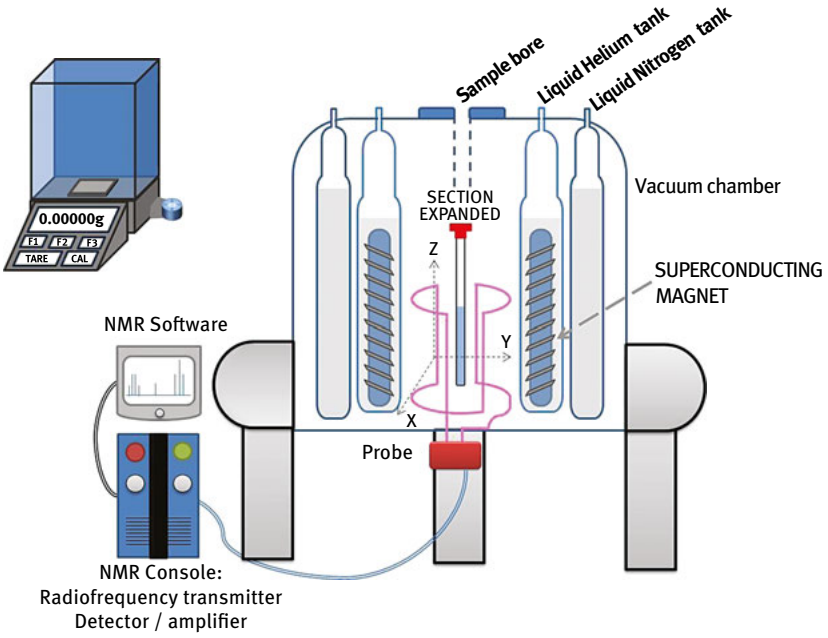

Figure 3.5: Representative figures of primary method equipment: balance for gravimetric measurements and nuclear magnetic resonance spectrometer.

Using such a primary CRM, for example a standard reference material from the US National Institute of Standards and Technology leads to traceability to the SI without the need for a reference standard of the same chemical structure as the sample [18].

qNMR experiment is composed of:

1. High-precision weighing of sample and standard with balance.
2. Addition of suitable deuterated NMR solvent.
3. Transfer to NMR tube.
4. Calibration of the measurement instrument parameters to assure that all protons across the spectrum become equally sensitive, so determination of quantitative results does not require the need for compound-specific extinction coefficients or calibrations.
5. Once those quantitation parameters are on the probe file, measurement takes about 15 min to complete. There is no maximum time.

The advantages of qNMR are:

1. It is capable of purity determination even for a compound without a RM.
2. Absolute quantification is not affected by impurities. At least one [1] H-qNMR signal must be not overlapped.

3. A calibration curve for quantification is not necessary.
4. The presence of a residual solvent easily missed in chromatography or gravimetry can be confirmed in qNMR.
5. Instrument stability.

While the disavadtages are:
1. Quantification is only possible for nonoverlapping peaks from NMR-active material.
2. Size of sample.

3.4 Qualitative methods: A new focus on analytical chemistry

A qualitative method can be defined [19] as an analytical method which identifies a substance based on its chemical, biological, or physical properties, whereas a quantitative method is an analytical method which determines the amount of a substance and expresses it as a numerical value and an appropriate unit.

A qualitative method tries to determine the presence or absence of one analyte in a sample (Figure 3.6) with respect to a specified level, below which the analyte is considered insignificant and above which the analyte is considered present. A quantitative method serves us to determine the concentration of that analyte in the sample. At the middle of these two types of methods, semiquantitative methods are situated [20], analytical methods which assign the test samples to a given class, for example high, medium, low, or very low. These methods provide some quantification of the analyte in the sample, useful when the accuracy of the measurement can be low.

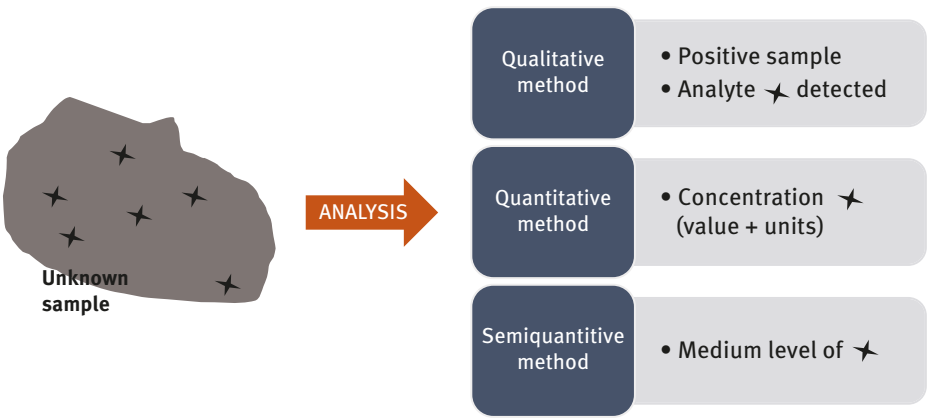

Figure 3.6: Differences in results from qualitative, quantitative, and semiquantitative methods.

During the last decades a lot of attention has been given to quantitative methods, their performance and validation, with qualitative methods being considered less interesting. Nevertheless, at this moment qualitative methods are being reconsidered due to their rapid and reliable response, which allows a high sample throughput in short time periods, and their adaptation to customer requirements. In certain cases, the most important issue is determining if a sample complies with a specific legislation and the accurate concentration result is not a key point. An example of this situation is the screening of a set of samples to determine the presence or absence of contaminants above or below the maximum permitted level (specification limit or threshold value) fixed by certain authority. In this situation customers need to know the classification of each sample and the quantification of each contaminant is a useless activity.

Different objectives can be listed for qualitative methods [21]: obtain information required for a specific purpose in a rapid procedure, eliminate as far as possible the pretreatment of the samples prior to their analysis (time-consuming and source of variability), or reduce the use of instruments (principally to avoid their costs and the qualified personnel involved). These methods are sometimes used to filter a set of samples and determine which ones must be analyzed by quantitative processes, so they are also called screening systems.

There are various classifications of sample screening systems, according to different criteria [21]. The relation between the analyte and the signal can be direct or due to a chemical, biochemical, or immunological reaction; the binary response obtained can discriminate one analyte or can be the same for various analytes from a chemical family; the state of the sample can be solid, liquid, or gaseous; the system can be used inside or outside the laboratory; and the screening can be independent or implemented during an analytical quantitative process.

According to the sample treatment, screening systems can be classified as [21] direct screening without sample treatment, screening after a simple sample treatment, and screening after a full sample treatment. In the last case, screening methods are preferred to minimize the use of expensive analytical equipment or to avoid another more complicated sample pretreatment.

Considering the type of detection, screening systems can be divided as sensorial and instrumental [20]. In sensorial detection, human senses are used to interpret the response of the method, the most common being vision. Responses are obtained due to the reaction between the analyte in the sample and a specific reagent involved in the procedure and the concentration of the analyte can be related directly or indirectly with the magnitude of the response. Different reaction concepts are used (chemical, immunological) and the final color can be determined either by visual inspection or by comparison with a color card, etc. In instrumental detection, comparison is made between the unknown sample and a reference sample which contains a specified level of the analyte, and one obtains an instrumental response, for example absorbance. The result of this kind of method is also

a binary response (yes/no) as in the sensorial detection, no calibration curves are made, and no quantification is obtained.

According to the fitness for purpose [22, 23], there are two main types of qualitative analyses. The first one is performed to classify the samples according to a specific criterion, provided by a regulation, a customer, or a laboratory specification and related to a single measurand or to a group of them. The second type pursues the identification of analytes or groups of analytes using techniques of separation and detection, for example chromatography and spectroscopy.

Considering the practical implementation of the qualitative method [23], different types of systems can be used: test kits and sensors which show the binary response directly (usually for sample classification) and conventional instrumentation which needs a treatment of the obtained data to convert it into a binary response (usually for identification of measurands). Maybe test kits are the most extended alternative, including a chemical reaction and an evaluation system, usually based on color.

The need to demonstrate traceability of results is essential in quantitative and in qualitative methods. This property assures that results can be related to an external reference (preferably a SI unit) through an unbroken chain of comparisons [10]. The final reference can be established using three different approaches [22]: comparing the results of the qualitative method with those obtained by a confirmatory, reference, or primary method; analyzing aliquots of CRMs; or participating in interlaboratory exercises (in this case one of the laboratories has established the traceability of the tested method).

Validation of qualitative methods is needed to apply them in solving several analytical problems. For example, in food chemistry these methods are used to detect contaminants (pesticides, mycotoxins, etc.) and the results have important consequences for producers, consumers, and authorities. In these applications, assuring that an analyte is present in a tested sample or that its concentration is above or below a specified level is very important and performance characteristics of the methods shall be determined.

A general procedure for validation of qualitative methods (Figure 3.7) includes different steps [22]. In the first one the analytical information that must be obtained by the method is fixed, according to customer's needs, objectives established by the laboratory, quantities provided by legislation or any other input, and the most appropriate method to solve the analytical problem is selected. In the following step, the performance characteristics of the method are determined, and the experimental processes needed to obtain them are designed. Finally, experimental results are obtained and compared with analytical information initially fixed as necessary and decision about validation of the method is made. To perform a complete validation of qualitative methods three types of samples must be available [22]: CRMs in an appropriate range of concentration, reliable blank samples, and unknown target samples.

The most important performance characteristics for qualitative methods (Figure 3.8) are specificity, LOD, reliability (false positive and false negative rates),

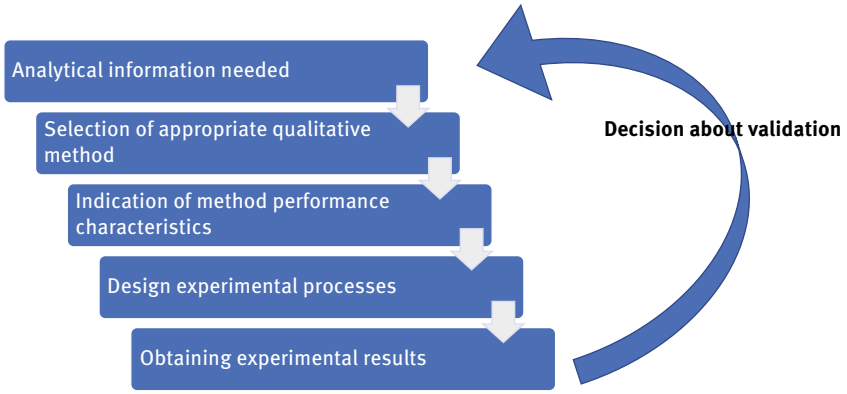

Figure 3.7: Steps of validation procedure for qualitative methods.

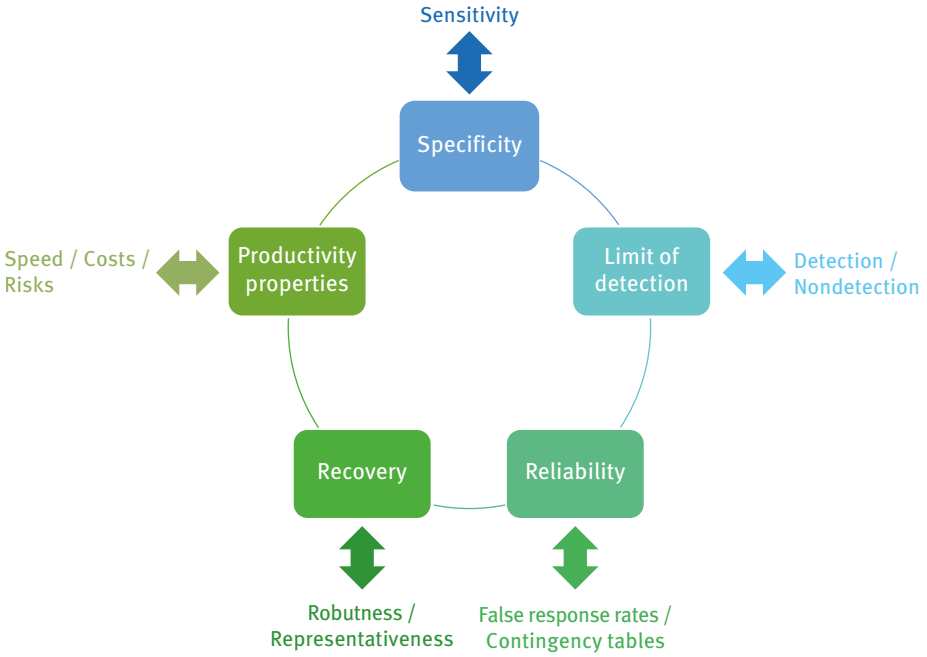

Figure 3.8: Main performance characteristics of qualitative methods.

and recovery. From a practical point of view the productivity-related properties (speed, costs, and risks) are also very important for a real implementation of qualitative methods [22], since a very expensive and slow method will not be selected by common users and it will be desirable to handle low-risk reagents.

Specificity [20] is the capacity of a qualitative method to classify known negative samples as negative, so it is the probability [24, 25] of achieving a negative result using

a known negative sample. This parameter is clearly related to sensitivity [20], the capacity of a qualitative method to classify known positive samples as positive, which is the probability [24, 25] of achieving a positive result using a known positive sample.

The LOD [22] is the amount of an analyte that provides a signal statistically different from the signal of a blank sample, and this property establishes the separation between zones of detection and nondetection

In validation of quantitative methods an essential parameter is the uncertainty [23], expressed as the concentration range within which the results can be expected, so it is a measure of the dispersion of the results [26]. In qualitative testing uncertainty is an indication [26, 27] of the probability of a sample classification being wrong. In this context, reliability [21, 22] is defined as the percentage of correct results provided by many independent tests using aliquots of the same sample (usually a CRM). This characteristic can be considered as a combination of accuracy and precision, both basic properties of quantitative analysis. Considering this, unreliability [20, 23] is the response range where the false responses (positive or negative) are produced. False negative and positive rates measure the probability that a known sample (positive or negative) is incorrectly classified (negative or positive, respectively) by the qualitative method. Bibliography refers to these terms with different definitions according to the specific author. For example [24], false negative rate can be the fraction of observed negatives that are false, the fraction of true positives that are considered negatives, or the fraction of all results that are false negatives. Different definitions correspond to different quantities, so the term must be correctly specified prior to the experimental design of the validation study.

Although false response rates are the most used form to express uncertainty in qualitative methods, other alternatives are possible [26, 27]:

- Contingency tables: In these tables the results of qualitative analysis are classified into categories, in the simplest case positive or negative. Some parameters, such as sensitivity and specificity, are calculated from the table and provide an overall idea of the performance of the assay and its reliability. They are also used to compare different assays, but cannot estimate the probability of obtaining a wrong result in an individual sample. The performance of this alternative depends on the sample size, but it is frequently used due to its easy application in a lot of different qualitative assays.
- Bayes' method: In this approximation the probabilities are estimated according to similar measurements under similar circumstances considering historical and conditional probabilities, obtaining a more complete measure of uncertainty than in the previous approach. This method is more complicated but provides an estimation of the uncertainty associated with a new measurement.
- Statistical intervals based on the normal or Gaussian distribution and its properties: A relationship is established between the specification limit (usually a concentration) and its instrumental response. Then the response of the unknown sample is compared to the one at the specification limit and the result is defined

with a calculated probability of committing errors. This method must be used with instrumental responses; it cannot be applied to binary approaches.

– Performance curves: These curves are established after the analysis of several samples with different concentrations using a screening method. The percentage of positive results above the specification limit is represented versus the concentration level and a cut-off concentration can be calculated. This is the value from which the response of the method is above the specification limit with a specified probability of error.

The recovery of a method needs to be determined if it includes extraction steps. One variable which must be considered to define these steps is the robustness of the qualitative method [21, 22], defined as the invariability of the sample results using slightly different experimental conditions (pH, temperature, etc.). For example, one parameter involved in robustness is the stability of the reagents used in the tests, which can be affected by variables as the temperature or the exact composition of the samples. On the other hand, the representativeness [22] of the sample is essential for obtaining reliable results, so correct sampling protocols must be established. Finally, depending on the selectivity of the method, the sample treatment will involve different number of steps, which will affect the recovery achieved.

Quality control procedures must be established to monitor the validity of the methods performed, either quantitative as qualitative. Important issues are the complete description of the parameter related to quality, the definition of the influences in quality and the features which determine the optimal quality. Typical approaches [28] are the analysis of RMs and blind samples, the use of control charts, the analysis of blank, duplicate, and spiked samples, and the proficiency testing. In qualitative analysis, quality control focuses on identifying false positives and false negatives. Internal quality control is related to the maintenance of validation conditions in the laboratory for a long time, whereas external quality control is used to compare results between laboratories.

Control charts [28] can be used for binary response, using a sample A that must produce a 100% positive response to detect false negative and a sample B that must produce a 100% negative response to detect false positive. In the simplest approach individual experimental data are represented and out-of-control situations are detected. If several analyses have been done, false rates can be represented in the control chart, for example one point for each experimental day. In this approach reference lines of the chart can be established, as an acceptance error level at 5% of false response, the warning limit at 10% of false response and the control limit at 15% of false response, and false responses include false positive and false negative. A more complex approach is used to represent false positives and false negatives independently, using a control chart with two axes and four regions: situation under control, false positive response, false negative response, and double false response (Figure 3.9). As in the previous case, results of each day must be plotted to detect

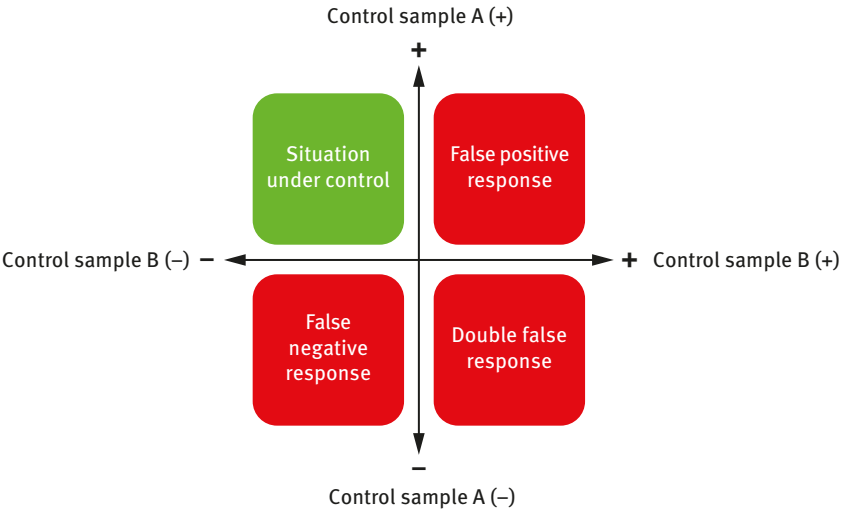

Figure 3.9: Control chart for qualitative analysis representing four regions: situation under control, false positive response, false negative response, and double false response.

out-of-control situations. In all approaches, the frequency of quality control must be established by the analyst, based on the number of samples used, the reliability of the method or the application of the results, but it is always recommended to use at least two control samples, one positive and one negative.

Proficiency testing programs have been adapted for qualitative analysis, showing its capacity to evaluate the comparability of the results produced by different laboratories. In these programs specificity and sensitivity of the method are estimated [24], employing samples at a representative range of concentrations and blank samples, all correctly characterized by quantitative methods.

CRMs are rather complicated to use in qualitative methods [20], because they must contain the analyte at a concentration level close to the limit of the method. If the concentration level is either far below or far above this limit, its use will only indicate if the method correctly classifies samples as negative or positive. For concentrations close to this level, the probabilities of false positive and false negative responses must be computed, so the comparison with a CRM must be in terms of probabilities, and cannot be in terms of concentrations.

Pure reference materials are analyzed by the instrumental techniques in qualitative methods used for identification [23] and their results are stored in libraries. After that, the results of the unknown sample are compared with these libraries and the identification of the compounds is performed using probabilistic techniques. Usually for classification methods a matrix RM must be used, since the interferences of the samples can modify the obtained results. Screening and confirmatory methods must be related to reference methods to demonstrate traceability, comparing the proportions of positive results.

In summary, qualitative methods are an often-forgotten part of analytical chemistry with very interesting properties to fulfill customer requirements, since they can provide a speedy answer to solve an analytical problem, without using expensive equipment and specialized personnel. Due to these reasons it is quite probable that in next years they will be developed and be used in every field of analytical measurements.

3.5 Reference materials: Characteristics, applications, and producers

A RM is a "material sufficiently homogeneous and stable with respect to one or more specified properties, which has been established to be fit for its intended use in a measurement process" [29]. Four important properties characterize all RMs (Figure 3.10):

- A property, qualitative or quantitative, defined by an element or a compound dissolved in an appropriate solvent or being part of a matrix.
- The homogeneity between all the units of the material, this characteristic is essential to guarantee the equivalence between measurements performed in several laboratories with different units of the material.
- The stability of the material in different temperature conditions along the time. The period of validity of the material and the appropriate transport conditions are defined from this characteristic.
- The intended use of the material, necessary for its initial design and final use.

RMs have been used for a long time, maybe the first one was issued by the Association of Official Agricultural Chemists in 1885, when they distributed six fertilizer samples with the aim of improving interlaboratory agreement [30].

Different forms of RMs can be available [31–33]:

- Pure substances, characterized by their purity or by the presence of trace impurities.
- Standard solutions and gas mixtures, mainly used for calibration purposes and usually prepared from pure substances using gravimetric steps.

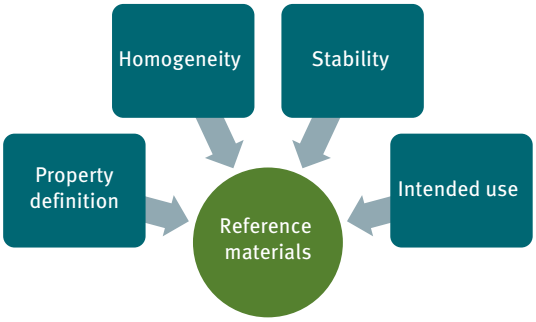

Figure 3.10: Properties of reference materials (certified and noncertified).

- Matrix RMs, prepared from natural matrices with the appropriate components or from fortified synthetic matrices, and characterized by the composition of certain constituents.
- Physicochemical RMs, defined by certain properties such as melting point, viscosity, pH, flash point hardness, etc.
- Methodologically defined RMs, characterized by an analytical protocol for certain parameters such as bioavailability of an element, soluble fraction, etc
- Reference objects or artifacts, defined by functional properties such as taste, odor, etc.
- RMs with an appropriate matrix composition, for the calibration of certain measuring instruments.

Also, RMs can be classified according to the method used for their characterization. Primary RMs are those materials established (and/or certified) using a primary method of measurement [34], which enables its traceability. Secondary RMs are defined according to primary RMs and are used to characterize working RMs.

Considering their composition (Figure 3.11), RMs can be single-substance RMs or matrix RMs [35, 36]. The first category is formed by pure chemicals or solutions of pure chemicals, usually with a low uncertainty associated which enables them to be used for calibration. In this case they are also called calibration standards or calibrators. The second category includes materials which contain the analytes in their natural form present in the environment, naturally collected or fortified, although spiked materials sometimes are not representative of real samples. The matrix is chosen to resemble the matrix of the samples that are going to be tested and the reference values of the analytes must be clearly established and correspond to the values of the problem samples. These RMs show higher uncertainties and are used to compare analytical methods and determine their characteristics, considering all the steps (Figure 3.12): extraction, cleanup, concentration, and measurement [36]. This is particularly important for analytes strongly bound to the matrix, for example for certain

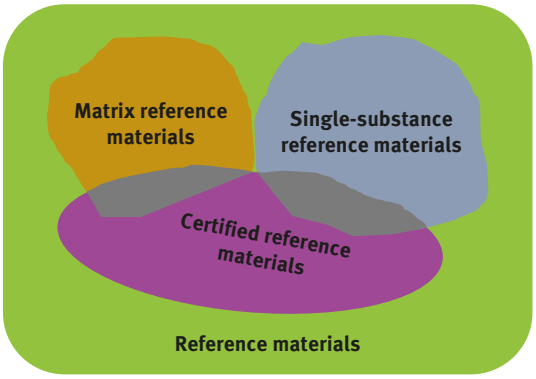

Figure 3.11: Types of reference materials.

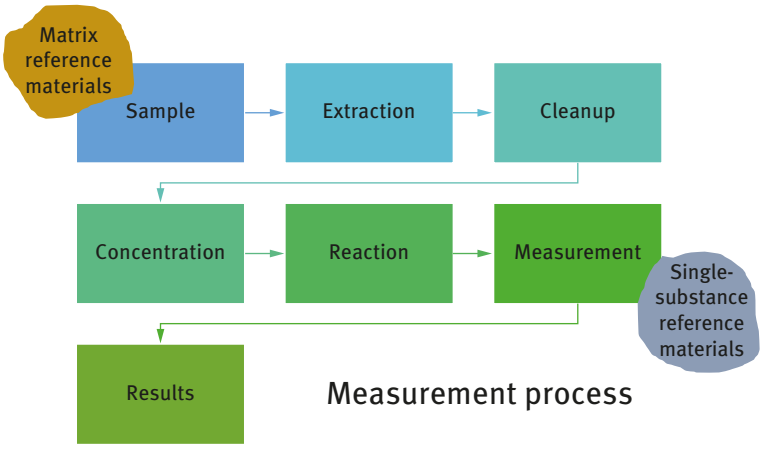

Figure 3.12: Use of single-substance reference materials and matrix reference materials in a measurement process.

contaminants present in food, because this influences the extraction efficiency [37], and for clinical RMs, which must show properties similar to those of human samples, i.e. commutability [38].

The suitability of a matrix RM will depend on its correspondence with the matrix of the routine samples. Due to this sometimes they are "fit for purpose" [39]. There are a lot of specific needs of matrix RMs in all the fields of analytical science and if the need is not common the end user must develop its own laboratory RM, stable and homogeneous, to carry out quality control checks. In this sense, European commission has supported the development of CRMs through consecutive Framework Programmes, obtaining a high number and variety of materials [40, 41].

In the field of food security, a complete composition analysis must be available to comply with the nutritional labeling laws, for exportation activities and to assist consumers in their dietary choices. For example, US foods sold must be labeled with calories, total carbohydrate, dietary fiber, sugars, total and saturated fat, cholesterol, protein and certain vitamins and minerals. AOAC International developed a fat-protein-carbohydrate triangle [42–44] with nine sectors that try to represent all the different food compositions and assist users in the accurate composition determination. CRM producers must develop materials for each sector, defined by an interval of percentages of fat, protein, and carbohydrate. The number of foods in each sector also influences the development of these materials; if more foods belong to a sector, the need of RMs will be higher. An important group of food with special needs is infant formula [45], to ensure the product safety for this special group of consumers.

Another important field affected by the availability of appropriate RMs is the clinical diagnostic field [46], which needs to apply the concept of traceability to *in vitro* measurements. In this case a complete traceability chain, with an unbroken chain of

comparison, should be established using the same measurement procedure in all the steps, to avoid slightly different definitions which are traduced in bias and imprecision. Matrices which fulfill the requirements of commutability with human samples must be produced and uncertainties of RMs should be low to increase their suitability for clinical measurements.

Sometimes RMs which are not accompanied by a certificate are defined by their use, for example proficiency testing materials, laboratory RMs, or quality control materials [35, 47]. Other names from the literature are in-house RMs or laboratory control materials, although these terms are associated to materials produced by an analytical laboratory for its own use rather than by an external provider [48].

To avoid increasing costs, final users must know the characteristics needed for their application and select the cheapest material that fulfills their requirements. For example, for analytical quality control an important characteristic will be the minimum sample intake, since this material is going to be used every day or, sometimes, in an even more frequent interval. Nevertheless, for calibration purposes this parameter will not be critical, since the frequency of use will be lesser [35]. In this last application a much more important characteristic is the uncertainty associated to the material, sometimes CRMs cannot be used for calibration purposes due to their very large uncertainty [47].

Some users suggest that the prices associated to RMs are very high and that the production of in-house RMs will be cheaper. This approach must be considered, because the production system needed to obtain a RM is complicated, with a lot of stages (Figure 3.13) performed by competent staff in calibrated equipment and several quality controls that guarantee the reliability of data associated to the material, necessary for its use. If an independent organization decides to produce its own in-house

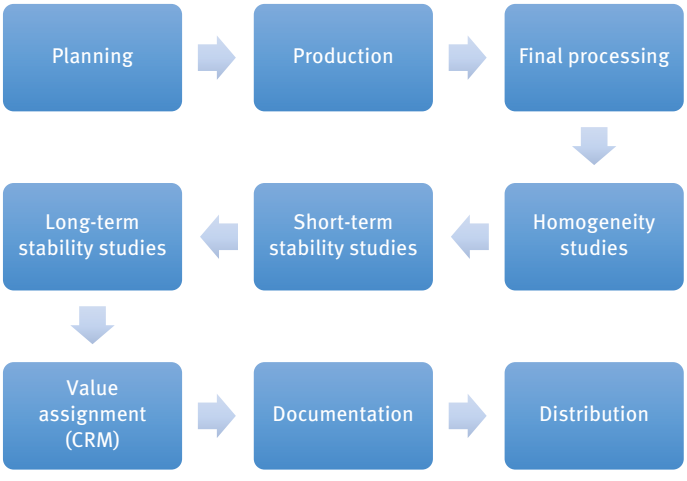

Figure 3.13: Steps of the process used to obtain reference materials.

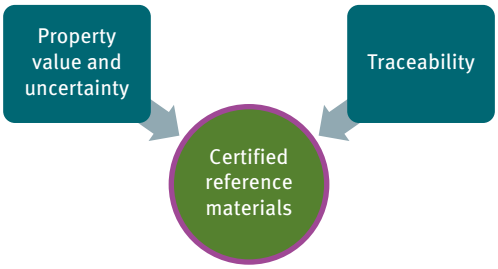

Figure 3.14: Additional properties of certified reference materials.

RMs, one must consider the cost associated with the work needed to obtain the materials and the time expended in these activities [35]. Also, the economic impact of using unsuitable materials must be considered, obtaining incorrect results and the loss on good image. Despite all this, as previously indicated, in some cases a laboratory needs to produce its own RMs due to various reasons, for example the lack of appropriate commercially available ones, a very usual situation in R&D laboratories, the need of a material that matches with a specific type of routine samples, etc.

CRMs are a group of RMs, defined as "reference material characterized by a metrologically valid procedure for one or more specified properties, accompanied by an RM certificate that provides the value of the specified property, its associated uncertainty, and a statement of metrological traceability" [29]. This group of RMs is characterized by two additional properties (Figure 3.14):

- A property value formed by a number with an associated uncertainty and the indication of its most relevant contributions, and the coverage factor used in the calculations. The reference value must be the best possible estimation of the true concentration of the material, being largely unbiased and highly precise [49]. The uncertainty must be small in comparison to the global uncertainty of the analytical method in which the material will be used, in the best cases it must be small enough to be ignored [30]. This is an important issue, since an analytical result can never be better than the RM on which it is based [36].
- The establishment of the metrological traceability, which assures that the property value can be related to the appropriate units or references (preferable SI units) through an unbroken chain of comparisons. This property guarantees that all the measurements of the same magnitude are comparable, because they share the same reference point, and that the uncertainty of the measurement can be correctly estimated [50]. Metrological traceability is indicated through the characterization method, the property and the reference used.

Appropriate references can be reference methods, RMs, or SI units, that must be clearly related to the property of the CRM produced. The unbroken chain of comparisons must be established to assure that there are not losses of information during the analytical procedure, especially if it requires successive experimental steps, long times of storage or sample collection. The uncertainty associated with each step of

the analytical procedure must be estimated and used to obtain the final uncertainty of the RM, this means that the smaller the chain of comparisons the better the final uncertainty and the more extensive the applications of the final material [51].

RMs must be sold accompanied by a document, sometimes called RM document or product information sheet [52], containing some mandatory items: title of the document; exact identification of the RM (example.g., indicating code and batch); name of the RM (which should provide a detailed description of the material); intended use; minimum sample size to be used (if it is applicable); period of validity; commutability (if it is required); and instructions for storage, handling and use. The version of this document must be indicated, together with the total number of pages, to guarantee the traceability of the included data. On the other hand, CRMs must be accompanied by a certificate of analysis, containing all the mandatory information previously listed and the following additional items: description of the material, property of interest, property value and associated uncertainty, a statement of metrological traceability, measurement methods (for method dependent measurands) and name and function of the approving personnel. Other information can be available in these documents, as the measurement methods used for method-independent measurands, health and safety information, use of subcontractors, indicative values, legal notice or reference to other documents. Users of RMs (certified or noncertified) must check that documents provided contain all the mandatory information, since its absence can be an indication that their quality and properties have not been correctly established.

Applications of RMs [32] are related to the previously commented characteristics and are different for certified and noncertified materials. The most important are:

- Calibration of methods and equipment.
- Method validation.
- Assignment of values to materials.
- Quality control.

Method validation includes determination of bias and precision. Bias is essential to guarantee the traceability of the results obtained whereas precision checks the within-laboratory standard deviation usually under repeatability conditions. These two characteristics can also be used in quality control, during the routine use of the method. Assignment of values to materials is a widely used application, for example pure materials are used to prepare solutions which are diluted to be used as calibrators, and the final concentration can be calculated using initial data and preparation done.

If the application needs an establishment of traceability only CRMs can be used, for example the calibration of equipment or the bias control. However, if the traceability is not necessary, for example in precision control, nonCRMs can be used (Figure 3.15).

To select an appropriate CRM for a specific measurement method, some characteristics must be checked: its concentration must be in the same level as the measurement process, its matrix must resemble as close as possible the matrix of the measured samples, its form should be the same as the samples, its quantity must be

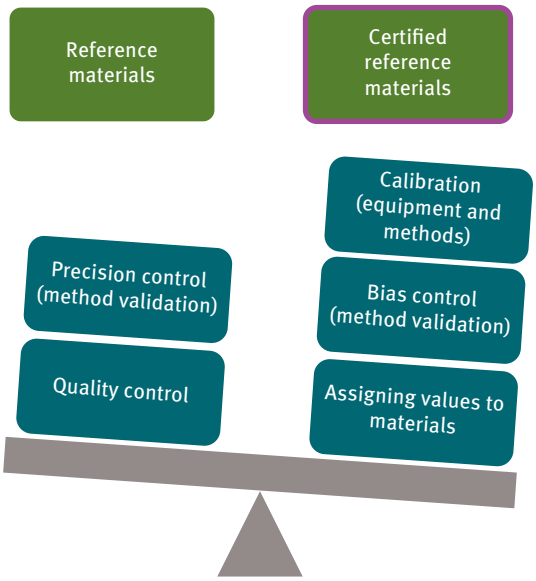

Figure 3.15: Applications of reference materials (certified and noncertified).

enough to perform a complete series of experiments, its properties should be stable during the experiments, its uncertainty must be compatible with the requirements of precision and trueness of the method and the commutability of the material, and the measured samples must be stated where applicable.

RMs can be found in catalogs and websites of producers. Nevertheless, some international databases have been developed to compare their characteristics and guarantee their confidence. COMAR (Code d'Indexation des Matériaux de Référence) database has been proposed in late 1970s and currently is free of charge for users and available through the web. It contains materials from several fields of application with complete information about description, form, chemical composition, physical properties, certificates and reports, status of availability, and producer. Signing up is mandatory and the search can be done according to the name of the material, physical and conventional property, fields of application, form and composition. Also, the Technical Division on Reference Material of AOAC International has developed its own RM database [53]. It is an online searchable database where RMs can be searched by analyte, by analyte and matrix and by their use in AOAC Official Methods of Analysis. This database provides useful information to compare different RMs and the information needed to obtain their complete certificates of analysis. Similar databases have been developed for specific materials and from private organizations, for example the Virtual Institute for Reference Materials database, an online database of RMs accessible by subscription.

Currently the interest in RMs is growing due to the new edition of ISO 17025 which will be available soon. In previous editions of this standard [4] the use of RMs was considered as optative and other types of materials were mentioned as appropriate,

without clear indications of the needed properties. Also, there were references to competent material providers without indication of their characteristics or the needed standards to comply.

In the last draft of the new edition of ISO 17025 [54] there appear references to the use of appropriate RMs and traceable RMs produced by providers according to the requirements indicated in ISO 17034, for equipment calibration, traceability establishment, and quality assurance. If the final new edition of the standard contains these considerations, all analysis and calibrations done in accordance with ISO 17025 will need RMs provided by externally accredited RM producers.

The accreditation is an independent declaration of the competence of an organization, the suitability of their methods, the appropriateness of their equipment and facilities and the assurance provided by their internal quality control. It also guarantees their technical competence and the independence of the personnel and the organization. Quality of analytical measurements guarantees consumer safety, environmental protection, and worldwide trade, while external accreditation assures that RMs meet their requirements.

Requirements that RM producers must fulfill were included in ISO Guide 34 [55], which is now obsolete and has been substituted by ISO 17034 [56]. Both documents are similar, but the new standard has been elaborated due to the increased interest in the production and use of RMs and the need for an international standard to be used by certain accreditation bodies [57]. A definition of RM producer appears in both documents, which includes their main functions: project planning and management, assignment and authorization of property values and related uncertainties, and issue of certificates and other relevant information which accompany the materials.

Requirements for RM producers were initially [55] divided as management system, technical and production requirements, whereas according to the new standard they are [56] general, structural, resource, technical, and production and management system (Figure 3.16). Requirements are very similar, but they are divided into different categories to adopt the common structure of other International standards about conformity assessment. RM producers are conformity assessment organisms, which guarantee that their products meet the relevant requirements, like calibration and testing laboratories, certification and inspection bodies or proficiency testing programs providers.

General, structural and management requirements are related to internal organization of producers, document control, customer service, internal structure, corrective actions, internal audits, and management reviews. In the new standard the main changes are the inclusion of requirements related to confidentiality and impartiality and the actions to address risks and opportunities, according to the new edition of ISO 9001 [58]. Management requirements can be demonstrated by a certificate according to ISO 9001 provided by an accredited certification body.

Resource, technical and production requirements include several points related with personnel, use of subcontractors, equipment, procurement of services and

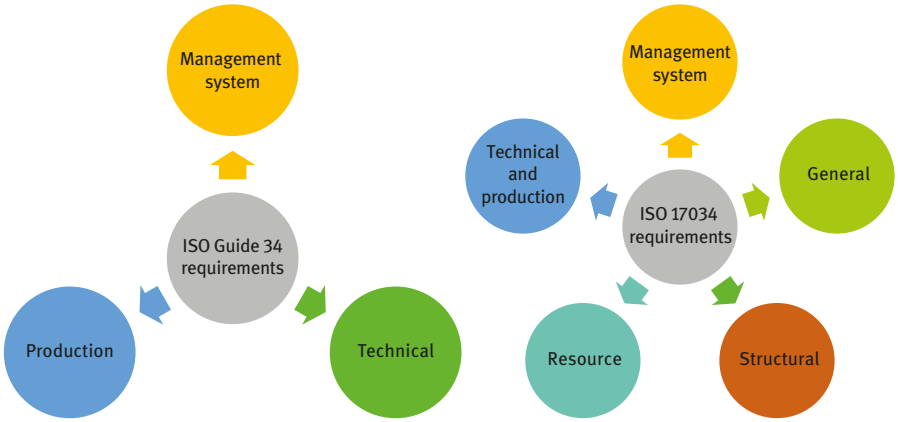

Figure 3.16: Requirements for reference material producers according to ISO Guide 34 and ISO 17034.

supplies, facilities, production planning and control, material handling and storage, characterization, homogeneity and stability of methods, assignment of values and uncertainties, traceability, RMs documents and labels, distribution services, quality and technical records, management of nonconforming work and complaints.

Several activities related to these requirements can be subcontracted to other organizations. In this case producer must demonstrate that requirements are achieved, and that essential information is provided to guarantee that activities are performed in an appropriate way. There are only four activities that should be always developed by the producer: production planning, selection of subcontractors, assignment and authorization of property values and uncertainties and authorization of certificates or other documents which accompany reference materials.

One important activity is the characterization of the materials, which can be done by different approaches, guaranteeing the traceability of the characterization. Some of the used schemes are [55, 56]:

- A single (primary) method in a single laboratory.
- Two or more independent reference methods in one or several laboratories.
- One or more methods of demonstrable accuracy, performed by a network of competent laboratories.
- An approach providing method-specific, operationally defined property values, using a network of competent laboratories.

The capability of a RMs producer is demonstrated by an external accreditation, completed with a technical annex or similar document with the extent of the accreditation and the list of included RMs.

In summary, RMs are a main topic in analytical research [59, 60], with several producers and international metrology organizations improving their characterization,

increasing their fields of utilization and trying to meet customer needs. It is supposed that future studies will expand their use to laboratories worldwide, due to their interesting characteristics and their broad range of applications.

Keywords: Primary method, reference material, calibrant, quantitation, quality control, monitoring, uncertainty

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M. Carmen Louzao and Paula Abal

4 Toxicological studies with animals

4.1 History of toxicity studies with animals and legislation

The history of toxicity studies begins with Paracelsus (1493–1541), physician, alchemist, and astrologer regarded as the father of toxicology, when he demonstrated the harmless and beneficial effects of toxins and proved dose-response relationships for the effects of drugs. Mateo Orfila (1787–1853), a Spanish physician and father of modern toxicology, determined the relationship between poisons and their biological properties and demonstrated specific organ damage caused by toxins [1].

In 1831, British physiologist Marshall Hall proposed five principles that should govern animal experimentation. First, an experiment should never be performed if the necessary information could be obtained by observations; second, no experiment should be performed without a clearly defined objective; third, scientists should be well informed about the work of their predecessors in order to avoid unnecessary repetition of an experiment; fourth, justifiable experiments should be carried out with the least possible infliction of suffering; and finally, every experiment should be performed under circumstances that would provide the clearest possible results, thereby diminishing the need for repetition of experiments. Many of his recommendations were formally instituted over a century later in the British Animals (Scientific Procedures) Act and the U.S. Animal Welfare Act.

Toxicological screening methods and toxicological research on individual substances were developed in the mid-1900s. The twentieth century brought the conception that health can be impaired as a result of the ingestion of a single dose but also of exposure to small chemical doses over an extended period of time. There was a shift in focus from acute to chronic toxicity [2]. The use of animals in toxicity studies began in 1920, when J. W. Trevan proposed the use of the 50% lethal dose (LD_{50}) test to determine the lethal dose of individual chemicals [3]. This test became accepted as the standard metric of toxicity. After the introduction of LD_{50}, a FDA scientist John Draize developed a method for testing eye and skin irritation using rabbits, and this method was widely accepted to evaluate the effects of chemicals and pharmaceuticals on the eye and skin [4]. Later, the US National Cancer Institute developed a test to identify carcinogenic chemicals through the daily dosing of rats and mice for 2 years.

In the early 1960s, thousands of babies were born with birth defects caused by thalidomide. After this, all the regulatory agencies concentrated on determining the toxicity profiles of all pharmaceutical substances available for regular patient use and made mandatory the submission of toxicity profiles of investigational new drugs. Toxicity testing with animals is necessary to prove that new drugs are safe before

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clinical trials and first administration to humans, to know side effects of drugs or to evaluate the harmful effects of toxins. Therefore, animal testing plays a large role in research and drug development and it occurs regularly throughout the EU. Rats and mice are the most commonly used animals in laboratories. Recent years have seen a surge in the use of zebrafish and nonhuman primates. However, while ethical concerns regarding zebrafish are low, those involving nonhuman primates are high. Therefore, the protection and welfare of animals used for scientific purposes is an area covered by EU laws.

The publication of "The Principles of Humane Experimental Technique" by W.M.S. Russell and R.L. Burch in 1959 marks the birth of the principle of the "Three Rs": the replacement, reduction, and refinement of animal testing. Directive 2010/63/EU [5] that updated and replaced the 1986 Directive 86/609/EEC is an indispensable tool at the EU level to protect experimental animals. The aim of the new directive is to strengthen legislation, and improve the welfare of those animals still needed to be used. In addition, the directive on the protection of animals used for scientific purposes [5] implements the Three Rs in Europe that took full effect on 1 January 2013.

4.1.1 The three Rs

The welfare of animals used in toxicology research is very important. There are good ethical, scientific, legal, and economic reasons for making sure that animals are looked after properly and used in minimum numbers. If an animal is suffering stress or pain it could affect the results of the research, therefore good animal welfare can improve the quality of science.

The guiding principles supporting the use of animals in scientific research are the Three Rs, replace, reduce, and refine:

- **Replace** the use of animals with alternative techniques, or avoid the use of animals altogether if that is possible.
- **Reduce** the number of animals used to a minimum, to obtain information from fewer animals or more information from the same number of animals.
- **Refine** the way experiments are carried out, to make sure animals suffer as little as possible. This includes better housing and improvements to procedures which minimize pain and suffering and/or improve animal welfare.

The principles of replacement, reduction, and refinement must be considered systematically when animals are used for the purposes of basic, translation and applied research, regulatory testing, and production as well as for the purposes of education and training in the EU. The facility, personnel involved, and experiments with animals should comply with the legislation on experimentation with animals. In some cases, it is possible to develop a whole new way of conducting a test involving

fewer animals. For example, the acute toxicity test with lots of animals was used for many years to find out how toxic chemicals are, but nowadays scientists are developing better tests, to do the same job but using fewer animals. The Joint Research Centre is host to the European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) that is actively involved in search for test methods for the Three Rs in animal testing. The EURL ECVAM Strategy papers cover areas such as acute mammalian systemic toxicity, aquatic toxicity and bioaccumulation, genotoxicity, skin sensitization and toxicokinetics (TK), and systemic toxicity.

In the late 1980s, the Organisation for Economic Cooperation and Development (OECD) and the International Conference on Harmonization (ICH) brought out the guidelines for toxicity testing of pharmaceutical substances. Test Guidelines Programme of the OECD has developed standardized methods of testing that are accepted by all OECD member countries through an agreement on the mutual acceptance of data. The OECD has substantially reduced the total number of animals used for certain standard tests. It also provides a focus for the introduction of new methods according to the Three Rs. Besides, regulatory agencies, such as OECD, EPA and FDA, administered the Good Laboratory Practices that are regulations to ensure the integrity of data from nonclinical studies.

In 2007, the National Research Council issued a report on toxicity testing that recommended move away from the use of animals in laboratory experiments [6]. However improved *in vitro*, *ex vivo*, *in silico*, or biomarker assays are needed to assess systemic interactions, downstream and adverse long-term effects without using animals before more reliance can be placed on those assays.

Toxicology studies are conducted to assess the degree to which compounds are toxic for organisms. Experimental animals usually are the biological systems to perform the toxicological testing since they can serve as accurate predictive models of toxicity in humans. Toxicity studies with animals are also needed to predict the therapeutic index of drugs and calculate the benefit-risk ratio and are becoming the "gold standard" for assessing human risk [7].

4.2 Experimental animals

The selection of the experimental animal should be made using a combination of considerations to obtain the best possible prediction of human response. The species selected should have the lowest welfare cost as a result of experimentation including transport to the laboratory, captivity, handling, and experimental procedures. The target receptor should be present in the test species and they should demonstrate appropriate pharmacodynamic response. The species chosen should be practical in terms of availability; husbandry; and ability to perform procedures and assess adverse effects, and meet compound requirements. In agreement with this, the experimental

animals suitable for toxicity studies are young and healthy, nulliparous and nonpregnant in the case of females. According to guidance documents, some tests should be conducted in two laboratory animal species, one rodent (rat, mouse) and one nonrodent (dog, pig, monkey, etc.) [8]. The choice of nonrodent species may be only justifiable if results with rodents are not conclusive or if special studies require them, in this case a smaller number of animals is used [5].

The animals most commonly used in experimentation are rodents due to low cost of maintenance, short life span, and the specific knowledge of their physiology. Rat is preferable when abundant biological samples are required (i. e., blood samples). The use of animals of large size is reserved for specific toxicity studies because they need a bigger comfortable resting area, more food, and a greater amount of test compounds increasing the cost of the study. For instance, rabbits are suitable for dermal (skin and eye) and reproductive toxicity testing and guinea pigs showed basic physiology more similar to humans than rodents. Dogs, commonly beagles, are often chosen for chronic toxicity while nowadays nonhuman primates are rarely used.

Moreover, the suitability of the experimental animal depends on the specific methods of housing, feeding, or handling. Personnel appropriately trained must handle the animals to minimize anxiety before, during, and after the study. Strict control of the environmental conditions as well as the use of appropriate animal care techniques specific to each species are fundamental in toxicology studies. The following parameters should be taken into account (Table 4.1) [9]:

1. Temperature, humidity, and ventilation
2. Light must be artificial and satisfy the biological requirements of each animal species
3. Feeding: Conventional laboratory diets along with water shall be provided *ad libitum*. The diet should cover all the nutritional requirements of the animal.
4. Housing options depends on the type of study and shall be adapted to the animal species, providing a comfortable resting area of adequate size, clean and dry. Some animals need social interaction and perform a specific activity. Depending on the study, the animals will be housed individually or in collective cages with no more than five animals per cage, all of the same sex. (Figure 4.1)

Transportation and introduction of animals into unfamiliar surroundings are potentially stressful events. An acclimation period allows animals time to stabilize in a new environment and promotes both animal welfare and reproducible experimental results. The acclimation period is 48 h for rodents and nonmammalian vertebrates (including birds, amphibians, and reptiles) and from 72 up to 5 days for larger mammals (including rabbits, cats, swine, sheep, and goats) [10]. During this time animals randomly selected and identified are caged in groups of the same age and sex. Weight variation should not exceed ± 20% of the mean weight of all the animals used in the study for each sex. If a fasting period prior to treatment is required, it should be appropriate to the animal species used.

Table 4.1: Specific conditions for housing different animal species and legal minimum standards applicable from 1 January 2017 [9].

	Mouse	Rat	Rabbit	Guinea Pigs	Dog	Cat	Non-human primates
Light	12 h/day	12 h/day	12 h/day	12 h/day	10–12 h/day	10–12 h/day	12h/day
Temperature	20–24°C	20–24°C	15–21°C	15–21°C	15–24°C	15–21°C	Not restricted
Humidity	45–65%	45–65%	>45%	45–75%	Not restricted	Not restricted	40–70%
Minimum floor cage area[a] (cm^2)	330	800 (<200–600 g) 1,500 (>600 g)	3,500 (<2 kg) 4,500–5,400 (2–6 kg) 6,600 (>6 kg)	1,800 (<150–450 g) 2,500 (450–>700 g)	45,000 (<10–20 kg) 80,000 (>20 kg)	15,000	Species-dependent
Minimum cage heigh (cm)	12	18 (<200–250 g) 20 (250–>600 g)	45 (<2–5 kg) 60 (5–>6 kg)	23	200	200	Species-dependent

[a]minimum floor area for one or more animals, except in the case of rabbit and cats (one animal) and dogs (one or two animals).

Figure 4.1: Mice housed in large cages.

4.3 Administration routes

Toxicology tests usually start with administration of a **Single Dose** to each animal. Those studies allow to determine the most appropriate dose range in the species to be tested, evaluate signs of toxicity, and get an idea of target organs. The route of administration of the test compound should approximate the normal exposure in humans as long as toxicity is route-dependent. To the choice of the administration route also determines the characterization of the test substance (physical and chemical properties, bioavailability, mode of action, etc.).

The maximum amount of test substance that can be administered depends on the species, animal size, and administration route (Table 4.2) [11]. Special consideration should be given to liquid agents administered by oral gavage or injection, which volume must not exceed 1 mL/100g body weight.

The main routes of administration are:

– **Oral:** via the diet, drinking water, gavage, or encapsulation.

The oral route is economical, convenient, relatively safe, and some animals can be trained to cooperate voluntarily in the administration. The taste and odor of the test substance may make the oral administration in diet or drinking water difficult. Capsules administration

Table 4.2: Administration volumes according to animal species and route of administration considered good practice [11].

Animal	Route and volumes (mL/100 g)				
	oral	s.c.	i.p.	i.m.	i.v.
Mouse	1	1	2	0.005	0.5
Rat	1	0.5	1	0.01	0.5
Rabbit	1	0.1	0.5	0.025	0.2
Minipig	1	0.1	0.1	0.025	2.5
Dog	0.5	0.1	0.1	0.025	0.25
Macaque	0.5	0.2	Not available	0.025	0.2

would be more appropriate in larger animals, such as dogs. The number and size of capsules administered should be proportional to the size of the animal to minimize regurgitation. Problems of stability and difficulty in determining the exact dose ingested may arise in oral administration. **Gavage** (oral intubation) is often used in research settings to ensure precise and accurate dosing of animals. Although this method could induce stress in daily handling of the animals, selection of appropriate tubing size for gavage is important to minimize discomfort while optimizing delivery of substances [12]. In oral administration, animals should be fasted prior to dosing the test substance.

Inhalation

Animals may be exposed to the test compound as a gas, vapor, aerosol, or a mixture thereof. Exposure is determined by the goal of the study and may be nose-only (head-only, nose-only, or snout-only) or whole-body [5]. For testing compounds as liquid, solid aerosols and vapors the preferred route of exposition is the nose-only (Figure 4.2A). Whole-body exposition implies single caged individuals, the test animal size should not exceed 5% of the total volume of the chamber to ensure atmosphere stability. Concentration of oxygen (at least 19%) and carbon dioxide (not exceeding 1%) as well as the relative humidity in the breathing zone should be controlled (Figure 4.2B). Inhalation time is adjusted for each animal species, 4–6 h in rodents.

Dermal

This route is adequate if the test substance penetrates the dermal layer. The test substance must be extended to 10% of the body surface area. This site is typically used for assessment of immune, inflammatory, or sensitization response [11]. Material may be formulated with an adjuvant. Volumes dependent upon the thickness of the skin but volumes of 0.05–0.1 mL can be used.

Intravenous

For this route, distinctions are made between bolus injection, slow intravenous injection, and intravenous infusion [11].

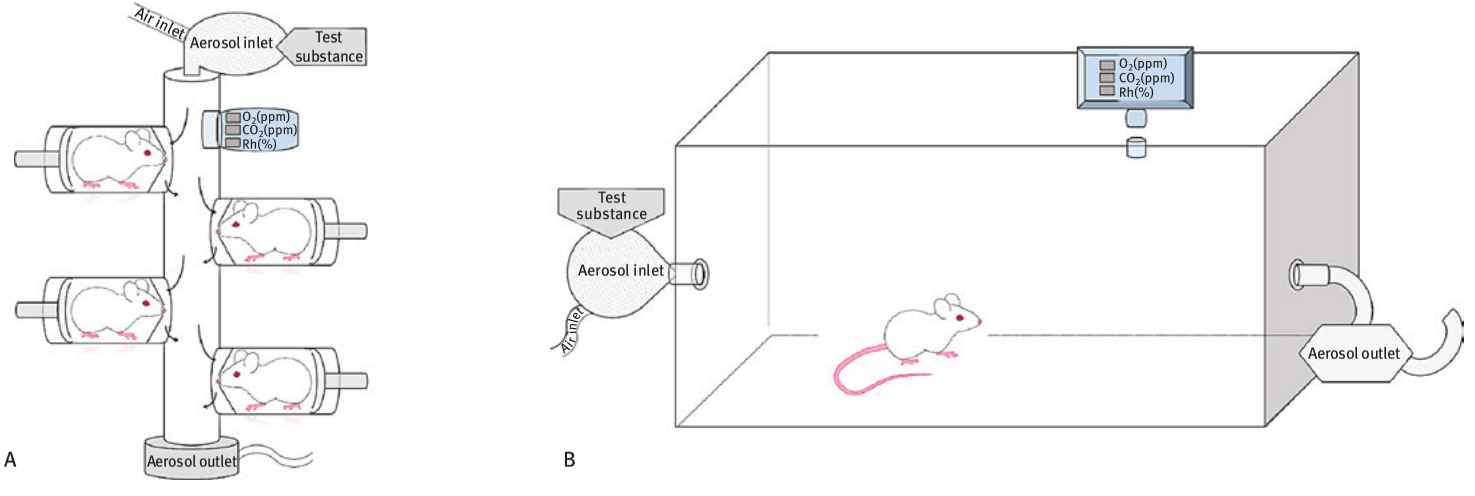

Figure 4.2: Shemes of inhalation chambers. (A) Scheme of the nose-only inhalation exposure tower configured with multiple chambers. Mice were immobilized inside individual compartments and exposed to the aerosolized/nebulized test substance, whose flow (arrows) only comes into contact the mice nose incorporating the excess to the internal flow of the camera. Levels of oxygen (ppm), carbon dioxide (ppm), and relative humidity are controlled. (B) Scheme of whole-body inhalation chamber. The test substance is aerosolized/nebulized prior to entry into the chamber. Single mouse exposure is recomended and atmosphere conditions (oxygen (ppm), carbon dioxide (ppm), relative humidity (%)) must be controlled.

Bolus injection: In most studies using the intravenous route the test substance is given over a short period of approximately 1 min. Such relatively rapid injections require the test substance to be compatible with blood and not too viscous. When large volumes are required to be given, the injection material should be warmed to body temperature. It is suggested that, for rodents, the rate of injection should not exceed 3 mL/min.

Slow intravenous injection: It may be necessary to consider administering substances by slow intravenous injection over the course of 5–10 min because of the expected clinical application of the compound, or because of limiting factors such as solubility or irritancy. In this case the possibility of extravascular injection of material should be minimized.

Continuous infusion: This intravenous administration could be chosen for solubility reasons or clinical indication. The volume and rate of administration will depend on the substance being given and the fluid therapy. As a guide, the volume administered on a single occasion will be <10% of the circulating blood volume over 2 h.

Intraperitoneal

In this route drug absorption from the peritoneal cavity after the administration of the compound as a suspension is dependent on the properties of the drug particles and the vehicle, and the drug may be absorbed into both systemic and portal circulations.

Intramuscular

Intramuscular injections may be painful because muscle fibers are necessarily placed under tension by the injected material. Sites need to be chosen to minimize the possibility of nerve damage. A distinction needs to be made between aqueous and oily formulations when speed of absorption is important.

Subcutaneous

This route is frequently used. The rate and extent of absorption depend on the formulation.

4.4 Types of toxicity studies with animals

The basic principles guiding toxicity test in animals are: to check the acute effect of the test substances on laboratory animals and to study the exposition of laboratory animals to high doses of test substances and over a long period of time in order to evaluate its possible hazard on humans. Toxicity studies can be divided into:

Acute toxicity studies: Examination of adverse effects that may occur on first exposure to a single dose of a substance.
Repeated dose, subchronic, or chronic toxicity studies: Identify whether toxicity occurs after continuous exposure to a substance.

The presence and severity of the toxic effect depends on the level (dose and length) along with route of exposure and animal species susceptibility. The minimum time for each type of study may vary according to regulations of different regions (United States, Europe, and Japan). The **dose** is the amount of test substance to which an individual is exposed. The test substances are administered in a **vehicle** whose selection is an important consideration in animal investigations. Vehicles themselves should offer optimal exposure but should not influence the results obtained for the compound under investigation, and they should be biologically inert, have no effect on the biophysical properties of the substance, and have no toxic effects on the animals [11]. Simple vehicles used to administer compounds include aqueous isotonic solutions, buffered solutions, co-solvent systems, suspensions, and oils. Therefore, toxicity studies require several groups of animals (controls and treated groups).

- Treatment groups of animals are administered with different doses of the compound tested
- Control groups of animals receive the vehicle to assure that it does not influence the results obtained for the compound under investigation

Animals for each group of the study must be randomly selected to ensure a representative population. All animals, control and treatment groups, should be handled in the same way. Clinical signs of toxicity, as well as their frequency, severity, and duration shall be recorded. In some toxicity studies, surviving animals must be humanly euthanized to avoid suffering [13]. All animals should be gross necropsied quickly and immediately after they are dead to identify target organs and examine morphopathological changes (Figure 4.3). Besides some samples of blood, urine, feces, and organ tissues are also collected to evaluate biochemical and ultrastructural changes induced by the substance tested.

Results from animal tests are used in combination with data on the efficacy of drugs in the risk-benefit assessment. For instance, they help to decide whether the beneficial effects of a treatment would outweigh the risks of adverse side effects. With regard to toxins, data of toxicological response with information on human exposure are integrated to produce a risk assessment and to identify control measures necessary to manage and reduce any identified risk. Besides, tests on species such as fish and amphibians are used to assess the potential environmental effects of chemicals.

There are different groups of toxicity studies with regard to duration and number of doses administered.

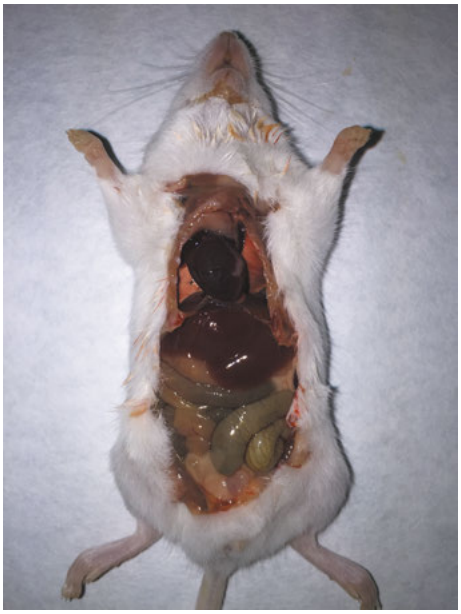

Figure 4.3: Gross necropsy of mouse: Abdominal and thoracic organs.

4.4.1 Acute toxicity testing

Acute toxicity studies are carried out to determine the effects of a single dose of compound on a particular animal species administered preferably by oral administration in a short period of exposition. However, other routes of exposure (inhalation, dermal, etc.) could also be used.

It could be recommended that acute toxicity testing be carried out in two different animal species [1]. The aims of acute toxicity testing are to define the nature and duration of any toxicity of the test substance, determine susceptible species, identify target organs, and provide information for risk assessment. Mortality during the exposure and observation period is recorded. Dead animals or animals sacrificed at the end of the study are examined for morphological, histological, and pathological changes (Figure 4.4).

Those tests also determine the lethal doses, and provide preliminary information relevant to single exposure or over-dosage in humans. Dose–response curves are obtained from results of the acute toxicity studies, and allow determination of the indicator of acute toxicity:

> **LD_{50}: 50% lethal dose** is a statistically derived single dose of a substance that can be expected to cause death in 50% of treated animals when administered by the given route of exposure. The LD50 value is expressed in terms of quantity of test substance per unit weight of test animal (mg/kg).

Lethal dose tests are still used to assess the safety of biological products such as certain foods (shellfish) for the presence of some toxins in EU [14]. Several methods

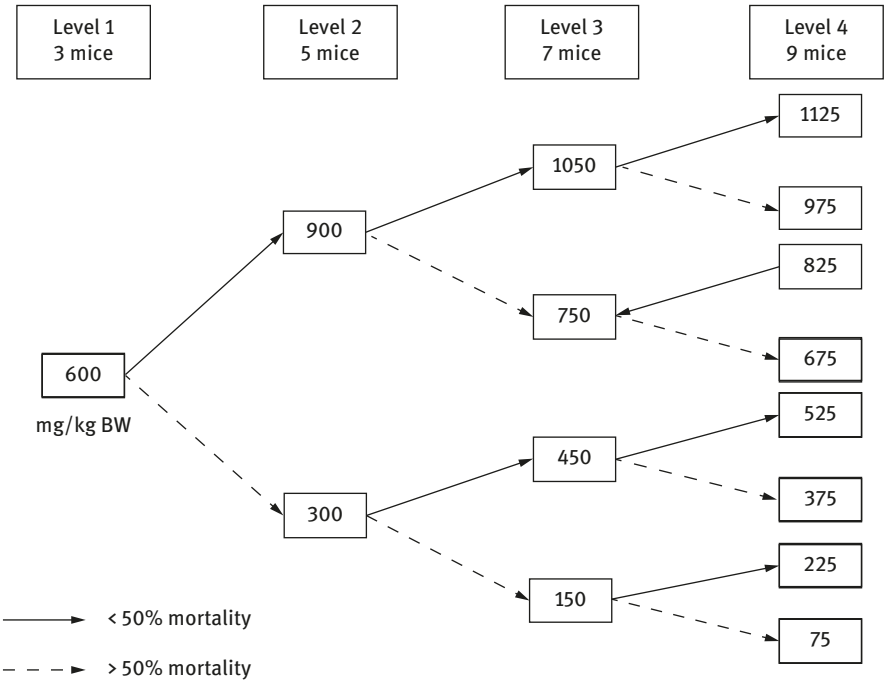

Figure 4.4: Representative 4-level up-and-down procedure for an acute toxicity testing. At each dose level the number of animals increased up to 9. If most of the mice survive after the treatment, the dose for the next level is increased (continuous arrows), if most of them died the dose was decreased (discontinuous arrow). The animals are observed for 24 or 48 h before dosing the next group.

have been developed to perform acute toxicity tests with the goal to use fewer animals and in some cases replace death as the endpoint with signs of significant toxicity [1].

Fixed dose procedure (FDP)

The FDP is frequently used to assess the nonlethal toxicity rather than the lethal dose [15]. Five animals group-caged compose each dose level. First, the dose to produce evident toxicity is selected by a sighting study from the fixed dose levels of 5, 50, 500, and 2,000 mg/kg body weight. When there is no previous information of the test substance, the starting dose will be 300 mg/kg. Higher or lower fixed doses will be administered depending on the presence or absence of signs of toxicity or mortality. The final point of the study is reached when toxicity is evident or no more than one death occurs.

Acute toxic class method (ATC)

The ATC method is a sequential procedure in which three animals of the same sex are used for each step [16]. Four preidentified starting doses may be used, and the test dose should be selected based on the Globally Harmonized Classification system [17]. When 2–3 animals die, the next level dose will be lower, but if 0–1 animal dies the

dose level is tested again and under the same result a higher dose level will be used next. The standard test for acute toxicity would use 60–200 animals, and requires death of animals as an endpoint.

Up-and-Down procedure (UDP)

Test Guidelines Programme of the OECD has developed standardized methods following the Three Rs. One of these is the UDP that substantially reduced the total number of animals used. This is the method developed and statistically evaluated for acute toxicity testing most often recommended by various regulatory agencies [18].

This method provides a way to determine the toxicity of chemicals while achieving significant reductions in animal use by performing sequential dosing steps [19]. The four-level UDP will require only three to nine animals in each dose level (Figure 4.4). Testing in females is recommended as they are generally more sensitive to toxicological studies. In the UDP, a small number of animals are dosed one at a time. If most of the animals survive, the dose for the next group of animals is increased; if most of them die, the dose is decreased. The animals (usually mouse or rats) are observed for 24 or 48 h before dosing the next group. The procedure for estimating the LD50 takes into account all deaths, and may be performed using widely available computer program packages (Figure 4.5). As a disadvantage this method should not be used for testing compounds where deaths could be beyond two days post dosing.

Acute toxicity testing for inhalation

Acute inhalation toxicity testing is performed for aerosol-like preparations [20]. Three animals per sex, preferably rats, or six animals of the susceptible sex, are exposed for 4 h to the test substance and then are monitored for 14 days. Mortality during the exposure and observation period is noted. Dead animals or animals sacrificed at the end of the study are examined for histological and pathological changes.

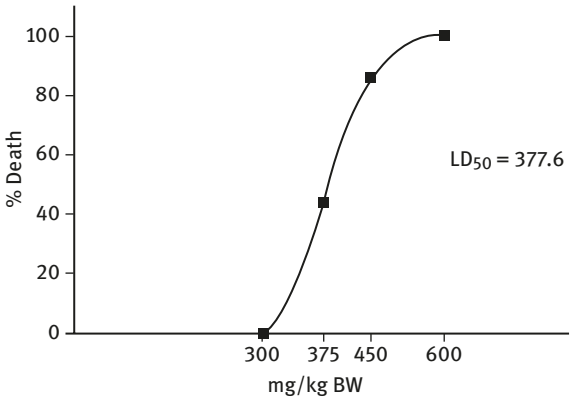

Figure 4.5: Dose–response curve of mortality and the LD50 generated.

Acute toxicity testing for topical preparations

The eye irritation test and skin irritation test are very important for topical preparations. The eye irritancy test and the skin irritancy test are used to measure the harmfulness of chemicals and pharmaceutical substances in rabbits and guinea pigs. At the end of the study, the animals are sacrificed and pathological changes are evaluated.

Acute eye irritation/corrosion

In this test the substance is administered to an animal's eyes, and the animal is restrained for 4 h [21]. Albino rabbit is the preferred animal. The test substance should be placed in the conjunctival sac of one eye of the animal, thus the other eye serves as a control. An initial study determines if the test substance is corrosive or a severe irritant. In case of negative response, a confirmatory test must be performed using two animals simultaneously; if the response is positive, the two animals' exposition will be sequential. Doses for liquid and solids testing are 0.1 mL and a maximum weight of 100 mg, respectively. For pressurized aerosol, the test substance is administered to the eye in a simple burst from 10 cm of the eye. The eye may be rinsed with saline or distilled water at 24 h or 1 h after the treatment if the solid test substance remains in contact with the eye. The administration of analgesics and anesthetics is needed before and after application of the test substance to minimize pain.

Acute dermal irritation/corrosion

Skin irritancy is normally assessed by applying the test substance to shaved areas of the backs of albino rabbits. Albino rabbits are normally exposed for 4 h to test substance, which then is removed when possible. Doses of 0.5 mL for liquid or 0.5 g for solid test substances are applied to the surface of the skin of animals individually housed. Generallys this study requires a initial test conducted in one animal by three sequential exposures to know if the test substance is corrosive and subsequently a confirmatory test with two animals. If the initial test is not performed, two or three animals may be treated with a single patch [22].

Acute Dermal Toxicity

The animals more used are adult rabbits, rats, mice, and guinea pigs. Five animals per sex compose each dose level. Female rats 8–10 weeks old are the preferred experimental animals. The test chemical is applied to the naked skin (10% of the total body surface area) with a porous gauze for 24 h. Each dose level is composed by two animals individually caged [23].

The local lymph node assay is a widely accepted test that meets regulatory requirements. In this test the substance is applied on the surface of the ears of a mouse for three consecutive days. At the end the mouse is euthanized and the early stages of sensitization are detected by measuring the proliferation of lymphocytes in the draining lymph node [1].

4.4.2 Repeated dose toxicity testing

Repeated dose toxicity testing is conducted to determine the existence of effects derived from an exposure of extended duration. Administration of the test substance is performed daily in the medium term, between 14 and 90 days depending on the purpose of the study and not lasting more than the 10% of the life span of the animal. Rats and mice are generally used but test may also be conducted in nonrodent animals such as the dogs, pigs, or macaques. At least 5 or 10 animals per sex should be used at each dose level. In the case of using nonrodent animals, only 4 per sex are needed. Repeated dose studies must be composed by at least three dose levels, and higher concentration should result in toxic effects without causing lingering signs or lethality and lower concentration should produce little or no evidence of toxicity, therefore these studies are also termed **Subacute Toxicity Testing** [24]. There is an extensive evaluation of toxic effects (with regard to body weights, clinical signs of toxicity, food consumption, clinical pathology, and biochemical parameters).

The interpretation of human safety details is essential in repeated dose toxicity studies. The test data allow an assessment of the parameter NOAEL:

> **NOAEL**: is the abbreviation for "no observed adverse effect level" and it is the highest dose without significant adverse effects.

NOEL is used in risk assessment [25]. For instance, in the case of food toxins, these studies are used to assign a reference dose to which safety factors are applied to give ADI:

> **ADI**: is the abbreviation for acceptable daily intake that is typically a hundredfold less than the observed NOAEL. ADI can be defined as the dose level to which humans may be exposed, with the practical certainty that no adverse health effects will ensue.

Another important concept is MTD:

> **MTD**: is the abbreviation for the maximum tolerated dose, it is the highest dose that produces toxicity but no death.

MTD studies often replace acute studies, especially in the case of large species such as dog and primates. They involve steadily increasing the dose given to an animal until adverse effects indicate that an MTD has been reached. This is normally determined by careful observation of the animals. Effects such as vomiting and convulsions are sometimes evaluated as signs of MTD.

These kind of repeated dose toxicity studies are used as preliminary to a long-term chronic toxicity studies and the preferred route is oral [26, 27], but the test substance may be administered by dermal [28], inhalation [29], or other parenteral routes.

Oral

Rodent species, preferably the rat, are used, although tests may be conducted also in pigs and dogs. Animals are treated daily, seven days each week. Dose levels are set by two to fourfold intervals.

Dermal

The adult rat, rabbit, or guinea pig may be used. In the case of skin-sensitization testing, multiple doses of the test substance are applied to the skin of guinea pigs to see if a later dose will cause a strong immune reaction, indicating sensitization to the chemical. Treatments are applied for at least 6 h per day, 7 days per week. The lowest dose level should not produce any evidence of toxicity.

Inhalation

The preferred species is the rat. Animals are exposed 6 h per day, 5 days per week. Changes in body temperature and respiration are characteristic.

In the repeated dose toxicity testing, blood removal could be a procedure performed on the animals. Table 4.3 indicates the maximum blood sample volumes that can be removed without significant disturbance to the animal's normal physiology. These values do not include a terminal sample, which can be taken when the animal is euthanized. Recommended routes for bleeding are the lateral tail vein, the sublingual vein, and the lateral tarsal vein for all rodents, and the marginal ear vein, central ear artery, and the jugular vein for rabbits while sampling by cardiac routes is only carried out as a terminal procedure under general anesthesia.

At the end of the repeated dose toxicity testing, tissues from most of the organs are removed, and histological changes are recorded (Figure 4.6). If possible, immunotoxicity (adverse effects on the immune system) studies are performed on the same animals. Immunotoxicological analysis is not feasible beyond the period of 14 days. Parameters such as delayed-type hypersensitivity, mitogen- or antigen-stimulated lymphocyte proliferative responses, macrophage function, and primary antibody response to T-cell dependent antigen are assessed in immunotoxicological studies.

Table 4.3: Total blood volume (mL) of the animal species more commonly used in experimentation and the maximum blood sample volumen recomended for extraction (mL).

Animal (weight)	Blood volumen (mL)	Maximum blood sample extraction (mL)
Mouse (25 g)	1.8	0.4
Rat (250 g)	16	3.2
Rabbit (4 kg)	224	45
Minipig (15 kg)	975	195
Dog (10 kg)	850	170
Macaque Rhesus (5 kg)	280	56
Macaque Cynomolgus (50 kg)	325	65

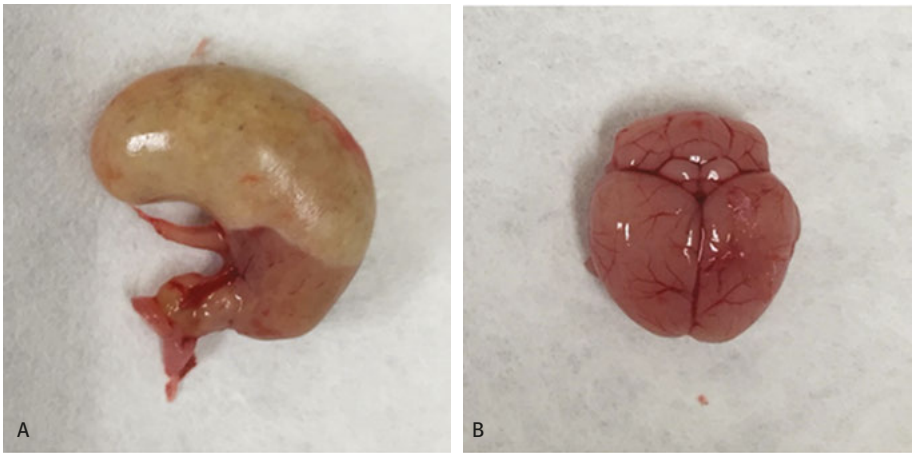

Figure 4.6: Mouse organs: (A) stomach, (B) brain.

Genotoxicity Testing

Genotoxicity refers to the induction of damage of the genetic material (DNA and chromosomes) and regulatory cellular components for the potential to cause heritable mutations [30]. In animal studies rodents are preferred, usually mice. The purpose is to demonstrate that the chemical can or cannot reach a sensitive tissue and cause genetic changes in the intact animal. When toxicity occurs, the dose levels should range from dose producing little or no toxicity to the MTD. If toxicity was previously not observed, the highest dose should be 1000 or 2000 mg/kg/body weight/day for administration periods of more than 14 days. The test substances may be administered as a single dose or separated doses within a maximum range of 2–3 h (split dose) to facilitate administration [31–33].

Mammalian erythrocyte micronucleus test

In this assay, erythrocytes from bone marrow or peripheral blood are analyzed in search of damage in the chromosomes or the mitotic apparatus of erythroblasts indicated by an increase formation of micronuclei [31]. Animals, usually rodents (rat or mice), are exposed to the test substance by a single dose or few daily doses. Control and each treated animal group must be composed of at least 5 animals/sex. The collection of samples will depend on the routine administration of the substance. After extraction, samples are stained and studied by microscopy or flow cytometer.

Mammalian bone marrow chromosomal aberration test

This *in vivo* test is an early predictor of carcinogenic activity [32]. Aberrations in chromosomes may be detected by this test in bone marrow cells. A single dose of compound is administrated to rats or mice that are killed either 24 or 48 h later for

examination of chromosomal changes in bone marrow cells. From bone marrow cells immediately obtained after euthanasia, fixed chromosome preparations are stained and analyzed. It is expected that the highest dose level used will show evidence of adverse effects if the substance is genotoxic, and the MTD is normally used to set this dose level.

In vivo mammalian alkaline comet assay

Single cells/nuclei suspensions are prepared from different tissues dissected for analyzing DNA strand breaks in animals, most often rodents (6–10 weeks old), although studies are carried out in other mammalian and nonmammalian species too [33].

Animal groups of five individuals of one sex or per sex and three for positive control group are required. Treatments are given daily for two or more days and samples collected once between 2 and 6 h after the last treatment. Dissected tissues should be analyzed by histopathology analysis.

Neurotoxicity studies

The effects of a test substance on the nervous system can be studied through neurotoxicity studies [34]. Structurally, the nervous system has two components: the central nervous system and the peripheral nervous system. The central nervous system is made up of of the brain, spinal cord, and nerves. The peripheral nervous system is further divided into the somatic and autonomic nervous systems. Neurotoxic studies may be employed to evaluate the specific histopathological and behavioral neurotoxicity of a chemical and are used to characterize neurotoxic responses such as neuropathological lesions and neurological dysfunctions (loss of memory, sensory defects, and learning and memory dysfunctions). Usually neurotoxicological studies are carried out in adult rodents. The test substance may be administered for 14 days or even more than 90 days, being in this case a subchronic toxicity testing, and neurological changes are evaluated [35].

4.4.3 Subchronic toxicity testing

The major difference between repeated dose and subchronic toxicity studies is the duration: repeated dose toxicity studies are conducted between 14 and 90 days, and subchronic toxicity studies are carried out over 90 days [36]. Rodents and nonrodents are used to study the subchronic toxicity of a substance. The test substance is administered preferably by oral route for 90 days but other routes could also be used [37, 38]. Weekly body weight variations, monthly biochemical and cardiovascular parameters changes, and behavioral changes are observed. At the end of the study, the experimental animals are sacrificed. Gross pathological changes are observed, and all the tissues are subjected to histopathological analyses. In the study protocol a control group and a high-dose group may be included.

4.4.4 Chronic toxicity testing

Chronic toxicity tests determine the effects of long-term exposure to a test substance [39]. Administration of the test substance is normally oral, although dermal and inhalation administrations are also used. Test substance is administered daily to animals for a period comprised between three months and up to two years depending on the life span of the animal. For dermal and inhalation administration, treatments are given for at least 6 h per day [40]. The duration of exposure has to be long enough to allow manifestation of effects due to cumulative toxicity.

Chronic toxicity studies are conducted with a minimum of one rodent and one nonrodent species. The studies must be composed of at least three dose levels, each one of 20 animals per sex for rodents or 4 per sex in nonrodent studies. Spacing of the dose levels is frequently of two–fourfold intervals, and the highest dose level should evidence toxicity but without suffering, morbidity, or death. During the study period, the animals are observed periodically for normal physiological functions, behavioral variations, and alterations in biochemical parameters. At the end of the study, the animals are sacrificed, and gross pathological changes are noted and histopathological studies are carried out. The report on chronic oral toxicity is essential for new drugs.

Carcinogenicity testing

Both rodents and nonrodent animal species may be used in carcinogenicity testing [41]. At least 50 animals should be used at each dose level. The tests are carried out over the greater portion of an animal's life span. During and after exposure to test substances, the experimental animals are observed for signs of toxicity and development of tumors. If these are not found, a test may be terminated after 18 months in the case of mice and hamsters and after 24 months with rats. If the animals are healthy, hematological analysis is performed after the 12 months and the 18 months, respectively, and the study is terminated.

Reproduction toxicity testing

These studies evaluate effects on reproductive function and the ability to produce birth defects. The test compound is administered to both male and female animals. Administration is for the duration of at least one complete spermatogenic cycle in male animals and for two complete estrous cycles for female animals. Rats and mice are most commonly used, but in some cases testing is carried out on other animals such as rabbits, guinea pigs, dogs, or primates. Adult animals, offspring, and fetuses are used in reproductive and development studies. These tests provide information on fertility, mating behavior, parenteral behavior, and development of the neonate adulthood [42].

Developmental toxicity/embriotoxicity studies

Studies on developmental toxicity provide specific information on the potential hazards to the unborn that may arise from exposure of the mother to a compound

during pregnancy. Rabbits are used in addition to rodents because they respond variably to the effects of potent human teratogens. Animals are treated orally between the 8th and 14th day of pregnancy or for up to the whole period of gestation. Urine contents, embryo or fetal death, altered growth, and structural changes in the fetus are evaluated. These results are used in hazard classification and risk assessment [42].

4.4.5 Toxicokinetic studies

TK studies that are an extension of pharmacokinetics of a substance are conducted to obtain information on its absorption, distribution, biotransformation, and excretion to aid in relating concentration to the observed toxicity [43]. Animal TK data help to understand the toxicology studies by demonstrating that the animals are systematically exposed to the test substance and by revealing which are the circulating moieties (parent substance/metabolites). In some situations, TK data can be collected as part of the evaluation in toxicological studies [44]. Basic TK parameters determined from these studies will also provide information on the potential accumulation of the test substance in tissues and/or organs and the potential for induction of biotransformation as a result of exposure to the test substance. TK can contribute to the assessment of the relevance of animal toxicity data for extrapolation to human hazard.

TK studies are preferably carried out in rodents but rabbits, dogs, nonhuman primates, and swine are also used. Oral administration is preferable, however other routes of administration (dermal, inhalation, intraperitoneal, and intravenous) may be applicable for certain chemicals [45]. Single dose may be adequate, however in some circumstances repeated dose administration may be needed. These studies may be conducted at one dose or, more likely, at two or more doses. Each animal is placed in a separate metabolic unit for collection of urine and feces (Figure 4.7).

The purpose of these studies is to obtain estimates of basic TK parameters for the test substance:

- **Cmax**: Maximal concentration in blood after administration or maximal excretion (in urine or faces) after administration
- **Tmax**: Time to reach Cmax
- **Half-life (t1/2):** The time taken for the concentration of the test substance to decrease by one-half in a compartment. It typically refers to plasma concentration or the amount of the test substance in the whole body
- **AUC**: Area under the curve in a plot of concentration of substance in plasma over time. It represents the total amount of substance absorbed by the body within a predetermined period of time.

Following administration of the test substance blood samples and also urine and fecal samples should be collected from each animal at suitable time points using

appropriate sampling methodology. Identification and quantitation of unchanged test substance and metabolites could be done by analytical systems such as HPLC, LC-MS, or NMR spectrometry.

Knowledge of tissue distribution of a test substance and its metabolites is important for the identification of target tissues, understanding of the mechanisms of toxicity, and the potential for test substance and metabolite accumulation and persistence. The per cent of the total dose in tissues as well as residual carcass should at a minimum be measured at the termination of the excretion experiment (e. g., typically up to 7 days post dose or less depending on test substance's specific behavior). Tissues that should be collected include liver, fat, GI tract, kidney, spleen, whole blood, residual carcass, target organ tissues, and any other tissues (e. g., thyroid, erythrocytes, reproductive organs, skin, eye, and brain) of potential significance in the toxicological evaluation of the test substance [46]. Analysis of additional tissues at the same time points should be considered to maximize utilization of animals.

TK models may have utility for various aspects of hazard and risk assessment, for example in the prediction of systemic exposure and internal tissue dose. Furthermore, specific questions on mode of action may be addressed, and these models can

Figure 4.7: Mouse in a metabolic cage.

provide a basis for extrapolation across species, routes of exposure, dosing patterns, and for human risk assessment.

4.5 Future of toxicity studies with animals

This chapter presents the toxicity studies with animals as a relatively standardized and guideline-driven process of testing compounds that relied on various *in vivo* studies in rodent and nonrodent animal species. Animal research faces the regulatory challenge to simultaneously improve the welfare of animal and keep the standard of science. The complexity of animal experiments can lead to the implementation of alternatives for these experiments. However, the most important obstacle for using alternatives to animal models is the lack of reliability of alternative methods. In the near-term future, *in vitro*, *ex vivo*, *in silico*, or biomarker assays as well as computational and informatics approaches to toxicity testing will contribute both to the winnowing and prioritizing of chemicals that need to be tested in animals Additionally, the information gleaned from these approaches will contribute to provide a basis for hypothesis-driven whole-animal testing. Besides, this will assist in reducing the number of animals needed and will potentially shorten the overall time necessary to complete safety assessments [47]. Although some progress in the field has already been achieved and several methods have been validated and are also used for regulatory toxicity testing of chemicals, a complete replacement of animals in toxicology studies is scientifically not justified [48]. Therefore, the ultimate adjudication of a chemical's toxic potential must reside in well-designed and conducted experiments using experimental animals.

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Keywords: pharmacokinetics, animal test species, administration route, acute testing, subchronic testing, chronic testing, toxicokinetics

Abbreviations: ADI: Acceptable daily intake; ATC: Acute toxic class method; AUC: Area under the curve; Cmax: maximal concentration; DNA: deoxyribonucleic acid; EPA: Environmental Protection Agency; EU: European Union; EURL ECVAM: European

Union Reference Laboratory for Alternatives to Animal Testing; FDA: Food and Drug Administration; FDP: Fixed dose procedure; GI: gastrointestinal; HPLC: high performance liquid chromatography; ICH: International Conference on Harmonization; i.p.: Intraperitoneal; i.v.: Intravenous; i.m.: Intramuscular; LD50: Lethal dose 50%; LC-MS: Liquid chromatography-mass spectrometry; MTD: Maximum tolerated dose; NMR: nuclear magnetic ressonance; NOAEL: No observed adverse effect level; OECD: Organisation for Economic Cooperation and Develop; s.c.: Subcutaneus; Tmax: Time to reach Cmax; TK: Toxicokinetics; UDP: Up-and-Down Procedure

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5 Toxicological studies with cells

5.1 Introduction

Cell culture yields a model system that provides direct access and evaluation of the effects of chemicals on tissues and constitutes a valuable tool to analyze cell toxicity mechanisms. *In vitro* model systems, in general, have been used to study the mechanism of action of chemicals and to analyze the cellular basis for compound-induced toxicity as well as to develop rapid and high-throughput screening systems for the evaluation of the toxicity of chemicals, which may complement *in vivo* toxicity testing or may replace some *in vivo* models if they are scientifically validated and accepted by regulatory agencies [1, 3].

The term *in vitro* ("in the glass") refers to the technique of performing a given experiment in a test tube or in a controlled environment outside a living organism. *In vitro* methods are based on the use of cells or tissues which are cultured under controlled conditions in flasks and plates. Cells/tissues are exposed to chemicals and their toxic or beneficial effect is measured. Increasingly, human cells are used since they better predict possible effects on humans. Commonly used *in vitro* models for assessing chemical toxicity include perfused organ preparations, isolated tissue preparations, single cell suspensions, and cell culture systems, such as primary cell cultures and mammalian cell lines. Of these *in vitro* models, cell culture systems have been widely used because they are reliable, reproducible, and relatively inexpensive experimental systems to assess chemical toxicity at the cellular level [21]. Nevertheless, it should be pointed out that whether *in vitro* tests are based on primary cells, immortalized (e.g., SV40 transformation) and cancer-derived cell lines, stem cells, or reconstituted tissue cultures, it is important to have *in vitro* systems that adequately mimic key events of in vivo mechanisms of action triggered in humans upon exposure to a toxic compound [25].

Safety testing of chemicals is required under several directives of the EU and international regulatory environments. At an international level, the OECD is developing a guidance document on good *in vitro* method practices (GIVIMP) [23], which aims to ensure the efficacy and efficiency of the process between *in vitro* method development and its implementation for regulatory use [10]. The first guidance report on good cell culture practice was released in 2005 [5] and a second one related with the use of stem cells in 2017 [24], and some of the recommendations exposed in these reports are summarized below.

5.2 *In vitro* culture conditions

Cells or tissues in culture require specific conditions that differ from *in vivo* systems. However, cell culture conditions vary for each cell type. The consequences of deviating

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from the culture conditions required for a particular cell type can range from the expression of aberrant phenotypes to a complete failure of the cell culture [5]. Specific elements of culture conditions include culture media, supplements and other additives, culture-ware, and incubation conditions.

- **Basal medium:** refers to a complex nutritive media designed to obtain a good cell viability or cell proliferation or to maintain a desired cell differentiation. Many solid or liquid medium formulations are commercially available with subtle changes in their medium formulation (including phenol red, glutamine and other additives). Most of the commercial media are derived from the Eagle's minimum essential medium which contains amino acids, salts, glucose, and vitamins, or its modifications such as Dulbecco's Modified Eagle's medium (DMEM) which contains a fourfold higher concentration of amino acids and vitamins, as well as additional supplementary components and was initially developed for the culture of embryonic mouse cells. The Ham's nutrient mixtures were originally developed to support the growth of several clones of Chinese hamster ovary (CHO) cells, as well as clones of HeLa and mouse L-cells. The RPMI media (developed by Moore and Hood [20] at Roswell Park Memorial Institute, hence the acronym RPMI), uses a bicarbonate buffering system and alterations in the concentrations of amino acids and vitamins and has been used for the culture of human normal and neoplasic leukocytes. However, their supplementation has demonstrated wide applicability for supporting growth of many types of cultured cells, including fresh human lymphocytes. However, even subtle changes in the media formulation can alter the characteristics of certain cells or tissues, therefore the medium to be used should be precisely specified.
- **Serum:** is a complex mixture essential for the maintenance and/or proliferation of many cell types. However, due to its complexity and batch-to-batch variations it introduces unknown variables in the culture system, and in addition it represents a potential source of microbiological contaminants such as mycoplasma and bovine viruses due to its animal origin [5].
- **Serum-free media:** are commercially available in order to decrease the batch-to-batch variability problems associated with the use of serum and offer better reproducibility and the potential for selective culture and differentiation of specific cell types such as cell lines [12]. However, serum-free supplements can also include poorly defined components such as pituitary extracts, chick embryo extracts, bovine milk fractions, or bovine serum albumin (BSA) (http://www.oecd.org/env/ehs/testing/OECD%20Draft%20GIVIMP_v05%20-%20clean.pdf) which can exhibit batch-to-batch variation in biological activity.
- **Antibiotics:** are agents used in cell culture to protect against contamination and for the selection of recombinant clones that express antibiotic-resistant genes. The use of antibiotics should be avoided if possible or minimized since those agents can interfere with normal cell biology [5].
- **Cell culture matrix**

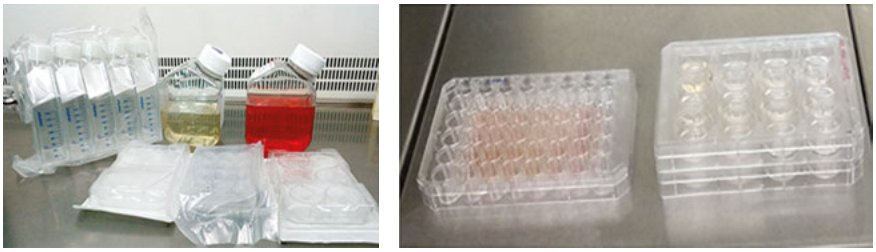


Figure 5.1: Images showing different formats of plastic culture vessels (flasks and plates) and commercial culture media (left panel) and precoated and washed culture plates ready for cell culture (right panel).

All cell culture plastic or glasses need to be sterile and are usually commercially available disposable plastic culture vessels. Most of the culture vessels are manufactured from polystyrene, a long carbon chain polymer with benzene rings attached to every other carbon. Polystyrene was chosen because it had excellent optical clarity and could be sterilized by irradiation. However, it is very hydrophobic and may make cell attachment difficult. Therefore, to increase cell attachment to the plastic surface, polystyrene is usually treated to create a more hydrophilic and negatively charged surface after medium addition. However, some cells are not able to attach in polystyrene-treated surfaces and require coating with biological materials including extracellular matrix, attachment and adhesion proteins (collagen, laminin, and fibronectin) and mucopolysaccharides, such as heparin sulfate, hyaluronate, and chondroitin sulfate, both individually and as mixtures. Another useful coating surface is the synthetic polymer poly-D-lysine (PDL) which creates a positive charge on polystyrene and consequently, for some cell types, enhances cell attachment, growth, and differentiation, especially in serum-free and low-serum conditions [27]. However, most of the coated culture surfaces need to be washed before cell seeding to avoid the possible toxicity of the coating material [5]. Figure 5.1 shows images of commercial presentations of plastic cultures (flask and plates), commercial culture medium, and culture plates after coating with laminin and PDL and washed with sterile phosphate buffered saline (PBS).

5.3 Handling and maintenance of cell cultures

Cell lines and primary tissues may contain microorganisms or pathogens able to cause human diseases or alter the *in vitro* results. To avoid these hazards, cell culture should be handled at biosafety (hazard) level 2. At a minimum, cell culture should be performed in a Class II biological safety cabinet (Figure 5.2). Class II cabinets are designated to prevent biological exposure to personnel and the environment and

to protect experimental material from being contaminated. Biological safety cabinets use high-efficiency particulate air (HEPA) filters in their exhaust and/or supply systems. These filtered cabinets are primarily designed to protect against exposure to toxicants, including biological agents used in the cabinet. Air flow is drawn from the room around the operator into the front grille of the cabinet, which provides personnel protection. In addition, the downward laminar flow of HEPA-filtered air provides protection for experimental material inside the cabinet. Because cabinet air has passed through the exhaust HEPA filter, it is contaminant-free, providing environmental protection, and may be recirculated back into the laboratory (Class II Type A biological safety cabinets) or ducted out of the building (Class II Type B biological safety cabinets). A scheme of the air flow through a biological safety cabinet is shown in Figure 5.2.

In addition, cell or tissues in culture should be kept in controlled ambient conditions. The optimal culture temperature depends on the cell type, thus insect cells have low optimal growth temperatures than mammalian cells, while most mammalian cells grow normally well at temperatures of 37°C [5]. Moreover, oxygen and carbon dioxide are vital for cell growth, and for most cell types the appropriate atmosphere is normally 5% v/v carbon dioxide in air. Cell culture incubators maintain a constant temperature and high humidity for the growth of tissue culture cells under a CO_2 atmosphere. Typical control of temperature settings in the cell culture incubator

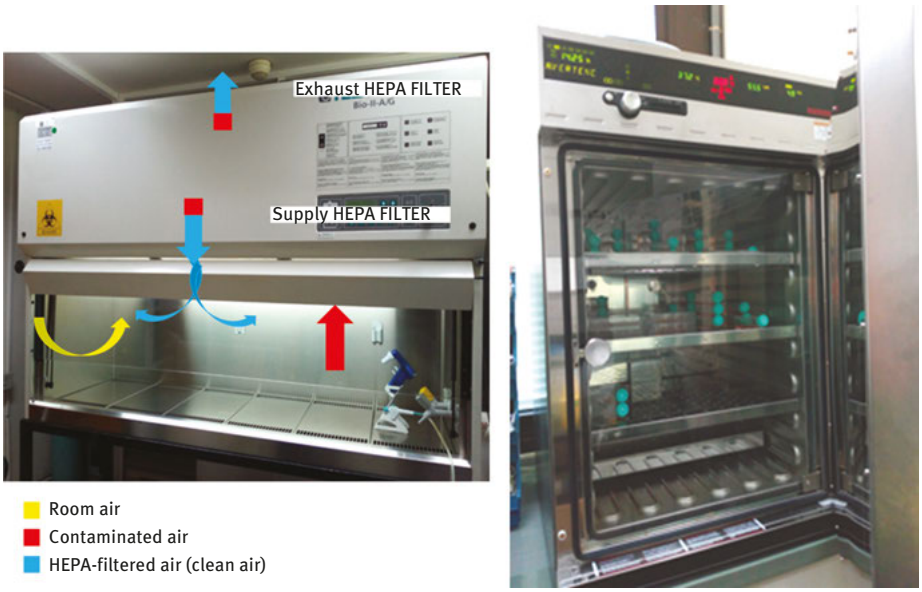


Figure 5.2: Images showing air flow through a biological safety cabinet for handling of cell cultures (left) and a culture incubator (right).

range from 4°C to 50°C, and CO_2 concentrations run from 0.3% to 19.9%. Moreover, the temperature is typically controlled either by a water bath that circulates through the walls of the cabinet, or by electric coils that give off radiant heat. Some units also include refrigeration for cooling. Relative humidity is maintained between 95% and 98% by an atomizer system or a water reservoir. An image of a cell culture incubator is shown in Figure 5.2.

5.4 *In vitro* culture models

In vitro model systems are frequently grouped in three broad groups listed below.

1. **Isolated organs or tissues:** These cells are normally obtained directly from an animal or a donor. These *in vitro* systems are widely used in toxicological applications and may be used in different preparations that include:
   a. **Slices of certain tissues** (such as liver, lung, kidney, and brain) that, temporarily, retain some structural and functional features of the original organ.
   b. **Isolation and reagregation of cells from different organs** that gives rise to two or three-dimensional cultures that also retain some functional properties of the original organ and tissue
   c. **Cells from blood or other body fluids** can be prepared as homogeneous preparations and kept *in vitro* for several days, or even used to generate stem cells (umbilical cord cells, bone marrow cells). A scheme illustrating the steps for purification of human T lymphocytes in our laboratory [26] as well as a confocal image for the expression of the voltage-dependent potassium channel KCNC1 (or Kv3.1) in control human T lymphocytes and in the same cells after 48 hours of treatment with the lectin concanavalin A at 50 µg/ml is shown in Figure 5.3.
2. **Primary cultures and early passage cultures:** These *in vitro* cultures are harvested cells and tissues obtained directly from animals or humans but maintained *in vitro* for several times depending on the cell type. Frequently, those cultures retain key morphological and functional features of the in vivo system and are widely used for basic research and in different *in vitro* applications. Depending on the tissue of origin, cells in primary culture can proliferate; however, their life span is limited and the cells may change their initial characteristics with time in culture. With some exceptions, these systems normally represent heterogeneous cell populations that may be maintained either in suspension or in monolayers in glass or plastic surfaces. Examples of such cultures are primary cultures of cortical or cerebellar neurons widely used for toxicity studies and mechanism of action of neurotoxic compounds [13] such as marine toxins [17–19]. Figure 5.4 shows a scheme for the isolation of primary cultures of cortical neurons and the *in vitro* development of such cells.

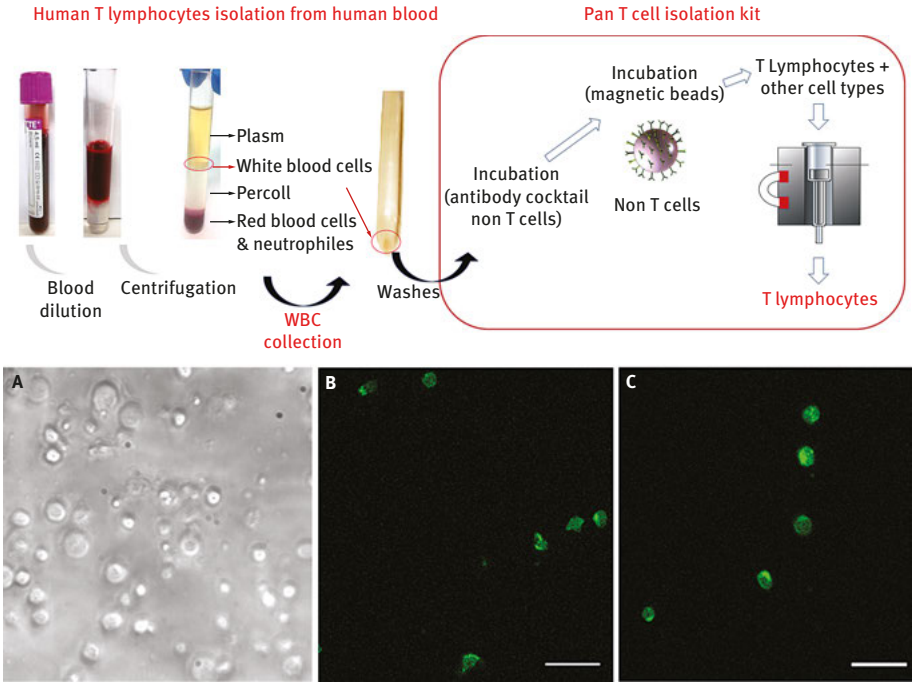


Figure 5.3: Upper panel: Scheme showing the isolation of human T lymphocytes from human blood donors. Lower panel: Phase contrast microscope image of purified human T lymphocytes (A). (B) Confocal image showing the expression of the Kv3.1 potassium channel in human T lymphocytes cultured for 48 h, and (C) the expression of the same channel after treatment of the cells for 48 h with concanavalin A. WBC; white blood cells.

3. **Cell lines:** This term refers to cells that are able to multiply *in vitro* for extended periods of time and can be maintained in subculture. Cell lines can be divided in finite cell lines, continuous cell lines, and stem cell lines.
   a. **Finite cell lines:** refer to cell lines that can be subcultured or passaged for a period of time; finally the cells stop to replicate but still maintain viability. Numerous finite cell lines have been established and they are genetically stable and remain diploid for many passages but generally reach senescence after 50–60 passages.
   b. **Continuous cell lines**: are cells that can be subcultured indefinitely and do not reach senescence. These cells are usually derived from tumors or normal embryonic tissues. Continuous cell lines can either generate spontaneously or may be produced through a variety of techniques including radiation or treatment with chemical mutagens or carcinogens, isolated from cultures infected with viruses, through genetic modification by transfection or obtained from transgenic animals.

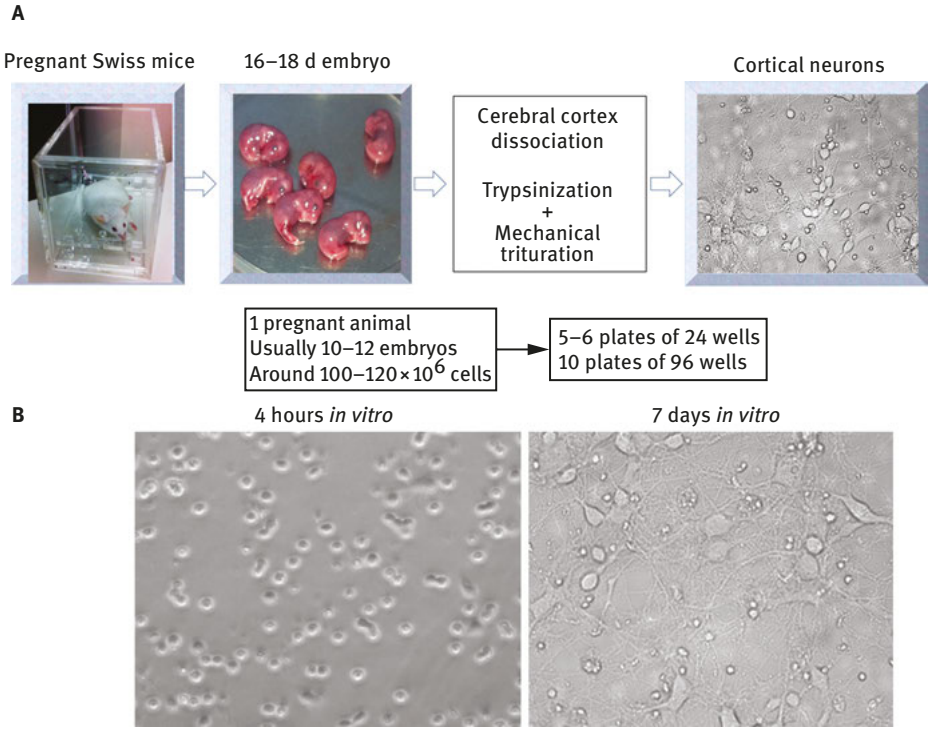


Figure 5.4: Scheme illustrating the procedure for the isolation of primary cultures of cortical neurons (A). Primary cultures of cortical neurons develop their typical morphology and functional characteristics with time in culture (B).

Widely used cell lines include epithelial cell lines obtained from the ovary of an adult Chinese hamster (CHO cells) or the human liver cancer cells HepG2 shown in Figure 5.5.

c. **Stem cell lines**: are continuous cell lines that retain the characteristics of stem cells and can produce diverse differentiated cell types. They require great care in their maintenance, handling, and preservation. One example of a human stem cell line is the CTX0E16 cell line which is a human neuronal stem cell line, obtained from the cerebral cortex of a fetus at 12 weeks of gestation and immortalized by the ectopic expression of the c-mycER^{TAM} transgene and kindly provided to our laboratory by a material transfer agreement with ReNeuron Limited (Guildford, Surrey GU2 7AF, U.K). Human neural progenitor cells CTX0E16 hNPCs were cultured following the provider instructions as previously reported [2]. Briefly, proliferating cells were maintained in reduced modified medium containing DMEM: F12 with 15 mM HEPES and sodium bicarbonate supplemented with 0.03% human serum albumin, 100 µg/ml

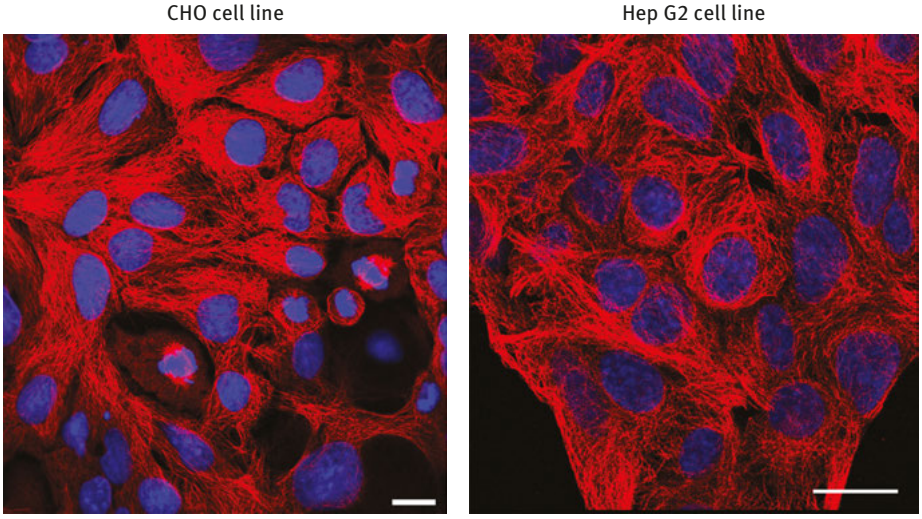


Figure 5.5: Confocal microscopy images showing adherent CHO cells and HepG2 cells after immunocytochemistry for the cytoskeleton marker tubulin (red). Nuclei were labeled with the nucleic acid stain Hoechst (blue). Images were kindly provided by Dr. J.A. Rubiolo (University of Santiago de Compostela).

apo-transferrin, 16.2 µg/ml putrescine, 5µg/ml human insulin, 60 ng/ml progesterone, 2 mM L-glutamine, and 40 ng/ml of sodium selenite. Under proliferative conditions cells were cultured in the presence of 10 ng/ml of human fibroblast growth factor, 20 ng/ml of human epidermal growth factor, and 100 nM hydroxytamoxifen. CTX0E16 hNPCs were seeded onto PDL (5 µg/cm^2), and laminin-coated (1 µg/cm^2) tissue culture flasks, with full media changes occurring every 2–3 days. Cells were passaged once they reached 70–80% confluence using Accutase and maintained between 25 and 30 passages. For differentiation CTX0E16 cultures were washed twice with nonsupplemented DMEM:F12 medium and passaged onto PDL and laminin-coated tissue culture plates or glass coverslips at a density of 50,000 cells per ml. Cells were then washed in warm Dulbecco's phosphate-buffered saline (DPBS) and maintained in neuronal differentiation media (Neurobasal Medium supplemented with 0.03% human serum albumin, 100 µg/ml apotransferrin, 16.2 µg/ml putrescine, 5 µg/ml human insulin, 60 ng/ml progesterone, 2 mM L-glutamine, 40 ng/ml sodium selenite, and 1 × B27 serum-free supplement. Half medium changes were performed every 2–3 days and cultures were differentiated for up to 60 days. Undifferentiated and differentiated phase contrast microscopy images of these human neuronal stem cells are shown in Figure 5.6.

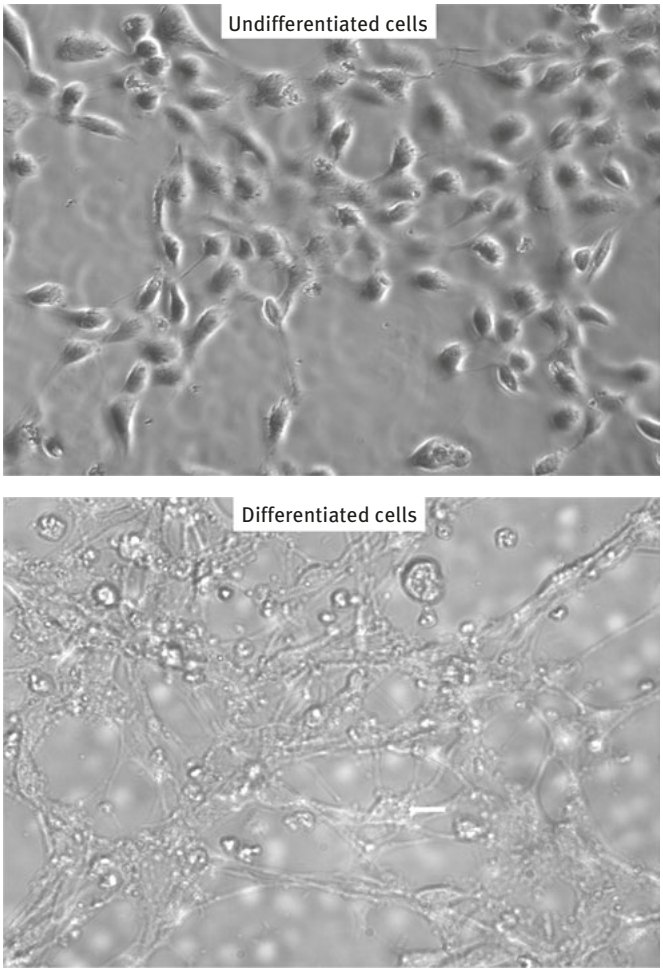


Figure 5.6: Phase contrast image showing undifferentiated (left) and differentiated human neuronal stem cells (right). Arrow heads indicate neuronal cell bodies.

5.5 *In vitro* cytotoxicity tests and methods to evaluate cellular function

In vitro tests may measure cellular function and cell death. Cell function is normally determined by the evaluation of the effect of chemicals on events or cellular signaling cascades that are related to cell injury or toxicity although several cell death tests are commonly used to evaluate cellular toxicity [21]. *In vitro* methods are common and widely used for screening and ranking chemicals, and have also been taken into account sporadically for risk assessment purposes in the case of food

additives; however, a major promise of *in vitro* systems is to obtain information on the mechanism of action of chemical which is considered pivotal for adequate risk assessment [7].

In Europe the European Union Reference Laboratory for alternatives to animal testing (EURL-ECVAM) has been established in 2011 and promotes the development and validation of alternative methods to animal testing. This laboratory has promoted the creation of the EURL ECVAM Database Service on Alternative Methods to Animal Experimentation (DB-ALM), a public and freely available service that provides evaluated information on the development and applications of advanced and alternative methods to animal experimentation in biomedical sciences and toxicology, both in research and for regulatory purposes (https://ecvam-dbalm.jrc.ec.europa.eu/). Actually this database has evaluated descriptions of more than 300 methods and almost 200 protocols, following the OECD guidelines. In total, detailed DB-ALM method descriptions, presented as comprehensive method summaries or individual protocols, are available for 26 different topic areas. Since, the recompilation of all the available *in vitro* methods is beyond the scope of this chapter, some of the methods are listed according to the proposed endpoint for DB-ALM classification and other methods are listed in more detail, focusing on those frequently used in our laboratory to analyze the toxicity of marine toxins.

Usually, cytotoxicity is considered primarily as the potential of a compound to induce cell death and is most frequently related to necrosis, a term used to define a class of irreversible cell death most often resulting from acute cellular injury that causes metabolic failure of the cell that coincides with rapid depletion of ATP [8]. Most *in vitro* cytotoxicity tests measure necrosis. However, an equally important mechanism of cell death is apoptosis, also known as "programmed cell death" which is a type of cell death that is mediated by a genetically controlled, energy-requiring program requiring different methods for its evaluation [8]. The inhibition of apoptosis is also of toxicological importance. Furthermore, detailed studies on dose and time dependence of toxic effects to cells, together with the observation of effects on the cell cycle and their reversibility, can provide valuable information about mechanisms and type of toxicity, including necrosis, apoptosis or other events [7]. It is widely accepted that *in vitro* cytotoxicity tests are useful and necessary to define basal cytotoxicity, for example the intrinsic ability of a compound to cause cell death as a consequence of damage to basic cellular functions. Cytotoxicity tests are also necessary to define the concentration range for further and more detailed *in vitro* testing to provide meaningful information on parameters such as genotoxicity, induction of mutations, or programmed cell death. By establishing the dose at which 50% of the cells are affected (i.e., TC_{50}; TC: toxic concentration), it is possible to compare quantitatively responses of single compounds in different systems or of several compounds in individual systems [7].

5.6 *In vitro* methods to evaluate the effects of compounds on reproduction

As mentioned above, the *in vitro* methods included in the DB-ALM database are listed in function of the endpoint analyzed, and each endpoint may include or not several subgroups. For example, the *in vitro* methods to evaluate the effects of compounds on reproduction are divided into three categories analyzing male and female fertility and developmental toxicity (Figure 5.7); however, as stated in the review document of these methods none of these *ex vivo/in vitro/in silico* methods have yet gained regulatory acceptance to date [9]. For this reason, these alternative methods do not allow for full replacement of animal testing. Therefore, these methods are mostly used for screening purposes and are intended to be applied in test batteries and as part of integrated testing strategies. However, with reference to "Reduction and Refinement" of animal use, the Extended One-Generation Reproductive Toxicity Study has been adopted as OECD Test

Figure 5.7: List of the reviewed *in vitro* methods to assess the effects of compounds on reproduction.

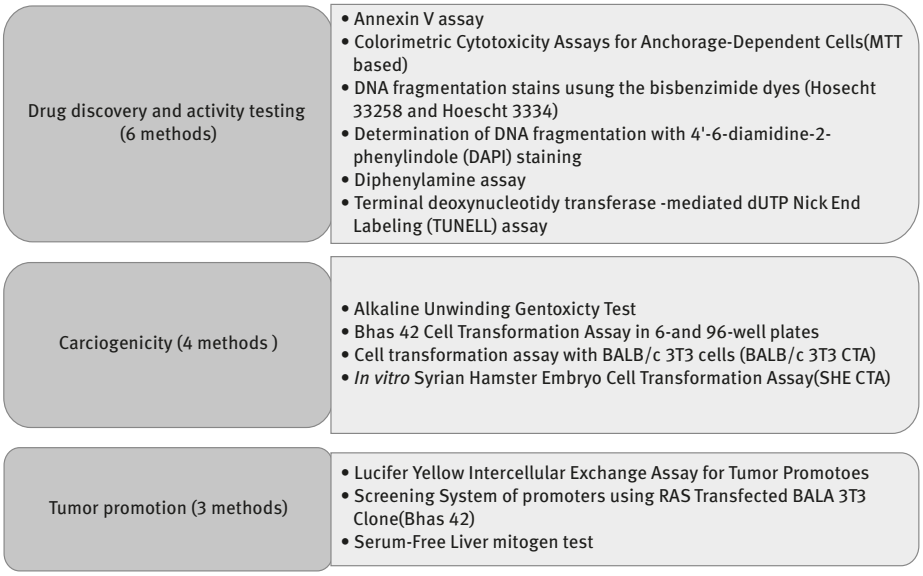


Figure 5.8: List of the reviewed *in vitro* methods for cancer research.

Guideline 443 [22]. For male and female fertility the list of cell-based protocols is summarized in Figure 5.7 while for developmental toxicity only the protocols are listed.

5.7 *In vitro* methods in cancer research

Cancer research and carcinogenicity and tumor promotion is one of the priority topics included in the DB-ALM. For the field of cancer research the methods are divided into three categories, which include drug discovery and activity testing (6 methods), carcinogenicity (4 methods), and tumor promotion (1 method). The methods included in this section are listed in Figure 5.8.

5.8 *In vitro* methods for environmental toxicity

For testing the environmental toxicity of chemicals and biological agents the DB-ALM provides several tests divided into different sections that include: Aquatic Short-Term Toxicity (6 methods), Genotoxicity/Mutagenicity (11 methods and 8 protocols), Hematotoxicity (1 methods and 1 protocol), Hepatotoxicity/Metabolism-mediated toxicity (31 methods and 9 protocols, however under this epigraph several methods for cell culture are included), and for immunotoxicity (1 method and 1 protocol). A summary of the DB-ALM methods to test for environmental toxicity is provided in Figure 5.9.

Figure 5.9: List of the reviewed *in vitro* methods to assess the environmental toxicity of compounds.

5.9 *In vitro* methods for basal toxicity and cytotoxicity

Under this section about 17 methods were reviewed and approved/recommended by the experts of the DB-ALM to test for the basal toxicity and cytotoxicity of compounds and biological agents, including marine toxins. These methods are detailed below summarizing the cell type employed and the cellular function or activity evaluated. Since this group of methods are usually employed as starting points to evaluate cellular toxicity are examined in more detail bellow.

CHO Cell Proliferation Test and CHO Cell Na^+/K^+/ATPase activity test: These are two different methods using the same cellular model. CHO cells constitute a system useful for ecotoxicological studies. The proliferation rate of CHO cells correlates with physiological membrane functions, such as adenosine uptake and the activity of Na^+/K^+/ATPase. In the proliferation assay CHO cells are cultured for one week in petri dishes with various concentrations of test substance and the number of cells is counted twice daily, and the proliferation rate is determined from the logarithmic growth phase. The CHO cell Na^+/K^+/ATPase test measures the activity of the enzyme in the membrane of CHO cells.

Cytoskeletal Alterations as a Parameter for Assessment of Toxicity: The method is based on the determination of changes of cytoskeletal proteins (α and β tubulin and vimentin) after exposure to compounds by indirect immunofluorescence microscopy or quantitative biochemical methods (extraction of tubulin from the cells and measuring the tubulin content of the extracts using a colchicine-binding assay). Immunocytochemistry and immunofluorescence microscopy are useful methods to evaluate the effects of marine toxins on cell integrity [31] and a protocol for such methods is shown in Figure 5.10.

The main advantage of this method is that microscope visualization of cytoskeletal proteins provides a detailed view of changes in morphology; however, the technique is not adequate to quantify changes in protein expression. An example of the results obtained after the treatment of neuronal cells with chemicals and the analysis of their effect on the cytoskeleton is shown in Figure 5.11. In the upper panel, control cortical neurons after 7 days in culture and age-matched neurons were treated with the marine ciguatoxin CTX3C for 24 hours and double staining was performed with antibodies against the neuronal microtubule associated protein MAP2 and against the GluR2/3 subunit of glutamate receptors. No apparent changes in protein expression or cell morphology were observed. In contrast, in the lower panel, human neuronal stem cells were treated with vehicle or with the active derivative of the neurotoxins 6-hydroxydopamine or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine known as 1-methyl-4-phenylpyridinium ion (MPP^+), which is capable of inducing degeneration in human neuronal stem cells [32] and subsequently were stained with the neuronal cytoskeletal marker β3-tubulin and the neuronal nuclear marker NeuN. As shown in Figure 5.11 morphological changes were evident in cells treated with the neurotoxin MPP^+.

As mentioned above, one the main disadvantage of immunocytochemistry is its difficulty to objectively quantify changes in protein expression levels. The data

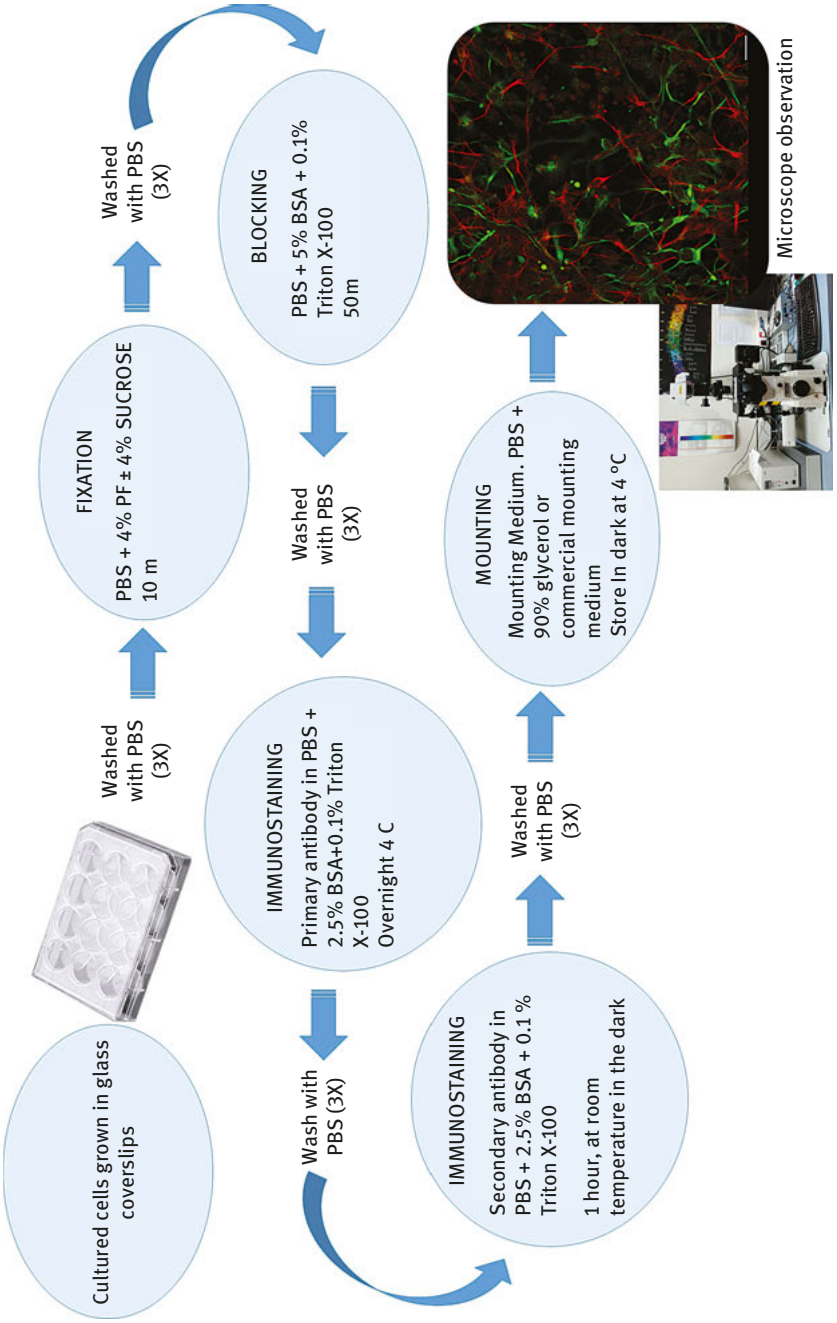


Figure 5.10: Scheme showing the protocol for immunostainig of adherent cells in culture.

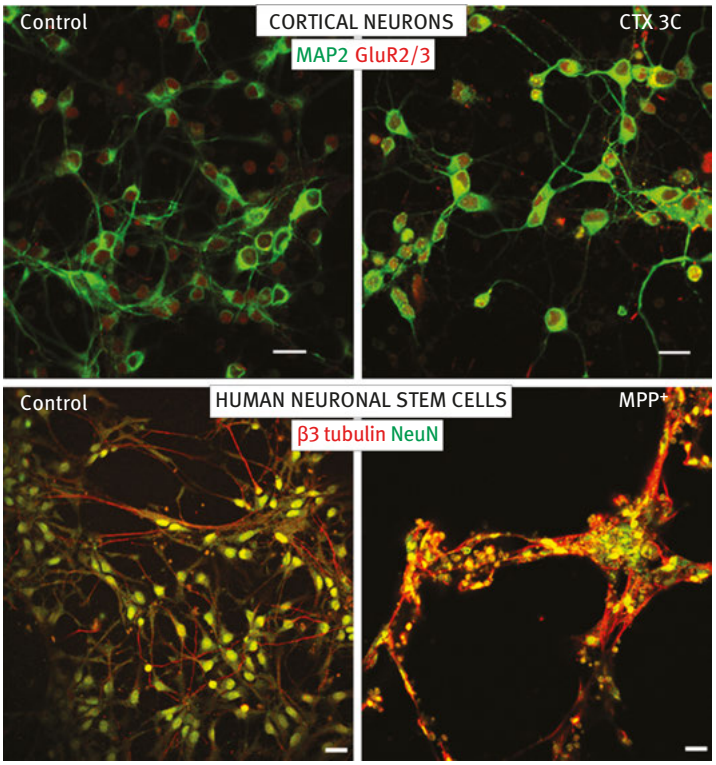


Figure 5.11: Confocal microscope images showing the staining of cytoskeletal proteins in primary cultures of cortical neurons (upper panel) and human neuronal stem cells (lower panel). Cultured cortical neurons not treated (control) and treated with the marine toxin CTX3C were stained with MAP2 (green) and the glutamate receptor subunit GluR2/3 (red), showing that the toxin did not alter neuronal morphology. In the lower panel human neuronal stem cells were exposed to the neurotoxin MPP^+ and stained with the neuronal cytoskeletal marker β3-tubulin (red) or the nuclear protein NeuN (green). MPP^+ treatment caused a dramatic change in cell morphology. Scale bar is 20 µm.

shown in the upper panel of Figure 5.11 do not indicate any change in the level of expression of the glutamate receptor subunit GluR2/3 after ciguatoxin treatment in cortical neurons. However, quantitative analysis of the expression levels of GluR2/3 by western blotting showed a significant decrease in GluR2/3 expression in cortical neurons after ciguatoxin treatment [18].

Western blotting is an important technique used in cell and molecular biology to identify specific proteins from a complex mixture of proteins extracted from cells and it allows to quantify the protein expression as well [16]. The technique uses three elements to identify a specific protein, namely:

- Separation of the protein by size or molecular weight
- Transfer of the proteins to a solid support
- Identification of proteins using primary and secondary antibodies.

Although several different methods can be used to quantify proteins in biological samples, sodium dodecyl sulfate polyacrylamide gel electrophoresis is the most widely used analytical method to resolve separate components of a protein mixture. The technique is used by many laboratories to investigate or demonstrate changes in expression of a given protein between control states and experimental conditions, and is still the method of choice for basic research; it is also a useful tool in clinical applications and may be applied to any biological sample including cell or tissue extracts and body fluids, such as plasma, serum, or urine [14]. A scheme of the procedure used for western blot in lysates from adherent cell cultures is shown in Figure 5.12. In brief, cell lysates are one of the most common systems used in western blot. Protein extraction from mammalian tissues usually requires mechanical disruption and the use of reagents containing detergents. Moreover, cell lysis should be performed at low temperature and with protease and phophatase inhibitors to prevent protein degradation. Once extracted, protein concentration is measured, usually with a spectrophotometer to quantify the amount of protein loaded into each well. Afterwards, the sample is diluted into a loading buffer, which contains glycerol that increases the density of the sample relative to the surrounding running buffer, making it easier to load in the well, a tracking dye (bromophenol blue) used to follow the run of protein sample on the gel. Sodium dodecyl sulfate (SDS) is added to denature proteins and load the proteins with a strong negative charge that will allow each protein to migrate in the electrophoretic field in a measure proportional to its size and furthermore thiol reagents (β-mercaptoethanol) are added also to reduce disulfide bonds. It is also very important to have positive and negative controls for the sample. For a positive control a known source of target protein, such as purified protein or a control lysate is used. This helps to confirm the identity of the protein, and the activity of the antibody. A negative control, which is either a null cell line for the specific protein or omission of the incubation step with the primary antibody is used as well to confirm that the staining is not nonspecific. Western blot uses two different types of agarose gels: stacking and separating gel. The gel in the top is the, stacking gel which is slightly acidic (pH 6.8) and has a lower acrylamide concentration making a porous gel, which separates protein poorly but allows them to form thin, sharply defined bands. The gel in the bottom part, called the separating or resolving gel is basic (pH 8.8), and has a higher polyacrylamide content, making the gel's pores narrower. Therefore, proteins are separated by their size in this gel since smaller proteins migrate more easily and hence more rapidly than larger proteins. After separating the protein mixture, it is transferred to a membrane (blotting) using an electric field oriented perpendicular to the surface of the gel, causing proteins to move out of the gel and onto the membrane. In this step it is necessary to ensure a close contact between the gel and the membrane and proper placement of the membrane between the gel and the positive electrode. The membrane must be placed as such, so that the negatively charged proteins can migrate from the gel to the membrane. This type of transfer is called electrophoretic transfer, and can be done in semi-dry or wet conditions. Wet conditions are usually more reliable as it is less likely to dry out the gel, and is preferred for larger proteins while semi-dry transfer is

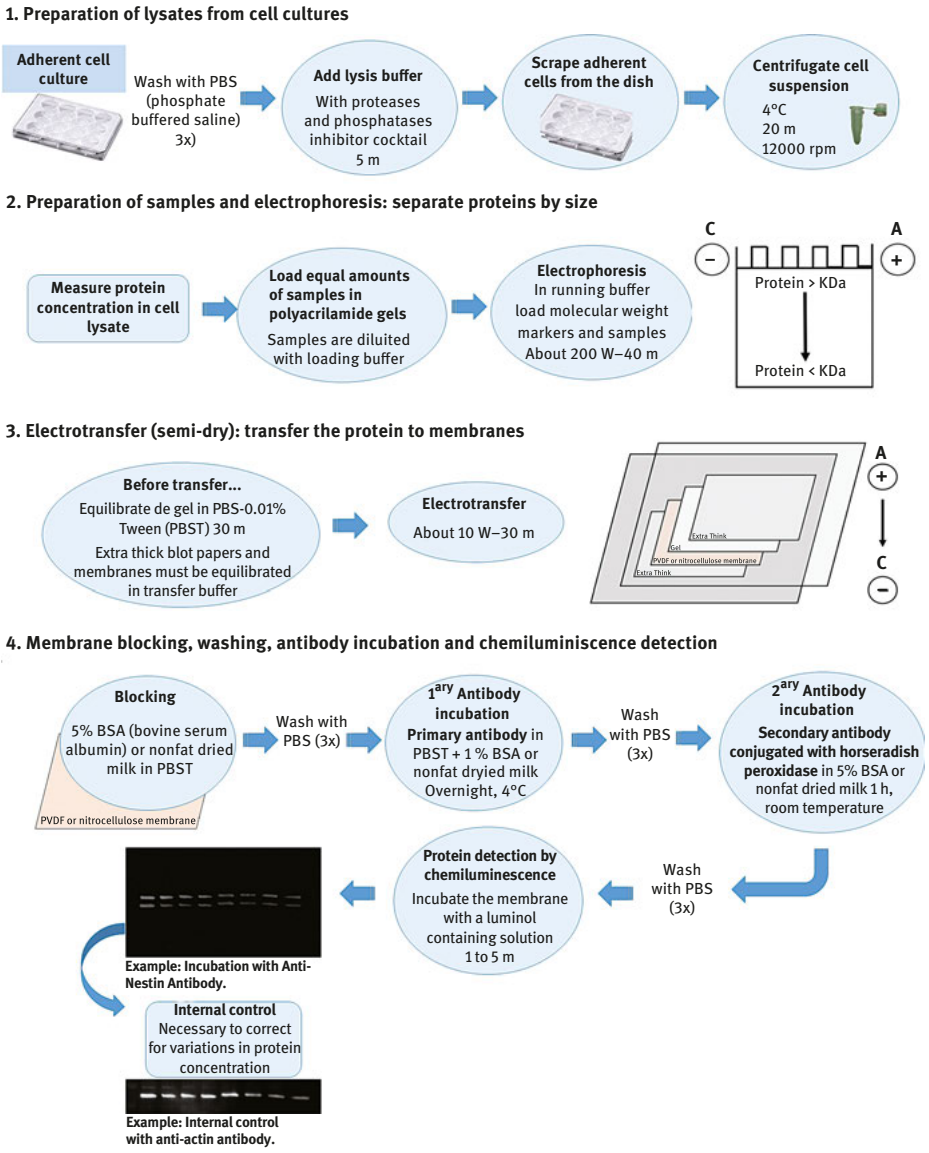


Figure 5.12: Schematic representation of a western blot protocol using cell lysates obtained from adherent cells.

faster. Membranes are made either of nitrocellulose or polyvinylidene difluoride (PVDF). Nitrocellulose is used for its high affinity for protein and its retention abilities but does not allow the membrane to be used for reprobing. In contrast, PVDF membranes provide better mechanical support and allow the blot to be reprobed and stored but have a higher background than nitrocellulose membranes. Regarding the washing, blocking,

and antibody incubation steps, it should be mentioned that blocking is very important to prevent antibodies from binding to the membrane in a nonspecific manner. Blocking is usually performed in 5% BSA or nonfat dried milk to reduce background. Frequently, the concentration of the antibody used is chosen following the instructions provided by the manufacturer, and primary and secondary antibodies are diluted in washing buffers, either PBS or tris buffered saline containing Tween 20. Afterwards, proteins in the membrane are usually detected with a secondary antibody linked to an enzyme such as horseradish peroxidase (HRP) and visualized by chemiluminescent detection. The use of chemiluminescence detection allows multiple film exposures and enables optimization of signal-to–noise ratio. Chemiluminescent detection occurs when energy from a chemical reaction is released in the form of light. The most popular chemiluminescent western blotting substrates are luminol-based. For example, in the presence of HRP and peroxide buffer, luminol oxidizes and forms an excited state product that emits light as it decays to the ground state. Light emission occurs only during the enzyme-substrate reaction; therefore, once the substrate in proximity to the enzyme is exhausted, signal output ceases. The two most common enzyme reporters that catalyze chemiluminescent reactions are HRP and alkaline phosphatase. Enzyme-conjugated secondary antibodies are used for western blotting, and light-producing reactions are captured with X-ray film or with charge-coupled device camera-based digital imaging instruments. The detection reagents can be removed and the entire blot reprobed to visualize another protein or to optimize detection of the first protein. Finally, it should be emphasized that quantification of protein levels is always semiquantitative because western blot data provide a relative comparison of protein levels, but not an absolute measure of quantity. The reason for the semiquantitative nature of western blot measurements is due to the variations in loading and transfer rates between the samples in separate lanes and to the fact that the signal generated by the detection is not linear across the concentration range of the samples [16]. However, the differences in protein loading are normally standardized, using internal control or loading control protein which are proteins derived from ubiquitously expressed “housekeeping” genes and have been widely used due to their presumed consistent level of expression across a diverse range of samples. Actin and tubulin are two of the most frequently used loading controls in biomedical research [6].

HEL-30 Cytotoxicity Test: Determines the anabolic competence of the cell. HEL-30 cells are incubated in the presence of radiolabeled leucine with or without test chemical for a short period of time. Uptake of the radio-labeled leucine is terminated by the addition of unlabeled leucine. Cell protein is precipitated with trichloroacetic acid and harvested onto glass-fiber filters. The radioactivity of the samples is measured by liquid scintillation counting.

Human Lymphocyte Cytotoxicity Assay: measures the leakage of DNA and lactate dehydrogenase (LDH, EC. 1.1.1 27) from lymphocytes into the surrounding medium as an indicator of cytotoxicity and also includes a measure of mitochondrial activity through the MTT assay. The method uses lymphocytes isolated from anticoagulated,

normal, human blood samples and grown for 5 days, centrifuged and resuspended in complete medium, aliquoted into 24-well plates and exposed to the test chemicals at appropriate dilutions.

LS-L929 Cytotoxicity Test: This test uses L-929 fibroblasts (mouse) maintained in suspension culture and incubated in the presence of test material in a range of concentrations for 4 hours. Cell viability is then determined by uptake of the dyes ethidium bromide and fluorescein acetate. A resultant cytotoxic effect is quantified using two complementary fluorimetric assay procedures. After exposure to a test compound, viable cells may be identified by their ability to accumulate fluorescein on incubation with fluorescein diacetate. The nucleic acid of both viable and nonviable cells can be stained with EB. Nonviable cells (stained only with EB) may be distinguished from viable cells (stained with ethidium bromide and with fluorescein diacetate) by the selective use of filters.

Laser Diffraction Measurement of Tumor Spheroids: Tumor cell lines cultured as aggregates can be utilized for *in vitro* radiosensitivity and/or chemosensitivity tests. Chemical effects are monitored by studying the changes in spheroid diameter measured by laser diffraction. This test evaluates cell viability and morphology and has been used with human cervical colorectal and lung cancer cell lines.

Quantitative Video Microscopy of Intracellular Motion and Mitochondria-Specific Fluorescence: the test uses either IMR-90 fibroblasts (human) or L-929 fibroblasts (mouse). IMR 90 cells are cultured on cover slips, mounted on slides and incubated for 1–24 hours in the presence or absence of test compounds. Movement of cell organelles is observed by means of video-enhanced contrast microscopy. The cells are maintained at a stable temperature and pH in an incubation chamber. At the same time the lysosomes and mitochondria are specifically stained with fluorescent vital dyes so that their number and morphology may be assessed. The analog video signal is enhanced, digitalized, and subjected to several steps of image processing. The final images are recorded and later analyzed to provide plots of organelle velocity versus incubation time.

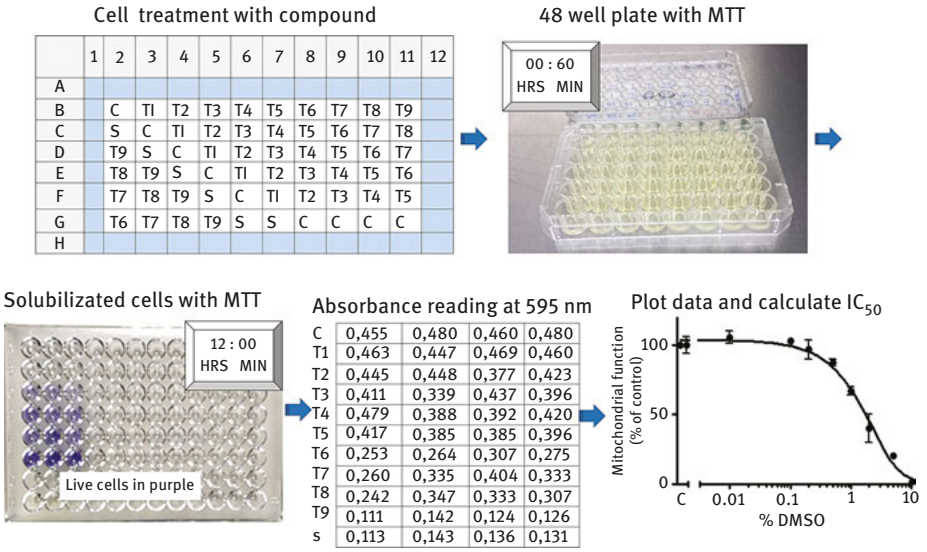
MTT Assay: The tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide (MTT) is taken up into cells and reduced in a mitochondria-dependent reaction to yield a formazan product [17, 19]. The product accumulates within the cell, due to the fact that it cannot pass through the plasma membrane. On solubilization of the cells, the product is liberated and can readily be detected and quantified by a simple colorimetric method. The method can be used almost with any cell type.

The ability of cells to reduce the MTT provides an indication of mitochondrial integrity and activity which, in turn, may be interpreted as a measure of viability and/or cell number. The test measures the tissue viability which is determined by the mitochondrial dehydrogenase activity and measured by formazan salt production from MTT. The result is expressed as % of the negative control. The endpoint value is the tissue viability (%) calculated as the ratio (Absorbance treated)/(Absorbance negative control) × 100. It is useful to determine the TC_{50} or the IC_{50} which is defined as the

concentration of a test substance that decreases the MTT reduction to formazan by 50%, from a dose–response curve.

The assay is useful to determine the viability of many cell types but not for cells with low mitochondrial activity. However, the number of cells initially plated, the period of exposure to chemicals, the concentration of MTT, the total duration of the experiment, etc. must be standardized for each cell line. The protocol used for the MTT assay in human neuronal CTX0E16 cells is summarized in Figure 5.13 and detailed bellow.

1. Cells seeded in 96- or 48-well plates are cultured for the desired time and treated with the compound of interest (in this case several DMSO concentrations: 0.001, 0.01, 0.1, 1, 2.5, 5 and 10% DMSO v/v) and control wells are treated with the corresponding vehicle used to solubilize the compound and are incubated in the culture incubator for the desired time (in this case 24 hours in the incubator).
2. Remove the culture medium and wash three times with Locke's buffer containing: 154 mM NaCl, 5.6 mM KCl, 1.3 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, and 5.6 mM glucose (pH 7.4) for 1 hour at 37°C.
3. Incubate the plates with 200 µl of 500 µg/ml MTT dissolved in Locke's buffer per well, for 60 min at 37°C. MTT solution should be prepared immediately before to the experiment, kept in the dark, and discarded after using.



	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		C	TI	T2	T3	T4	T5	T6	T7	T8	T9	
C		S	C	TI	T2	T3	T4	T5	T6	T7	T8	
D		T9	S	C	TI	T2	T3	T4	T5	T6	T7	
E		T8	T9	S	C	TI	T2	T3	T4	T5	T6	
F		T7	T8	T9	S	C	TI	T2	T3	T4	T5	
G		T6	T7	T8	T9	S	S	C	C	C	C	
H												

C	0,455	0,480	0,460	0,480
T1	0,463	0,447	0,469	0,460
T2	0,445	0,448	0,377	0,423
T3	0,411	0,339	0,437	0,396
T4	0,479	0,388	0,392	0,420
T5	0,417	0,385	0,385	0,396
T6	0,253	0,264	0,307	0,275
T7	0,260	0,335	0,404	0,333
T8	0,242	0,347	0,333	0,307
T9	0,111	0,142	0,124	0,126
s	0,113	0,143	0,136	0,131

Figure 5.13: MTT test to measure cell viability in CTX0E16 human neuronal stem cells: Experimental design is shown in the left upper panel (in blue are empty wells): C = control cells, T = treated cells. Treatments: T1 = DMSO 0, 001%, T2 = DMSO 0.01%, T3 = DMSO 0,1%, T4 = DMSO 0,2%, T5 = DMSO 0,5%, T6 = DMSO 1%, T7 = DMSO 2%, T8 = DMSO 5%, T9 = DMSO 10%; S = saponin (cell death control). Upper right panel: addition of MTT to treated cells. Lower left panel: MTT colored formazan salt in solubilizated cells. Middle lower panel: 595 nm absorbance values reading with the corresponding treatment for one representative experiment. Right lower panel: Dose–response plot showing the means of at least three independent experiments.

4. Carefully wash off excess MTT in order to avoid cell detachment and add 200 µl of 5% SDS at 5%, 200 µl per well to disaggregate the cells and keep the plate overnight in the dark.
5. Transfer disaggregated cells from each well to 96-well plates and treat with 10 µl isopropanol to eliminate air bubbles
6. Read the absorbance of the colored formazan salt at 595 nm in a spectrophotometer plate reader

Resazurin (Alamar Blue^{TM}) Cytotoxicity Assay: This test is also used to determine the viability of cells in culture after exposure to chemicals. Resazurin (7-hydroxy-3H-phenoxazin-3-one 10-oxide, also called Alamar Blue^{TM} by some authors) is a nontoxic, nonfluorescent blue dye which is reduced by metabolically active cells to the fluorescent red dye resorufin. This redox indicator is a widely used nontoxic reagent that exhibits both fluorimetric and colorimetric properties in response to metabolic activity. Resorufin has a pink color and is highly fluorescent. As the number of living cell decrease, the intensity of the fluorescence also decreases. The reduction-induced color change varies proportionately with cell number and time, changing from a nonfluorescent blue color to a reduced pink fluorescent form [4]. A scheme of the procedure used to evaluate cytotoxicity using the resazurin assay is shown in Figure 5.14.

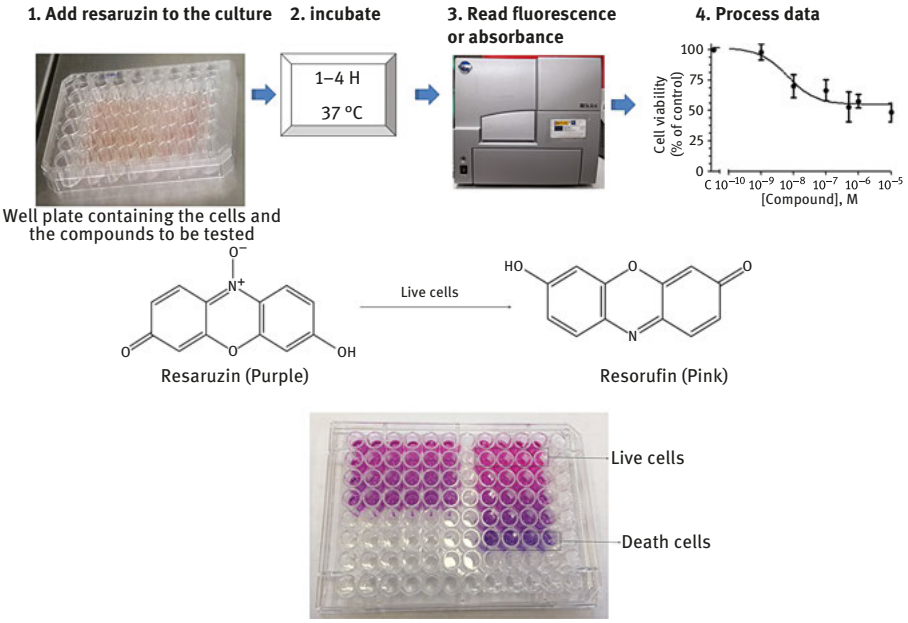


Figure 5.14: Scheme showing the protocol to evaluate cytotoxicity using the resazurin cytotoxicity assay.

Table 5.1: Summary of the different endpoints proposed to evaluate *in vitro* cytotoxicity as well as their main advantages and disadvantages.

Endpoint	Assay	Mechanism	Advantages/Disadvantages
1.Structural cell damage (noninvasive)	Evaluation of overall cell shape, cytoplasmic structure, flatness and outline properties on a good phase contrast light microscope	Screening assay	**Advantages:**, noninvasive **Disadvantages:** qualitative data, no exact cell death definition
	LDH-release test	Lactate dehydrogenase (LDH) enzyme is released to the culture medium when cell membranes rupture (nonviable cells), and the enzyme can then be measured in the supernatant.	**Advantages:** Measurement of a definite/unambiguous cell death endpoint; can be combined with cell function assays. Allows cells to be used for other purposes, if only supernatant is sampled. **Disadvantages:** Normalization necessary (extra wells for controls). Frequently high-background LDH levels are observed (e.g., from serum components).
2. Structural cell damage (invasive)	Membrane penetration by dyes to detect "cytotoxicity" (e.g., naphthalene black, trypan blue, propidium iodide, ethidium bromide, EH-1)	Involves the use of dyes that stain nonviable cells, but do not enter viable cells with an intact cell membrane.	**Advantages:** Rapid and usually easy to interpret. Gives information on the single cell level. **Disadvantages:** May overestimate viability since apoptotic cells continue to have intact membranes and may appear viable. Some dyes (e.g., trypan blue, H-33342) are cytotoxic, so that the evaluation has to be performed rapidly. Usually needs to be combined with dyes that stain both live and dead cells
	Retention of dyes within intact cells to detect "viability" (e.g., fluorescein diacetate or calcein-AM)	The lipid-soluble dyes are transformed by cellular enzymes (esterases) into lipid-insoluble fluorescent compounds that cannot escape from cells with intact membranes.	**Advantages:** Rapid and usually easy to interpret. **Disadvantages:** Some cells leak the dyes and some dyes and some dyes can suffer photo-bleaching.
	Evaluation of programmed cell death/apoptosis markers	Activation of caspases (enzymatic analysis or staining)	**Advantages:** Adds mechanistic information to cytotoxicity data.

(continued)

Table 5.1: (continued)

Endpoint	Assay	Mechanism	Advantages/Disadvantages
		Activation or endonucleases (detectable as DNA fragmentation). Chromatin condensation (detectable by DNA stains) Detection of phosphatidylserine on the outside of the plasma membrane (annexin staining)	**Disadvantages:** Not all types of cell death may be detected by a given endpoint. Needs to be combined with a general cytotoxicity test. Some endpoints are prone to artifacts (annexin staining) and some staining techniques (TUNEL, caspase-3) lead to an un-intentional selection of subpopulations. Caspase activity measurement does not easily yield a prediction model for the extent of cell death.
3. Cell growth	Cell counting	For some cell populations impaired growth is considered as a reduction of viability.	**Advantages:** growth can be a sensitive parameter of cell well-being. **Disadvantages:** growth is not necessarily linked to cytotoxicity; artifacts. Needs careful control in combination with cytotoxicity assays.
	BrdU or EdU incorporation	Measures new DNA synthesis based on incorporation of the easily detectable nucleoside analogs BrdU (or EdU) into DNA.	**Advantages**: Measurement on single cell level. Easy to quantify. **Disadvantages**: BrdU/EdU can be cytotoxic; high cost and effort compared to counting.
	Staining of cellular components that are proportional to overall cell mass (proteins by e.g., sulforhodamine B or crystal violet; DNA by Hoechst H-33342)	These assays evaluate a surrogate measure of overall cell mass and assume that it correlates with total cell number	**Advantages**: Simple and cheap; lots of historical data **Disadvantages:** Mostly not a single cell measure but only population level. Protein staining is only a surrogate endpoint of real cell number. For DNA quantification with Hoechst 33342: fluorescent probe penetration, bleaching, and cytotoxicity are issues to be considered.
4. Cellular metabolism	3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium (MTT) assay, or similar tetrazolium dye reduction assays	Measures the reduction of the tetrazolium dye by viable cells	**Advantages:** High throughput, easy, robust, low cost. Used in several ISO standards and OECD test guidelines. High sensitivity. Can be used for tissue constructs. **Disadvantages:** Measures amount of viable cells and needs control for contribution of proliferation. Cells with reduced mitochondrial function may appear nonviable. Measurement usually not on single cells.

Resazurin (Alamar blue) reduction assay	Fluorescent resorufin is formed from resazurin through mitochondrial metabolism of viable cells.	**Advantages:** Many tests can be performed rapidly in multi-well dishes and cells can be tested repeatedly (noninvasive measurement). High sensitivity. **Disadvantages:** Cells with reduced mitochondrial function may appear nonviable. Some test items interfere with the assay (e.g., superoxide also reduces the dye)
Mitochondrial depolarization assays (based on fluorescent indicator dyes)	Measurement of mitochondrial membrane potential by addition of potential sensing fluorescent dyes like JC-1, TMRE, MitoTracker, etc.	**Advantages**: fast, cheap, high throughput; single cell information. **Disadvantages**: as for MTT (measures cell function, not cytotoxicity). Artifacts by test items that affect mitochondria specifically. Artifacts by test items that affect plasma membrane potential, bleaching, quenching and unquenching, and shape changes and clustering of mitochondria.
Neutral red assay (ISO 10993)	Active cells accumulate the red dye in lysosomes and the dye incorporation is measured by spectrophotometric analysis.	**Advantages:** Low cost. Used in several ISO standards and OECD test guidelines. **Disadvantages:** Normalization required for quantitative measurement, e.g., with protein content or number of cells. Gives usually information only at the population level. Not suited for tissue constructs and certain cell lines.
ATP assays	Measurement of the total ATP content. Dying cells fail to produce ATP, have an increased ATP consumption, and may lose ATP through perforations of the plasma membrane. For the test, cell lysates are prepared, and the ATP content is assessed by a luminometric assay.	**Advantages:** fast, high throughput **Disadvantages:** no single cell data, expensive, not a direct measure of cytotoxicity.

Adapted from OECD [23].

Finally, in this section it should be mentioned that a draft of GIVIMP for the development and implementation of *in vitro* methods for regulatory use in human safety assessment has been developed by the European validation body EURL ECVAM and accepted on the work plan of the OECD test guideline program since April 2015 [23] and has been reviewed in 2017 [33]. This draft provides a list of several endpoints useful to evaluate cell toxicity, and the proposed tests are summarized in Table 5.1.

5.10 *In vitro* methods to test cellular function

To evaluate cellular function in living cells there are multiple techniques and several of them including cytosolic calcium determination and electrophysiological measurements are commonly used to evaluate the effects of marine compounds [17–19, 26, 28, 29]. Due to the complex equipment and specialized personnel required to perform electrophysiological determinations, only the basic principles of electrophysiology are going to be summarized. In brief, electrophysiological recordings allow gaining insight into the effect of compounds on voltage-gated and

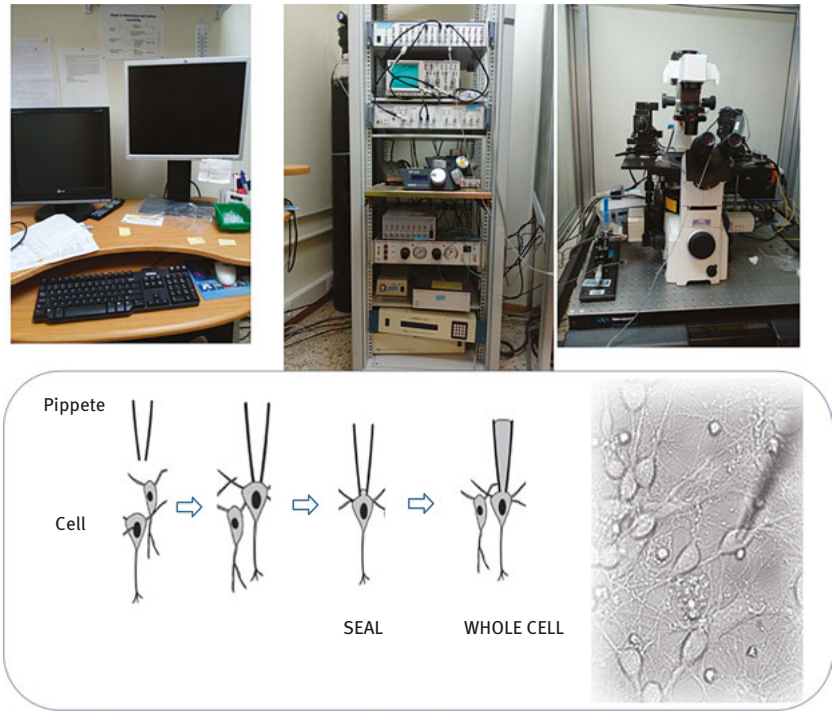


Figure 5.15: Electrophysiological equipment and schematic representation of the procedure to obtain a seal between the recording pipette and the cell.

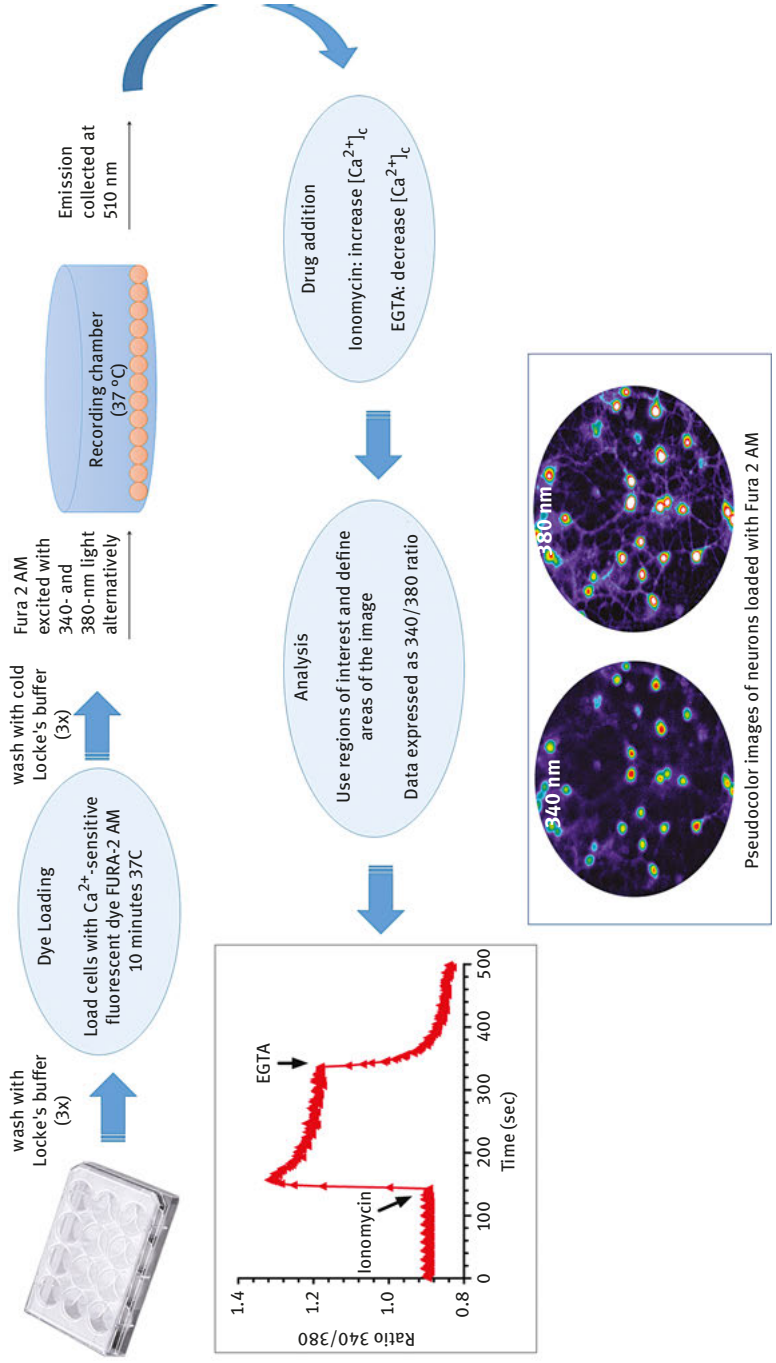


Figure 5.16: Schematic representation of the protocol for the determination of the cytosolic calcium concentration in primary cultures of cerebellar granule cells using the cell-permeable calcium-sensitive dye Fura-2 AM.

ligand-gated ion channels. The technique is based on the use of a recording pipette in narrow contact with the cell membrane (seal) and applying negative pressure in order to break the cell membrane and be able to measure either the current (in the modality of voltage clamp) or the voltage (in the modality of current clamp) flowing through the cell membrane. A scheme showing some of the equipment required in an electrophysiology setup as well as the basis of the procedure to obtain access to the cell is shown in Figure 5.15.

Another powerful technique to assess cell function after chemical application is cytosolic calcium imaging. The increase in cytosolic calcium concentration has been shown to play an important role in vital cellular functions such as muscle contraction, cell secretion, oocyte fertilization, nerve conduction, embryo development, and apoptosis in animals, plants and microbes, and in the invasion of mammalian cells by parasites, bacteria, and viruses. Therefore, live cell imaging of increases in cytosolic calcium concentration in cellular compartments has been investigated intensively. Multiple calcium imaging systems are now available commercially [11]. Among the first calcium indicators used for monitoring the dynamics of cellular calcium signaling were bioluminescent calcium-binding photoproteins, such as aequorin. However, for calcium imaging in living cells the most important achievement was the development of more sensitive and versatile fluorescent calcium indicators and buffers by Roger Tsien and colleagues [30]. These indicators were the result of the hybridization of highly calcium-selective chelators like ethylene glycol tetraacetic acid (EGTA) or 1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA) with a fluorescent chromophore. The first generation of fluorescent calcium indicators consisted of quin-2, fura-2, indo-1, and fluo-3. Quin-2 is excited by ultraviolet light (339nm) and was the first dye of this group to be used in biological experiments (see [15] for review). The widely used calcium-sensitive fura-2 AM is a combination of calcium chelator and fluorophore. It is excitable by ultraviolet light (e.g., 350/380 nm) and its emission peak is between 505 and 520 nm [30]. The binding of calcium ions causes intramolecular conformational changes that lead to a change in the emitted fluorescence. A summary illustrating the protocol for cytosolic calcium imaging in primary cultures of cerebellar granule cells is shown in Figure 5.16.

Acknowledgments

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Keywords: Cytotoxicity, *in vitro*, neuronal cultures, immunocytochemistry, MTT, western blot

Abbreviations: OECD: Organisation for Economic Cooperation and Development; GIVIMP: Guidance Document on Good *In Vitro* Method Practices; DMEM: Dulbecco's Modified Eagle's medium; BSA: bovine serum albumin; PDL: poly-D-lysine; PBS: phosphate buffered saline; HEPA: high-efficiency particulate air; DPBS: Dulbecco's phosphate-buffered saline; EURL-ECVAM: European Union Reference Laboratory for alternatives to animal testing. DB-ALM: EURL ECVAM DataBase Service on ALternative Methods to animal experimentation; PF: paraformaldehyde; SDS: Sodium dodecyl sulfate; MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MPP^+: 1-methyl-4-phenylpyridinium ion; PVDF: polyvinylidene difluoride; HRP: horseradish peroxidase; LDH: Lactate dehydrogenase; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide;EGTA: ethylene glycol tetraacetic acid; BAPTA: or 1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid

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Eva Alonso and Rebeca Alvariño

6 Marine Toxins

6.1 Introduction

Out of the 2,000 known species of microalgae dinoflagellates, marine phycotoxins ("phyco," from algae) are toxic secondary metabolites produced by about 200 species. Dinoflagellates are the first step of the food chain, and their toxins accumulate in filter-feeding mollusks and some fish. These microscopic organisms are mostly unicellular and can be found in freshwater and marine waters. Most of them are classified as photosynthetic and planktonic and live as free organisms in the water. However, studies on benthic species are increasing due to their high toxin production proportion [1]. These molecules seem to be harmless for those organisms that act as mere vectors. Phycotoxins can be also detected in other species such as gastropods, crustaceans, and fishes, especially in tropical waters, but more and more frequently in temperate waters Figure 6.1. The term secondary metabolite is rather ambiguous, and to date there is no clear explanation about the biological role of these marine toxins. Under specific ecological sea conditions (water temperature, salinity, turbidity, light irradiation, nutrients), explosive growths of dinoflagellates may occur, causing staggering accumulations of toxins if seafood. This increase in the unicellular algae concentration is known as "harmful algal blooms" (HABs), a phenomenon where microalgae accumulation draws big, colored stains in the water. Depending on the organisms, these blooms can be green, brown, orange, or red. Figures 6.2 and 6.3. Since these toxins are pharmacologically very potent, they pose an acute risk to consumers. Some of them, such as ciguatoxins, cause food intoxications so frequently that they are considered as a potential neglected third-world disease. Some of the phycotoxins, such are palytoxin or maitotoxin, are among the deadliest compounds in nature [2].

Not all the marine phycotoxins are due to dinoflagellates, some human poisoning has also been related to macroalgal consumption. At least two edible red alga consumption, *Acanthophora spicifera* and *Gracilaria edulis*, were associated with human deaths in Asia. The toxic compound polycavernoside A was then reported as the responsible compound [3].

A great diversity of molecules with complex chemical structures, different mechanism of action and biological activity can be found among marine phycotoxins. Neuronal and gastrointestinal symptoms can be observed in human intoxications. In fact, marine toxin classification used to be referred to human symptoms (neurotoxic, amnesic and paralytic toxins) but nowadays marine toxin groups are instead classified by their chemical characteristics as lipophilic, hydrophilic, and amphiphilic toxins, see Table 6.1. Some toxins are produced by different species, while, on

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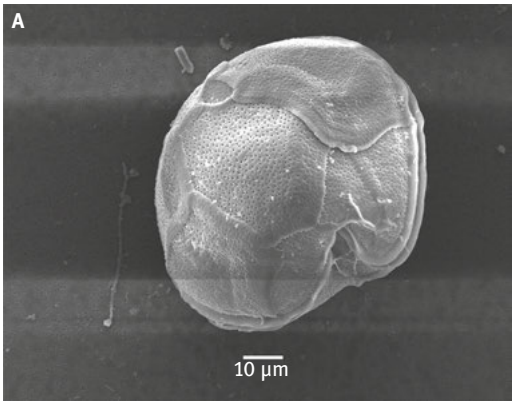

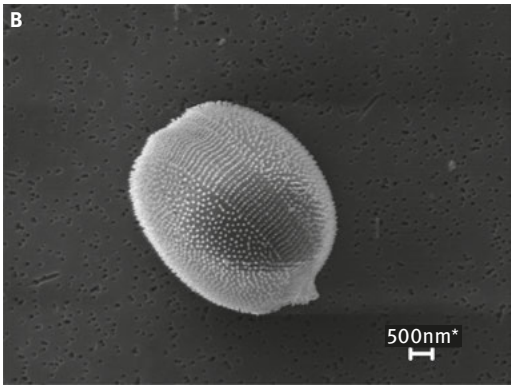

Figure 6.1: Dinoflagellates from *Gambierdiscus* and and Prorocentrum species. (A) *Gambierdiscus toxicus*. (B) *Prorocentrum minimum*.

Figure 6.2: Algal bloom. Red Tide.

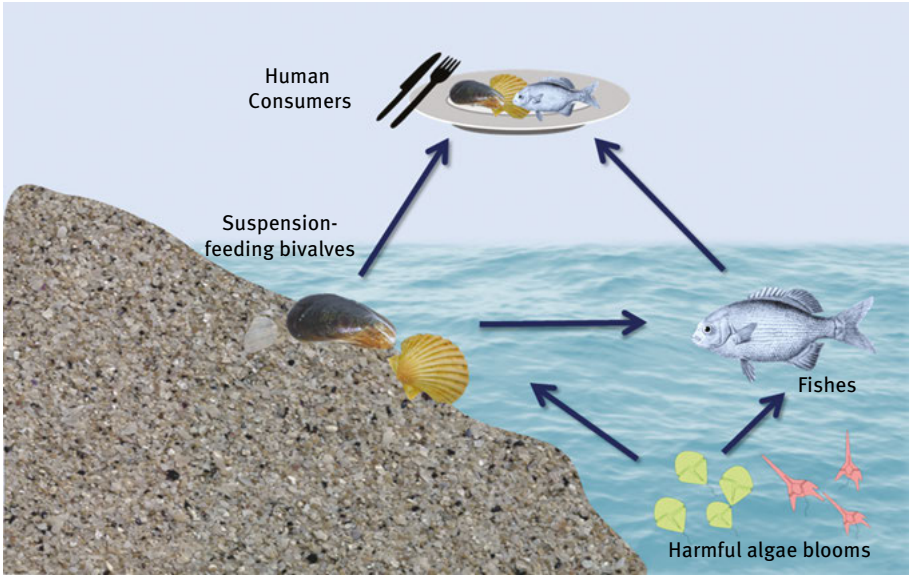

Figure 6.3: Trophic chain.

the other hand, one species may produce several toxin groups [4] (i.e., *Gambierdiscus* may produce ciguatoxins, gambierol, gambierone, and maitotoxin [5]).

With the unquestionable health risk, there is a high economic cost for the fishery industry. HABs provoke the closure of fishing areas, increase the costs for the monitoring of toxin levels, and some of the toxins (such as Ichthyotoxins) are harmful for fishing and marine cultures. Due to this, the control and legislation of marine toxins is of great importance. Countries have administrations that look after food safety and public health and establish the legal levels of toxins in marine food. In Europe, the European Food Safety authority "EFSA" and in United States, the US Food and Drug Administration "FDA" issue the guidelines for marine toxin detection. For each legislated toxin, a maximun amount of toxin per kilogram of flesh and a detection method is established. The international limits for marine biotoxin trade are set by the Codex Committee on Fish and Fishery Products. There is a "Standard for Live and raw Bivalve Mollusks," where a maximum level in mollusk flesh for five groups of toxins is established [10]. Table 6.2 shows the toxic limits set by Codex Alimentarius.

Although tetrodotoxin and ciguatoxin are not in the list of regulated marine toxins, the EU regulation states that poisonous fish derivatives and/or contaminated products should not be placed on the market. For ciguatoxins a maximum level in fish has been established in Japan, Mexico, and the United States [11]. Altough there are no worldwide agreement about toxin limits and analytical methods for their detection, most of the countries tend to a consensus in the monitoring programs procedures.

Table 6.1: Classification of marine toxins.

Marine Phycotoxins	Group [6]	Reference Compound [6]	Mechanism of Action [6]	Analogs [6]	Producer Organism (genus) [4]
Lipophilic Toxins	**Ciguatoxins**	Ciguatoxin-1 (CTX1)	Voltage dependent Na^+ channel activation	Ciguatoxins	*Gambierdiscus*
	Brevetoxins	Brevetoxin B (PbTx2) Brevetoxin A (PbTx1)	Voltage dependent Na^+ channel activation	PbTx 3–14 Brevenal	*Karenia* *Chatonella*
	Gambierol		Voltage dependent K^+ channel blockage		*Gambierdiscus*
	Okadaic Acid and Dinophyisis toxins (DTX)	Okadaic Acid	Protein phosphatase 1 and 2 inhibition	DTX 1–6	*Dynophysis* *Prorocentrum*
	Pectenotoxins	Pectenotoxin 2 (PTX2)	Actin inhibition	PTX 1–14 PTX seco acid	*Dynophysis*
	Yessotoxins	Yessotoxin (YTXs)	Phosphodiesterase 4A activation	YTX analogs: HidroxiYTX; CarboxyYTX; HomoYTX; KetoYTX; NoroxoYTX	*Protoceratium* *Lyngulodinium* *Gonyaulux*
	Azaspiracids	Azaspiracid 1 (AZA1)	Unknown	Azaspiracids 1-11	*Azadinium spinosum*
	Cyclic Imines	Gymnodimine A Spirolide (SPX)	Reversible blockage of cholinergic receptors	Gymnodimine A,B; SPX A-G (y desmethil SPX); Pinnatoxin A-D; Pteriatoxin A-C; Pinnamine; Prorocentrolide A-B; Spiroprorocentrimine C; Symbioimines, Portimine [7].	*Gymnodinium* *Karenia* *Alexandrium* *Prorocentrum*

(continued)

Table 6.1: (Continued)

Marine Phycotoxins	Group [6]	Reference Compound [6]	Mechanism of Action [6]	Analogs [6]	Producer Organism (genus) [4]
Hydrophilic Toxins	**Domoic acid**	Domoic acid	Kainate receptor activation		Diatoms of genus *Pseudo-Nitzschia* and *Nitzschia*
	Paralytic Toxins (PSP)	Saxitoxin	Site-1 voltage -dependent Na^+ channel inhibition	Carbamates (GTX 1-4, NeoSTX); N-Sulfocarbamoil (C1-4, GTX5-6); Decarbamoil (dcGTX1-4, dcSTX, dcNeo); Benzoates (GC1-6)	*Alexandrium* *Gymnodinium* *Pyrodinium* *Cyanobacteria*
	Tetrodotoxin, TTX		Same as saxitoxin		Several bacterial species [8].
Amphiphilic Toxins	**Maitotoxins**	Maitotoxin	TRP channel activation [9] Ca^{2+} entry activation	2^{99}possible isomers	*Gambierdiscus*
	Palytoxins	Palytoxin	Na^+/K^+ ATPase blockage	10^{21} possible isomers , Ostreocins, Ovatoxins	*Ostreopsis*

Table 6.2: Toxin limits set by Codex Alimentarius.

Saxitoxin	<0.8 mg saxitoxin equivalents/kg
Okadaic acid	<0.16 mg okadaic acid equivalents/kg
Domoic acid	<20 mg domoic acid/kg
Brevetoxin	200 mice units or <equivalent/kg (in legislated countries, tropical and subtropical areas)
Azaspiracid	0.16 mg/kg (in legislated countries)

There are diverse methods for marine toxin detection and quantification that will be revised in this book (see related chapters). Mouse bioassay (MBA) was the official method for paralytic and lipophilic toxins for several years but the guidelines for regulation and reduction in the use of experimental animal have tipped the balance in favor of the analytical methods such as HPLC combined with MS/MS or with UV detection. In MBA, seafood extracts are administered intraperitoneally to 20 g mice and the number of dead animals, death time, and symptoms allow a sample toxicity prediction. In analytical methods a certified standard is needed to detect and quantify the toxins in the samples. Moreover, the advances in understanding the toxin mechanism of action and in new technologies provide high-throughput functional detection methods based in the cellular target of the marine compounds [11]. Figure 6.4 shows the methods used for the monitoring of these toxins:

The following section shows a brief review about the origin, structure, and mechanism of action of the different marine phycotoxins:

6.2 Lipophilic toxins (Figure 6.5)

- Pectenotoxins
- Yessotoxins
- Azaspiracids
- Okadaic acid
- Dinophysis toxins
- Ciguatoxins
- Brevetoxins
- Gambierol
- Cyclic Imines

6.2.1 Pectenotoxins

Pectenotoxins (PTXs) are polyether macrolides isolated from *Dinophysis* genus organisms with a worldwide distribution. More than 20 pectenotoxin analogs have been identified in algae and shellfish but most of them are products of shellfish metabolism [12].

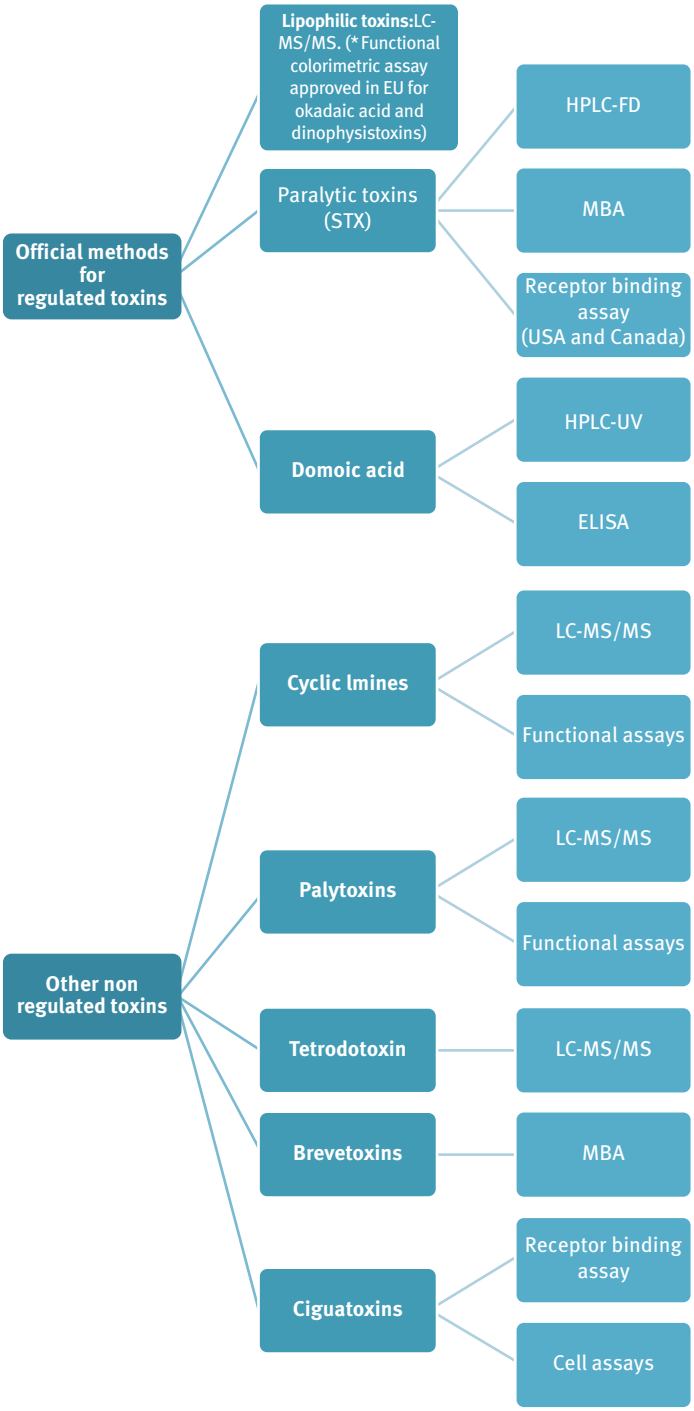

Figure 6.4: Methods used for the monitoring of regulated and nonregulated marine toxins.

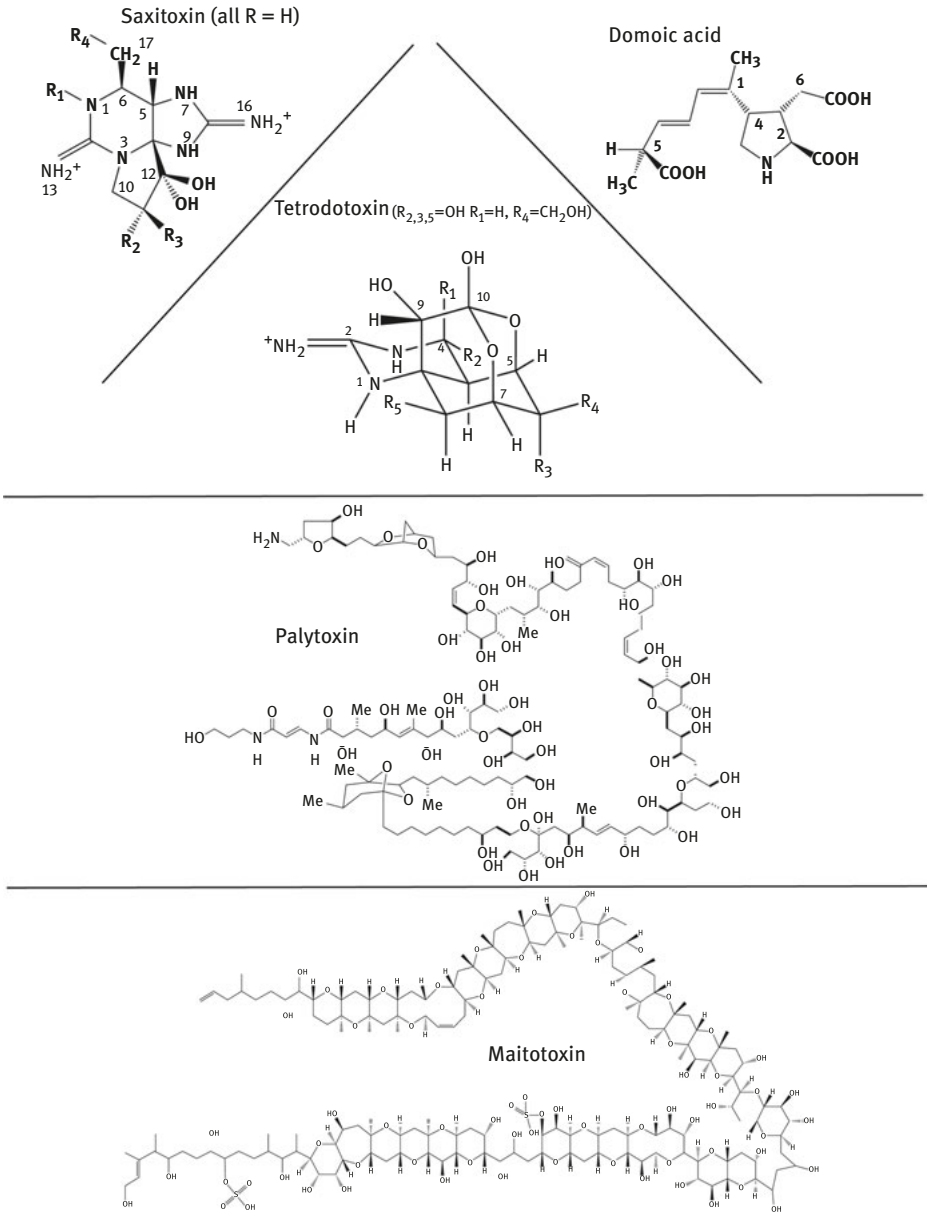

Figure 6.5: Hydrophilic (upper part) and Lipophilic toxins' structures.

PTX-1, PTX-2, PTX-3, and PTX-11 have similar toxicity in mice (LD_{50} 200-400 µg/kg) and the targeted organ seems to be the liver where macroscopic damage can be observed after intraperitoneal injection. Their toxicity is lower orally due to a poor intestinal absorption. No human poisoning has been associated to PTXs. Some diarrheic episodes were related in the past with PTX-2 but later the coexistence of okadaic acid was

settled as the cause responsible. The mechanism of action of this group of phycotoxins is the interaction with the actin skeleton of cells and the consequent destabilization of its structure. Actin is one of the most abundant cytoskeletal proteins that has a key role in important processes such as cell growth, signaling, and maintenance of cell shape. *In vitro* studies report that PTX-2 inhibits actin polymerization [13].

Although there is no clear link between human poisoning and episodes related to PTX, and most of the available data were obtained in *in vitro* and in vivo experiments, this group of toxins is still regulated in Europe with a limit of 160 μg PTX-2 equivalents/kg shellfish meat.

6.2.2 Yessotoxins

Yessotoxin (YTX) is a polyether compound firstly described in Japanese waters after a food intoxication outbreak with diarrheic symptoms. Due to the symptomatology and that it was often isolated with okadaic acid, it was originally classified among diarrheic toxins. Lately, it has been classified as a separate group for its biologic origin and effects [14]. This phycotoxin, with more than 100 analogs, is produced by different dinoflagellate species from the genera *Protoceratium, Lingulodinium*, and *Gonyaulux* [15–17], some of them are produced directly in the dinoflagellate and other analogs after shellfish metabolism. It is formed by 11 adjacent rings, an unsaturated lateral chain, and two sulfate groups [18]. While the carbonate skeleton is liposoluble, the sulfate groups confer to the molecule amphiphilic characteristics, making it the most polar of the liphophilic toxins [19].

YTX is accumulated in the mussel and scallop digestive gland but it can be also detected in the muscle [20]. MBA results in a high mortality when the toxin is administered intraperitoneally but no mortality is registered orally [14]. This is due to the amphiphilic characteristics that modified the intestinal absorption. Controversial effects are observed in vivo with different LD_{50} and tissue alterations, the most relevant effect being in the heart [21–25]. EFSA originally proposed a 25 μg YTZ equivalents/kg body weight as a reference limit but recently the toxin limit has been set at 3.75 mg of YTX equivalents/kg body weight due to the absence of human intoxications [26, 27].

Several intracellular pathways are modified by YTX and different effects are observed depending on the cellular type used. YTX targets, among other biological effects, calcium signaling, cAMP levels, PDEs, AKAP, and PKC expression and activates programmed cellular death [28]. For example, it is known that YTX activates phosphodiesterase in human lymphocytes followed by a calcium-dependent cAMP decrease [29]; furthermore, it does not interact with protein phosphatases, which are the targets of okadaic acid. This PDE–YTX interaction was also confirmed by biosensor techniques [30]. Moreover, this compound potently induced cell death in several cell lines and primary cultures [28, 31].

6.2.3 Azaspiracid

Azaspiracids (AZA) are polyether compounds synthesized by the dinoflagellate *Azadinium spinosum* and firstly reported in mussels from Ireland [32]. At least AZA-1 and AZA-2 are produced by the dinoflagellate and some analogs such as AZA-3, AZA-4, and AZA-5 are metabolites isolated in seafood, although more than 20 analogs have been described in the literature. No human deaths have been reported and only diarrheic episodes (nausea, vomiting, diarrhea, and stomach cramps) are related with AZA-containing mussels. In fact, there are no appropriate LD_{50} for these compounds, the available LD_{50} in mice data report a highest toxicity for AZA-2 and AZA-3 followed by AZA-1 and AZA-4, all of them above 100 µg/Kg body weight and the least toxic being AZA-5. After AZA administration, several organ damages are observed, including intestinal swelling, fatty acid deposition in liver, spleen and thymus damage, and even tumorogenic effects [33]. Recently, neuro and cardiotoxic effects have been also related to AZA exposure [34, 35]. Currently, the regulatory limit in Europe is set at 160 µg of AZA-1 equivalents/Kg shellfish meat.

The biological target of these group of compounds is still unknown but several studies describe cytotoxicity in several cell lines, effects on actin cytoskeleton, and effects over intracellular signaling molecules such as MAP kinases, Ca^{2+}, and cAMP [32]. Moreover, different studies also report effects of AZAs on *human ether-á-go-go related gene* potassium channel (hERG K^+), where inhibition of the open state of hERG K^+ channels has been observed [36, 37].

6.2.4 Okadaic acid and dinophysistoxins

Okadaic acid and its analogs, dinophysis toxins (DTXs), are isolated from several dinoflagellates from the genera *Prorocentrum* and *Dinophysis*. These polyether toxins have a wide distribution and can be found in the microorganism itself or in the shellfish as acylated forms. These compounds are responsible for the Diarrhetic Shellfish Poisoning (DSP) syndrome: nausea, vomiting, diarrhea, and abdominal pain observed after contaminated shellfish consumption. The symptoms are usually minor with a full recovery period of 2-3 days. These compounds are strong inhibitors of the serine/threonine phosphatase 1, 2A, and 3, with 2A being the most potently inhibited protein phosphatase. These phosphatases are important signal-transducer proteins involved in the control of several cellular processes. The repetitive exposure to these phycotoxins has been also associated with a tumor-promoting effect in mice and even studies about the relationship of human colorectal cancer risk and okadaic acid and analogs contaminated shellfish consumption have been carried out [38, 39]. They are regulated toxins and the actual European regulatory limit is set at 160 µg/Kg shellfish meat. The toxicity equivalency factors (TEFs) for this group of phycotoxins are 1, 1, and 0.6 for okadaic acid, DTX-1, and DTX-2 respectively. These values were calculated based on intraperitoneal administrations and only a TEF study on DTX-2 by the

oral route has been published [40]. Although the mode of action of the toxins is well defined, the diarrheic effect is not fully understood. It was originally thought to be caused by disruptions of the tight junctions on the intestinal cells, but several works question this [41], and now propose a neuroactive effect based on neuropeptide Y [42].

6.2.5 Ciguatoxins

Ciguatera is an endemic food intoxication observed after tropical and subtropical fish consumption in Indo-Pacific Oceans and Caribbean Sea areas. However, *Gambierdiscus* spp. have been observed in Mediterranean and Atlantic areas in the last years in accordance with the spread of this benthic producer organism attributable to climate warming [43]. Ciguatera symptoms include gastrointestinal and neurological manifestations, such as diarrhea, nausea, vomiting, paresthesia, myalgia, headache, muscular weakness, and cold allodynia. Usually gastrointestinal manifestations appear before neurological symptoms but the neurological ones are the most characteristic of this food intoxication and specifically cold allodynia is considered pathognomonic of ciguatera. Lethality is low, but in some cases ciguatera symptoms persist or reappear several years after intoxication due to the accumulation of the toxin in adipose tissue and long-term effect in the nervous system [44–46].

Dinoflagellates from *Gambierdiscus* spp. produce several polyether toxins, the most studied are ciguatoxins and also gambieric acid, gambierol, and maitotoxin. Different ciguatoxin structures are observed depending on dinoflagellate location and therefore the prefix P-, C-, or I- is added to identify Pacific, Caribbean, or Indic molecules, respectively. Pacific toxins are the most toxic. These compounds are cyclic polyethers and are thermally stable, liposoluble, and highly oxygenated. These compounds potently interact with sodium channels. They are strong activators of voltage-gated sodium channels through the competitive binding to receptor site 5 at low nanomolar concentrations. This channel activation leads to cell depolarization, spontaneous nerve firing, elevation of intracellular free Ca^{2+} concentration, and even neurotransmitter release. Toxin binding to site 5 produces a shift in the voltage dependence activation of Na^{+} channels causing a Na^{+} influx at membrane potentials where these channels would be usually inactivated. However, other properties of these channels remain unaffected as the peak Na^{+} current size [47].

Although there is no regulatory limit in the fish in Europe, only ciguatoxin-free fish are allowed to reach the market.

6.2.6 Brevetoxins

Species from the genus *Karenia* are the responsible for Brevetoxins (BTXs) production, mainly *Karenia brevis*. Although these dinoflagellates have worldwide distribution, BTX intoxication has been only reported in United States, Mexico, and New Zealand.

Their structure is similar to those of CTXs with a ladder-like fused oxygen containing ring skeleton. Based on their backbone structure BTXs can be classified as A-type (BTX-1, BTX-7, and BTX-10) and B-type (BTX-2, BTX-3, BTX-5, BTX-6, BTX-8, and BTX-9) with BTX-2 being the most common. This group of toxins binds to site 5 of voltage-gated sodium channels producing a Na^+ ion influx.

Limited human data are available with BTX and no regulatory limit exists in Europe but in some countries (United States, New Zealand, and Australia) the limit is fixed at 200 mice units or 0.8 mg BTX-2 equivalents/kg shellfish meat. BTX intoxication is responsible for neurotoxic shellfish poisoning, leading to general gastrointestinal symptoms and neurological effects such as paresthesia, ataxia, disorientation, and reversal temperature sensation (similar to the observed with ciguatoxins). Severe intoxication can end in limb paralysis and respiratory distress; however, no deaths have been reported and recovery is observed in few days. Although toxic effects in humans after ingestion are rare, BTX-related effects after inhalation are the most common. Exposure to BTX-containing aerosols provokes respiratory tract and conjunctival irritation [48–50].

BTXs are extensively studied for their ichthyotoxicity. They are absorbed through the gill membranes and produce serious behavioral and developmental damages due to their interaction with nerve functioning and neuromuscular transmission [51].

6.2.7 Gambierol

Gambierol has been isolated from *Gambierdiscus toxicus* dinoflagellate as a part of the ciguatoxin group. It is also a cyclic polyether, but it differs in the mechanism of action, human symptoms induced, and potency. This toxin has a ladder-shaped octacyclic ether skeleton with a partially conjugated triene side chain [52]. This family of ladder-shaped polyether toxins targets transmembrane proteins within the lipid bilayer [53]. It elicited a high toxicity in mice with a LD_{50} of 50 and 150 μg/kg after intraperitoneal and oral administration, respectively, and neurological symptoms [54]. Due to the structural similitude with ciguatoxins, it was believed that both phycotoxins share the mechanism of action but, while ciguatoxins are strong modulators of voltage-gated sodium channels with little effect in voltage-gated potassium channels [55–57], gambierol does not block or affect sodium channels in the nanomolar range but, instead, it efficiently inhibits potassium channels in several cellular models [58–61] at nanomolar concentrations. Kopljar et al described that gambierol anchors the gating machinery of Kv in its resting state, requiring stronger depolarizations for the channel opening, and depolarizations above the physiological range [60].

6.2.8 Cyclic Imine toxins

This group of lipophilic toxins has a macrocyclic structure with an imino functional group. Among them, we can find spirolides, gymnodimine, pinnatoxins, pteriatoxins,

prorocentrolides spiro-prorocentrimines, and portimine, all of them with a common imino functional group and similar intraperitoneal toxicity in mouse and characteristic neurological manifestation [62, 63]. While spirolides are worldwide distributed and no human poisoning has been reported, pinnatoxins, pteriatoxins, prorocentrolides, and spiroprorocentrimines are only reported in Japan, China, and Taiwan areas. Pinnatoxin A was in fact responsible for one of the main food intoxications in Japan and China [64]. Similar to spirolides, gymnodimine has not been connected with human intoxications but intraperitoneal injection in mouse has resulted in animal death and its geographic distribution is also more limited [65].

As previously stated, spirolides are the most widely distributed cyclic imines. They have been reported in North American, Canadian, and European coasts [66]. They were firstly isolated in New Scotland (Canada) in a routine monitoring and later *Alexandrium ostenfeldii* was identified as the responsible dinoflagellate [67, 68]. Fourteen compounds have been identified till now and classified in three different groups. Spirolides A-d in the first, E and F in the second, and spirolide G is the latest [69].

These toxins act through cholinergic receptors interaction. 13-desmethil spirolide C produces a muscarinic and nicotinic receptor overexpression in rats after high-dose administration [70]. *In vitro*, a high affinity in competitive binding and electrophysiology studies for muscular and neuronal nicotinic receptors at nanomolar concentrations has demonstrated [71]. In human neuroblastoma cells this toxin induced a dose-dependet internalization of muscarinic M3 receptor [72].

Gymnodimine was isolated in 1994 as a metabolite from the dinoflagellate *Gymnodinium,* later *Karenia Selliformis* [73] was identified as the real producer. As spirolides, gymnodimine has a low oral toxicity besides the intraperitoneal route, which is followed by neurological symptoms and leads to fast animal death. The blockage of the nicotinic receptors in the neuromuscular junction was concluded to be its mechanism of action [74] and inhibition of acetylcholine currents in Xenopus oocytes tranfected with α7 nicotinic receptor has been also described [71].

6.3 Hydrophilic toxins (Figure 6.6)

- Saxitoxin
- Domoic acid
- Tetrodotoxin

6.3.1 Saxitoxin and analogs

These nonpeptide neurotoxins are produced by several species of *Alexandrium* genus and two specific dinoflagellates species, *Gymnodinium catenatum* and and *Pyrodinium bahamense*. Their poisoning is lethal, and it is characterized by neurological symptoms

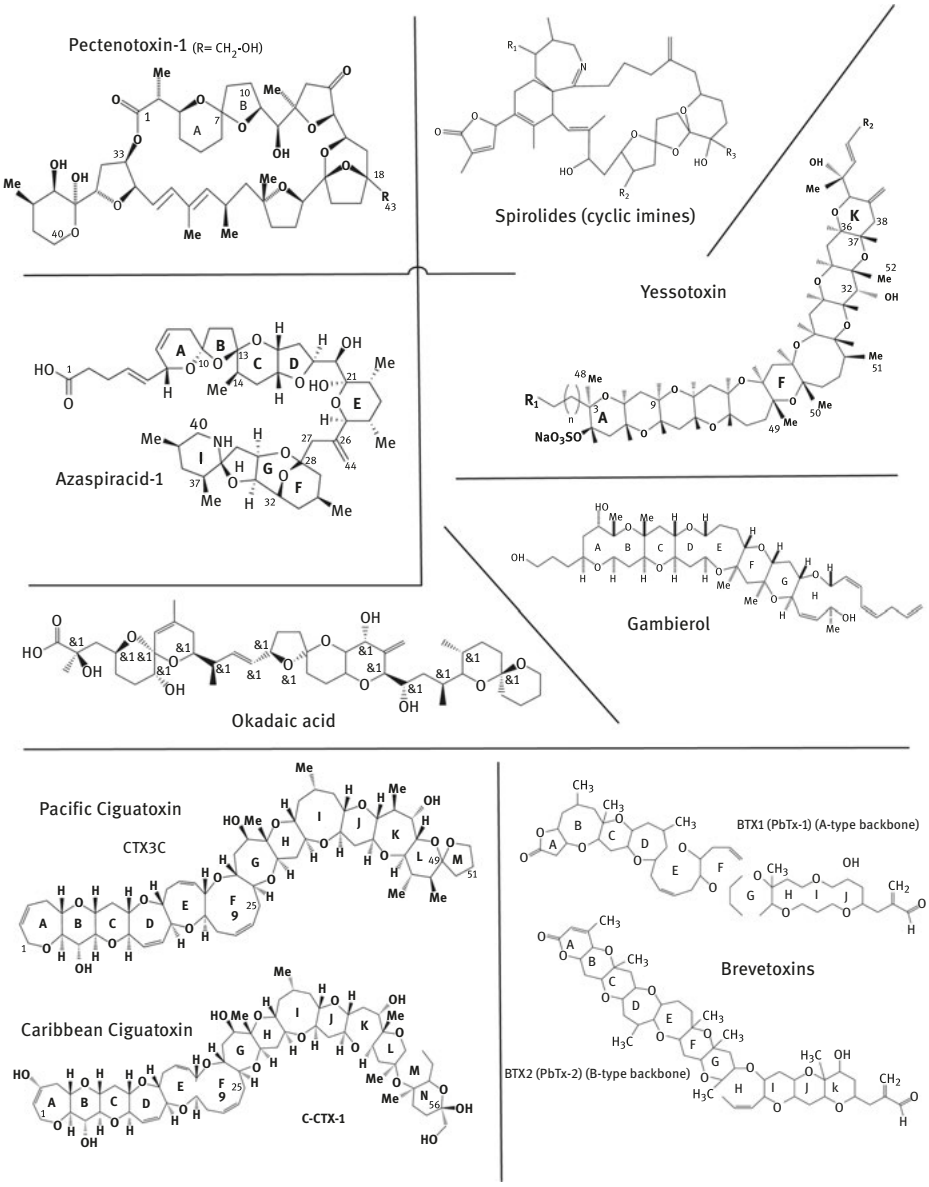

Figure 6.6: Lipophilic and amphiphilic toxins' structures.

such as paralysis, dizziness, headache, and respiratory arrest, owing to the mechanism of action of this group of toxins, the blockage of site 1 sodium channels in excitable cells (similar to tetrodotoxin), that interferes with the normal nervous transmission [75, 76]. The intoxication symptoms appear rather fast, but with artificial ventilation the patients can recover in few days, as the binding to the receptors is reversible.

They are included in the paralytic shellfish poisoning group (PSP toxins) and are formed by more than 57 tetrahydropurines, closely related among them, and represented by saxitoxin (STX) [77]. The basic structure of STX is a 3,4-propinoperhydropurine which can be modified by addition of hydroxyl, carbamyl, N-sulfocarbamoyl, or sulfate groups, producing several analogs with different chemical properties and potency. However, they share the same mechanism of action. The legal limit for this group of toxins is 800 µg of STX equivalents/Kg or 4 mouse units/Kg of meat, which corresponds to 18 µg of STX as the lethal amount needed for a 20 g mouse in 15 min [78].

6.3.2 Domoic acid

Domoic acid is a worldwide distributed toxin firstly isolated from the red macroalga *Chondria armata*, later it was described that this compound was also produced by several *Pseudo-nitzschia* diatom species.

This water-soluble compound is a toxic glutamate analog with high affinity for the a-amino-5-hydroxy-3-methyl-4-isoxazole propionic acid (AMPA) and kainate neuronal glutamate ionotropic receptors. Hippocampus has a high concentration of AMPA/Kainate receptors and therefore pyramidal hippocampal neurons are the main target of this toxin, specifically the CA3 area. The activation of these receptors leads to decrease in ATP levels and increase of intracellular calcium levels. These effects are followed by a reactive oxygen and nitrogen species generation [79].

In 1987 a serious food poisoning incidence was observed in Canada after consumption of domoic acid-contaminated mussel. Gastrointestinal and serious and uncommon neurological symptoms were reported. Some patients showed confusion, disorientation, seizures, and even epileptic processes and coma. Among all the affected individuals, three died some days after the poisoning and other two died 3 months and 2 years later respectively for poisoning related matters. Due to this, the toxicity of domoic acid in animals has been extensively studied. After intraperitoneal injection a characteristic behavior is observed in rodents. Lethargy is followed by forelimb paralysis, vigorous scratching, and circular movements. Progressively, animals lose postural control and in the final stages, seizures and tremors are observed prior to death. The complexity of neurological damage includes also damage to the hypophysis and endocrine alterations [80]. The regulatory limit for this toxin is 20 mg/kg shellfish meat.

6.3.3 Tetrodotoxin

Tetrodotoxin (TTX) is one of the most important neurotoxins known to block site-1 sodium channels and thus this compound inhibits the propagation of action potentials in muscle and nerve cells. This heterocyclic compound consists of a guanidinium moiety connected to an oxygenated backbone with a

2,4-dioxaadamantanestructure with six hydroxyl groups [81]. It has 25 naturally analogs that are classified in 4 groups depending on their chemical properties: analogs chemically equivalent to TTX, deoxy compounds, one oxidized analog, and C11-lacking analogs. Intoxication symptoms appear between 10 and 45 min. They include perioral paresthesia, numbness, and in severe cases respiratory distress, cyanosis, and paralysis that might lead to respiratory failure and death. This compound binds to receptor site 1 on sodium channels and has been used to identify and classify the voltaged-dependent sodium channel subtypes with respect to their TTX sensitivity [82]. Consistently, sodium channels are classified as TTX-resistant subtypes, Nav 1.8 and Nav 1.9 (implicated in neuropathic pain states [83]) and TTX-sensitive subtypes, Nav 1.7, 1.3, 1.2, and 1.1, which are implicated in inflammation, epilepsy, or neuropathic pain. Due to this, TTX is being used in several pharmacological researches for its therapeutic potential in curing migraines, addictions, or as an anesthetic agent for pain [84].

The median lethal dose for TTX in rodents is between 9 and 10 μg/kg intraperitoneally and 232 orally [85]. TTX has been described in different animals like fishes, arthropods, echinoderms, and mollusks among others, especially the pufferfish. It is as well reported in several bacterial species and dinoflagellates [84, 86, 87]. However, bacteria might be the primary source of TTX [88]. Although TTX presence was traditionally linked to Asian countries, in the last years it has been reported in bivalve mollusks and gastropods in Europe and even human intoxication has been reported in Spain, and this is attributable to global warming [89–92]. No European regulatory limit exists for TTX but pufferfish family species should not be marketed [93]. In Asian countries there is regulation on the use of this compound.

6.4 Amphiphilic toxins

- Maitotoxin
- Palitoxin

6.4.1 Maitotoxin

Maitotoxin is together with ciguatoxins responsible for ciguatera poisoning. However, it is believed that ciguatoxins are the main toxins responsible for neurological symptoms, whereas maitotoxin is less implicated because of it low oral absorption. Nevertheless, this compound is the most toxic secondary metabolite isolated to date with an intraperitoneal LD_{50} of 50 ng/kg; however, its oral toxicity is far lower. This huge polyketide-derived polycyclic ether of 3,422 Da (the largest and most complex known) is produced by *Gambierdiscus toxicus* and accumulates in carnivorous fishes,

mainly in their liver and viscera but not in their flesh as ciguatoxins. Four different compounds have been isolated: maitotoxin-1, maitotoxin-2, maitotoxin-3, and maitotoxin-4 [94].

This compound exhibits a calcium uptake interaction in many cellular types and due to this it has been used as a pharmacological tool for calcium-dependent cellular process studies. This intracellular calcium influx leads to several cellular responses such as depolarization, muscle contraction, neurotransmitters release, and sperm acrosome changes among others [94]. However, its exact mechanism of action remains unknown.

6.4.2 Palytoxin

Palytoxin (PLTX) was first isolated from *Palythoa* spp. in 1971 in Hawaii. After that, palytoxin has been reported in *Palythoa* spp. from other temperate and tropical waters, a sea anemone and the red alga *Chondria armata*. Moreover, PLTX derivatives are also isolated in dinoflagellates from the genus *Ostreopsis*, a dinoflagellate with a worldwide distribution in temperate and tropical waters which due to global warming is spreading to new areas [95]. This large compound has a lipophilic and a hydrophilic area with 64 stereogenic centers. Molecular weight and formula differ depending on the producer species.

The main mechanism of action of PLTX is the disruption of the mammalian cell Na^+-K^+-adenosine triphosphatase (ATPase) pump. PLTX binds to the ATPase pump and turn it in a nonspecific ion channel that is open permanently. This pump is one of the most important transporters of the cellular membrane and its dysfunction leads to membrane potential alterations. In humans, PLTX poisoning is characterized by myalgia, myoglobinuria, respiratory distress, and cyanosis, while alterations in creatine phosphokinase, AST, ALT, and lactate deshydrogenase levels are also observed. Furthermore, in coincidence with *Ostreopsis* blooms, several episodes of respiratory, ocular, and skin damages have been reported. In these cases, the exposure to toxins in aerosols was by inhalation or skin contact. If death occurs, it may be by respiratory failure. In rodents, ataxia and paralysis are the first symptoms observed after intraperitoneal administration. As in humans, death occurs by respiratory arrest and increased levels of creatine phosphokinase, AST, ALT, and lactate dehydrogenase [96, 97].

PLTX levels are not currently regulated. However, EFSA recommended its regulation in 2009 with a limit of 30 μg/kg but due to its chemical properties and large weight the intestinal absorption rate is poor. Given the large number of potential analogs, including ovatoxins and ostreocins, their presence will be a matter of concern in the future. Since their growth is associated to warm waters, and they seem to be affected by global warming, this family of compounds will need a follow up study in the future [98].

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Eva Cagide and Mercedes Álvarez

7 Cyanobacterial toxins

7.1 Cyanobacteria and algal blooms

Cyanobacteria are microorganisms traditionally called "blue-green algae," due to their color, despite the fact that they are not related to algae. They belong to the *Bacteria* domain (Figure 7.1) [1–4], therefore they are not eukaryotes, but share some characteristics resembling those of algae, such as oxygenic photosynthesis and pigments; in fact, a common pigment is the blue-colored phycocyanin, which together with the green pigment chlorophyll a, is the reason for the former term.

Cyanobacteria are the only prokaryotes able to produce oxygen by photosynthesis, and are considered responsible for the increased production of oxygen in the

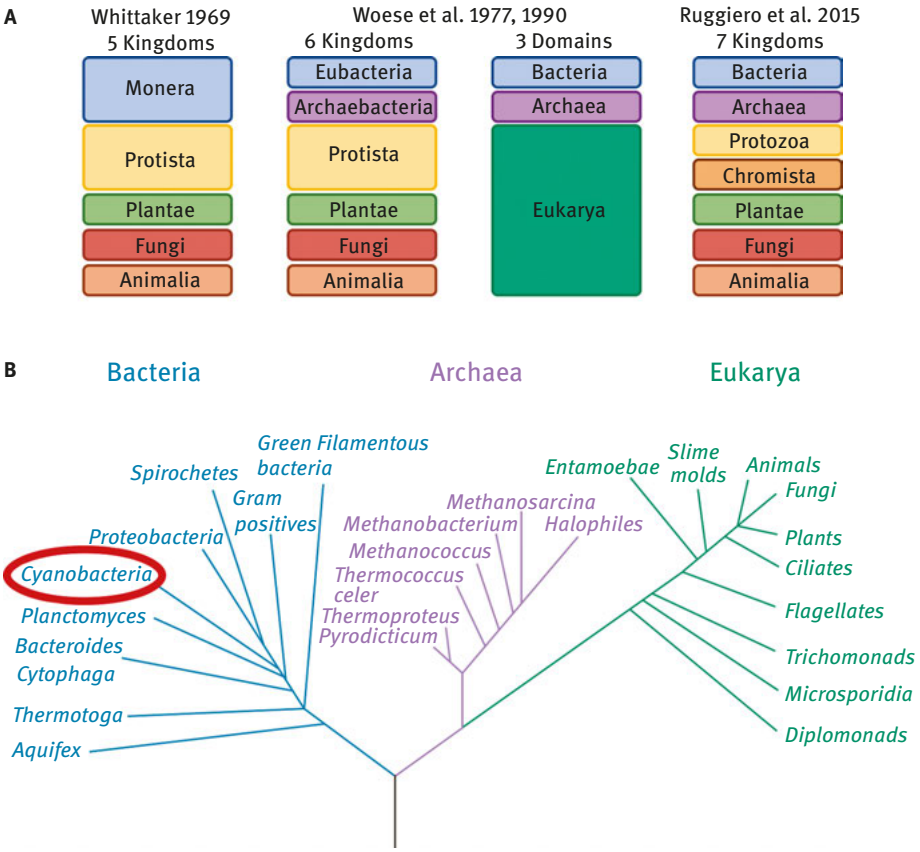

Figure 7.1: (A) Cyanobacteria classification according to Whittaker [1], Woese and Fox [3] and Woese et al. [2], and Ruggiero et al. [4]. (B) Phylogenetic tree according to Woese's 3 domains.

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Earth's atmosphere when the Great Oxidation Event occurred during the early Proterozoic, 2.5–2.3 billion years ago (2.5–2.3 Ga), contributing to the creation ofthe current atmosphere and allowing the great biodiversity of aerobic organisms [5].

In fact, the widely accepted endosymbiosis theory of Lynn Margulis explains the origin of eukaryotic cell organelles through the symbiosis of prokaryotic progenitors [6]. Specifically, the origins of plastids have been related to *Cyanobacteria*, probably nitrogen-fixing filamentous cyanobacteria [7]. This allowed the emergence of different lineages, making it one of the most significant evolutionary events with extraordinary importance for life on Earth.

The great morphological diversity of cyanobacteria is the basis for their classification in Bergey's Manual of Systematic Bacteriology [8, 9], which has divided cyanobacteria into five subsections:

I (= Order *Chroococcales*)
II (= Order *Pleurocapsales*)
III (= Order *Oscillatoriales*)
IV (= Order *Nostocales*)
V (= Order *Stigonematales*)

Subsections I and II are unicellular cyanobacteria, usually aggregated in colonies by secreting mucopolysaccharides, whereas subsections III–V include filamentous cyanobacteria. Like most bacteria, subsection I reproduces by binary fission or budding [10], while subsection II reproduces by multiple fission and endospore release (baeocytes) [11].

Subsections III–V include filamentous cyanobacteria where reproduction takes place by fragmentation and formation of short motile filaments called hormogonia. Subsection III is formed by simple unidirectional filamentous cyanobacteria, and subsections IV and V have the ability to form differentiated cells: the metabolically specialized heterocysts (for nitrogen fixation, in anaerobic conditions, Figure 7.2) and the resting cells (akinetes) [12]. In addition, subsection V consists of the only cyanobacteria that produce "true branches," growing multiseriate filaments and in several planes, and are the most evolved prokaryotes with regard to thallus organization and cell differentiation [13].

The cyanobacteria inhabit aquatic and terrestrial environments [14], surviving under a wide range of physicochemical conditions of pH, salinity, temperature,

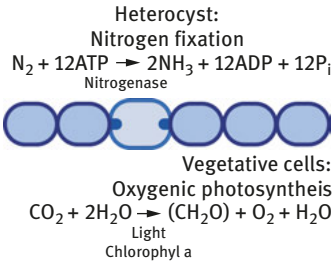


Figure 7.2: Processes of Nitrogen fixation and Oxygen production in cyanobacteria.

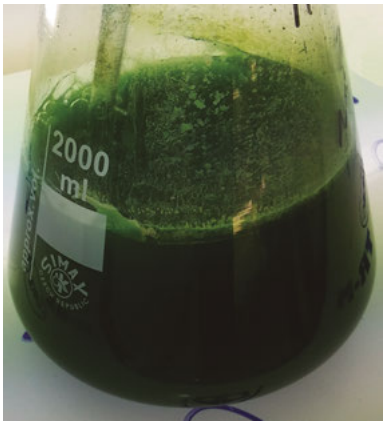


Figure 7.3: Cyanobacteria in culture, showing a high cellular proliferation.

etc., and are naturally found in almost all environments on Earth, from desert to hot springs and ice-cold water [15]. Under specific conditions (mainly of temperature and nutrients), these organisms can massively proliferate and accumulate in the water (Figure 7.3), causing a negative impact to natural resources or humans [16]. These episodes are known as harmful algal blooms (HABs), which in some cases produce toxic compounds named cyanotoxins due to the producing organisms [17]. The main parameters favoring the occurrence of cyanobacterial blooms include [13, 18]:

- increased nutrients (nitrogen and phosphorus),
- warm water temperature,
- high intensity and duration of sunlight,
- quiescent surface water,
- water column stratification,
- changes in water pH, and
- occurrence of trace metals.

Also, human activities leading to eutrophication and global climate change have been related to an increase in geographical distribution and frequency of HABs [19].

These HABs have a considerable impact on recreational water quality and have become a public health concern because the toxins that some species of cyanobacteria produce may provoke harmful effects on human consumers and wild or domestic animals, through drinking water, or recreational/direct exposure [20].

7.2 Cyanotoxins

Cyanobacteria are capable of producing a great range of bioactive secondary metabolites including substances with antitumor, antiviral, anticancer, antibiotic, and antifungal activity; UV protectants; and specific inhibitors of enzymes [21].

In addition, several strains [22] of these microorganisms are capable of producing substances, which are highly toxic to humans and other animals and have also been implicated in cases of human illness; these compounds are called cyanotoxins. The cyanotoxins are secondary metabolites, which are not essential for primary metabolism but synthesized within the cells of some species.

The harmful effects of cyanotoxins include risk of mortality at naturally relevant concentrations. The first documented lethal intoxication of livestock due to drinking water contaminated with cyanobacteria was published the 19th century. Since then, human illnesses associated to drinking water; inhalation, contact, or ingestion of contaminated recreational water; and even because of the water used for hemodialysis have been reported. However, many cases go unreported because of the lack of knowledge associating the symptoms with the toxicity of cyanobacteria [23].

While it is unclear why certain strains of cyanobacteria are able to produce toxins, it has long been speculated that they may have a role as protective compounds, since there have been reports that some cyanotoxins are potent inhibitors of invertebrate grazers in the aquatic environment.

Cyanobacterial populations can be dominated by a single species or be composed by a variety of species, some of which can be toxic. Moreover, a cyanobacterial bloom of a single species may have a mixture of toxic and nontoxic strains. Some strains are much more toxic than others, sometimes for more than three orders of magnitude. This may mean that an extremely toxic strain, even when it occurs in small amounts among a greater number of nontoxic strains, can transform the algal bloom into a toxic one [24].

Toxic and nontoxic strains from the same species of cyanobacteria cannot be separated by microscopic identification. To confirm that a particular strain of cyanobacteria is a producer of toxins, it is important to isolate a pure culture of that strain, preferably free of other bacteria and to detect and quantify the concentrations of toxin in the pure culture (either by bioassays or chemical analyzes); and, if possible, to purify and fully characterize the cyanotoxins.

Cyanotoxins are a diverse group of natural toxins, both from the chemical and the toxicological points of view [25–29]. In fact, these bioactive compounds exhibit a wide range of effects depending on their toxic mechanism of action, with the most commonly recognized cyanotoxins produced by cyanobacteria classified according to their chemical structure and target organs (Table 7.1).

From a toxicological point of view, cyanotoxins can be classified into five major classes [29]:

- *Neurotoxins*: Anatoxins, Saxitoxins (STXs), and β-N-methylamino-L-alanine (BMAA).
- *Hepatotoxins*: Microcystins (MCs) and Nodularins (NODs).
- *Cytotoxins*: Cylindrospermopsins (CYNs) .
- *Dermatoxin*s: Aplysiatoxins and Lyngbyatoxin.
- *Irritant and gastrointestinal toxins*: Lipopolysaccharide (LPS) endotoxins.

Table 7.1: General features of cyanotoxins.

Structure	Cyanotoxin	Primary target organ Mode of action	Cyanobacterial genera
Hepatoxic Cyclic peptides	Microcystins	Liver Protein phosphatase inhibition	*Microcystis, Anabaena, Planktothrix (Oscillatoria), Nostoc, Hapalosiphon, Anabaenopsis, Phormidium, Phanocapsa*
	Nodularins	Liver Protein phosphatase inhibition	*Nodularia*
Neurotoxic Alkaloids	Anatoxin-a Homoanatoxin-a	Nerve synapse Agonist of nicotinic acetylcholine receptor	*Anabaena, Planktothrix (Oscillatoria), Aphanizomenon, Cylindrospermum*
	Anatoxin-a (S)	Nerve synapse Acetylcholinesterase inhibition	*Anabaena*
	Saxitoxins	Nerve axons Blockage of voltage-gated sodium channels	*Anabaena, Aphanizomenon, Lyngbya, Cylindrospermopsis, Oscillatoria, Phormidium, Planktothrix*
Cytotoxic Alkaloids	Cylindrospermopsins	Liver, kidneys, lungs, spleen, intestine Protein synthesis inhibition	*Cylindrospermopsis, Anabaena, Aphanizomenon, Umezakia, Lyngbya, Oscillatoria, Raphidiopsis*
Dermatoxic Alkaloids	Lyngbyatoxin-a	Skin, gastrointestinal tract Potentiation of protein kinase C	*Lyngbya*
	Aplysiatoxins	Skin Potentiation of protein kinase C	*Lyngbya, Schizothrix, Planktothrix (Oscillatoria)*
Lipopolysaccharides		Potential irritant; affects any exposed tissue	*All*
Amino acids	BMAA (β-N-methylamino-L-alanine)	Potentially related to neurodegenerative diseases	*Anabaena, Cylindrospermopsis, Microcystis, Nostoc, Planktothrix (Oscillatoria)*

Structurally, the cyanotoxins are quite diverse [14, 25, 30, 31], and they can be categorized into four large groups according to their chemical structures:

- *Cyclic peptides*: MCs and NODs.
- *Alkaloids*: Anatoxins, STXs, CYNs, Lyngbyatoxins, and Aplysiatoxins.
- *Lipopolysaccharides*.
- *Other bioactive compounds*: Neurotoxic amino acids, different from the 20 standard amino acids that comprise proteins (BMAA, β-N-methylamino-L-alanine).

7.2.1 Microcystins

7.2.1.1 Mode of action

These compounds have been named according to the first cyanobacteria associated to their production, *Microcystis* (specifically *Microcystis aeruginosa*) [32, 33]. However, these toxins have been found to be synthetized by other species of *Microcystis*, and also by genus *Anabaena*, *Planktothrix* (previously known as *Oscillatoria*), *Nostoc*, *Anabaenopsis*, *Haphalosiphon*, etc. [25, 30]. The diversity of producers makes MCs the most widespread cyanotoxins in nature [34].

The presence of MCs has been widely reported, with the most important and serious case of MC intoxication being the one that occurred at a hemodialysis clinic in Caruaru, Pernambuco State (Brazil), in February 1996, where 116 patients were intoxicated and experienced visual disturbances, nausea, vomiting, and muscle weakness after routine dialysis, and 52 died of a syndrome now called *Caruaru Syndrome*. Since then, incidence of several human and animal poisoning due to MCs has been described, with gastroenteritis being the major clinical manifestation [35–37].

The biological target of MCs are the serine/threonine protein phosphatases (PP1 and PP2A), and these cyanotoxins are considered primarily hepatotoxic. The organic anion-transporting polypeptides are responsible for the absorption and distribution of MCs to the liver, where they exert their toxicity through inhibition of PP1 and PP2A. These transporting proteins are more abundant in liver because they are responsible for the transmembrane transport of bile acids and hormones in hepatocytes, but they are also expressed in the gastrointestinal tract, kidney and brain, therefore these are vulnerable organs as well [14, 38, 39].

Inhibition of protein phosphatases mediated by MCs can lead to a hyperphosphorylation of the cytoskeleton structural filaments, provoking cytoskeletal degradation, deformation of hepatocytes, and, ultimately, liver damage and organ failure. In addition, the cellular alterations that MCs cause may also provoke genomic instability. It has been demonstrated that the inhibitory action of microcystin-LR on ser/thr protein phosphatases leads to the production of reactive oxygen substances inducing DNA damage with genotoxic effects. Moreover, this toxin also interferes with DNA damage

repair pathways, contributing also to their carcinogenicity. In fact, the International Agency for Research on Cancer (IARC, a branch of the World Health Organization (WHO)) has included Microcystin-LR in Group 2B, as a possibly carcinogenic substance to humans [40].

7.2.1.2 Chemical structure

The first chemical structures of cyanobacterial cyclic peptide toxins were identified in the early 1980s and the number of fully characterized toxin variants has greatly increased during the 1990s. The first of such compounds found in freshwater cyanobacteria were cyclic heptapeptides, which are the most prevalent class of cyanotoxins and the most frequently studied (see Table 7.2) [34].

MCs are cyclic heptapeptides composed of protein and nonprotein amino acids with molecular masses around 1,000 Da (Figure 7.4). The seven amino acids that are involved in the formation of of a MC include a unique β-amino acid (Adda, (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid)), as well as alanine (D-Ala), D-erythro-β-methylaspartic acid (D-MeAsp), glutamic acid (D-Glu), and *N*-methyl dehydroalanine (Mdha).

Structural variations have been reported in all seven amino acids, but most frequently with substitution of L-amino acids in the two variable positions 2 and 4, demethylation of amino acids at positions 3 (Masp) and/or 7 (Mdha), and methyl-esterification of D-Glu at position 6 (Figure 7.4). More than two hundred structural variants of MCs have been described to date, see Table 7.2 [41]. The general structure consists of:

Cyclo-[D-Ala(1)]-[X(2)]-[D-MeAsp(3)]-[Z(4)]-[Adda(5)]-[D-Glu(6)]-[Mdha(7)]

where X and Z are the variable residues. The most common isoform is microcystin-LR, where X and Z are respectively leucine and arginine:

Cyclo-[D-Ala(1)]-[L-Leu(2)]-[MAsp(3)]-[L-Arg(4)]-[Adda(5)]-[D-Glu(6)]-[Mdha(7)]

The main structural variations can be:

- MCs contain two variable residues, which make the differentiation between variants of MCs. These two variable elements are always standard L-amino acids (X and Z).
  - At the X-position the most common variable amino acids are leucine (Leu), arginine (Arg), and tyrosine (Tyr).
  - At the Z-position the most common amino acid is arginine (Arg).
- Presence or absence of the methyl groups on D-MeAsp and/or Mdha.
- Presence or absence of methyl ester on D-Glu.

MCs are relatively polar molecules due to the presence of carboxylic acids at 3 and 6 positions and the frequent presence of arginine at positions 2 and 4. But some variants contain hydrophobic nonpolar amino acids (Ala, Ile, and Val) and amino

Table 7.2: Some structural variations of microcystins reported in the scientific literature. Amino acid variations in the structures were present at sites 1-2-3-4-5-6-7.

Microcystin	MW/g·mol^{-1}	LD_{50}[a]	1	2	3/R^2	4	5/R^3	6	7/R^1
MC-LR	994	50	Ala	Leu	Me	Arg	Me	Glu	Mdha
[D-Asp^3]MC-LR	980	160–300	Ala	Leu	H	Arg	Me	Glu	Mdha
[Dha^7]MC-LR	980	250	Ala	Leu	Me	Arg	Me	Glu	Dha
[D-Asp^3, Dha^7]MC-LR	966	+	Ala	Leu	H	Arg	Me	Glu	Dha
[DMAdda^5]MC-LR	980	90–100	Ala	Leu	Me	Arg	H	Glu	Mdha
[(6Z)-Adda^5]MC-LR	994	>1200	Ala	Leu	Me	Arg	Me	Glu	Mdha
[L-Ser^7]MC-LR	998	+	Ala	Leu	Me	Arg	Me	Glu	Ser
[D-Glu(OCH$_3$)^6]MC-LR	1008	>1000	Ala	Leu	Me	Arg	Me	Glu-OMe	Mdha
[DAsp^3, D-Glu(OCH$_3$)^6]MC-LR	994	NR	Ala	Leu	H	Arg	Me	Glu-OMe	Mdha
[ADMAdda^5]MC-LR	1022	60	Ala	Leu	Me	Arg	$COCH_3$	Glu	Mdha
[D-Asp^3, ADMAdda^5, Dhb^7]MC-LR	1009	+	Ala	Leu	H	Arg	$COCH_3$	Glu	Dhb
[L-MeSer^7]MC-LR	1012	150	Ala	Leu	Me	Arg	Me	Glu	Mser
[D-Asp^3, ADMAdda^5]MC-LR	1008	160	Ala	Leu	H	Arg	$COCH_3$	Glu	Mdha
[D-Ser^1, ADMAdda^5]MC-LR	1038	+	Ser	Leu	Me	Arg	$COCH_3$	Glu	Mdha
[ADMAdda^5, MeSer^7]MC-LR	1040	+	Ala	Leu	Me	Arg	$COCH_3$	Glu	Mser
[L-MeLan^7]MC-LR	1115	1000	Ala	Leu	Me	Arg	Me	Glu	MeLan
[D-Leu^1]MC-LR	1036		Leu	Leu	Me	Arg	Me	Glu	Mdha
[D-Asp^3]MC-RR	1023	250	Ala	Arg	H	Arg	Me	Glu	Mdha
[Dha^7]MC-RR	1023	180	Ala	Arg	Me	Arg	Me	Glu	Dha
[D-Asp^3, Dha^7]MC-RR	1009	+	Ala	Arg	H	Arg	Me	Glu	Dha
[(6Z)-Adda^5]MC-RR	1037	>1200	Ala	Arg	Me	Arg	Me	Glu	Mdha
[L-Ser^7]MC-RR	1041	+	Ala	Arg	Me	Arg	Me	Glu	Ser
[D-Asp^3, MeSer^7]MC-RR	1041	+	Ala	Arg	H	Arg	Me	Glu	Mser
[D-Asp^3, ADMAdda^5, Dhb^7]MC-RR	1052	+	Ala	Arg	H	Arg	$COCH_3$	Glu	Dhb

(continued)

Table 7.2: (Continued)

Microcystin	MW/g·mol^{-1}	LD_{50}[a]	1	2	3/R^2	4	5/R^3	6	7/R^1
MC-YR	1044	70	Ala	Tyr	Me	Arg	Me	Glu	Mdha
[Dha^7]MC-YR	1030	+	Ala	Tyr	Me	Arg	Me	Glu	Dha
[D-Asp^3]MC-YR	1030	+	Ala	Tyr	H	Arg	Me	Glu	Mdha
MC-LA	909	50	Ala	Leu	Me	Ala	Me	Glu	Mdha
MC-LF	985	+	Ala	Leu	Me	Phe	Me	Glu	Mdha
MC-LY	1001	90	Ala	Leu	Me	Tyr	Me	Glu	Mdha
MC-LW	1024	NR	Ala	Leu	Me	Trp	Me	Glu	Mdha
MC-WR	1067	150–200	Ala	Trp	Me	Arg	Me	Glu	Mdha
MC-HilR	1008	100	Ala	Hil	Me	Arg	Me	Glu	Mdha
MC-HtyR	1058	80–100	Ala	Homo-tyr	Me	Arg	Me	Glu	Mdha
MC-LAba	923	NR	Ala	Leu	Me	Aba	Me	Glu	Mdha
MC-LL	951	+	Ala	Leu	Me	Leu	Me	Glu	Mdha
MC-AR	953	250	Ala	Ala	Me	Arg	Me	Glu	Mdha
MC-YA	959	NR	Ala	Tyr	Me	Ala	Me	Glu	Mdha
MC-VF	971	NR	Ala	-	Me	Phe	Me	Glu	Mdha
MC-FR	1028	250	Ala	Phe	Me	Arg	Me	Glu	Mdha
[Dha^7]MC-FR	1014	NR	Ala	Phe	Me	Arg	Me	Glu	Dha
[D-Asp^3, $ADMAdda^5$]MC-LHar	1022	+	Ala	Leu	H	Homo-Arg	$COCH_3$	Glu	Mdha
[D-Asp^3, Dha^7]MC-E(OMe)E(OMe)	983	+	Ala	Glu(OMe)	H	Glu(OMe)	Me	Glu	Dha
[D-Asp^3, Dha^7]MC-EE(OMe)	969	+	Ala	Glu	H	Glu(OMe)	Me	Glu	Dha
[Dha^7]MC-HphR	1028	+	Ala	Homo-Phe	Me	Arg	Me	Glu	Dha
MC-M(O)R	1028	700–800	Ala	Met(O)	Me	Arg	Me	Glu	Mdha
MC-XR	–	NR	Ala	X	Me	Arg	Me	Glu	Mdha
MC-LZ	–	NR	Ala	Leu	Me	Z	Me	Glu	Mdha

[a]Toxicity determined intraperitoneal mouse (μg/Kg); the LD_{50} value is the dose of toxin that kills 50% of exposed animals; a "+" denotes a toxic result in a non-quantitative mouse bioassay or inhibition of protein phosphatase and "NR" denotes "Not reported".

Aba	Aminoisobutyric acid	E(OMe)	Glutamic acid methyl ester Δ
ADMAdda	*O*-Acetyl-*O*-demethylAdda	(H_4)Y	1,2,3,4,-tetrahydrotyrosine
Dha	Dehydroalanine	Har	Homoarginine
Dhb	Dehydrobutyrine	Hil	Homoisoleucine
DMAdda	*O*-DemethylAdda	Hph	Homophenylalanine
Hty	Homotyrosine	MeSer	*N*-Methylserine
MeLan	*N*-Methyllanthionine	(6Z)-Adda	Stereoisomer of Adda at the Δ^6 double bond
M(O)	Methionine-S-oxide	X, Z	Unknow amino acids

This table does not intend to be a full description of all microcystins described in the literature. Revision of more than 200 analogs of microcystins can be checked in Appendix 3, Tables of Microcystins and Nodularins [41].

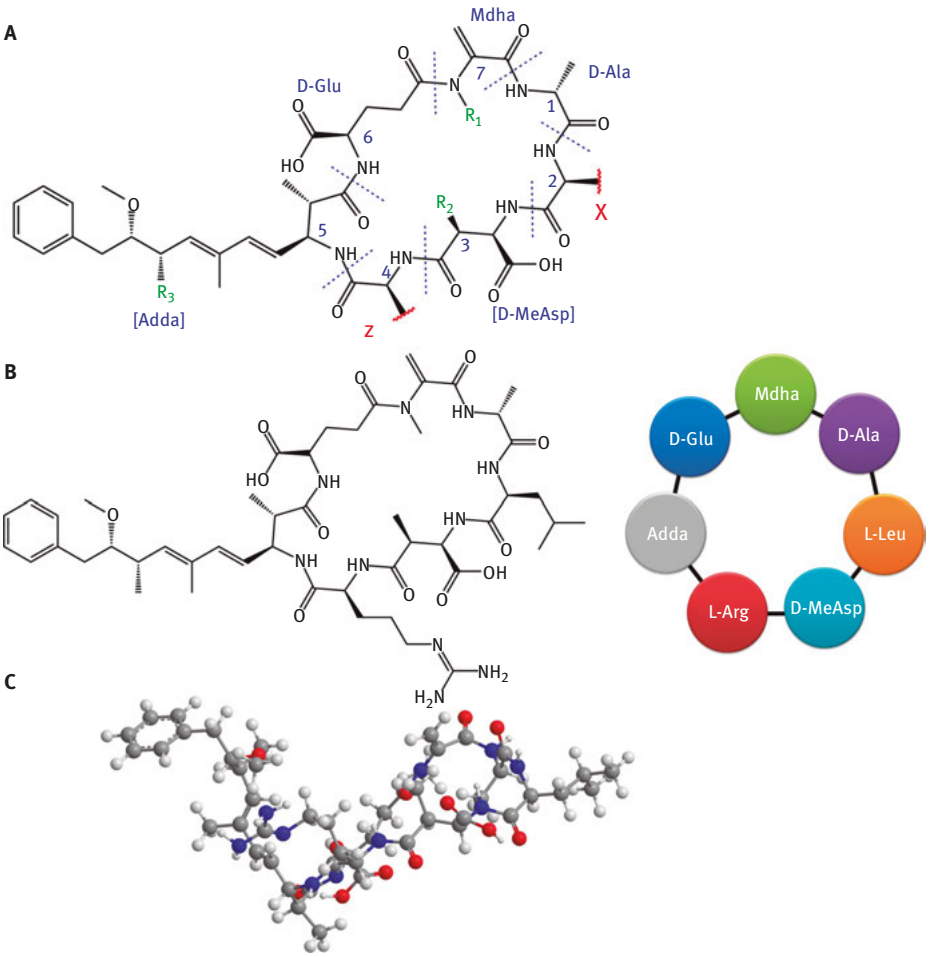
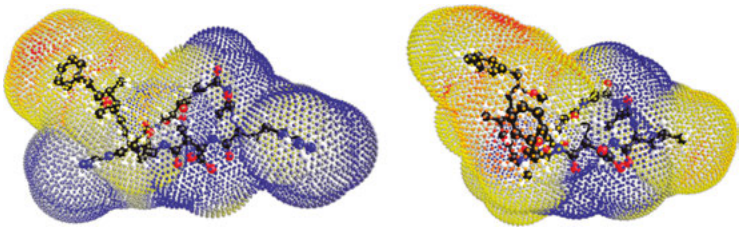


Figure 7.4: (A) General structure of microcystin. General numbering of residues is indicated. (B) 2D and dummy structure of microcystin-LR where **X** is L-Leu, **Z** is L-Arg and R_1, R_2, R_3 are respectively methyl groups. (C) 3D image of Microcystin-LR.

acid residues in the highly variable parts of the molecule. In MC-LF and MC-LW, the more hydrophobic phenylalanine (F) and tryptophan (W), respectively, have replaced arginine (R) in MC-LR, see Figure 7.5 to compare polarity. Depending on the structure, MCs are expected to have different in vivo toxicity and bioavailability, but only a few studies have considered the toxic properties of the more hydrophobic variants.

As previously indicated, MCs disrupt the proper functioning of an important group of cell enzymes, protein phosphatases. Inhibition occurs by binding to the protein phosphatases through a carboxyl group, two hydrogen bonds, and an aliphatic chain (Adda). If any of the three components is missing or altered, then MCs fail to bind to



MC-RR MC-LF

Figure 7.5: Molecular Lipophilicity Potential (MLP) surface of polar Microcystin-RR and more hydrophobic Microcystin-LF. Blue color represents molar polar region, while the red color represents more lipophilic region. Although all MCs are soluble in water the hydrophobicity varies considerably, which is primarily important for the uptake of the toxins by organisms and cells.

the receptors [42]. The configuration of the Adda–Glu part of the toxins is essential for their activity. Studies on the structure–activity relationship showed that formation of the [6(Z)Adda] stereoisomer, saturation of the diene in Adda, methylation of glutamic acid or linearization render the compounds nontoxic or lead to a significant decrease in toxicity. On the other hand, the demethylation of amino acid residues in NODs and MCs exerts little effect on their toxicity [43].

More than 200 structural variants of MC have been characterized so far from bloom samples and isolated strains of cyanobacteria (see Table 7.2). However, four microcystins (LR, RR, LA, and YR) are of special concern to the US Environmental Protection Agency (US EPA) and are on US EPA Contaminant Candidate List III, which was developed through a series of international panel discussions [42]. The WHO has set a provisional drinking water guideline value (GV) of 1 µg/L for microcystin-LR. Their high stability under extreme conditions and high solubility in water are attributed to their cyclic chemical structure and their functional groups, respectively. Being naturally occurring organic compounds, MCs can undergo biodegradation. Because of their stable structure, they can persist in aquatic systems for days before any significant degradation occurs. Of the four priority MC variants, only the arginine and tyrosine amino acid side chains are potentially vulnerable to oxidation.

7.2.2 Nodularins

7.2.2.1 Mode of action

NODs are structurally similar to MCs and can induce similar toxic effects. They are produced by the brackish water filamentous, planktonic cyanobacterium *Nodularia spumigena*. Although these toxins are not as frequent as the widespread MCs, they have been implicated in the deaths of different organisms, including wild and domestic animals [14].

The mode of action of NODs is analogous to that of MCs, via inhibition of the protein phosphatases (PP1, PP2A, and PP3) and triggering a functional and structural disruption of the liver. Therefore, they are also considered as hepatotoxins. These toxins are also transported into the hepatocytes through the bile acid carriers (organic anion-transporting polypeptides), where they induce damages such as aggregation of the cytoskeleton, lipid peroxidation, loss of membrane integrity, DNA fragmentation and strand breaks, cell blebbing, cellular disruption, and intrahepatic bleeding, which may result in hemorrhagic shock [44].

As MCs, NODs have been demonstrated to be genotoxic, inducing DNA strand breakage, chromosomal damage, and producing reactive oxygen species, resulting in oxidative stress. In addition, NODs may be a carcinogenic threat due to their suspected initiating and tumor-promoting activity. However, the specific details of this activity have not been fully elucidated, and IARC has included NODs in Group 3, as a substance not classifiable as to its carcinogenicity to humans [40].

7.2.2.2 Chemical structure

Structurally, NOD is a cyclic pentapeptide composed of protein and nonprotein amino acids with molecular masses around 800 Da (Figure 7.6). The five amino acids that are involved in the NOD structure include: Adda, D-glutamic acid (D-Glu), N-methyldehydrobutyrine (MeDhb), D-erythro-β-methylaspartic acid (D-MeAsp) and L-arginine (L-Arg).

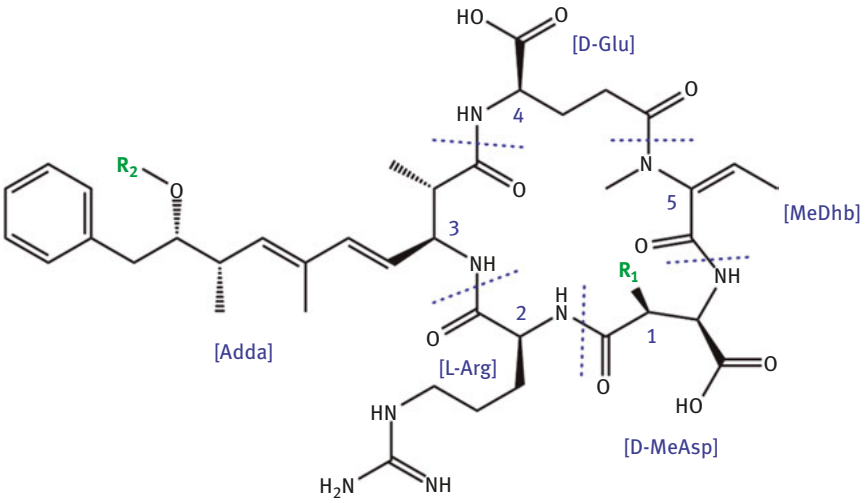


Figure 7.6: Structure of nodularin (NOD), R_1=R_2=CH_3. General numbering of residues is indicated. The L-Arg residue of NOD may be replaced with a homoarginine (nodularin-Har) or valine residue (motuporin). The molecular weight of NOD variants ranges between 760 and 840 Da.

Table 7.3: Structural variants of NOD reported in the scientific literature. Amino acid variations in the structures were present at sites 1-2-3-4-5.

Nodularin	MW/g·mol^{-1}	LD_{50}[a]	1/R^1	2	3/R^2	4	5
Nodularin (NOD-R)	825	50	Me	Arg	Me	Glu	Mdhb
Motuporin (NOD-V) ([l-Val^2]NOD)	768	NR	Ala	Val	Me	Glu	Mdhb
[d-Asp^1]NOD	811	NR	H	Arg	Me	Glu	Mdhb
[DMAdda^3]NOD	811	NR	Me	Arg	H	Glu	Mdhb
[dhb^5]NOD	811	NR	Me	Arg	Me	Glu	Dhb
[6(Z)-Adda^3]NOD	825	nontoxic	Me	Arg	Me	Glu	Mdhb
[D-Glu(OCH_3)^4]NOD	839	NR	Me	Arg	Me	Glu-OMe	Mdhb
[l-Har^2]NOD	839	NR	Me	Har	Me	Glu	Mdhb
[MeAdda^3]NOD	839	NR	Me	Arg	Me + extraMe	Glu	Mdhb
Linear Nodularin	843	Adda-d-Glu(γ)-Mdhb-d-MeAsp(β)-l-Arg-OH					

[a]The mean dose which kills 50% of the animals

The structure of NOD is:

Cyclo–[[D–MeAsp(1)]–[L-Arg(2)]–[Adda(3)]–[D-Glu(4)]–[Mdhb(5)]]

Structural variations have been reported in all five amino acids, two of these variants have alterations within the Adda residue, which reduces or abolishes the toxicity of the compound. The D-Glu residue is essential for toxicity of NOD so the esterification of the free carboxyl abolishes toxicity. However, the substitution at position 1 has little effect on toxicity. The other two isoforms, nodularin-Har and motuporin, are variable at position 2. Ten naturally occurring analogs of NOD have been reported to date versus more than two hundred structural variants of MCs (see Table 7.3).

Both, MCs and NODs, are chemically very stable, considering their peptidic nature. Spontaneous hydrolysis apparently occurs only at negligible rates. Boiling of MC at neutral pH does not lead to considerable decay for weeks, and even at pH 1 and 40°C the half-life time is around three weeks. Further, MCs were found to be resistant to enzymatic cleavage by common proteases like trypsins. Since MCs and NODs do not absorb UV light in the spectrum of natural sunlight ($\lambda > 290$ nm) no photolysis of the pure compounds occurs. Indirect photodegradation occurs in the presence of photosynthetical pigments or humic substances under natural conditions [45].

7.2.3 Neurotoxic alkaloids: anatoxins and saxitoxins

Mass occurrences of neurotoxic cyanobacteria have been reported from North America, Europe, and Australia, where they have caused animal poisoning. Three families of cyanobacterial neurotoxins are known [25]:

- anatoxin-a and homoanatoxin-a,
- anatoxin-a(S), and

– STXs, also known as paralytic shellfish poisons (PSPs) in the marine literature, which block nerve cell sodium channels.

Anatoxin-a has been found in *Anabaena*, *Oscillatoria,* and *Aphanizomenon*; homoanatoxin-a from *Oscillatoria*; anatoxin-a(S) from *Anabaena*; and STXs from *Aphanizomenon*, *Anabaena*, *Lyngbya,* and *Cylindrospermopsis* [22, 29].

7.2.4 Anatoxin-a and homoanatoxin-a

7.2.4.1 Mode of action

Anatoxins were discovered as a result of their involvement, during the '60, in the poisoning of cattle during a massive bloom of *Anabaena flos-aquae* in Saskatchewan Lake, Canada [46]. The production of anatoxins is linked to several genera of cyanobacteria, not only *Anabaena*, but also *Aphanizomenon, Planktothrix, Oscillatoria,* or *Phormidium* [47, 48].

Many case reports of wildlife, dogs, and livestock poisoning have been related to anatoxins since its discovery. Main signs of toxicity were neurological, with muscle convulsions, and resulting in death in the most fatal cases due to respiratory paralysis.

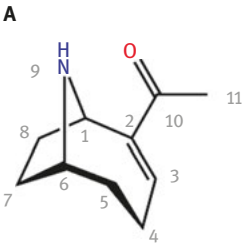
Therefore, anatoxins are primarily neurotoxic substances, exerting their toxicity at cellular level by binding the acetylcholine receptors, which causes a continuous nerve depolarization, resulting in the blockage of further impulse transmission. The evidenced clinical signs of neurotoxicity include loss of coordination, cyanosis, convulsions, and death from respiratory paralysis, since neurotransmission is critical for the activity of skeletal muscle [49].

7.2.4.2 Chemical structure

Anatoxin-a (ATXa) is a low-molecular weight alkaloid ($C_{10}H_{15}NO$, MW = 165 Da), a secondary amine, 2-acetyl-9-azabicyclo(4-2-1)non-2-ene (Figure 7.7). Anatoxin-a is found in nature as the (+)-anatoxin-a enantiomer, whereas much research has been done to investigate the synthesis of both (+)- and (–)-anatoxin-a enantiomers [50], with the racemic mixture being the most commercially sold.

Homoanatoxin-a ($C_{11}H_{17}NO$, MW = 179) is an anatoxin-a homolog with a propionyl group at C-2 instead of the acetyl group in anatoxin-a. The LD_{50} of anatoxin-a is 200 μg/Kg, whereas homoanatoxin-a possesses one-tenth the toxicity of anatoxin-a.

Both are highly soluble in water. Anatoxin and homoanatoxin are unstable and are converted into epoxy and dihydro degradation products, processes that are dependent on a number of environmental parameters, especially pH and light (see Figure 7.8) [51].



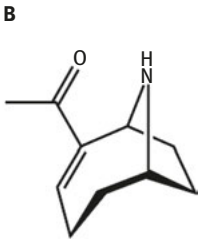


Figure 7.7: Structure of (A) natural (+)-anatoxin-a and structure of (B) synthetic (–)-anatoxin-a.

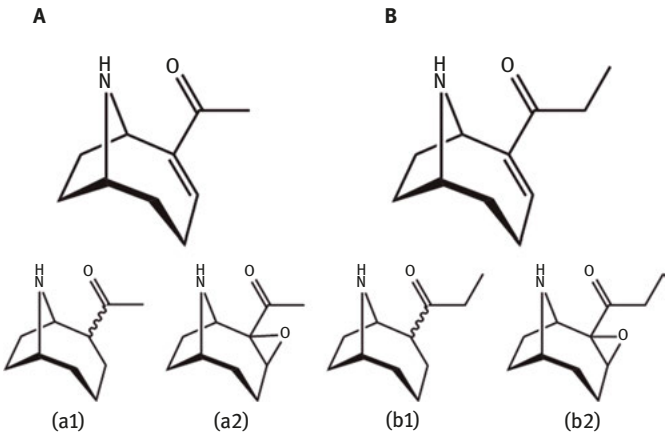


Figure 7.8: Structure of (A) (+)-anatoxin-a, (B) homoanatoxin-a, and degradation products: a1) dihydroanatoxin-a, a2) epoxyanatoxin-a, b1) dihydrohomoanatoxin-a, and b2) epoxyhomoanatoxin-a.

While these reaction products have reduced toxicity or are nontoxic, the detection of dihydroanatoxin-a, epoxyanatoxin-a, dihydrohomoanatoxin-a, and/or epoxyhomoanatoxin-a during routine screening would be useful in alerting the presence of lower, but still toxic, levels of parent toxins, or in forensic investigations to determine the previous presence of parent toxins.

7.2.5 Anatoxin-a(S)

7.2.5.1 Mode of action

Anatoxin-a(s) is a natural compound also produced by *Anabaena*, although it is an organophosphate alkaloid, therefore structurally unrelated to anatoxins. This cyanotoxin was named due to salivation produced in intoxicated animals as a consequence of the acetylcholinesterase inhibition [52]. This mechanism of action is similar to that of the organophosphorus insecticides, although restricting its toxicity to the periphery, maintain brain and retinal cholinesterase activities normal [49]. Acetylcholinesterase blockage inhibits the acetylcholine hydrolization, avoiding the postsynaptic membrane

repolarization. Therefore, in addition to hypersalivation, clinical signs of intoxication include lacrimation, mucoid nasal discharge, tremors, ataxia, diarrhea, muscle weakness, respiratory distress (dyspnea), and convulsions preceding death [53].

7.2.5.2 Chemical structure

Anatoxin-a(S) is a unique phosphate ester of a cyclic N-hydroxyguanine (MW = 252) (Figure 7.9). The LD_{50} of anatoxin-a(S) is 20 µg/Kg bw (i.p. mouse). Structural variants of anatoxin-a(S) have not been detected.

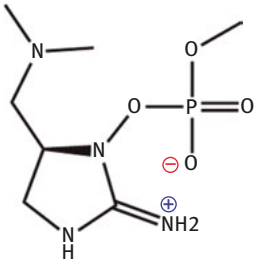


Figure 7.9: Structure of anatoxin-a(S).

7.2.6 Saxitoxins

7.2.6.1 Mode of action

STX receives its name from the mollusk, *Saxidomus giganteus*, from which it was isolated and originally was identified, although these toxins are produced not only by cyanobacteria but also by marine dinoflagellate species belonging to the genus *Alexandrium*, *Gymnodinium*, and *Pyrodinium*. The main cyanobacterial organisms related to their production are *Anabaena*, *Aphanizomenon*, *Lyngbya*, *Planktothrix*, or *Cylindrospermopsis* [49].

The main poisoning produced by STXs is known as Paralytic Shellfish Poisoning, caused by these neurotoxins affecting the peripheral nervous system. Symptoms start with tingling, burning, and numbness of the lips and fingertips, which spread across the face and neck to the extremities, and continue with loss of muscle coordination, respiratory distress, and, in the most severe cases, total paralysis, which may lead to death due to respiratory muscle paralysis [14].

At the cellular level, this group of toxins bind selectively and reversibly to the extracellular side of the voltage-dependent Na^+ channels present in the excitable cells, more specifically, to site 1. Although STX could also act on other targets, such as Ca^{2+} and K^+ channels. Binding to voltage-dependent Na^+ channels prevents both

the entry and exit of Na^+ ions of the cell, which inhibits the transmission of nervous impulses in the peripheral nerves and skeletal muscle, causing the interruption of a wide variety of cellular functions, including muscle functions and leading to death in animals by respiratory arrest [54, 55].

7.2.6.2 Chemical structure

STX is the parent molecule in a class of compounds, collectively termed paralytic shellfish poisons (PSPs). Structurally, PSPs are a group of carbamate alkaloids sharing a common trialkyl tetrahydropurine tricyclic skeleton with two guanidinium moieties, which are responsible for their high polarity, Figure 7.10 [56].

Variations in functional groups at five defined positions around the ring categorize into subgroups according to functional group at the R_4 position (Table 7.4):

- Carbamate toxins have a carbamoyl moiety.
- Decarbamoyl toxins only have a hydroxyl group.
- *N*-sulfocarbamate toxins possess a *N*-sulfocarbamoyl.
- Deoxydecarbamoyl toxins with no functional group.
- Hydroxybenzoate toxins are 4-hydroxybenzoate ester derivatives.
- Acetate toxins are acetate ester derivatives.

Other variations are N-oxidation at N-1 and sulfate substitution at C-11. The latter results in α/β epimeric pairs, i.e., C1/C2, GTX1/GTX4, GTX2/GTX3, dcGTX2/dcGTX3 which are in equilibrium through keto-enol taumerism. Both variations result in a decrease in toxicity relative to STX except GTX1, which exhibits toxicity comparable to that of STX. The toxicity of the derivatives varies by approximately two

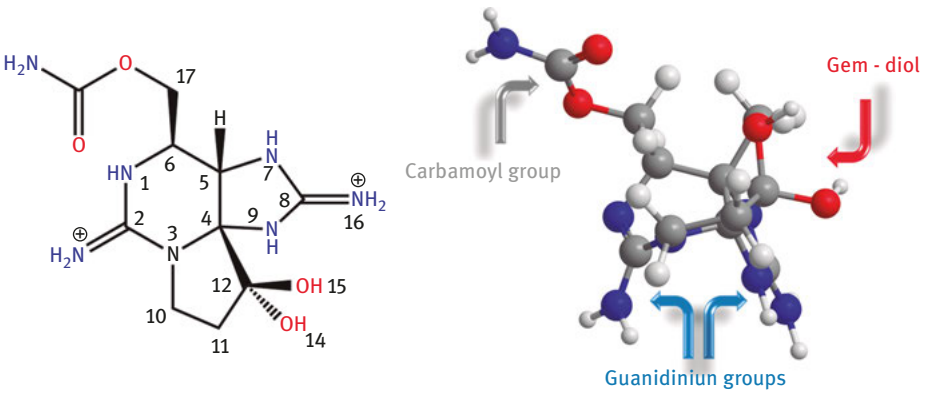
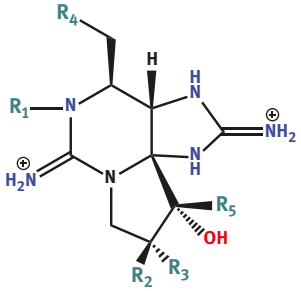


Figure 7.10: Chemical structure of saxitoxin. It possesses two pKas of 8.22 and 11.28, which belong to the 7,8,9 and 1,2,3 guanidinium groups, respectively [56].

Table 7.4: Saxitoxins and analogs reported from cyanobacterial strains and bloom samples.

TOXIN	Variable chemical groups in toxins					Cyanobacteria				TEF[e]
	R_1	R_2	R_3	R_4	R_5	Aph[a]	Ana[b]	Lyn[c]	Cyl[d]	
STX	H	H	H	$OCONH_2$	OH	+	+		+	1.0
GTX2	H	H	OSO_3^-	$OCONH_2$	OH		+			0.4
GTX3	H	OSO_3^-	H	$OCONH_2$	OH		+			0.6
GTX5	H	H	H	$OCONHSO_3^-$	OH		+			0.1
C1	H	H	OSO_3^-	$OCONHSO_3^-$	OH		+			–
C2	H	OSO_3^-	H	$OCONHSO_3^-$	OH		+			0.1
NEO	OH	H	H	$OCONH_2$	OH	+			+	1.0
GTX1	OH	H	OSO_3^-	$OCONH_2$	OH		*			1.0
GTX4	OH	OSO_3^-	H	$OCONH_2$	OH		*			0.7
GTX6	OH	H	H	$OCONHSO_3^-$	OH		*			0.1
dcSTX	H	H	H	H	OH		+	+		1.0
dcGTX2	H	H	OSO_3^-	H	OH		+	+		0.2
dcGTX3	H	OSO_3^-	H	H	OH		+	+		0.4
LWTX1	H	OSO_3^-	H	$OCOCH_3$	H			+		–
LWTX2	H	OSO_3^-	H	$OCOCH_3$	OH			+		–
LWTX3	H	H	OSO_3^-	$OCOCH_3$	OH			+		–
LWTX4	H	H	H	H	H			+		–
LWTX5	H	H	H	$OCOCH_3$	OH			+		–
LWTX6	H	H	H	$OCOCH_3$	H			+		–

STX: Saxitoxin
GTX: Gonyautoxins
C: C-toxins
dcSTX: Decarbamoylsaxitoxin
LWTX: Lyngbya-wollei-toxins
[a]Toxins found in *Aphanizomenon flos-aquae*, New Hampshire, USA [60, 61].
[b]Toxins reported in an *Anabaena circinalis* strain and bloom samples, Australia [62–64]. dcGTX2 and dcGTX3 are probably break down products of C1 and C2 in this species [65]. An asterisk in this column denotes toxins reported by Humpage et al. [62] for *Anabaena circinalis* based on retention time data, but not confirmed by mass spectrometry, and not found in subsequent studies.
[c]Toxins detected in *Lyngbya wollei*, USA [66].
[d]Toxins thus far found in *Cylindrospermopsis raciborskii*, Brazil [67].
[e]Toxicity equivalency factor expresses the toxicity of PSPs in terms of the saxitoxin dihydrochloride form recommended by the European Food Safety Authority (EFSA).



orders of magnitude, with STX being the most toxic, followed by neosaxitoxin and gonyautoxins 1 and 3. Different values have been reported in the literature; as these values are dependent in part upon the purity of the compounds, it is likely these differences are simply a result of differences in the purities of the toxin preparations.

Currently, PSPs include more than 50 components [57–67]. Due to structural differences, each analog has a slightly different affinity to the binding site of voltage-gated sodium channels, and thus justify particular toxicity.

Lyngbya-wollei-toxins (LWTXs) do not appear to be as toxic as other STXs, the presence of acetate in the side chain resulted in a 7-fold to 17-fold decrease in mouse toxicity compared to their carbamoyl counterparts, while the reduction at C-12 resulted in a complete loss of mouse toxicity [66]. However, given their structural similarity to STX, abiotic or biotic transformation of LWTXs to other more toxic analogs cannot be ruled out without additional bioassays. This type of transformation has been observed for N-sulfocarbamoyl STXs (C1 and C2) which can be converted to the more toxic decarbamoylgonyautoxins [65].

7.2.7 Cylindrospermopsin

7.2.7.1 Mode of action

CYN was identified for the first time in 1979 after the hospitalization of 148 people with symptoms of hepato-enteritis in Isla Palmera, Australia, after a HAB in the local drinking water supply where the predominant species was *Cylindrospermopsis raciborskii* [25, 68]. Currently, other genera of cyanobacteria are known to produce CYN: *Anabaena*, *Aphanizomenon*, *Lyngbya*, *Raphidiopsis*, *Umezakia*, and *Oscillatoria* [14].

The CYN target organ is the liver, but it also affects kidneys, adrenals, lung, heart, spleen, and thymus. The proposed mechanism of toxicity is the inhibition of protein synthesis, with the inhibition of glutathione protein synthesis and activation of cytochrome P450 being of primary importance [69, 70]. First clinical signs of poisoning include liver and kidney failure. In fact, it can cause general cytotoxicity, and depending on the injured cells, provoke, hepatitis, renal malfunction, gastroenteritis, and hemorrhage from blood vessels. Finally, CYN is also genotoxic and can cause chromosome loss and DNA strand breakage, so it has been suggested to be a potential carcinogen [49].

7.2.7.2 Chemical structure

CYN is a polyketide-derived alkaloid with a central functional guanidino moiety and a hydroxymethyluracil attached to the tricyclic carbon skeleton with a molecular weight of 415 (Figure 7.11). The compound is zwitterionic and highly water

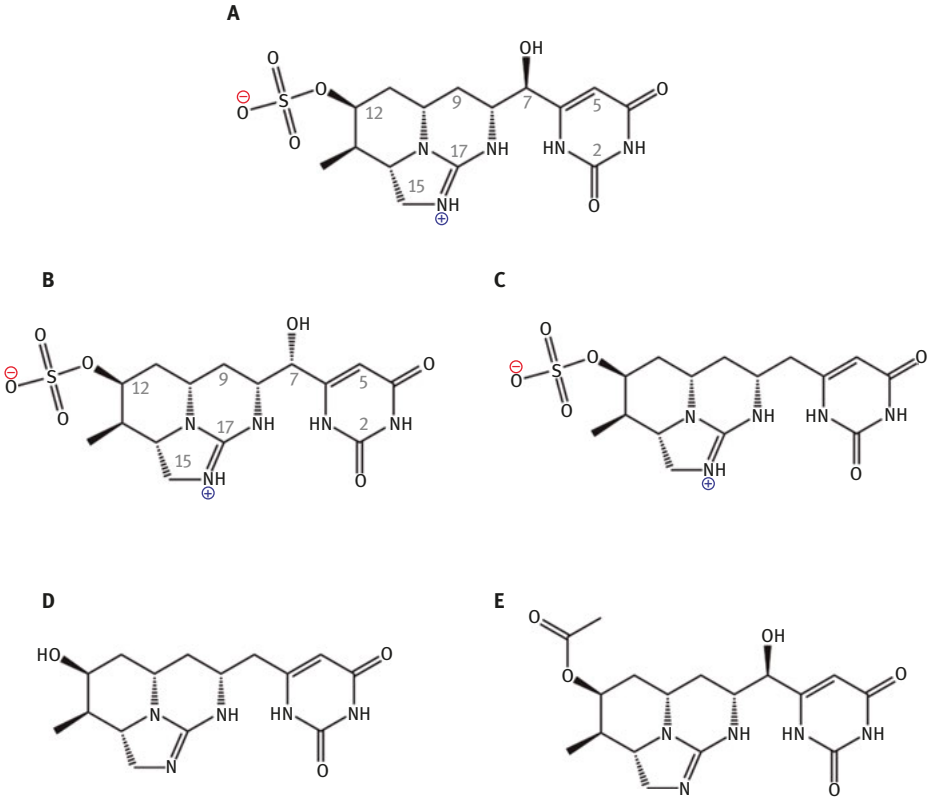


Figure 7.11: Structure of (A) cylindrospermopsin, (B) 7-epicylindrospermopsin, (C) deoxy-cylindrospermopsin, (D) 7-deoxy-desulfo-cylindrospermopsin, and (E) 7-deoxy-desulfo-12-acetylcylindrospermopsin.

soluble. CYN is stable to extreme temperatures (no degradation at 100°C for 15 min) and pH [71].

Two naturally occurring analogs of CYN, 7-epicylindrospermopsin (7-Epi-CYN), and deoxy-cylindrospermopsin (7-deoxy-CYN) have been also identified [72]. But, two new analogs of CYN have been reported in this decade: 7-deoxy-desulfo-cylindrospermopsin and 7-deoxy-desulfo-12-acetylcylindrospermopsin [73]. Based on their structural features, it is likely that these new analogs also possess the harmful biological activities displayed by the rest of the CYN family.

The uracil moiety as well as the hydroxyl at C7 are crucial for toxicity, making CYNs very important because of their potent toxicity as well as potential chronic effects at exposure levels below the toxicity threshold.

There is concern about the way CYN bioaccumulates in freshwater organisms. Toxic blooms of genera which produce CYN are most commonly found in tropical,

subtropical, and arid zone water bodies, and have recently been found in Australia, Europe, Israel, Japan, and the USA.

7.2.8 Aplysiatoxins and lyngbyatoxins

7.2.8.1 Mode of action

Aplysiatoxins were originally isolated from the *Aplysiidae* sea hare *Stylocheilus longicauda* [74], although the cyanobacteria *Lyngbya* was later reported to be the producing organism [75, 76]. Since then, aplysiatoxins have been isolated from other cyanobacteria, such as *Schizothrix* and *Oscillatoria* [77].

Aplysiatoxins are responsible for severe cases of contact dermatitis, causing the "swimmers itch," due to their inflammatory activity. Moreover, it has been probed that aplysiatoxins have tumor-promoting activity through the activation of protein kinase C, in a similar way as 12-O-tetradecanoylphorbol-13-acetate (TPA), binding to the phorbol receptor on the cell membrane [78].

Lyngbyatoxins were also isolated from the cyanobacterium *Lyngbya*, and, as in the case of aplysiatoxins, these toxins possess inflammatory activity [79].

They are also responsible for dermatitis and have been associated with oral and gastrointestinal inflammation [49]. Their mechanism of action is the same as that of a plysiatoxins, through activation of protein kinase C, and they are also considered tumor promoters [80].

7.2.8.2 Chemical structure

The chemical structure of these molecules can be seen in Figure 7.12.

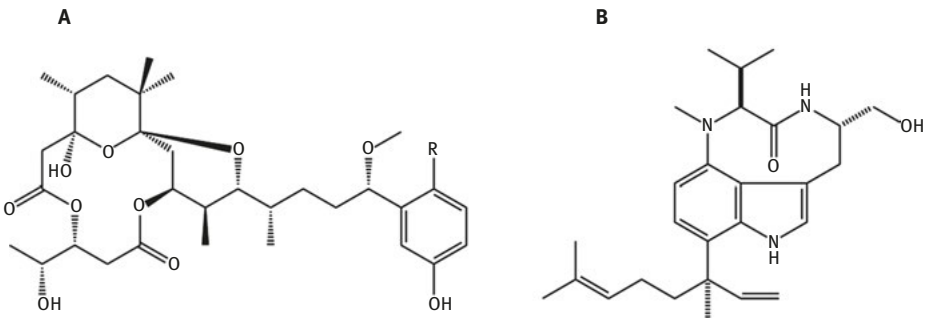


Figure 7.12: (A) Structure of aplysiatoxin (R = Br) and debromoaplysiatoxin (R = H). (B) Structure of lyngbyatoxin-a.

7.2.9 Lipopolysaccharides

7.2.9.1 Mode of action

LPSs are a common irritant that usually form part of outer membrane of the cell wall in Gram-negative bacteria, therefore they are also found in cyanobacteria, although usually possess less potency [24].

These substances contribute to membrane stability and integrity of bacteria, but they also act as a protection system. They has been described to activate the innate immune response, binding and activating the Toll-like receptor 4 (TLR-4) therefore triggering the proinflammatory cytokine cascade [81].

The effects provoked by LPSs include gastrointestinal symptoms, fever, headache, cutaneous signs, allergy or respiratory diseases, contributing to inflammatory and gastrointestinal incidents in humans [82].

7.2.9.2 Chemical structure

LPSs, as the name implies, are condensed products of a sugar, usually a hexose, and a lipid, normally a hydroxy C_{14}-C_{18} fatty acid. It is generally the fatty acid component of the LPS molecule that elicits an irritant of allergenic response in humans and mammals. Though not as potent as other cyanotoxins, some researchers have claimed that all LPSs in cyanobacteria can irritate the skin, while other researchers doubt the toxic effects are that generalized.

7.2.10 BMAA

7.2.10.1 Mode of action

Most groups of cyanobacteria, including cyanobacterial symbionts of the genus *Nostoc*, and free-living cyanobacteria from the five cyanobacterial subsections may produce the neurotoxic amino acid β-methylamino-L-alanine (BMAA) [83].

The neurotoxicity of BMAA was first demonstrated during the '60 [84], when this compound was proposed to lead to amyotrophic lateral sclerosis or Parkinsonism dementia in Guam, due to consumption of cycad flour (*Cycas circinalis*, now known as *Cycas micronesica*) [85, 86].

On a cellular level, this cyanotoxin acts as a glutamate agonist at AMPA, kainate, and NMDA receptors, increasing the intracellular concentration of Ca^{2+} and inducing neuronal activity by hyperexcitation. In fact, it has been pointed out that BMAA could mimic the amino acid L-serine, and cause misfolded proteins in brain after chronic exposure, causing those neurological illnesses [87]. The role of BMAA at the onset as well as progression of neurodegenerative diseases including Alzheimer's disease (AD) is strongly supported by many studies [88–93].

7.2.10.2 Chemical structure

BMAA is a nonproteinogenic amino acid ubiquitously produced by cyanobacteria in marine, freshwater, brackish, and terrestrial environments.

The structure can be seen in Figure 7.13.

This compound is being investigated for its potential as an environmental risk factor for neurodegenerative diseases, including amyotrophic lateral sclerosis, Parkinson's disease and AD, since the higher than normal incidence of the Amyotrophic Lateral Sclerosis/Parkinsonism Dementia complex, particularly within the local Chamorro people of Guam, is associated with the presence of BMAA [94, 95].

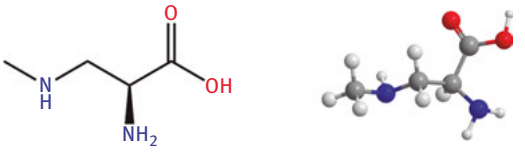


Figure 7.13: Structure of BMAA.

7.3 Detection methods

Several methodologies have been described for the analysis of cyanotoxins in water that are based on different principles. The use of one or another methodology is linked to the use to be made of the analytical results [96, 97]. Table 7.5 summarizes the main characteristics of the methodologies used in the cyanotoxins analysis. Table 7.6 shows different approaches to detect most relevant cyanotoxins in freshwater.

Developing analytical methods to detect cyanotoxins reliably and routinely in naturally matrices must be based on three practical criteria: (a) reliability (accurate and precise), (b) the speed and consequently also to a large degree the cost of the analysis, and (c) the possibility to conduct an "on-site" analysis. Based on the above-mentioned criteria, techniques for analysis can be divided into *screening methods* (mouse bioassay, ELISA, receptor assays and qPCR) and *confirmatory methods* (allow an unambiguous identification of the analyte): instrumental methods such as chromatography (GC/MS, HPLC-UV/PDA, HPLC-MS/MS).

The first group of methods are mainly aimed at the qualitative detection of toxins as fast as possible, preferably already in the field, i.e., have a "yes" or "no" answer.

The methods in the second group are predominantly used to confirm a positive result by means of a rapid method and/or to provide a more accurate quantitative result ("how much of the toxin is actually in there?").

As a premise, the screening methods must be sufficiently sensitive to avoid false negative results, in addition, they must be simple and fast, and allow the analysis of a large number of samples. While, the main disadvantage of instrumental methods is that they require the existence of certified reference material for each molecule to be unequivocally identified and this is not always possible, especially in the case of

Table 7.5: ☑ Advantages and ☒ disadvantages of principal analytical methods to detect cyanotoxins.

Analytical method	Performance
Mouse Bioassay (MBA)	☑ Measurement of real toxicity. Biological test system used to directly demonstrate the presence of unknown toxins. ☒ Not specific. ☒ Conflict with Animal Welfare Directive. Death of animals is method endpoint. Therefore, it is not respectful of animal welfare. ☒ Test cannot be validated. Large variation in results between laboratories. Sex, weight, and strain of mice influence the test results.
Receptor-Based Assay (RBA)	☑ The fastest detection method possible with high sample throughput. ☑ Specific, sensitive, and reproducible. ☑ Measurement of real toxicity. ☑ Good comparability with chromatographic methods and Mouse Bioassay. ☒ May overestimate the toxin concentration. Not specific to each analog, and may indicate presence of other substances interfering with receptor. ☒ Does not show the same sensitivity for all cyanotoxin variants.
LC-UV, FLD LC-MS/MS	☑ Rapid and accurate identification and quantitative determination of toxins: suitable for detection of all cyanotoxins. ☑ More sensitive than Mouse Bioassay. ☑ High selectivity, suitable for monitoring as preventive measure. ☑ Specific. ☒ Reference materials not available for all toxins. ☒ Indirect measurement of toxin analogs: concentrations calculated by reference to a standard corresponding to the parental compound. In UV, absorption, reflects overall concentration as toxin equivalents. ☒ NO Measurement of real toxicity. In general, structurally unrelated, unknown toxins cannot be detected. ☒ Expensive and requires highly skilled analysts. ☒ No standard methods, expensive, requires complex data interpretation, time-consuming.
ELISA	☑ Relatively inexpensive. ☑ Kits available, relatively easy to use. ☑ Fast and sensitive. ☒ Indirect measurement of the toxin. ☒ Irregular cross-reactivity between congeners. ELISA assay might over or underestimate the amount of cyanotoxins present in the sample resulting in both false positives and false negatives. ☒ NO Measurement of real toxicity. Total results reported as ”Toxin equivalents” irrespective of the congeners present.
Molecular methods	☑ Time efficiency. ☑ Comparability of results for toxic versus toxin-producing cells. ☑ Specific – no gene = no toxin ☒ Irregular False Positives and False negative results.

(continued)

Table 7.5: (continued)

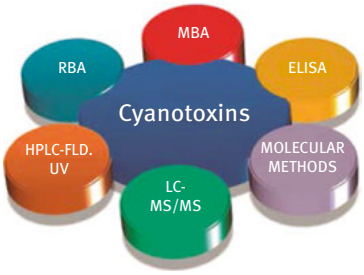


Table 7.6: Typical detection methods for cyanotoxins.

	ATXs	CYNs	MCs	NODs	STXs
Biological Assays					
MBA	Y	Y	Y	Y	Y
RBA	N	N	Y	Y	N
Neurochemical	Y	N	N	N	Y
ELISA	Y	Y	Y	Y	Y
Molecular Methods (nontoxin methods)					
qPCR	Y	Y	Y	Y	Y
DNA microarray chips	N	Y	Y	Y	?
Chromatographic methods					
GC/MS	Y	N	N	N	N
LC/UV	Y	Y	Y	Y	Y
LC/FLD	Y	N	N	N	Y
LC/MS	Y	Y	Y	Y	Y
LC-MS/MS	Y	Y	Y	Y	Y
Y: implemented method		**N: no implemented method**		**?: not confirmed**	

MCs (there are more than 100 identified variants). Often more than one toxin may be present in a sample. The consensus among those using analytical methods is that a single method will not suffice. The best approach for monitoring is to use a combination of screening and more sophisticated and cost-specific chemical methods.

In any case, it is clear that both strategies are perfectly complementary, allowing an initial screening of the samples by a rapid method based on the toxigenic potential, that is, in a detection proportional to the toxicity of the molecule and not to its structural properties. The samples whose results have been positive to the biological screening test can be later analyzed for the identification of the specific toxin responsible for water contamination by an instrumental method. In some cases positive samples in the screening stage may not be confirmed with the instrumental method due to the aforementioned problem of the absence of reference patterns for many of the cyanotoxins discovered so far [98].

Both methods have in common that they require a prior sample preparation step. Cyanotoxins can be found intracellularly and must be released prior to their analysis by an appropriate procedure. Thus, sample preparation may need to include sonication (to break up cells) and a variety of extraction procedures in order to isolate the different (i.e., more lipophilic or polar) compounds. In the scientific literature there are many protocols for lysis and extraction of toxins. So far, published studies on the levels of cyanotoxins in water supplies have generally not clearly indicated whether total or free toxins were measured. Moreover, many of them have not been perfectly evaluated in terms of their capacity to recover and therefore their application can generate an underestimation of the toxin content that can put the health of the water at risk. In addition, the extraction method must be compatible with the analysis technique used.

On the other hand, the methods for the detection of cyanotoxins can also be classified as *structural methods*, *functional methods*, and *molecular methods*.

The first group is based on the physicochemical properties of the molecule and among them are HPLC [99, 100] and immunochemical methods. Functional methods use the biological target on which the toxin acts, and therefore, they are an indication of the toxigenic potential of the molecule [101, 102]. The latter includes quantitative polymerase chain reaction (qPCR) which simultaneously quantifies total cyanobacteria along with genes responsible for toxin production. Positive detections indicate that the gene is available in the bloom material and may produce toxins (the test does not indicate that the cells are actively producing the toxin). Negative detections indicate that the gene for a particular toxin is not in the bloom material (captured by the sample) and likely not produced.

7.4 Health aspects: Guidelines and legislation

Toxic cyanobacteria are encountered around the world, and problems related to safe drinking water production are common. The presence of cyanobacterial toxins in drinking and bathing waters has been recognized as a human health hazard by the WHO and a provisional GV for the common hepatotoxin, microcystin-LR in drinking water has been established [103]. National legislation has been recently introduced in some European countries and elsewhere to control MC levels [42, 104].

MC-LR is the most toxic and most frequently found derivative of MCs in water resources. Health-related episodes in humans and animals caused by MC-LR contamination have been reported in several countries, including the United States, Australia, China, Great Britain, and Brazil.

Sufficient oral toxicity data on cyanotoxins in mammals to permit GVs [105, 106] to be derived with some confidence for human drinking water are only available for microcystin-LR [24] and more recently, for CYN [107] but are insufficient for the derivation of concentration limits.

After the first human fatal incident occurred in Brazil in 1996, the WHO set the provisional microcystin-LR GV in potable water to 1 μg/litere [24, 108]. The WHO has

also established the tolerable daily intake to 0.04 μg kg^{-1} day^{-1} since cyanobacteial toxins bioaccumulate in aquatic microorganisms that humans consume and because of their use as dietary supplements. A similar GV for CYN in drinking water may be appropriate [107]. Since microcystin-LR appears to be one of the most toxic of known cyanotoxins to mammals, and adequate GVs for other cyanotoxins are currently lacking, then it seems prudent to apply the microcystin-LR GV to other cyanotoxins until further data are available. Inevitably, the significance of cyanotoxin data is being interpreted in terms of the microcystin-LR GV. However, the interpretation of such data for risk management is a developing practice. Several countries throughout the world have already adopted or adapted the WHO GV for microcystin-LR into national water legislation. Others prefer to use the GV for guidance only, taking into account that safety factors are built into GV derivation [24, 29]. It is not appropriate here to favor the regulatory or guidance approach, but rather to emphasize that the purpose and scope of a GV for drinking water [108] needs to be recognized. Thus for a cyanotoxin in drinking water the GV is:

- an estimate of the concentration of cyanotoxin which would not result in a significant risk to a consumer over a lifetime of drinking water consumption.
- advisory.
- derived to accommodate uncertainties and safety factors in its derivation.
- provisional and subject to revision in response to further advances in basic knowledge and practical experience.
- not intended as a recommended concentration to which cyanotoxin-containing water can be allowed to degrade.
- a tool for use in the development and application of cyanotoxin risk management approaches, taking into account practicality, feasibility, and the protection of health and water resources.

Legislation governing the occurrence and concentrations of specific cyanotoxins for health protection is not only limited to MCs but also to STXs. Regulations governing STX concentrations were first introduced for health protection from eating shellfish potentially contaminated with STXs. The EU specifies that shellfish should not contain over 80 μg STX per 100 g of mussel meat [109]. However, these neurotoxins are known to be produced by several cyanobacteria genera.

Keywords: BMAA, anatoxin, aplysiatoxin, blue-green algae, cyanobacteria, cyanotoxin, cylindrospermopsin, freshwater toxins, guideline values, legislation, lipopolysaccharides, lyngbyatoxin, microcystin, nodularin, PSP, saxitoxin

Abbreviations: Adda: 2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid; BMAA: β-N-methylamino-L-alanine; Ala: alanine; AD: Alzheimer's disease; ATXa: anatoxin-a; Arg: arginine; CYNs: cylindrospermopsins; GC: Gas Chromatography; Glu: glutamic acid; GV: guideline value; HABs: harmful algal blooms;

HPLC: High Performance Liquid Chromatography; Har: homoarginin; IARC: International Agency for Research on Cancer; Leu: leucine; LPS: lipopolysaccharide; LWTXs: Lyngbya-wollei-toxins; MS: Mass Spectrometry; Mdha: methyl dehydroalanine; MeAsp: methylaspartic acid; MeDhb: methyldehydrobutyrine; MCs: microcystins; MLP: Molecular Lipophilicity Potential; MBA: Mouse Bioassay; NODs: nodularins; PSPs: paralytic shellfish poisons; PDA: Photodiode Array; PP: protein phosphatase; RBA: Receptor-Based Assay; STXs: saxitoxins; Tyr: tyrosine; UV: Ultraviolet; US EPA: US Environmental Protection Agency; WHO: World Health Organization

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8 Isolation, characterization, and identification of mycotoxin-producing fungi

8.1 Introduction

Filamentous fungi produce a wide diversity of compounds with biological activity. Some of these compounds may be toxic to humans and other vertebrates (other mammals, poultry and fish), and are called mycotoxins [1, 2]. The term mycotoxin is restricted to toxic low-molecular weight metabolites produced by species of microscopic filamentous fungi (commonly known as molds) belonging to several genera in the phylum Ascomycota, primarily *Fusarium, Aspergillus,* and *Penicillium*, which can colonize plants cultivated for human and animal consumption [3]. Some species of Basidiomycota, such as *Amanita phalloides*, *Amanita muscaria*, *Clytocybe fragrans*, *Cortinarius orellanus*, and *Paxillus involutus*, and Ascomycota, such as *Gyromitra esculenta*, that form mushrooms, also produce toxic metabolites in their fruiting bodies, but they are not considered mycotoxins. The distinction between a mycotoxin and a mushroom toxin is mainly based on human intention: mushrooms poisons are usually deliberately ingested by humans, frequently as a result of mistaken identity [4], and mycotoxin exposure is almost always accidental [2].

Mycotoxins are produced by the fungal mycelium and secreted into the substrate, which is usually an agricultural commodity susceptible to mold colonization, but can also be formed in the reproductive structures and then be present in asexual and sexual spores.

Exposure to mycotoxins is mostly by ingestion of contaminated food and feed [5]. The main sources of mycotoxin contamination in the human food chain are cereals and their by-products (Table 8.1), either directly through the consumption of contaminated cereal-based food or indirectly through the intake of residues and metabolites of mycotoxins present in milk and other animal products obtained from livestock given contaminated feeds [1, 6]. It should be noted that cereals are the most important energy source in animal feed. Among cereals, maize is widely considered to be one of the most susceptible crops to mycotoxins, and rice among the least susceptible ones.

Mycotoxins can also be found in grapes, coffee, cocoa, groundnuts, tree nuts, some fruits, and other food commodities, and in animal feeds, as spoiled stored fodder (like silage), cereal by-products used in feed processing, etc. [7].

Contamination of food and feed with mycotoxins is a threat to human and animal health [10]. This can occur either pre-harvest when grain and forage crops are growing in the field or post-harvest during handling, transportation, storage, and processing of raw materials. Mycotoxins are thermostable, have great chemical stability, and withstand

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Table 8.1: Major mycotoxins in food and feed, important mycotoxigenic fungi that produce them, and EU limits [8, 9].

Mycotoxin	Food/feed commodity	Main producing fungi	EU limits[a] (µg/kg)
Aflatoxins B_1, B_2, G_1, G_2	Cereals (maize, wheat, rice, sorghum), peanut, pistachio, figs, dry fruits, almond, ground nuts, tree nuts, cottonseed, spices	*Aspergillus flavus* *Aspergillus parasiticus*	2–8 for B_1 4–15 for total (B_1+ B_2+G_1+ G_2)
Aflatoxin M_1	Milk, milk products		0.05 in milk 0.025 in infant formulae and infant milk
Ochratoxin A	Barley, oats, grapes, wine, cocoa, coffee, beans, cheese	*Aspergillus ochraceus* *Penicillium verrucosum* *Aspergillus carbonarius*	200–1,750
Deoxynivalenol	Cereals (barley, maize, oats, wheat), safflower seeds	*Fusarium graminearum* *Fusarium culmorum*	200–4,000
Zearalenone	Cereals	*Fusarium graminearum* *Fusarium culmorum*	20–400
T-2 and HT-2 toxins	Cereals (maize, barley, wheat, oats)	*Fusarium sporotrichioides* *Fusarium poae*	15–2,000[b] (Recommendations)
Fumonisins B_1, B_2, B_3	Maize, maize products, sorghum, asparagus	*Fusarium verticillioides* *Fusarium proliferatum*	0,5–10
Patulin	Apples, products derived from apples, pears, cherries	*Penicillium expansum*	10–50

[a]Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs; Commission Regulation (EC) No 1126/2007 of 28 September 2007 amending Regulation (EC) No 1881/2006 setting maximum levels for certain contaminants in foodstuffs as regards *Fusarium* toxins in maize and maize products.
[b]Commission Recommendation of 27 March 2013 on the presence of T-2 and HT-2 toxins in cereals and cereal products.

industrial processing, so that all products made from contaminated raw materials are likely to contain these compounds. Apart from *Fusarium*, *Aspergillus,* and *Penicillium*, some other microscopic ascomycetes (*e.g.*, species of *Alternaria* and *Claviceps*) also produce mycotoxins in the field but they are not identified as food safety hazards, possibly because they generally do not accumulate to toxic levels in agricultural products.

Most mycotoxins are produced in the field [11]. Key factors that influence the natural infection of plants by mycotoxigenic fungi are environmental conditions during plant growth: temperature and/or water stress, high moisture content (water activity), and insect attacks [12]. These environmental conditions may vary from year to year, resulting in differences in the fungal species that colonize the plants and thus in the type and amount of mycotoxins which contaminate the whole food chain [13].

Figure 8.1: Different *Aspergillus* species, as noted by different colors of their asexual reproductive structures, growing from a kernel of maize on dichloran glycerol agar (DG18) medium.

Cereals, and other crops, may be invaded either pre-harvest or post-harvest by several mycotoxigenic fungi (Figure 8.1), each frequently able to produce several mycotoxins, increasing significantly the potential for multi-toxin contamination [7, 14].

Mycotoxins can also be produced by indoor molds growing on building materials. The most toxic are those from species of *Stachybotrys*. Adverse health effects may appear when mycotoxins are released from fungal spores and colony fragments after inhalation [15].

8.2 Major mycotoxins and their toxicity

More than 400 mycotoxins and derivatives have been described, of which around 20 can be present in foods and feeds at significant levels and often enough to constitute a food safety problem and be considered important in human and animal health [16]. In most countries, there are specific regulations, with those of EU being presented in Table 8.1, that set limits to the levels of the mycotoxins most frequently detected in products intended for human consumption: aflatoxins (aflatoxin B_1, B_2, G_1, G_2, M_1), ochratoxin A (OTA), trichothecenes (deoxynivalenol (DON), T2 and HT-2 toxins), zearalenone (ZEN), fumonisins (FB_1, FB_2, FB_3), and patulin (PAT). Aflatoxins, OTA, and PAT are produced by *Aspergillus* and *Penicillium* species, and trichothecenes, ZEN, and fumonisins by *Fusarium*.

The only major mycotoxin not produced by fungi is aflatoxin M_1, which is the principal hydroxylated metabolite of aflatoxin B_1 produced in the liver of dairy cows fed with aflatoxin B_1-contaminated feedstuffs. Aflatoxin M_1 is secreted in the milk and can be found in milk and other dairy products [17].

Animal feed legislation usually only regulates the maximum permitted levels of aflatoxin B_1, while makes recommendations for other mycotoxins [3] (Table 8.2).

Other toxins of interest, although less frequently accumulated to toxic levels in harvested agricultural products and not regulated, are alternariol, altenuene, altertoxins I-III, and tenuazonic acid, produced by *Alternaria* species, HT-2 toxin produced by certain species of *Fusarium*, sterigmatocystin produced by *Aspergillus* species, cyclopiazonic acid (CPA) produced by both *Aspergillus* and *Penicillium* species, and ergot alkaloids formed by species of *Claviceps* [3, 18].

Table 8.2: EU limits for major mycotoxins in feeds. Legislated limits are only for aflatoxin B_1. For the remaining mycotoxins, limits are recommendations.

Mycotoxin	Animal feeds	EU limit (mg/kg)[a]
Aflatoxin B_1	All feed materials	0.02
	Complementary and complete feed	0.01
	with the exception of:	
	– compound feed for dairy cattle and calves, dairy sheep and lambs, dairy goats and kids, piglets and young poultry animals	0.005
	– compound feed for other cattle, sheep, goats, pigs and poultry	0.02
Deoxynivalenol	Cereals and cereal products	8
	Maize by-products	12
	Complementary and complete feedingstuffs with the exception of:	5
	– complementary and complete feedingstuffs for pigs	0.9
	– complementary and complete feedingstuffs for calves, lambs and kids	2
Zearalenone	Cereals and cereal products	2
	Maize by-products	3
	Complementary and complete feedingstuffs for piglets and gilts	0.1
	Complementary and complete feedingstuffs for sows and fattening pigs	0.25
	Complementary and complete feedingstuffs for calves, dairy cattle, sheep and goats	0.5
Fumonisins B_1+B_2	Maize and maize products	60
	Complementary and complete feedingstuffs for:	
	– pigs, horses, rabbits and pet animals	5
	– fish	10
	– poultry, calves, lambs and kids	20
	– adult ruminants and mink	50
Ochratoxin A	Cereals and cereal products	0.25
	Complementary and complete feedingstuffs for pigs	0.05
	Complementary and complete feedingstuffs for poultry	0.1

[a]Commission Regulation (EU) N° 574/2011 of 16 June 2011 amending Annex I to Directive 2002/32/EC of 7 May 2002 on undesirable substances in animal feed; Commission Recommendation 2006/576/EC of 17 August 2006 on the presence of deoxynivalenol, zearalenone, ochratoxin A, T-2 and HT-2 and fumonisins in products intended for animal feeding.

In the last years, increasing attention has been given to the so-called emerging toxins, including enniatins, beauvericin, fusaproliferin and moniliformin, and to modified and matrix-associated forms of DON, ZEN, fumonisins and T-2 and HT-2 toxins [19],

Table 8.3: Toxic effects of major mycotoxins found in food and feed [8, 9, 27, 28].

Mycotoxin	Toxic effects
Aflatoxins B_1, B_2, G_1, G_2	Liver cirrhosis, immunosuppression and cancer.
Aflatoxin M_1	
Ochratoxin A	Liver and kidney damage.
Deoxynivalenol	Emesis, feed refusal and immunosuppression.
Zearalenone	Estrogenic activity.
T-2 and HT-2 toxins	Immunosuppression, causative agent in alimentary toxic aleukia (ATA).
Fumonisins B_1, B_2, B_3	Neural tube defects, causative agent in leukoencephalomalacia.
Patulin	Inflammatory alterations of the gastrointestinal tract.

initially called masked mycotoxins, all mainly produced on cereal crops by *Fusarium* species and not subjected to any regulation. The term masked mycotoxin was coined for metabolites of a parent mycotoxin conjugated or attached to certain molecules, and formed in the plant or fungus. Recent works show that some emerging mycotoxins are present in high concentrations in food and feed, which increases the interest in studying their toxicity, whereas the existence of modified and matrix-associated mycotoxins means that actual amounts of the parent mycotoxins in food and feedstuffs might be higher than that currently determined by routine and conventional analytical methods [20, 21, 22]. New conjugated mycotoxins and analogs are continually being described [23], raising the need for studying whether they might contribute to toxicity either directly or indirectly through the release of the parent mycotoxins.

Carcinogenicity, nephrotoxicity, hepatotoxicity, reproductive problems, gastrointestinal effects, immunosuppression, dermal effects, and central nervous system disorders are toxic effects associated to these fungal metabolites (Table 8.3). The highest acute and chronic toxicity in humans and animals is produced by aflatoxins, especially the most predominant aflatoxin B_1, which are potent carcinogens [13]. Aflatoxins B_1, B_2, G_1, G_2, and M_1 have been classified as carcinogenic to humans by the International Agency for Research on Cancer [24]. OTA, FB_1, and FB_2 are possibly carcinogenic to humans [25]. ZEN has estrogenic and anabolic activity, and DON, the most abundant trichothecene, has been related to immunosuppression, reproductive disorders, vomiting, and other symptoms in humans and animals [26].

Toxic effects associated to mycotoxin exposure may be acute or chronic. These effects depend on the mycotoxin type, the level and duration of exposure, and, in production animals, the animal species that is exposed and the age of the animal [29]. Although acute mycotoxicoses produced by high doses of some mycotoxins in humans and animals (*e.g.*, turkey X disease, human ergotism) are the best known, they are rare. Ingestion of low to moderate amounts of mycotoxins, particularly those produced by *Fusarium* in cereals in the field, is common and generally

does not result in obvious intoxication. However, the greatest risk to human and animal health is related to chronic exposure (*e.g.,* cancer induction, immune suppression) [2].

Diseases caused by exposure to mycotoxins are collectively called mycotoxicoses. They are mostly the result of eating contaminated food, although skin contact with mold-infected substrates and inhalation of spore-borne mycotoxins are also sources of exposure. Mycotoxin exposure is more likely in parts of the world where poor methods of food handling and storage are common, where problems of malnutrition are important, in buildings harboring high levels of molds, and in countries with few mycotoxin regulations [2].

8.2.1 Aflatoxins

Aflatoxins are difurocoumarin derivatives produced by *Aspergillus* species, predominantly *Aspergillus flavus* and *Aspergillus parasiticus* [30]. The aflatoxins group was identified as causative agent of "turkey X" disease in the 1960s after the death of thousands of turkey poults, ducklings, and chicks fed with contaminated peanut meal in England [3].

Aflatoxins are readily absorbed from the gastrointestinal tract and reach the systemic circulation [31]. In this way, aflatoxins are distributed to various body organs, especially the liver in which they are bio-transformed trough hydroxylation, hydration, demethylation, and epoxidation. The epoxidation of aflatoxin B_1 is more efficient than the other naturally occurring aflatoxins and, therefore, it is the most toxic compound [32]. The resultant metabolites are excreted in bile and urine after conjugation [33]. Acute effects of aflatoxins are mediated by one of these metabolites, namely aflatoxin B_1 exo-8,9-epoxide, which cause impairment of protein synthesis and binds to proteins in the blood serum. Consequently, a reduction in protein content in body tissues is observed which has been associated with liver and kidney necrosis [31]. Carcinogenic activity of aflatoxins is also mediated by aflatoxin B_1 exo-8,9-epoxide, which reacts with the guanine bases of liver cells forming a DNA adduct (aflatoxin-N7-guanine). If the DNA adduct is not repaired, it causes mutations in p53 tumor suppressor gene which results in carcinogenesis [34].

The mycotoxicosis that results from the ingestion of aflatoxins is called aflatoxicosis. Acute aflatoxicosis is caused by the intake of large doses of aflatoxins, which result in direct damage to the liver, usually through liver cirrhosis [35]. In some cases, the illness leads to death. In this sense, 125 people died during one of the latest aflatoxicosis outbreaks that happened in Kenya in 2004 [36]. Chronic exposition to sub-lethal doses of aflatoxins has been related with immunosuppression and nutritional alterations [35]. Long exposure to aflatoxins in the diet causes cancer in many animal species. The carcinogenic activity of aflatoxin B_1 is well established, the liver is the primary target; however, tumors have been found in other sites such as kidney or colon.

8.2.2 Ochratoxins

Ochratoxins are produced by some *Aspergillus* and *Penicillium* species. Members of the ochratoxin group are dihydrocoumarines linked to a molecule of L-β-phenylalanine via an amide bond. OTA is the most produced and most toxic analog of the ochratoxin family. It has been implicated in a diverse range of toxicological effects in both animals and humans [37].

The main target organ of OTA is the kidney. In mammalians, the ingestion of OTA causes nephropathy in a dose- and time-dependent way [38]. Kidney alterations can be a consequence of both acute and chronic exposure to this environmental toxicant. In animals, the administration of acute lethal doses of OTA produces hemorrhages, intravascular coagulation and necrosis of liver, kidney, and lymphoid organs [39]. In humans, the inhalation of OTA produced by *Aspergillus ochraceus* was related with the development of acute renal failure [40]. Toxicity resulting from chronic exposure seems to be the most significant since acute exposure is rare. In this regard, exposure to low doses of this toxin over long periods of time leads to characteristic toxic effects, which are manifested through kidney damage. OTA is also an immunotoxic and hepatotoxic agent. The immunosuppressant activity of OTA is characterized by a significant reduction of immune organs and changes in the number and functions of immune cells [41]. Hepatotoxicity is mediated by the production of reactive oxygen species (ROS), which cause DNA damage, leading to apoptosis in hepatic cells [42].

In addition to the described effects, OTA can cross the placenta from mother to fetus, resulting in embryotoxic and teratogenic effects in animals. The most common symptoms are reduced birth weight and craniofacial abnormalities [43].

8.2.3 Trichothecenes

Trichothecenes are a group of sesquiterpenes which include more than 180 analogs, all of them containing an epoxide group. According to their structure, these compounds are divided into four groups, namely types A, B, C, and D [44]. Trichothecenes are produced mostly by *Fusarium* species, which produce type A and type B trichotecenes.

Type A trichothecenes include diacetoxyscirpenol, HT-2 toxin, and T-2 toxin, the last compound being considered as the most toxic trichothecene. Strains of *Fusarium sporotrichioides* and *Fusarium poae* are the main producers of these toxins [45]. Type B trichothecenes include DON and its derivative forms: 3-acetyl DON and 15-acetyl DON. Strains of *Fusarium culmorum* and *Fusarium graminearum* are the major producers [46].

Within the group of type A trichothecenes, most data on toxicokinetics and mechanism of action deal with T-2 toxin. Absorption, distribution, and excretion of T-2 toxin are rapid [47]. This mycotoxin is metabolized in the liver and other organs. Although

many products have been reported from T-2 metabolism, HT-2 is the major toxin. T-2 toxin and its metabolites are excreted in urine and feces [48].

Available data on type B trichothecenes are focused on DON in production animals. The oral absorption is low for ruminants and poultry, while swine and rodents readily absorb this mycotoxin. In pigs, up to 82% of the orally administered DON reaches the systemic circulation. In general, trichothecenes bind to the 60S subunit of ribosomes, leading to toxic effects [49].

Consumption of food contaminated with type A trichothecenes (mainly T2 toxin) leads to the development of alimentary toxic aleukia. This mycotoxicosis is characterized by leukopenia, agranulocytosis, exhaustion of the bone marrow, necrotic angina, and death [50]. Chronic exposure to type A trichothecenes is associated with anorexia, reduced body weight gain, and lesions in the upper digestive tract [51].

Gastrointestinal tract toxicity is the most characteristic effect of type B trichothecenes. Acute intoxication with DON (also known as vomitoxin) leads to anorexia and emesis. Other symptoms include abdominal distress, increased salivation and diarrhea. Only exposure to extremely high concentrations produces mortality or marked tissue injury. Prolonged dietary exposure to low concentrations of DON produces feed refusal and weight-gain suppression. Animals, especially swine, exposed to high doses of type B trichothecenes for prolonged periods of time develop lesions in the gastrointestinal tract and lymphoid tissues. Moreover, in these animals, leukocyte apoptosis and immunosuppression are observed [52, 53].

8.2.4 Zearalenone

ZEN is a phenolic resorcyclic acid lactone produced by *Fusarium* species [54]. ZEN and its analogs alpha-zearalenol (α-ZOL) and beta-zearalenol (β-ZOL) are found in the field, usually as cereal contaminants. These mycotoxins are, in many cases, responsible for estrogen-related diseases observed in farm animals [55].

ZEN is readily absorbed after oral ingestion. However, this toxin is subject to an extensive pre-systemic metabolism and only low amounts reach the systemic circulation [56]. ZEN is bio-transformed in the liver and intestines where is converted to α-ZOL and β-ZOL by an enzymatic reaction catalyzed by hydroxysteroid dehydrogenases. Thereafter, α-ZOL and β-ZOL are transformed into α-zearalanol and β-zearalanol (α-ZAL and β-ZAL) [57]. These metabolites are subsequently conjugated by UDP-glucuronyl transferases, and afterwards excreted in urine and bile [58].

The mechanism of action of ZEN and its metabolites is mediated by their binding affinity to estrogen receptors, which yields estrogenic effects by the activation of gene transcription [59]. Not all compounds have the same affinity for estrogen receptors, with the extent of affinity decreasing as follows: α-ZOL>ZEN>β-ZOL [60]. Swine is the most sensitive species to ZEN exposition since it shows a preferential conversion of ZEN to α-ZOL, which is the metabolite with the highest affinity for estrogen receptors [61].

ZEN exhibits low acute toxicity after oral ingestion. Chronic exposure to ZEN has well-established effects on the endocrine and reproductive systems. This mycotoxin induces alterations in the reproductive tract, weight changes in some endocrine glands (adrenal, thyroid and pituitary), and changes in serum levels of ovary hormones with decreased fertility rates. However, no teratogenic effects have been reported so far. In pigs, ZEN causes severe signs of hyperestrogenism [52]. The main effect observed in long-term rodent studies is low body weight gain. In mice, ZEN induces pituitary adenomas and produces liver lesions which evolve to hepatocellular adenomas. Moreover, alterations in the mammary gland and fibrosis of the uterus and cystic ducts have been reported [62].

8.2.5 Fumonisins

Fumonisins are a family of mycotoxins produced by several field fungi belonging to the genera *Fusarium*, primarily *Fusarium verticillioides* and *Fusarium proliferatum,* which frequently infect maize and other crops [63, 64]. The most prevalent and toxic analogs are fumonisin B_1 (FB_1) and fumonisin B_2 (FB_2) [55].

Fumonisins are poorly absorbed as less than 5% of the ingested fumonisins reaches the blood stream [65]. There is no in vivo evidence of significant FB_1 metabolism [65]. Toxic effects of fumonisins are mediated by the inhibition of the enzyme ceramide synthase due to the structural similarity that this family of mycotoxins has with sphinganine. Toxin ingestion leads to an increase in the amount of sphinganine in serum, tissues, and urine. In addition, depletion of ceramide, sphingomyelin, and glycosphingolipids may occur [66–68].

Fumonisins cause a neurotoxic disease of horses known as equine leukoencephalomalacia. This is a fatal illness characterized by liquefactive necrosis of the cerebral white matter involving frontal and parietal lobes [69]. In swine, fumonisins induce pulmonary edema probably because of an acute left-sided heart failure [70]. Other toxic effects such as nephrotoxicity and hepatotoxicity have been observed in laboratory animals [71]. In humans, epidemiological studies relate these mycotoxins with neural tube defects and esophageal cancer [68, 72].

8.2.6 Patulin

PAT is a heterocyclic lactone produced by various species of *Penicillium*, *Aspergillus,* and *Byssochlamys* [73]. This compound was isolated in 1943 as an antibiotic effective against gram-positive and gram-negative organisms [74]. However, later studies showed that it was toxic to humans and animals. Nowadays, PAT is one of the best characterized mycotoxins found in agricultural products [75].

There is still little data about the absorption and metabolism of PAT. This toxin is mainly accumulated in the red blood cells [76]. PAT inhibits many enzymes since it is a highly reactive molecule with affinity for sulfhydryl-containing compounds such as cysteine or glutathione [77]. One important aspect of PAT toxicity is its ability for suppressing catalase activity, which results in the increase of ROS generation, leading to oxidative stress [78].

Acute toxic effects of PAT are associated with inflammatory alterations of the gastrointestinal tract, producing ulceration and inflammation of the mucosa of the stomach [79]. This may be caused by the destruction of the tight junctions in the epithelial cell layer [28]. Other clinicopathological alterations may include agitation, convulsions, metabolic alkalosis, pulmonary congestion, edema, oliguria, reduced plasma protein, and neutrophilia [77, 79]. Chronic exposure to PAT may involve neurotoxic and immunosuppressive effects [80].

8.3 Main mycotoxin-producing fungi

Although more than fifty fungal genera are known to include mycotoxigenic species, the vast majority and most important mycotoxigenic species are found in three fungal genera belonging to two orders in the Ascomycota: the genus *Fusarium* in the order Hypocreales and the genera *Aspergillus* and *Penicillium* in the order Eurotiales.

Species of *Fusarium* colonize forage and grain crops in the field, some of them causing severe diseases and other living endophytically (growing in a plant without affecting it), and produce mycotoxins before harvest. *Aspergillus* and *Penicillium* species generally grow on foodstuff and feed under inadequate conditions of drying, transport, storage, and processing. An exception is *Aspergillus flavus*, which can be a pathogen or an endophyte in the field, and a storage fungus, and can produce mycotoxins in the three cases.

The production of mycotoxins in the field by species of *Fusarium* is practically unavoidable because they are common components of the epiphytic and endophytic microflora in many crops. The importance of mycotoxin contamination by *Fusarium* will depend on environmental conditions during the crop-growing season and the subsequent food storage stage. However, colonization of stored agricultural commodities by *Penicillium* and *Aspergillus* species can be prevented by drying crops at harvest and/or by controlling environmental conditions of storage [14].

The identification and classification of mycotoxigenic species require some knowledge on the life cycle of filamentous ascomycetes, and especially on the morphological characters of sexual and asexual reproductive structures, together with the application of molecular methods and phylogenetic analysis developed in the 2000s.

8.3.1 Fungi: An overview

The kingdom Fungi, placed together with the animals in the eukaryotic supergroup Opisthokonta [81], is one of the largest and ubiquitous groups of living organisms on Earth. The number of extant fungal species has been estimated to range from ~1.5 to 5.1 million, although only about 135,000 have been formally classified and named by taxonomists [82–84]. Fungi are unicellular or multicellular nonchlorophyllic organisms, most of them obligate aerobes. The cell wall consists of various layers, mainly composed of chitin and glucan [85]. Unicellular fungi are commonly referred to as yeasts. The vast majority of fungi are multicellular and exist as filamentous forms which undergo a vegetative and a reproductive stage. The vegetative body of a fungus is the thallus, which may be unicellular (in yeasts) or filamentous and multinucleated (in most fungi). Some fungi are dimorphic as they can exist either in yeast or filamentous form.

All fungal species are heterotrophic. Considering the nutrient source, they can be classified as: biotrophs, when nutrients are from a living host (plant or animal); saprotrophs, when nutrients are obtained from dead or decomposing organic matter, mainly dead plants but also dead animals; and necrotrophs, when the fungus infect a living host and kill host cells to get the nutrients [86].

Appropriate conditions of temperature, water availability, and pH are essential for fungal growth. Even though the temperature range is quite wide, most species grow well at temperatures around 25°C. Most fungi are acidophilic, growing well between pH 4 and 6, and require a high water activity (a_w), with a minimum a_w of around 0.65 [87].

Fungi are currently classified, according to macro- and microscopic morphological characteristics of their reproductive structures, and molecular, phylogenetic and phylogenomic analyses, in the following divisions (phyla): Ascomycota, Basidiomycota, Blastocladiomycota, Chytridiomycota, Cryptomicota, Microsporidia, Mucoromycota, Neocallimastigomycota, and Zoopagomycota [88–91]. The majority of the named fungal species are Ascomycota (64,163 species), followed by Basidiomycota (31,515 species) [92], both phyla grouped in the subkingdom Dikarya as they have dikaryotic hyphae.

Within the Ascomycota, most known species (roughly 90%) belong to the subphylum Pezizomycotina (ca. 59,000), which likely comprises the largest fraction of unknown fungal diversity [93].

Known species of ascomycetes are mainly terrestrial although more than 3,000 species occur in freshwater habitats and there are more than 1,500 marine species [82].

Most species are decomposers that enzymatically digest organic compounds from the dead substrate where they live on and absorb the released nutrients through the chitinous cell walls of their hyphae. Their role in breaking down organic compounds have made them important contributors to nutrient recycling in ecosystems. Some

members of the division Ascomycota are biotrophs that may colonize roots of most terrestrial plants and form mutualistic symbiosis called mycorrrhizas (from the Greek *mykes* = fungus and *rhiza* = root). Both symbionts benefit from the association: the plant provides a carbon source to the fungus and the fungus absorbs phosphate ions and other mineral nutrients from the soil and transfers them to the plant. Also many species of Ascomycota live in a symbiotic relationship with an alga or cyanobacteria forming lichens. Other ascomycetes are used in the food industry (for making bread, cheeses, alcoholic beverages) and for production of medicinally important compounds, such as antibiotics.

The phylum Ascomycota also includes species that cause disease in animals, humans, and plants. Many agriculturally important plant pathogens are ascomycetes.

Like the great majority of Basidiomycota, some Ascomycota (like morels and truffles) form macroscopic sexual fruiting bodies, commonly known as mushrooms. However most ascomycetes develop microscopic fruiting bodies and are referred to as microfungi [92].

8.3.2 Life cycle of Ascomycota

Although some Ascomycota, such as yeasts, are unicellular organisms, the great majority grows as filamentous forms. In the vegetative stage, spores of filamentous microfungi germinate and form long thread-like walled filaments termed hyphae which extend, branch and intertwine within the supporting substrate as a network, denominated mycelium (the thallus). Hyphae are made from tubular cells attached to one another. Growth takes place at the tips of hyphae.

If nutrients are available from the substrate and environmental conditions are favorable, the hyphae of individual fungi extend endlessly via apical growth outward in all directions from the center, showing a radially expanding colonial growth, until nutrient sources are exhausted.

Hyphae are divided into individual cells by internal cross walls called septa (sing., septum), which are perforated by a single, central pore. Fungal cells contain haploid nuclei.

Filamentous microfungi reproduce by means of spores either sexually, with homothallic or heterothallic mycelia depending on the species, or asexually (Figure 8.2).

The sexual reproductive state in the Ascomycota is referred to as the teleomorph, typically a fruiting body, while the asexual state is called the anamorph. Some species produce more than one morphologically distinct anamorphs, which are then called synanamorphs. Fungal species that present a sexual state and one or more asexual states are pleomorphic.

Another frequent term is holomorph, which refers to the whole life cycle of a fungus, including both the anamorph and the teleomorph.

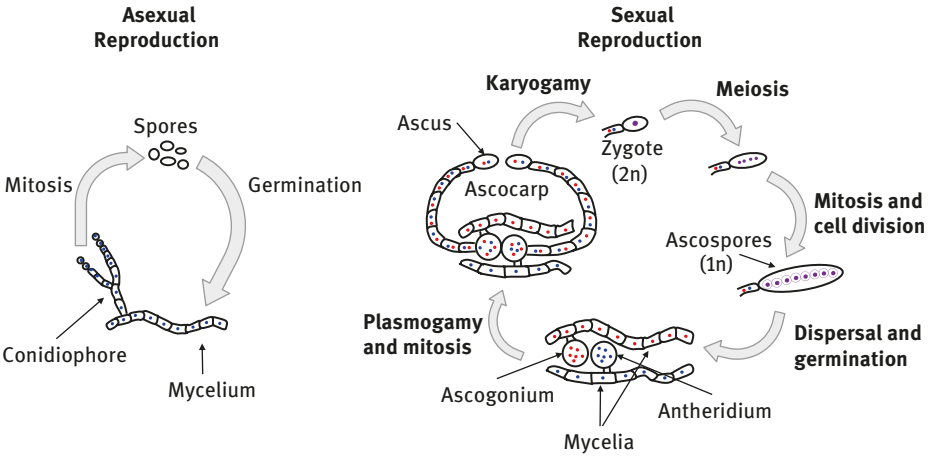

Figure 8.2: Life cycle of filamentous Ascomycota. Adapted from Zeeshan 93 – Own work, CC BY-SA 4.0, https://commons.wikimedia.org/w/index.php?curid=46636026.

8.3.2.1 Sexual reproduction

For sexual reproduction, the haploid hyphae of two mating fungal strains lie side by side, and then each of them develop a projection toward the other. The projections, which are sexual reproductive structures called gametangia (antheridium – ♂, and ascogonium – ♀), fuse together, and then cell walls break down, leading to the production of dikaryotic hyphal strands. The tips of these strands eventually form sac-like structures called asci (sing. ascus). The sac-like structures are typical of Ascomycota, which are often referred to as sac fungi. In the ascus, the two haploid nuclei from the parent strains fuse and form a diploid nucleus, which undergoes meiosis and forms four haploid nuclei. These haploid nuclei divide by mitosis to form eight haploid ascospores.

Asci may be protected by differentiated haploid hyphae, tightly interwoven, which form fruiting bodies called ascocarps (also known as ascomata). Ascocarps may be spherical (cleistothecia, sing. cleistothecium), globular or flask-like with an apical narrow opening (perithecia, sing. perithecium), or commonly stalked and broadly open like a disk or a cup (apothecia, sing. apothecium). When ascospores are released form ascocarps and germinate, they will develop into new haploid hyphae.

Homothallic fungi have both male and female nuclei derived from the same individual (the same thallus/mycelium) for sexual reproduction. Thus, two types of haploid nuclei are produced from the same mycelium to form a diploid nucleus. This means that they reproduce sexually by self-fertilization. They can also reproduce sexually by mating a partner (*i.e.*, another fungal individual of the same species) of compatible mating type.

Heterothallic fungi rely on outcrossing. This is because each individual produces only one type of mating haploid nucleus and needs a mating partner with compatible

mycelium, for sexual reproduction. In this case two different mycelia (thalli) are needed to form a zygote. Some heterothallic fungi can also present self-fertilization under certain environmental conditions.

8.3.2.2 Asexual reproduction

Asexual reproduction is the main form of propagation in the Ascomycota. The fungal spore is a haploid cell produced by mitosis from a haploid parent cell, being therefore genetically identical to the parent cell. Asexually produced spores are usually called conidia. They are formed exogenously by fragmentation of the tips of specialized hyphae called conidiophores. Conidiophores may be from short hyphal-like structures to complex branched structures differing notably in appearance from the hyphae. Moreover, conidiophores may branch off from the mycelia, being arranged singly, or may be organized into four types of asexual structures: sporodochia (sing. sporodochium), which are cushion-shaped containers composed of a mass of hyphae covered with conidiophores; pycnidia (sing. pycnidium), hollow round to flask-shaped structures lined with conidiophores; acervuli (sing. acervulus), piles of hyphae bearing a compact layer of conidiophores; and synnemata (sing. synnema), columnar structures of united conidiophores bearing conidia principally at the apex [94].

In conidiophores arranged singly, the hyphal tip can be very similar to a normal hyphal tip or, commonly, be differentiated into a bottle-shaped cell called phialide, from which spores are produced.

Spores are dispersed by wind, water, and animals, and when they germinate they form a hypha which will develop into mycelium.

8.3.3 Naming of filamentous Ascomycota

Mycotoxigenic fungi have been traditionally identified and classified according to the morphological characteristics of their sexual reproductive structures, observed in the teleomorphic state. However, although the anamorphic and the teleomorphic states are known for most ascomycetes, many species have only been found as anamorphs (asexual fungi) and they were identified attending at the asexual reproductive characteristics.

Until recently, pleomorphic fungal species might have two scientific names (termed dual nomenclature): one for the asexual morph (anamorph) and another for the sexual morph (teleomorph), which might cause confusion. In July 2011, experts attending the International Botanical Congress in Melbourne determined to abandon the dual nomenclature for pleomorphic fungi. Consequently, the International Code of Nomenclature for Algae, Fungi and Plants adopted the single name nomenclature. On 1 January 2013, dual naming of fungi ended. Since then, one fungus can only have one name [95]. This change was possible thanks to methods of DNA sequencing and

phylogenetic analysis for fungal identification: the anamorph and the teleomorph of the same fungal species have the same DNA sequence data. The 'One Fungus = One Name' nomenclature was applied to all fungal genera, including of course those harboring major mycotoxigenic fungi: *Fusarium*, *Aspergillus*, and *Penicillium* are anamorph names that were chosen over the teleomorph names.

8.3.4 *Fusarium*

The genus *Fusarium* belongs to the family Nectriaceae, order Hypocreales, in the phylum Ascomycota. *Fusarium* is one of the largest genera of fungi. It comprises nearly 1,500 species, subspecies, varieties, and *formae speciales* [96]. Some species of *Fusarium* are among the most economically important plant pathogens affecting agricultural crops worldwide, causing a number of diseases which not only reduce crop quality, since they produce mycotoxins, and yield, but also can lead to the death of plants [97]. This is the case of head blight of wheat, Fusarium ear rot of maize, and Fusarium stalk rot of maize.

8.3.4.1 Morphological characteristics of *Fusarium* species

From a morphological point of view, *Fusarium* isolates are identified and classified based on the characteristics of the asexual reproductive structures. The size, number of septa, general shape, and shape of apical and basal cells of macroconidia are important features for the identification of *Fusarium* species (Figure 8.3). When looking at a macroconidium, the more curved portion of the cell is to the top, the apical cell is to the left, and the basal cell is to the right [98].

Other useful characters for morphological identification of *Fusarium* isolates are the size, number of septa, and shape of microconidia, if present since not all *Fusarium* species produce them. Common shapes of microconidia are: oval, kidney-shaped, obovoid with a truncate base, pyriform, napiform, globose, spherical, and fusiform (Figure 8.3).

In Figure 8.4, images of asexual structures of some important mycotoxigenic *Fusarium* species are shown.

Apart from microconidia, the conidiogenous cell on which they are borne, and the arrangement of microconidia on and around the conidiogenous cell are also important characteristics for species identification [98].

Two basic types of conidiogenous cells are distinguished: monophialides, which have a single opening per cell through which conidia are produced, and polyphialides, which have more than one opening. In the phialides, microconidia may be arranged singly, like in polyphialides, in chains, or in false heads, like in monophialides. The term false head is due to the fact that, despite they superficially resemble

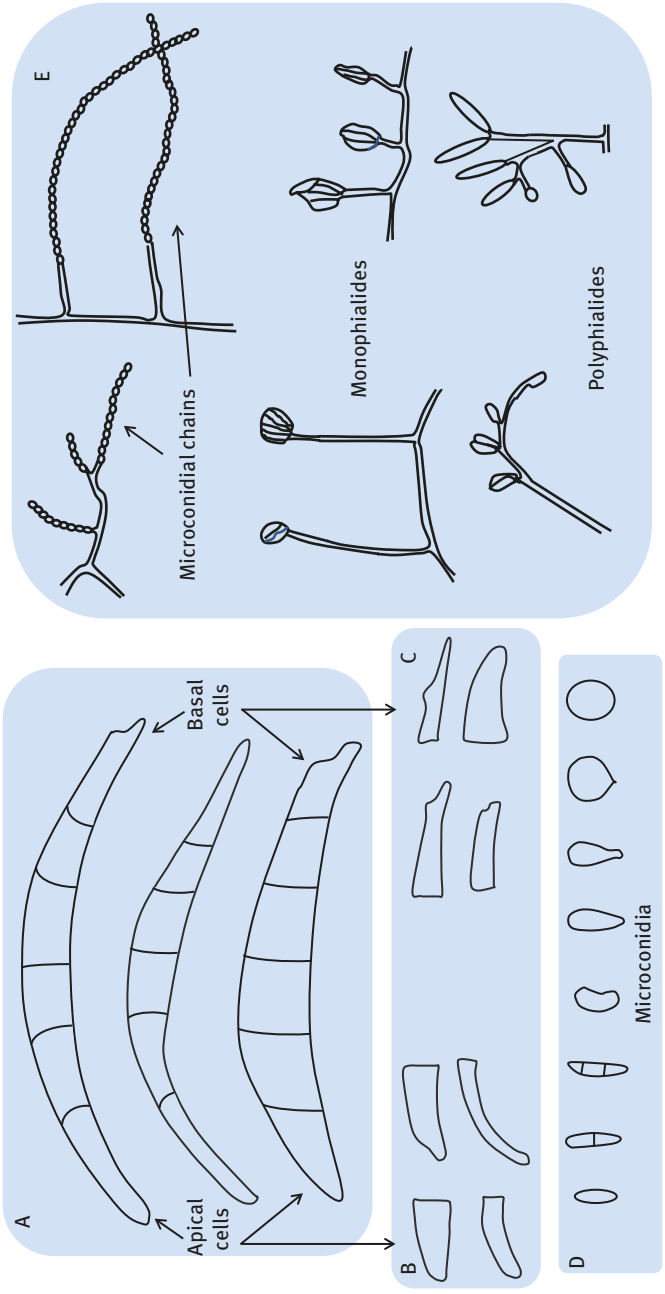

Figure 8.3: Morphological characteristics of macroconidia (A), apical and basal cells of macroconidia (B,C), microconidia (D), and phialides (E) in the genus *Fusarium* (adapted from Leslie and Summerell [98]). Drawings not to scale.

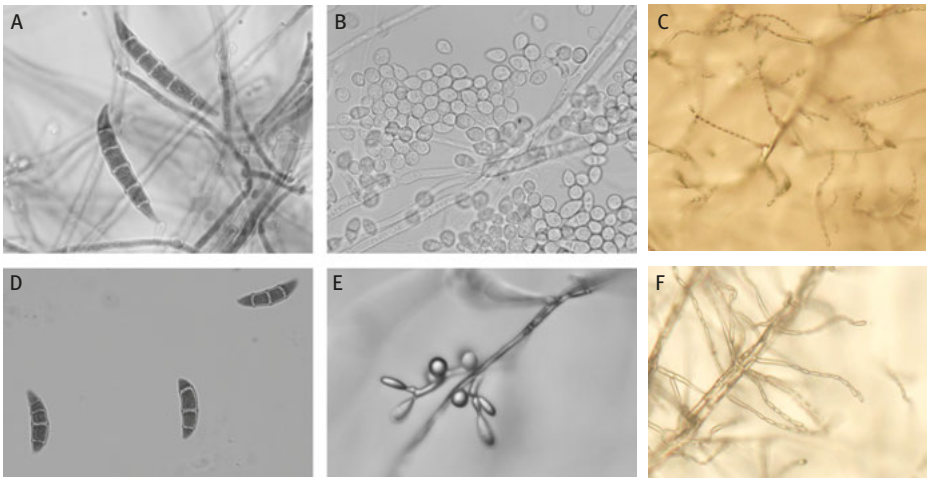

Figure 8.4: Morphological characteristics of asexual structures in the genus *Fusarium*. (A) macroconidia of *Fusarium graminearum*; (B) microconidia of *Fusarium poae*; (C, F) microconidial chains of *Fusarium verticillioides*; (D) macroconidia of *Fusarium culmorum*; (E) polyphialides of *Fusarium sporotrichioides*.

spore heads, *Fusarium* species do not form true heads of conidia as happens in *Aspergillus*, for instance, but clumps of spores at the end of the phialides [98].

Some *Fusarium* species produce also chlamydospores, which are enlarged thick-walled vegetative cells that form within hyphae (intercalary) or at hyphal tip (terminally). They separate from the parent hypha and behave as resting spores, surviving in unfavorable conditions. Chlamydospores may be born singly, doubly, in clumps, and in chains [98].

In some mycotoxigenic *Fusarium* species, such as *Fusarium avenaceum*, *Fusarium sporotrichioides,* and *Fusarium subglutinans,* fusoid conidia with up to 3–4 septa, called mesoconidia, are formed from polyphialides in the aerial mycelium, but not in sporodochia. They are larger than microconidia and often lack a notched basal cell.

Pigmentation of colonies growing on agar media (Figure 8.5), either on plates or slants, and growth rate in PDA medium at either 25°C or 30°C, are also used as significant characters in the identification of *Fusarium* species.

Many *Fusarium* species produce several mycotoxins. Also some mycotoxins are produced by several *Fusarium* species. However, the mycotoxigenic profile can be used as a secondary character for identification of some species.

Up to the introduction of the single nomenclature for fungal species names, several teleomorph genera were associated with species of *Fusarium*, the most common being the genus *Gibberella*. Some *Gibberella* species, which currently are named with the anamorph name, are important pathogens on cereals: *Gibberella zeae* = *Fusarium graminearum*, *G. moniliformis* = *Fusarium verticillioides* [97].

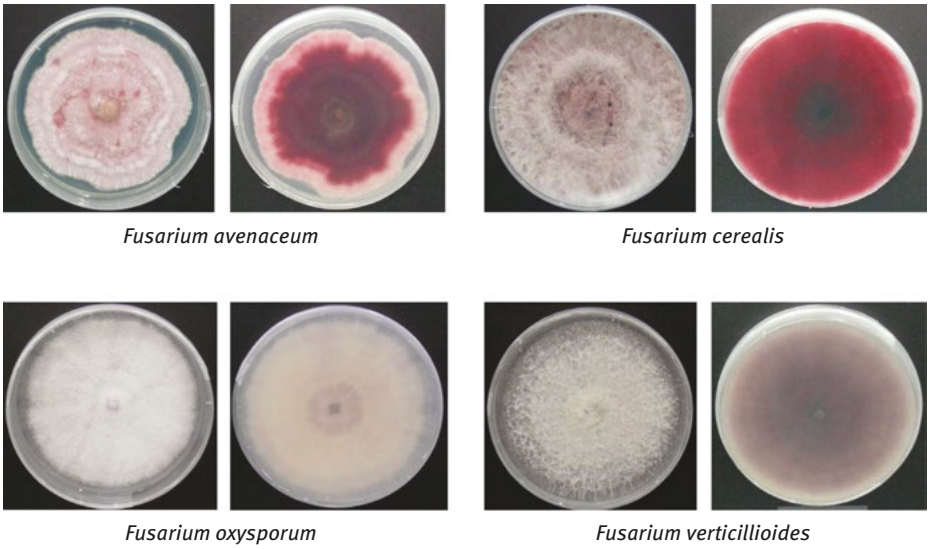

Figure 8.5: Colony surface (left) and reverse (right) of isolates of *Fusarium avenaceum*, *Fusarium cerealis*, *Fusarium oxysporum,* and *Fusarium verticillioides* grown on PDA at 25°C.

8.3.5 *Aspergillus* and *Penicillium*

The genera *Aspergillus* and *Penicillium* belong to the family Aspergillaceae, order Eurotiales, in the phylum Ascomycota. They are characterized by the formation of flask-shaped or cylindrical phialides, by the production of asci inside cleistothecia or surrounded by Hülle cells, and by ascospores mainly having a furrow or slit [99].

8.3.5.1 *Aspergillus*

The genus *Aspergillus* comprises approximately 350 accepted species which share an asexual spore-forming structure called aspergillum [100, 101]. The name of the genus was decided by an Italian priest and biologist, Pier Antonio Micheli, in 1729, after viewing the fungal asexual structures of an isolate under a microscope, since they reminded him of the shape of an *aspergillum*, a Latin work to design a holy water sprinkler that derives from the verb *aspergere* (to sprinkle) [102].

Aspergillus species are currently classified into four subgenera (namely *Aspergillus*, *Circumdati*, *Fumigati*, and *Nidulantes)* and 19 sections, each including related species [99], based on morphological characters but that largely correspond with the current published phylogenies [100].

Of particular interest is the section *Flavi* because it includes the two species most commonly implicated as causal agents of aflatoxin contamination: *Aspergillus flavus* and *Aspergillus parasiticus* [103]. *Aspergillus flavus* produces aflatoxins B_1

and B_2, and CPA, which is a mycotoxin that has neurotoxic effects [104]. Two types of *Aspergillus flavus* strains have been found: the S-type, which produces numerous small sclerotia (<400 mm in diameter), relatively few conidia and high levels of aflatoxins (B_1 and B_2), and the L-type, characterized by producing fewer, larger sclerotia (>400 mm in diameter), more conidia and less aflatoxins than the S-type [105]. *Aspergillus parasiticus* produces both aflatoxins B_1 and B_2 and aflatoxins G_1 and G_2, but no CPA.

The genus *Aspergillus* is widely distributed worldwide, but most species, particularly those of the section *Flavi* are mainly found in temperate and subtropical climates between latitudes 26 and 35° north and south of the Equator [106], and therefore the risk of aflatoxin contamination in food and feed commodities is greater in those regions.

8.3.5.2 Main morphological characteristics of *Aspergillus* species

The morphology of the conidiophore is the predominant microscopic character used for the characterization and identification of *Aspergillus* species (Figure 8.6).

In most aspergilli, the conidiophore has a foot cell at the base and a long stipe (usually non-septate), which is a thick-walled hyphal branch which arises perpendicularly from the foot cell. The tip of the stipe is swollen forming a structure called vesicle. Conidiogenous cells, termed phialides, develop on the vesicle surface. In some species, the phialides are the only layer of supporting cells on the surface of the vesicle, and then it is said that the conidiophore has an uniseriate head. In other species, there is a layer of supporting cells, called metulae, on the surface of the vesicle and, over them, a second layer with the phialides. When metulae and phialides,

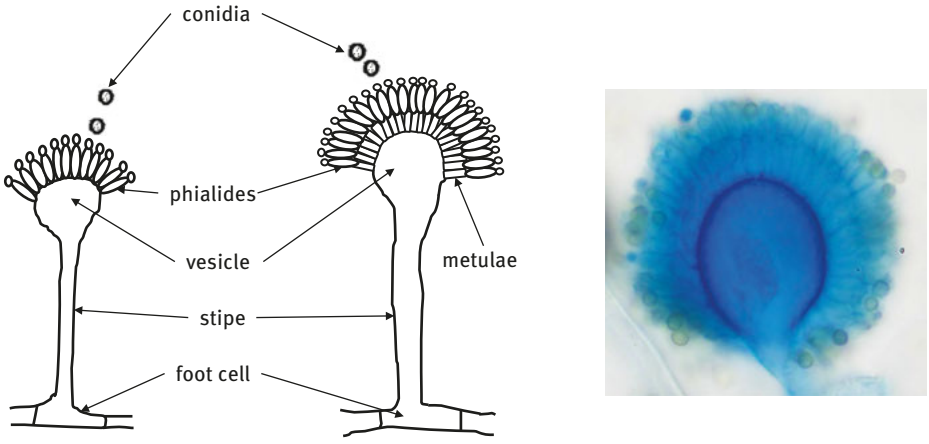

Figure 8.6: On the left, morphological characteristics of the conidiophores in the genus *Aspergillus* (drawings not to scale). On the right, photo of a conidiophore of *Aspergillus flavus* stained with cotton blue.

which are borne simultaneously, are present, the conidiophore has a biseriate head. Conidia form by budding of the cytoplasm from the phialide cells. Additional phenotypic characters used for species identification are conidial color and ornamentation, growth rate on agar media, and growth rate at different temperatures and water activities [99, 107]. As an example, *Aspergillus* section *Flavi* includes species with conidial heads in shades of yellow-green (Figure 8.7).

In addition to the conidial state (anamorph) characteristic of the genus, approximately one-third of *Aspergillus* species also have a sexual state (*i.e.*, a teleomorph), all but five of which are homothallic [108]. For sexual reproduction, *Aspergillus* form cleistothecia, which are ascocarps (fruiting bodies) containing numerous asci. Meiospores called ascospores are formed within the asci. The ascocarps produced by the sexual states associated with *Aspergillus* are so different in morphology that the teleomorphs were until recently classified in eleven different genera, reflecting an enormous degree of phylogenetic and biological diversity [108].

Traditionally nine teleomorph genera were linked to *Aspergillus* anamorphs: *Chaetosartorya*, *Emericella*, *Eurotium*, *Fennellia*, *Hemicarpenteles* (now considered to belong to *Penicillium*), *Neosartorya*, *Petromyces*, *Sclerocleista* and *Stilbothamnium*, and two more were added at the beginning of the 2000s: *Neopetromyces* and *Neocarpenteles*. When the single name system came into use all the teleomorph genera were synonymized with *Aspergillus* [100].

Some aspergilli form masses of Hülle cells associated with cleistothecia. Hülle cells are thick-walled specialized multinucleate cells that originate from a nest-like

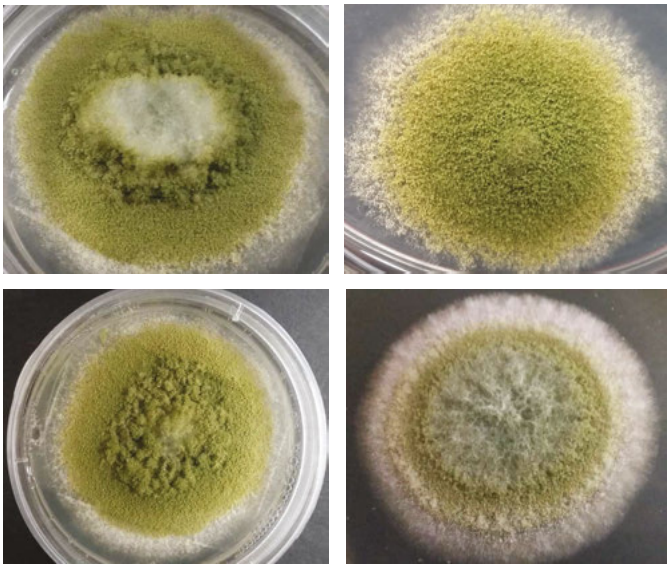

Figure 8.7: Colony surface of isolates of *Aspergillus* spp. belonging to section *Flavi* grown on PDA (top left and right, bottom left) and MEA (bottom right).

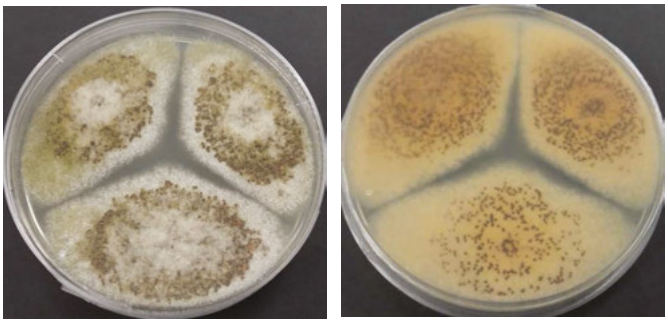

Figure 8.8: Formation of sclerotia by an isolate of *Aspergillus* sp. of section *Flavi* grown on CYA.

aggregation of hyphae during sexual development. They serve as nurse cells to the developing cleistothecium. Other structures found in *Aspergillus* are sclerotia, which are asexual hardened masses of hyphae, yellow to black in color, capable of surviving dormant in soil in harsh environments for long periods until conditions are favorable for growth. Species of *Aspergillus* section *Flavi* form dark sclerotia (Figure 8.8). In some species cleistothecia form embedded within the sclerotia [109].

8.3.5.3 *Penicillium*

The genus *Penicillium* includes over 360 species [99]. Most decompose organic materials and cause devastating rots as pre- and post-harvest pathogens on food crops, producing a wide range of mycotoxins, while other are common indoor air allergens [110]. *Penicillium* species contaminate a wide variety of foods and are capable of growing at refrigeration temperatures, thus frequently spoiling refrigerated food products.

The name *Penicillium* is derived from the Latin word *penicillus*, which means little brush. It was introduced by Link in 1809 due to the similarity of the asexual structures of these fungi with a brush.

8.3.5.4 Main morphological characteristics of *Penicillium* species

The conidiophores of *Penicillium* branch near the apex, forming a brush-like structure (Figure 8.9). Enlarged cells, called metulae, are formed at the apex of the conidiophore. The conidiophores exhibit a well-defined cluster of phialides that are either directly attached to a stipe, or through one or more stages of branching, depending on the species [111].

The conidia of the penicillia are colored, mostly in shades of grey to blue to blue-green (Figure 8.10).

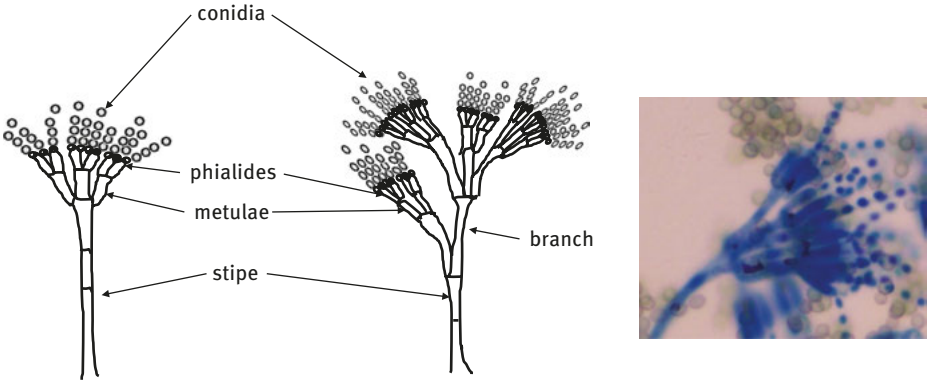

Figure 8.9: On the left, morphological characteristics of the conidiophores in the genus *Penicillium* (drawings not to scale). On the right, photo of a conidiophore of *Talaromyces purpureogenus* (formerly *Penicillium purpureogenum*) stained with cotton blue.

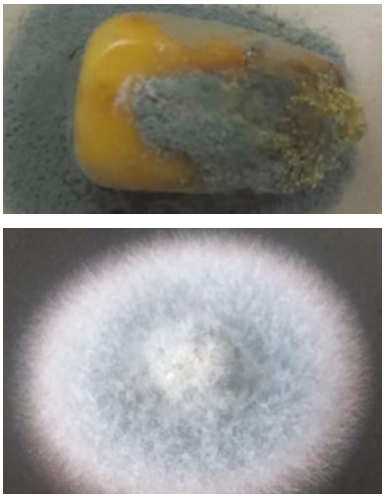

Figure 8.10: Above, blue-gray colonies of *Penicillium* spp. growing from a maize kernel on PDA (yellowish green colonies on the kernel tip correspond to *Aspergillus* sp. of section *Flavi*). Below, colony of a monosporic isolate of *Penicillium* sp. grown on MEA at 25°C.

Some species of *Penicillium* have a sexual state, forming ascospores in cleistothecia. *Eupenicillium*, *Chromocleista*, and *Hemicarpenteles* are teleomorph genera currently included in the one name *Penicillium*. However the teleomorph genus *Talaromyces*, characterized by acerose phialides and usually symmetrical branched conidiophores, was not included and now it is acknowledged as a separate fungal genus [99].

Aspergillus and *Penicillium* are phylogenetically closely related [100], but can be easily differentiated by the morphological features of their conidiophores: the conidiophore of *Aspergillus* has a well defined foot cell and an unbranched often non-septate stipe, while the conidiophore of *Penicillium* is branched, has a septated stipe and lacks a distinct foot cell. In addition, the phialides in *Penicillium* are born successively and not simultaneously as in *Aspergillus* [99].

8.4 Methods for isolation, identification, and characterization of mycotoxigenic fungi

Taxonomic identification of fungi is essential when working with isolates that contaminate food and feed products with mycotoxins, not only for assessing food quality but also for the development of control strategies for ensuring food safety.

Identification of mycotoxigenic species is initially based on the observation of morphological characteristics of isolates, specifically macroscopic characters of colonies grown on culture media and microscopic features of the sexual and/or asexual structures. However, although classical identification methods can be useful to identify some fungi, they frequently provide poor identification sensitivity, have long turnaround times, and can lack specificity for species-level identification [112].

For these reasons, the traditional phenotypic identification is currently completed with molecular techniques, which include extraction of fungal DNA, polymerase chain reaction (PCR) amplification, and sequencing of certain regions of the fungal genome, and phylogenetic analysis. In many occassions molecular techniques are essential for species identification. In biology, phylogenetics is the study of evolutionary relatedness among groups of organisms (*e.g.*, species, populations), which are discovered through molecular sequencing data and morphological data matrices.

The classification and identification of *Fusarium*, *Aspergillus,* and *Penicillium* species were based on phenotypic characters until the early 1990s, but, since then, a polyphasic approach has been implemented in the taxonomy of the three genera. The polyphasic taxonomy integrates several kinds of data and information on the fungal isolates, as phenotypic (macro-morphology of colonies and micro-morphology of fungal structures) and physiological (*e.g.,* growth on different cultivation media at different temperatures and water activity) data, extrolite profiles (in the case of *Aspergillus* and *Penicillium*), and multigene phylogenetic analysis [99]. Extrolites are secondary metabolites, including mycotoxins.

The extrolite profiles are acquiring more and more importance in the polyphasic taxonomy, since they are species-specific [113]. Nevertheless, current identification of *Aspergillus* and *Penicillium* is strongly supported by molecular and phylogenetical analysis and still to a lesser extent by chemotaxonomy [100].

For phylogenetic species recognition of isolates from the three genera, fungal DNA is first extracted and then usually two to four loci are amplified and sequenced. The genes most often used are ITS, beta-tubulin (*BenA*), calmodulin (*CaM*), translation elongation factor 1 alpha (*TEF1a*), RNA polymerase II largest subunit (*RPB1*), RNA polymerase II second largest subunit (*RPB2*), and actin (*Act*). Sequences from the amplified genes are then used to construct gene genealogies and compare them. For a given fungal isolate, the concordance of two or more gene genealogies allows species recognition. The phylogenetic approach that

recognizes fungal species based on concordance of multiple gene genealogies is known as Genealogical Concordance Phylogenetic Species Recognition, and was first endorsed by [114].

There has been a steep increase in the number of accepted *Fusarium*, *Aspergillus*, and *Penicillium* species in the last ten years, and this will probably continue into the next years [99]. This has been related to the introduction of molecular techniques and phylogenetic analysis in many laboratories worldwide.

8.4.1 Detection and isolation of mycotoxigenic fungi

Mycotoxigenic fungi must be isolated from representative food and feed samples properly collected and handled. At the time of collection, it is important to identify clearly each sample with a label. In case samples are perishable, they must be transported to the laboratory in portable coolers.

The detection of mycotoxigenic fungi in plant samples from the field, and in food and feed products, requires the following preliminary procedures:

1. Use a laminar flow hood. Prepare a safe and sterile workspace. Keep all surfaces dry and clean. Keep in mind that surfaces at the back of the hood and objects close to the table surface are more likely to remain sterile. When handling material potentially contaminated with mycotoxigenic fungi, wearing of a laboratory coat, disposable gloves, and safety glasses is recommended.
2. Prepare agar media for fungal cultivation (Figure 8.11). All ingredients must be mixed up and then heated to dissolve. Afterwards, the media must be sterilized in an autoclave at 121°C for 15 min. Verify for correct pH before autoclaving.

   The agar medium should be tempered to 45–50°C before dispensing it aseptically into the base of petri dishes of 90 mm diameter. This will minimize condensation in the lid of the dish. Also it is an essential step if a heat-sensitive solution (*e.g.*, an antibiotic) is to be added [98]. Pass the neck of agar bottle through flame, open petri dish lid as little as possible and dispense approximately 20 mL of agar medium per petri dish of 90 mm diameter. Wait until the medium solidifies. Petri plates can be stored in an inverted position in a refrigerator until use, in order to prevent drying of the agar.

   Although agar media in petri dishes are usually employed, the same media can also be used in agar slants (agar slope) in test tubes. A slant is a tube placed at an angle during cooling to give a large slanted surface for fungal inoculation. In this case, once prepared, the medium is dispensed in to the slants with a syringe or some other repeating dispenser before autoclaving. Test tubes of 10 × 75, 13 × 100, and 16 × 150 mm, receiving 1.25, 2.5, and 6.0 mL of medium per slant, respectively, are most commonly used [98]. After adding the medium, tubes are stoppered with plastic foam plugs or aluminum foil, placed in a rack,

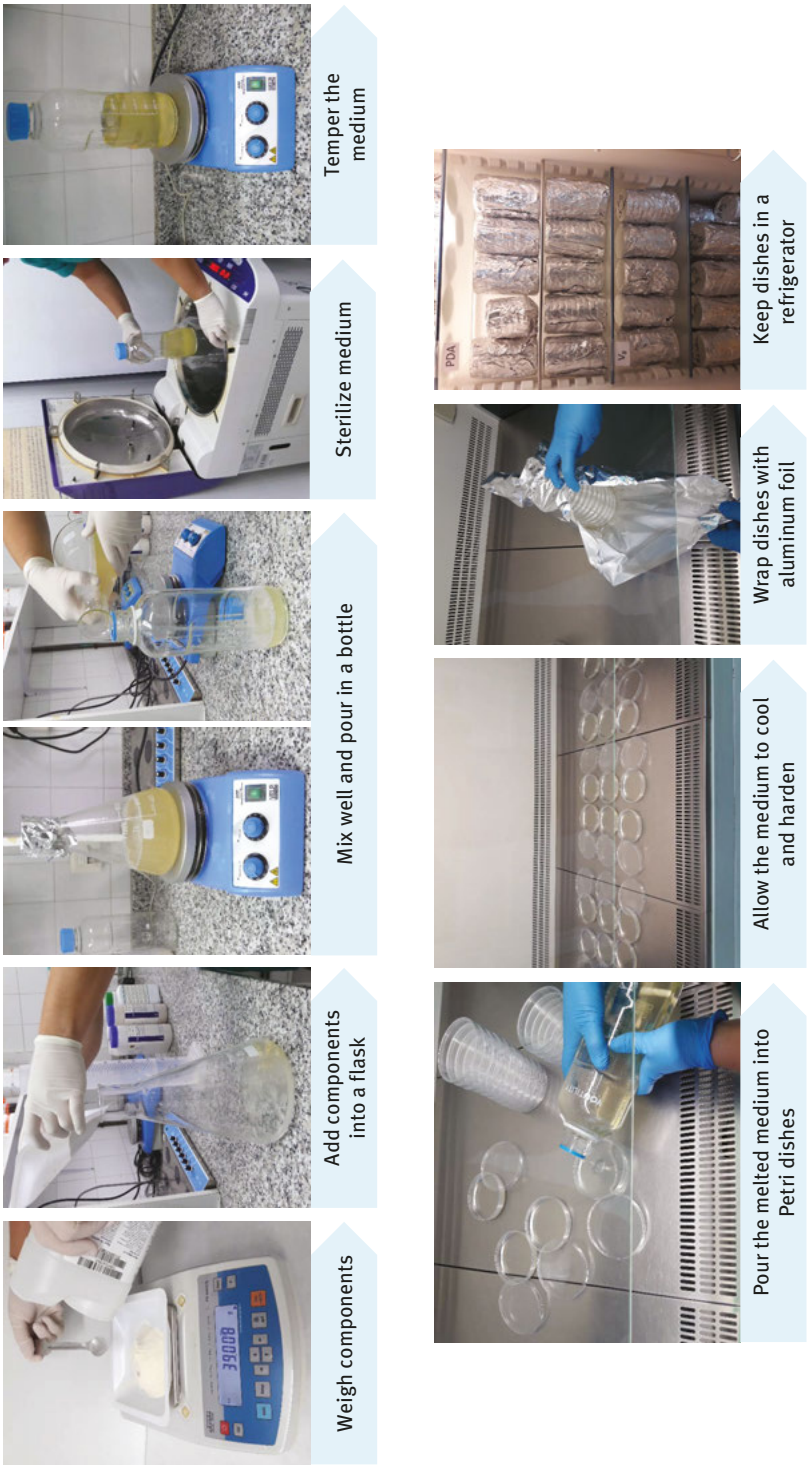

Figure 8.11: Procedure for preparing agar media for fungal cultivation.

and autoclaved. Afterwards the medium in the test tube is allowed to harden in a slanted position (some test tubes are specifically set up for this), laying on their sides using a pipet to keep them tilted up. Slants should then be stored in a vertical position in a refrigerator until use.

3. Sterilize not only solutions and media but all instruments before use. Autoclave all material at 121°C for 15 min.

8.4.1.1 Media for isolation of *Fusarium, Aspergillus*, and *Penicillium* species

The standard media used for isolation of *Fusarium* are potato dextrose agar (PDA), which is a general-purpose media for growing a wide range of fungi, and Komada, which is a *Fusarium*-selective medium. Also, malt extract agar (MEA) and water agar can be used. Spezieller Nährstoffarmer Agar (SNA) and Carnation Leaf-piece Agar (CLA) are also employed, together with PDA, for species identification [98].

PDA, MEA, and more complex agar media such as Czapek agar (CZ) and Czapek yeast Autolysate agar (CYA) are commonly used for the isolation of *Aspergillus* and *Penicillium* species.

Antibiotics, such as chloramphenicol and streptomycin, may be added to molten agar media (after autoclaving) to inhibit bacterial growth on plates. Also dichloran, Rose Bengal, or NaCl are routinely added to agar media to inhibit fast-growing molds, such as *Rhizopus* and *Mucor* species [109].

In Table 8.4, the composition of media recommended for isolation and identification of species of *Fusarium*, *Aspergillus,* and *Penicillium* is shown.

8.4.1.2 Plating methods

For the detection of mycotoxigenic fungi in food and feed products, dilution plating and direct plating in general and specific cultivation media are the most widely used methods [92].

In the dilution plating method, a food homogenate is prepared and then serial dilutions of the homogenate are made followed by plating. For this one gram (dry weight) of the sample is ground up (if needed) and dispersed in 9 mL of sterile water. One milliliter of this solution is transferred to a second tube containing 9 mL of sterile water, resulting in a 0.01 dilution of the spore mass in the original sample. This process is repeated to yield dilutions of 0.001, 0.0001, etc. For plating, either the pour-plate method or the spread-plate method can be used. In the pour-plate method, the diluted sample is pipetted into a sterile petri dish, and then a sterile melted medium is added and mixed with the sample. In the spread-plate method, the diluted sample is pipetted and spread evenly onto the surface of the agar plate with the help of a sterile glass spreader.

Table 8.4: Media for fungal cultivation. Adapted in part from [100,110].

Potato Dextrose Agar (PDA)	
White potatoes	250 g
Dextrose,	20 g
Agar	20 g
Distilled water	1,000 mL

Czapek stock solution (concentrate)	
$NaNO_3$	30 g
KCl	5 g
$MgSO_4 \cdot 7H_2O$	5 g
$FeSO_4 \cdot 7H_2O$	0.1 g
Distilled water	100 mL

Trace elements stock solution	
$CuSO_4 \cdot 5H_2O$	0.5 g
$ZnSO_4 \cdot 7H_2O$	0.1 g
Distilled water	100 mL

Czapek's Agar (CZ)	
Czapek concentrate	10 mL
Sucrose	30 g
$CuSO_4 \cdot 5H_2O$	0.005 g
$ZnSO_4 \cdot 7H_2O$	0.001 g
Agar	20 g
Distilled water	1,000 mL

Czapek Yeast Autolysate Agar (CYA)	
Czapek concentrate	10 mL
Sucrose	30 g
Yeast extract	5 g
$CuSO_4 \cdot 5H_2O$	0.005 g
$ZnSO_4 \cdot 7H_2O$	0.01 g
Agar	20 g
Distilled water	1,000 mL
pH 6.2 ± 0.2	

Malt Extract Agar (MEA)	
Malt extract	50 g
$CuSO_4 \cdot 5H_2O$	0.005 g
$ZnSO_4 \cdot 7H_2O$	0.01 g
Agar	20 g
Distilled water	1,000 mL

Malt Extract 20% Sucrose Agar (M20S)	
Malt extract	50 g
Sucrose	200 g
$CuSO_4 \cdot 5H_2O$	0.005 g
$ZnSO_4 \cdot 7H_2O$	0.01 g
Agar	20 g
Distilled water	1,000 mL
pH 5.4 ± 0.2	

Yeast Extract Sucrose Agar (YES)	
Yeast extract	20 g
Sucrose	150 g
$MgSO_4 \cdot 7H_2O$	0.5 g
$CuSO_4 \cdot 5H_2O$	0.005 g
$ZnSO_4 \cdot 7H_2O$	0.001 g
Agar	20 g
Distilled water	885 mL
pH 6.5 ± 0.2	

Spezieller Nährstoffarmer agar (SNA)	
Sucrose	0.2 g
Glucose	0.2 g
KH_2PO_4	1 g
KNO_3	1 g
$MgSO_4 \cdot 7H_2O$	0.5 g
KCl	0.5 g
Agar	20 g
Distilled water	1,000 mL

Table 8.4: (continued)

Creatine Sucrose Agar (CREA)	
Sucrose	30 g
Creatine·$5H_2O$	3 g
$K_3PO_4 \cdot 7H_2O$	1.6 g
$MgSO_4 \cdot 7H_2O$	0.5 g
KCl	0.5 g
$FeSO_4 \cdot 7H_2O$	0.01 g
$CuSO_4 \cdot 5H_2O$	0.005 g
$ZnSO_4 \cdot 7H_2O$	0.01 g
Bromocresol purple	0.05 g
Agar	20 g
Distilled water	1,000 mL
pH 8 ± 0.2	

Oatmeal agar	
Oatmeal flakes	30 g
$CuSO_4 \cdot 5H_2O$	0.005 g
$ZnSO_4 \cdot 7H_2O$	0.01 g
Agar	20 g
Distilled water	1,000 mL
pH 6.5 ± 0.2	

Autoclave flakes (121°C for 15 min) in 1,000 mL of distilled H_2O. Squeeze mixture through cheese cloth and use flow through, topping up to 1,000 ml with distilled water with 20 g agar.

Komada-*Fusarium* medium	
Part 1	
$Na_2B_4O_7 \cdot 10H_2O$	1 g
K_2HPO_4	1 g
KCl	0.5 g
$MgSO_4 \cdot 7H_2O$	0.5 g
Fe-Na-EDTA	0.001 g
D-Galactose	20 g
L-Asparagine	2 g
Agar	15 g
Distilled water	1,000 mL
Part 2	
PCNB (Pentachloronitrobenzene) (Terraclor 75%)	1 g
Oxgall (Bile Bovine)	0.5 g
Streptomycin sulfate	0.3 g
pH 3.9 ± 0.1	

Mix components of part 1 and boil to melt agar. Then, cool the medium until 50–55°C and add components of part 2.

Dichloran Glycerol Agar (DG18)	
Peptone	5 g
Glucose	10 g
KH_2PO_4	1 g
$MgSO_4 \cdot 7H_2O$	0.5 g
Dichloran	0.002 g
Chloramphenicol	0.1 g
Agar	15 g
Distilled water	1,000 mL
pH 5.6 ± 0.2	

In the direct plating method, the sample is usually surface disinfected with 5% sodium hypochlorite or 70% ethanol–water solution before plating. Working in a laminar airflow hood, forceps, sterilized by passing them through a flame and allowed to cool, should be used to remove pieces of the sample from the disinfecting solution. Then the sample pieces should be blotted on a sterile paper towel. In case samples are not surface disinfected, it is advisable to keep them at –20°C to kill mites and insects that might interfere with fungal isolation [109]. Plating consists of placing pieces of the sample on the surface of the agar in petri dishes (Figure 8.12). Whatever the plating method used, agar plates are then incubated at 25°C in the dark for seven days or longer.

8.4.2 Obtaining monosporic fungal isolates

Fungal colonies grown from the sample pieces in agar plates will have to be subcultured until obtaining pure cultures. For species identification, it is essential to obtain pure cultures of the mycotoxigenic fungi that may contaminate food and feed commodities.

Small pieces of agar containing hyphal tips should be cut from the growing edge of every distinct colony visible on the plate and transferred to another agar plate and incubated again until obtaining pure cultures, with no contamination of other fungal isolates or bacteria. This can be done with the help of a dissecting microscope placed inside the laminar airflow hood.

Another way to obtain a pure culture of a fungal isolate is to try to remove single germinated conidia with the help of a sterile needle, previously dipped in sterile distilled water, and place them onto agar medium (in a petri dish or a slant). When dealing with *Fusarium* isolates, monosporic cultures are obtained, as a standard, from an agar piece carrying a single germinated conidium that is placed in the center of an agar plate or the center of the surface in an agar slant (Figure 8.13). Monosporic isolates can be stored at 4°C on agar plates.

8.4.3 Morphological characterization

Once monosporic cultures of *Fusarium*, *Aspergillus* and *Penicillium* are obtained, the next step is made subcultures on specific media for morphological characterization.
Morphological identification of *Fusarium* species generally is based on characters observed in pure cultures of isolates grown on PDA, SNA, or CLA. Colony morphology, pigmentation, and growth rates must be observed in PDA plates. Shape and size of macroconidia; shape, size, and formation of microconidia; and production of chlamydospores can be recorded in colonies growing in PDA but especially in SNA and CLA [98]. Some *Fusarium* species have particular morphological features that allow their

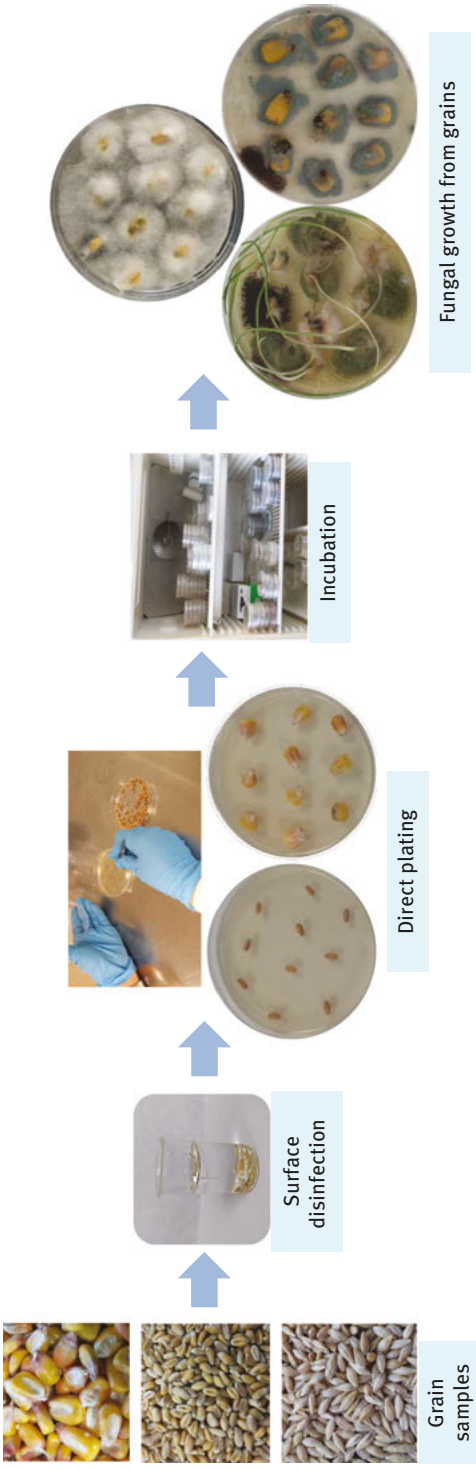

Figure 8.12: Isolation of mycotoxigenic fungi by direct plating method. On the left (from top to bottom): grains of maize, wheat, and barley. On the right: above, colonies of *Fusarium* spp. growing from barley grains; below, colonies of *Aspergillus* spp. (green and black mycelia) and *Fusarium* spp. (pinkish white mycelia) growing from wheat grains (left), and colonies of *Penicillium* spp. (blue grayish mycelia) and *Aspergillus* spp. (black mycelia) growing from maize kernels (right).

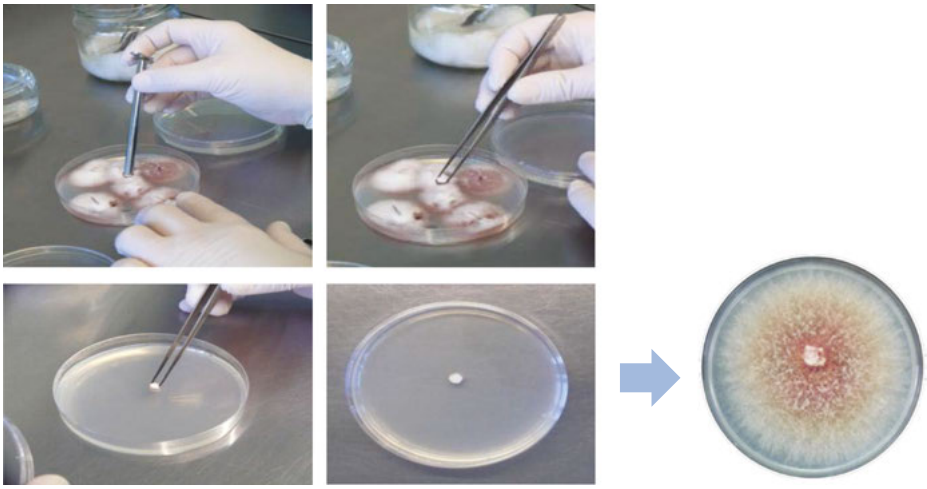

Figure 8.13: Subcultivation of fungal isolates grown from wheat grains for obtaining monosporic cultures. On the right, a monosporic isolate of *Fusarium culmorum* grown on PDA.

identification, but many do not. In any case, morphological characterization of *Fusarium* species is currently complemented with molecular and phylogenetic analysis.

Samson et al. [100] and Visagie et al. [110] recently recommended the use of the following standardized methods for the subculture of *Aspergillus* and *Penicillium* isolates for their identification and characterization based on morphological characters:

1. Prepare spore suspensions in a 30% glycerol SS-buffer (0.5 g/L agar, 0.5 g/L Tween 80) solution, which can be stored at −80°C. Spore suspensions can be also made in a 0.2% agar and 0.05% Tween 80 solution, and stored at 4°C.
2. Prepare cultivation media in 90 mm petri dishes (preferentially vented) with a volume of 20 mL of medium per plate. Media recommended as standard for *Aspergillus* and *Penicillium* are CYA and MEA. Additional media, such as CZ, Yeast Extract Sucrose agar, Dichloran 18% Glycerol agar (DG18), oatmeal agar, and creatine sucrose agar (CREA), among others, can be used to observe a wider range of morphological characters. Some morphological characters of *Aspergillus* isolates may vary depending on the cultivation media and incubation conditions.
3. Inoculate the corresponding spore suspension in CYA and MEA plates in three-point pattern (Figure 8.14) using a micropipette (0.5–1 µl per spot). Do not wrap plates with Parafilm®, since air exchange restriction often inhibits growth and sporulation [115].
4. After inoculation, incubate CYA and MEA plates reverse side up at 25°C for seven days in the dark, setting additional CYA plates incubated at 30 and 37°C that are useful to distinguish between species. For *Aspergillus* isolates of the section *Circumdati* additional CYA plates incubated at 30°C are recommended, while for the section *Fumigati* 45–50°C are recommended.

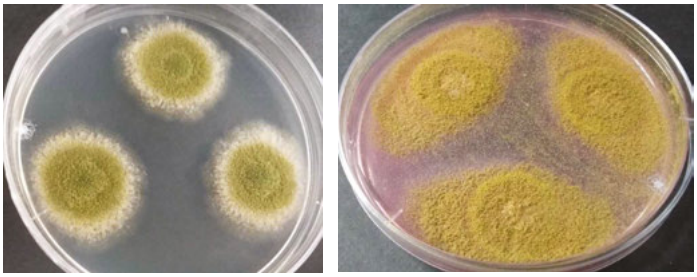

Figure 8.14: Colonies of two isolates of *Aspergillus* spp. of section *Flavi* grown from spore suspensions inoculated on CYA (left) and DG18 (right) plates in three-point pattern.

After seven days of growth, macroscopic and microscopic features of the isolates must be recorded. Macroscopic characteristics of *Aspergillus* are: colony growth rates, degree of sporulation, production of sclerotia or cleistothecia, colors of mycelia, formation of soluble pigments, exudates, colony reverses, Hülle cells, and cleistothecia [100].

In *Penicillium* isolates, important macroscopic characters are: colony texture, degree of sporulation, the color of conidia, the abundance, texture and color of mycelia, the presence and colors of soluble pigments and exudates, colony reverse colors, and degree of growth and acid production on CREA. As in the case of *Aspergillus*, some species may produce cleistothecia or sclerotia, but mostly after longer incubation times, especially on oatmeal agar. Thus oatmeal agar plates should be incubated for prolonged periods [110].

Microscopic characteristics of asexual and sexual reproductive structures of *Aspergillus* and *Penicillium* are important diagnostic characters. In *Aspergillus* isolates, the shape of conidial heads; the presence or absence of metulae between vesicle and phialides (i.e., uniseriate or biseriate); color of stipes; and the dimension, shape, and texture of stipes, vesicles, metulae (when present), phialides, conidia, and Hülle cells (when present) should be recorded. The same must be done for cleistothecia, asci, and ascospores. Also important for identifying species are the size, morphology, and, especially, ornamentation of ascospores.

The same applies for *Penicillium* isolates. Microscopic characteristics of conidiophores and cleistothecia (when produced) should be observed. It is important to accurately describe the branching patterns of conidiophores, to record the wall texture/ornamentation of stipes and conidia, and the dimension, shape, and texture of stipes, vesicles, metulae/branches (when present), phialides, conidia, cleistothecia, asci, and ascospores (when present).

For observing microscopic characteristics, preparations are usually made from fungal cultures on PDA or SNA in the case of *Fusarium*, and on MEA in that of *Aspergillus* and *Penicillium*, after 7–10 days of growth, although other media can also be used. In fact, oatmeal agar is a good medium for observing cleistothecia. As a mounting fluid for preparations, the use of lactic acid (60–70%) or Shear's solution or lactic

Table 8.5: Mounting media.

Shear's mounting medium	
Potassium acetate	6 g
Glycerine	120 mL
Ethanol 95%	180 mL
Distilled water	300 mL
Ink blue	1 g

Lactofuchsin	
Acid fuchsin	0.1 g
Lactic acid (85%)	100 mL

Cotton blue in lactic acid	
Cotton blue	0.01 g
Lactic acid (85%)	100 mL

acid with cotton blue is recommended. Lactofuchsin can be used for *Fusarium* cultures. The composition of mounting fluids is presented in Table 8.5.

For staining fungal structures of *Fusarium*, *Aspergillus*, and *Penicilllium* isolates, a small drop of mounting medium can be first placed on a microscope slide (Figure 8.15). Then, using a sterilized dissecting or inoculating needle, a small portion of the colony must be removed and placed in the mounting medium, and, with a second needle, teased it out. Mounts can be washed with drops of 70% ethanol to prevent air bubbles and wash away excess conidia in the case of *Aspergillus* isolates. Then a cover-slip should be carefully placed over the mount, lowering one edge to the slide before the other.

When possible, the use of differential interference contrast light microscopy (= Nomarski) is highly recommended for best observation of conidial ornamentation and conidiophore characters [100, 110], since this technique produces high-contrast optical images of the edges of objects and fine structural detail within transparent specimens (Figure 8.16).

8.4.4 Molecular and phylogenetic identification

For phylogenetic identification of fungal species, the following molecular techniques have to be carried out (Figure 8.17): extraction of fungal DNA, PCR amplification, and sequencing of certain regions of the fungal genome that are associated with certain locations/functions, generally referred to as molecular markers.

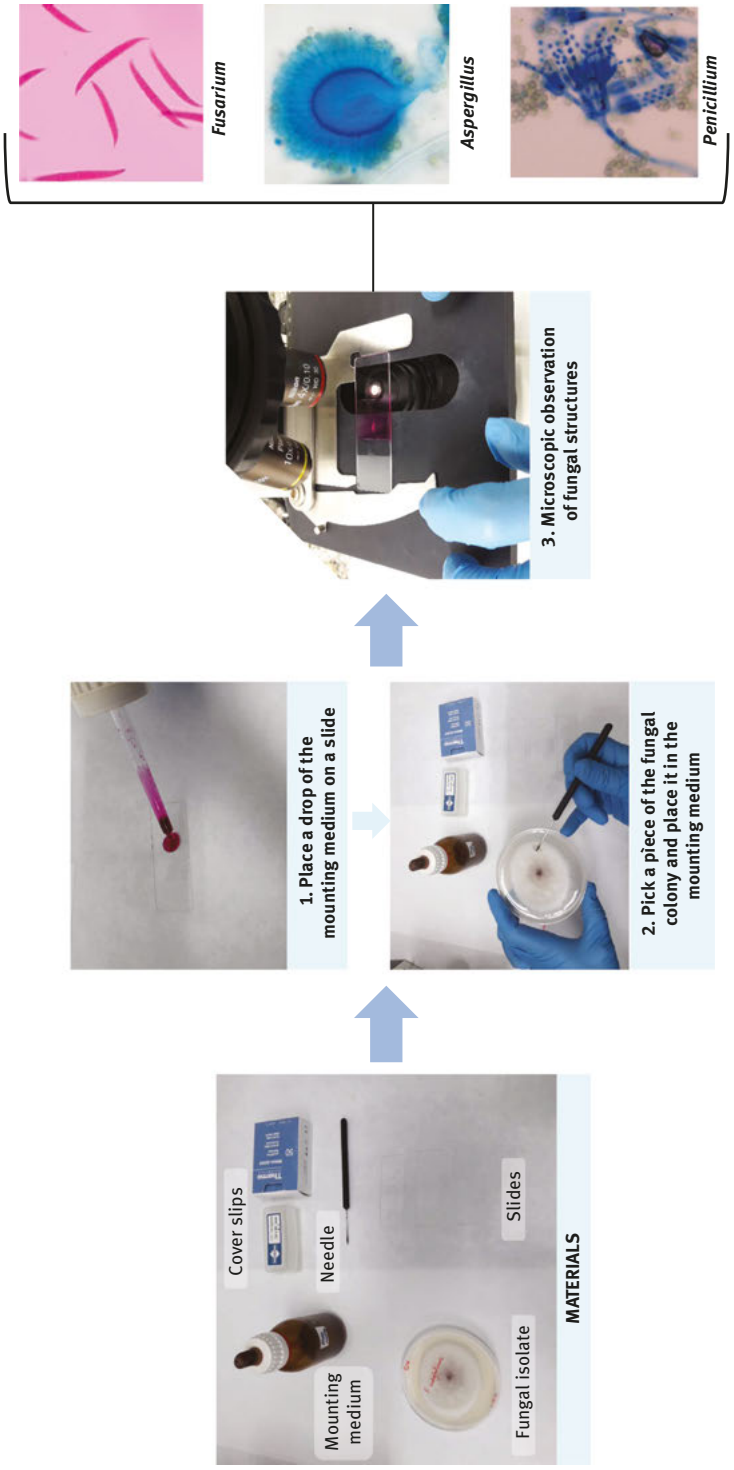

Figure 8.15: Procedure for staining and mounting slides for microscopic observation of fungal isolates. On the right (from top to bottom), macroconidia of *Fusarium graminearum*, a conidiophore of *Aspergillus flavus*, and a conidiophore of *Talaromyces purpureogenus* (formerly *Penicillium purpureogenum*) are shown.

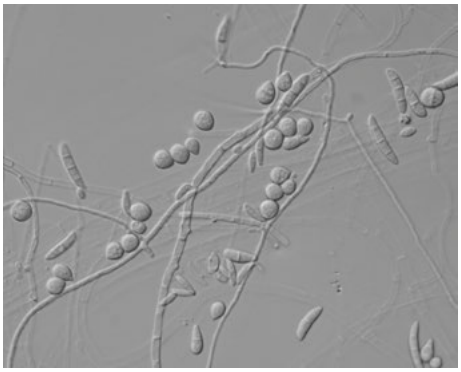

Figure 8.16: Microconidia of *Fusarium sporotrichioides* observed in a microscope by Nomarski interference contrast.

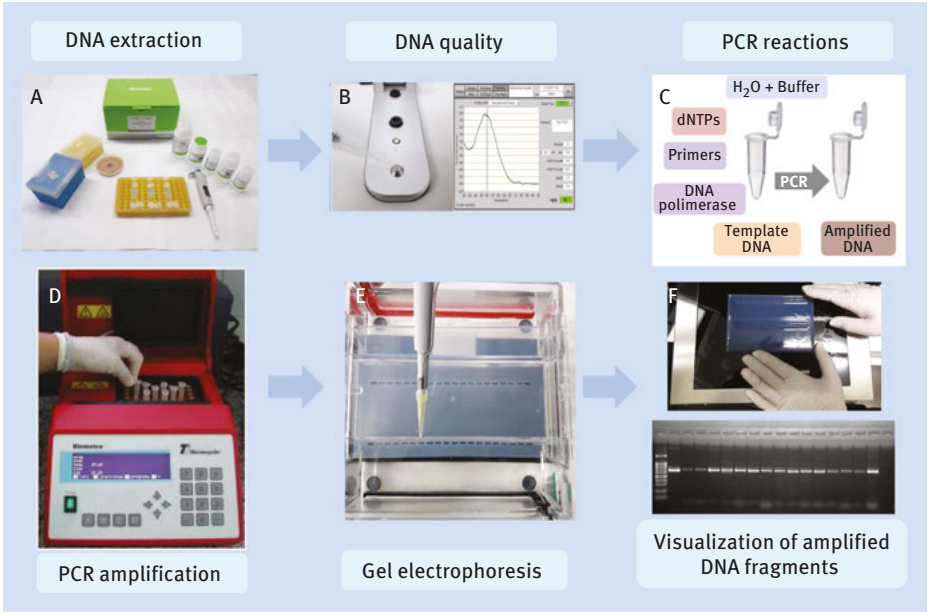

Figure 8.17: Methods for molecular identification of mycotoxigenic fungi: extraction of fungal DNA (A), determination of quality and quantity of template DNA (B), PCR amplification of certain regions of the fungal genome (C,D), electrophoresis of PCR products in agarose gels (E), and examination of amplified DNA products under a UV transilluminator (F).

8.4.4.1 Extraction of genomic DNA

Accurate identification of mycotoxigenic fungi using a sequence-based approach requires an extraction method that yields template DNA pure enough for PCR or other types of amplification [116]. There are commercial kits that enable rapid extraction of fungal DNA extraction. Quantity, quality, and integrity of

template DNA must be subsequently determined. The most common technique to determine DNA concentration and purity is measurement of absorbance (optical density) using an UV spectrophotometer. The DNA concentration is determined from the absorbance at 260 nm. The most common method to assess purity is to calculate the ratio of the absorbance at 260 nm divided by the reading at 280 nm, which gives an indicator of purity from protein contaminants (protein absorbs at 280 nm while nucleic acids absorb at 260 nm). Good-quality DNA will have an A_{260} /A_{280} ratio of 1.7–2.0. Lower values may indicate protein contamination. Another indicator of sample purity is the 260/230 ratio, which should be around 2.0–2.2. Low A_{260} /A_{230} ratios are related to contamination with buffer salts or organic compounds. DNA quantity and integrity can be determined by agarose (0.5% wt/vol) gel electrophoresis at 60 V using an appropriate DNA ladder as molecular weight markers, followed by gel staining and visualization under UV light.

8.4.4.2 PCR amplification and sequencing

Once good-quality fungal DNA is extracted, two to four loci are usually amplified and sequenced (Figure 8.18). The internal transcribed spacer region of the nuclear rDNA, which encompasses the noncoding transcribed spacers ITS1 and ITS2 and the 5.8S rDNA gene (ITS1-5.8S-ITS2), is the official barcode for fungi, because it has primers that work universally [117]. However, in *Fusarium*, *Aspergillus*, *Penicillium* and many other genera of ascomycetes, the ITS region is not variable enough for distinguishing closely related species. In addition, the ITS region is characterized by frequent size variation and the presence of repeated sequences, which hinders sequence alignment above the genus level [118].

As secondary marker, the more commonly sequenced gene for *Fusarium* is the translation elongation factor 1 alpha (*TEF1a*), although other genomic DNA genes, such as beta-tubulin and histone H3, and mitochondrial DNA genes have also been used. *TEF1a*, which encodes an essential part of the protein translation machinery, shows a high level of sequence polymorphism among closely related species, even in comparison to the intron-rich portions of protein-coding genes such as calmodulin, beta-tubulin and histone H3. Thus, *TEF1a* has high phylogenetic utility because it is highly informative [119].

Samson et al. [100] proposed the use of the calmodulin gene (*CaM*) as a temporary secondary identification marker in *Aspergillus*, whereas Visagie et al. [110] proposed the beta-tubulin (*BenA*) for routine identification of isolates in the case of *Penicillium*. ITS1-ITS4 are primers used for amplification of the ITS region [120]), CMD5-CMD6 [121]) and CF1-CF4 [122] for the *CaM* gene, and Bt_2a-Bt_2b for the *BenA* gene [123]. For amplification of ITS, *CaM* and *BenA* genes, the following conditions are used: one cycle at 94°C for 5 min; 35 cycles at 94°C for 45 s, 55°C for 45 s, 72°C for 1 min; and a final cycle at 72°C for 7 min.

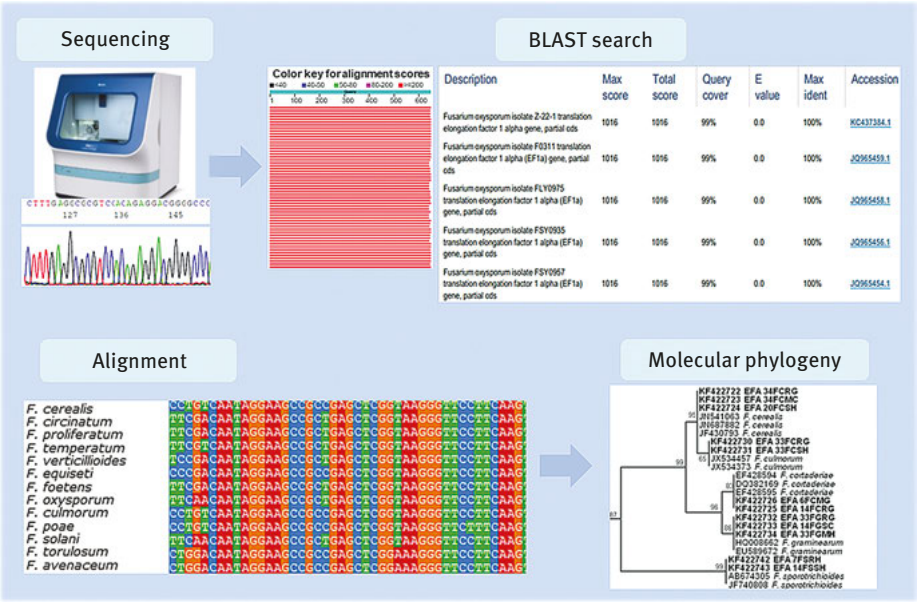

Figure 8.18: Methods for molecular phylogenetic identification of mycotoxigenic fungi: sequencing of amplified DNA regions, comparison of sequences by BLAST search and alignment, and construction and study of phylogenetic trees for species identification. At the bottom right, example of a phylogenetic tree (dendrogram) for identification of *Fusarium* isolates.

The PCR products are electrophoresed in agarose gels that are subsequently stained and examined under a UV transilluminator (Figure 8.17). Products of PCR are then purified and sequenced. Sequences are usually compared with related fungal ITS, *TEF1a*, *CaM*, and *BenA* sequences using the BLAST search on NCBI's GenBank sequence database or other curated specialized databases containing fungal sequences (*e.g.*, FUSARIUM-ID, RefSeq Target Loci (RTL)). Phylogenetic analyses of nucleotide sequences of ITS, *TEF1a*, *CaM*, and *BenA* genes are finally carried out for isolate identification. Sequences are used to construct and study phylogenetic trees (Figure 8.18). A phylogeny (also called a dendrogram) is a graph-like structure whose topology describes the inferred evolutionary history among a set of biological entities, such as species or DNA sequences.

As revised by Niessen [124], molecular studies on mycotoxigenic fungi are not only focused on species identification, what is important, but many are addressed to study mycotoxin pathway gene sequences that could allow to identify fungal species and their toxigenic potential. The genetics and regulation of trichothecene biosynthesis have received much attention and currently several genes from the trichothecene biosynthesis cluster (such as *tri5*, *tri6*, *tri7*) are used to design species- and group-specific PCR primers. These studies have resulted in the knowledge of different chemotypes

in *Fusarium graminearum*. Similar research has been undertaken for fumonisin-producing species. It has been shown that genes *fum1* (= *fum5*), *fum6,* and *fum8* are only present in *Fusarium verticillioides, Fusarium proliferatum, Fusarium fujikuroi* and *Fusarium nygamai*, which represent the principle producers of fumonisins within the *Fusarium fujikuroi* complex.

Other studies have aimed to combine qualitative and quantitative methods for detecting the toxigenic potential of fungal species colonizing food crops. One of the approaches is based on multiplex real-time PCR which have been used to detect and quantify fungal species in cereal grains by using markers targeting the trichothecene synthase (*tri5*) gene in trichothecene-producing *Fusarium* sp. isolates, the rRNA gene in *Penicillium verrucosum,* and the polyketide synthase gene (*Pks*) in *Aspergillus ochraceus* [125].

Molecular detection of mycotoxigenic fungi is thus a valuable tool that can help to optimize food and feed production processes for minimized risk of mycotoxin production [124].

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Abbreviations: a_w: water activity; α-ZOL: alpha-zearalenol; α-ZAL: alpha-zearalanol; β-ZAL: beta-zearalanol; β-ZOL: beta-zearalenol; BenA: beta-tubulin gene; Ca M: calmodulin gener; CLA: carnation leaf-piece agar; CREA: creatine sucrose agar; CYA: Czapek yeast Autolysate agar; CPA: cyclopiazonic acid; CZ: Czapek agar; DG18: dichloran glycerol agar; DON: deoxynivalenol; FB_1: fumonisin B_1; FB_2: fumonisin B_2; FB_3: fumonisin B_3; ITS: internal transcribed spacer region; MEA: malt extract agar; M20S: malt extract 20% sucrose agar; OTA: ochratoxin A; PAT: patulin; PCR: polymerase chain reaction; PDA: potato dextrose agar; SNA: spezieller nährstoffarmer agar; TEF1α: translation elongation factor 1 alpha gene; YES: yeast extract sucrose agar; ZEN: zearalenone

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9 Analysis of environmental toxicants

9.1 Introduction

The movement and fate of environmental contaminants are key aspects to determine their impact in the environment. As a general principle, the transportation and fate of contaminants is controlled by their physical transport and reactivity. It involves either chemical or biochemical reactions or other physical interactions with other phases, therefore those chemicals released into the environment rarely remain in the form, or at the location, of release. Figure 9.1 shows the main ways involved in transport and chemical fate. Most often, those substances are originated in the anthroposphere and they can pass to the air, earth, water (either surface or ground waters), sediments, and to the biota (plants and animals).

The dilution of the toxicant in question as well as transfer among living creatures can happen and therefore concentration or bioaccumulation takes place, while at the same time most transport between environmental phases results in wider dissemination; for instance, lipid-soluble toxicants are readily taken up by organisms following exposure in air, water, or soil. They can persist in the tissues long enough to be transferred to the next trophic level, unless they are quickly metabolized. At this moment, if the organism is more susceptible than those at the previous level, the toxicant can become deleterious.

In order to study toxicants from the point of view of the analytical methodologies to identify them, it is convenient to classify them according to their chemical behavior. However, no single classification method is applicable for the entire spectrum of toxic agents present in the environment. In this chapter the classification is based on the analytical behavior of toxicants due to the fact that toxicants can be grouped according to the analytical procedures involved to measure them. In the Stas-Otto scheme [1], toxicants have been divided into the following groups:

(a) Volatile toxicants, e. g., hydrocianic acid, alcohols, acetone, phenol, chloral hydrate.
(b) Extractive toxicants:
    (i) Toxicants extractable by ether from acid solution, e. g., organic acids, nitro compounds.
    (ii) Toxicants extractable by ether from alkaline solution, e. g., alkaloids.
    (iii) Metals and metalloids, e. g., copper, mercury, zinc, silver, antimony.

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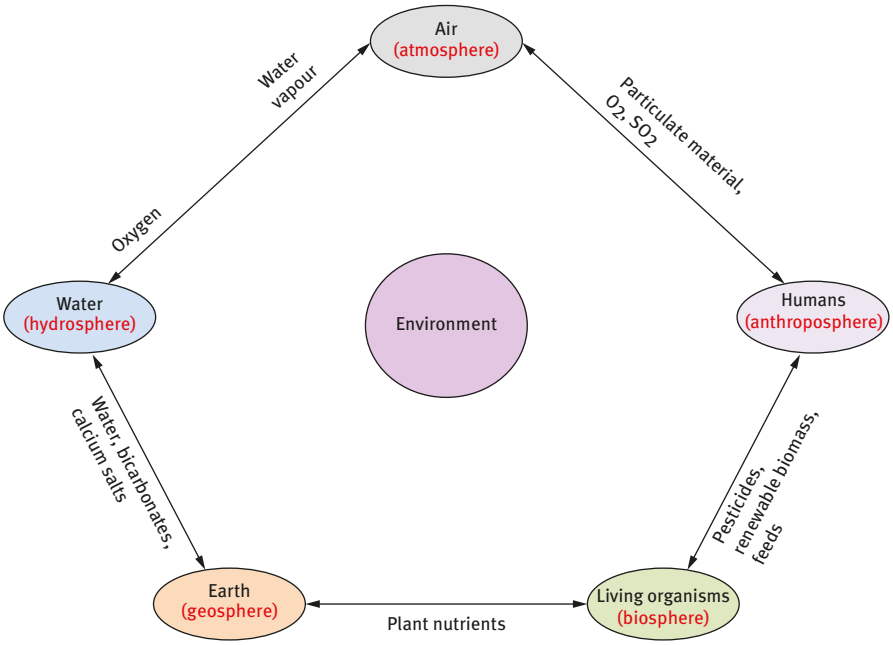

Figure 9.1: Interchange of some environmental toxicants delivered between anthroposphere and the other environmental sections that are involved in chemical fate.

9.2 General guidelines of chemical analysis in the environment

Chemical analysis of toxicants in the environment can be carried out in two different ways, depending on: weather toxicants are in high concentrations or in low concentrations. The second possibility is worth looking at in much detail, because the analytical procedures involved will be much more complex. In order to be able to determine very small amounts (very low concentrations) of chemicals in the environment, it is necessary to follow a series of operations (Figure 9.2):

1. isolation (extraction and separation) of the chemicals of interest from sample matrix (air, water, sediment, living beings, etc.)
2. separation and purification of the chemical of interest from other co-extracted chemicals (sample cleanup)
3. if necessary, sample concentration
4. measurement by highly selective and sensitive analytical equipment.

Occasionally, it is also necessary to derivatize (chemically modify) the chemicals of interest prior to their analysis.

Within the broad range of instrumental methods used in environmental analysis, there are three methods which are the most commonly implemented ones, namely:

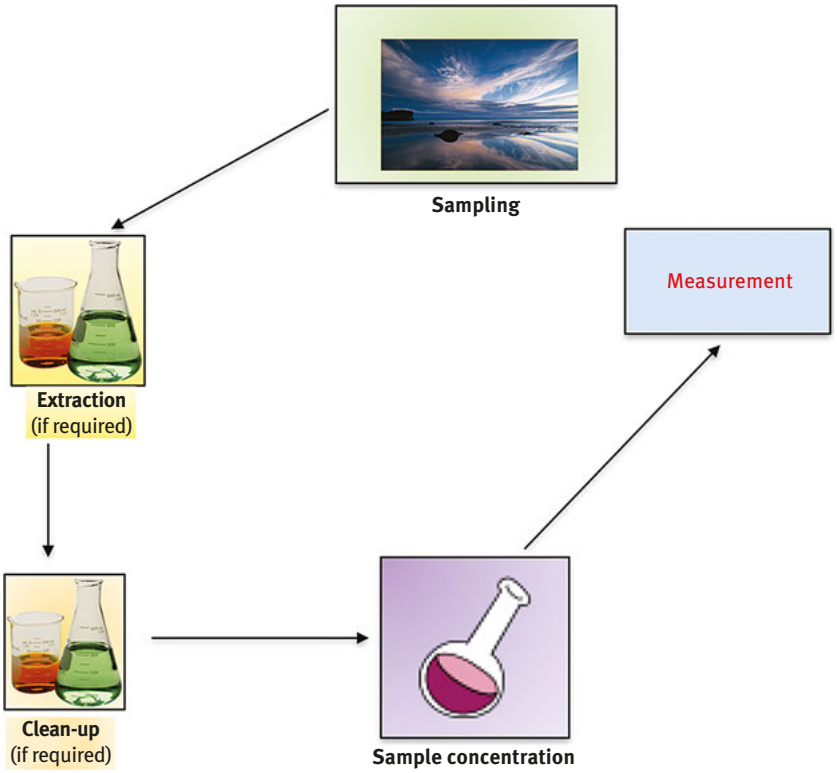

Figure 9.2: General guidelines in chemical analysis of environmental samples.

atomic spectrometry, GC, and LC. The following section describes the series of operations in more detail, focusing on these three groups of instrumental methods.

9.2.1 Isolation (extraction and separation)

The methods for separation of toxicants depend on the type of sample, and in this section samples are divided into three types: water samples; sediment, soil, and biological samples; and air samples. In Figure 9.3 the extraction and separation methods regarding the type of sample are shown.

9.2.2 Separation and purification

Extracts from environmental samples can be complicated mixtures. Components of these mixtures can interfere with the analytical methods to be used, especially in the case of GC and LC analyses by giving poor separation because of over separation

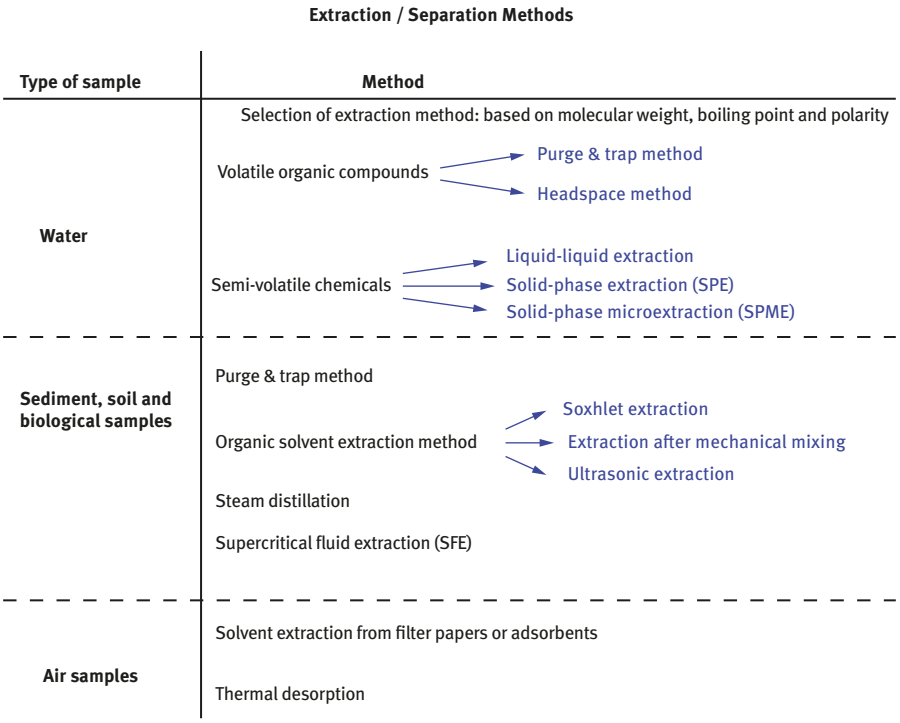

Figure 9.3: Extraction and separation methods regarding the type of sample.

capacity of a column or by containing compounds that elute at the same time as the peaks of the compounds of interest. It is, therefore, necessary to clean up, or remove, those co-extracted compounds as much as possible. Cleanup methods include acid-alkaline partition, acetonitrile-hexane partition, and column chromatography. Column chromatography separation can involve adsorption, partition, gel permeation, ion exchange, etc. Figure 9.4 summarizes all the possibilities.

9.2.3 Sample concentration

When sample needs to be concentrated, either a concentrator (like a Kuderna-Danish concentrator) or rotary evaporator can be used: it depends on the boiling point of the compounds of interest, their sublimation character, timeframe for analysis, etc.

The concentration setup takes longer than the evaporation setup, and is applicable to low-boiling point compounds as well as to high-boiling point compounds. The rotary evaporation can concentrate large volumes of samples in a relatively short period of time, although it causes big evaporative losses and is not suitable for low-boiling point compounds. However, for further concentration one must use a micro-column or evaporate under a stream of nitrogen.

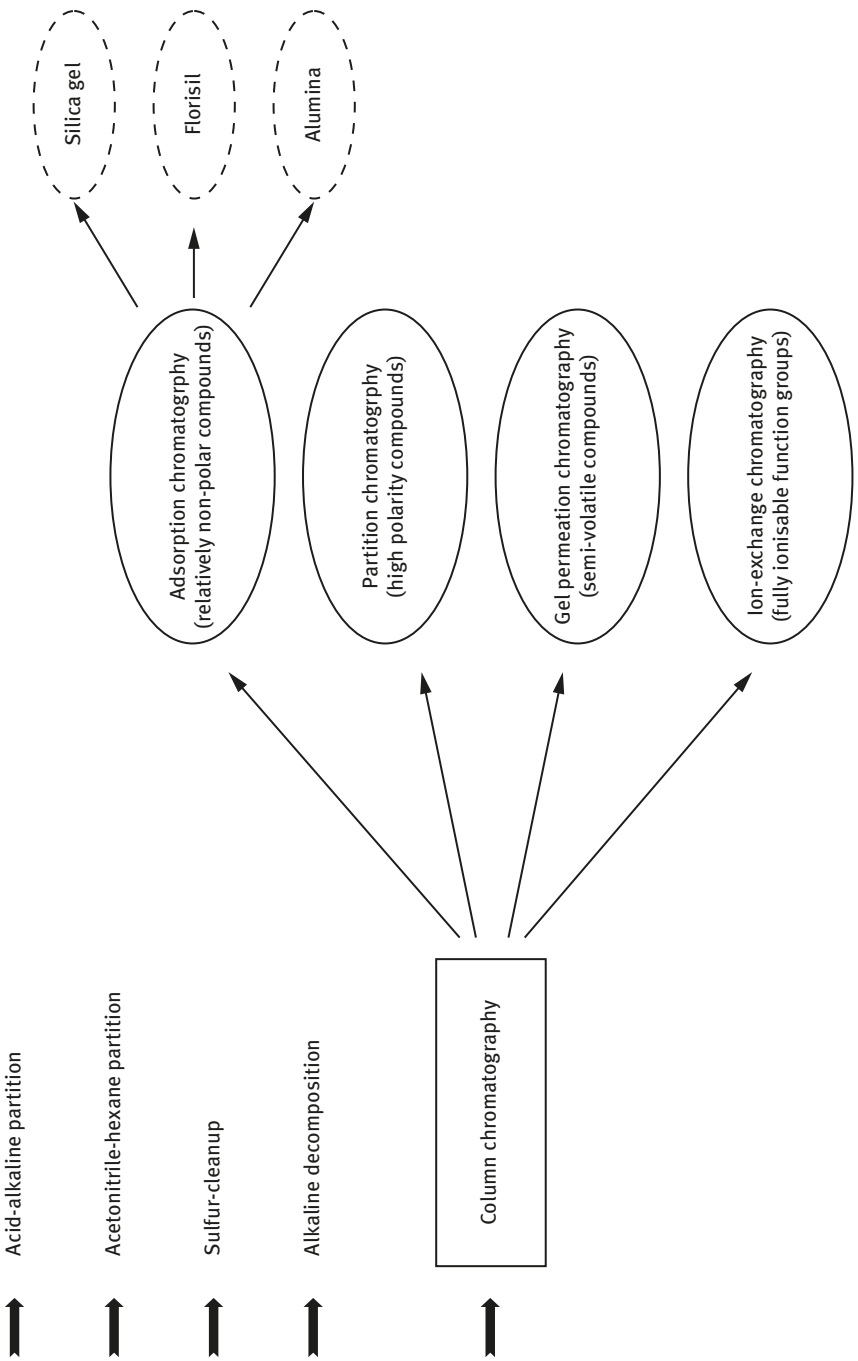

Figure 9.4: Clean-up methods to be used after extraction in environmental samples.

9.2.4 Measurement: Instrumental analytical methods

Modern instrumental techniques of analysis are essential for determining environmental toxicants. We are in a very exciting period in the evolution of analytical chemistry, where the development and optimization of new and improved analytical techniques are taking place. They allow the detection of much lower amounts of chemical compounds and so it is possible to determine contaminants that would not have been possible to detect otherwise. Although it is quite common in literature to find the instrumental method of analysis related to some specific type of samples, in this chapter the analytical techniques are described as well as their properties: in each case the nature of samples that may be analyzed is mentioned.

There are three important groups of analytical techniques to take into consideration: atomic spectrometry, GC, and LC. A better sensitivity is acquired in many cases with the use of hyphenated techniques, therefore they will also be mentioned in each group.

9.3 Atomic spectrometry

Atomic spectrometry studies those elements that can be analyzed as atoms. Out of 118 identified elements, about 91 of them are called metals. These metallic elements are traditionally analyzed with this group of techniques and can be divided into two classes: those that are essential for survival, such as iron and calcium, and those that are nonessential or toxic, such as cadmium and lead. These toxic metals, unlike some organic substances, are not metabolically degradable and their accumulation in living tissues can cause death or serious health threats. Furthermore, these metals, dissolved in wastewaters and discharged into surface waters, will be concentrated as they travel up the food chain. Eventually, extremely poisonous levels of toxins can migrate to the immediate environment of the public. Metals that seep into groundwaters will contaminate drinking water wells and harm the consumers of that water. Pollution from manmade sources can easily create local conditions of elevated presence, which could lead to disastrous effects on animals and humans. Actually, man's exploitation of the world's mineral resources and the technological activities tend to unearth, dislodge, and disperse chemicals, particularly metallic elements, which have recently been brought into the environment in unprecedented quantities and concentrations and at extreme rates.

Heavy metals can be defined in several ways. One possible definition is the following: heavy metals form positive ions in solution and they have a density five times greater than that of water. They are of particular toxicological importance. Many metallic elements play an essential role in the function of living organisms. Humans receive their allocation of trace elements from food and water, an indispensable link in the food chain being plant life, which also supports animal life. It is well established that assimilation of metals takes place in the microbial world as well as in

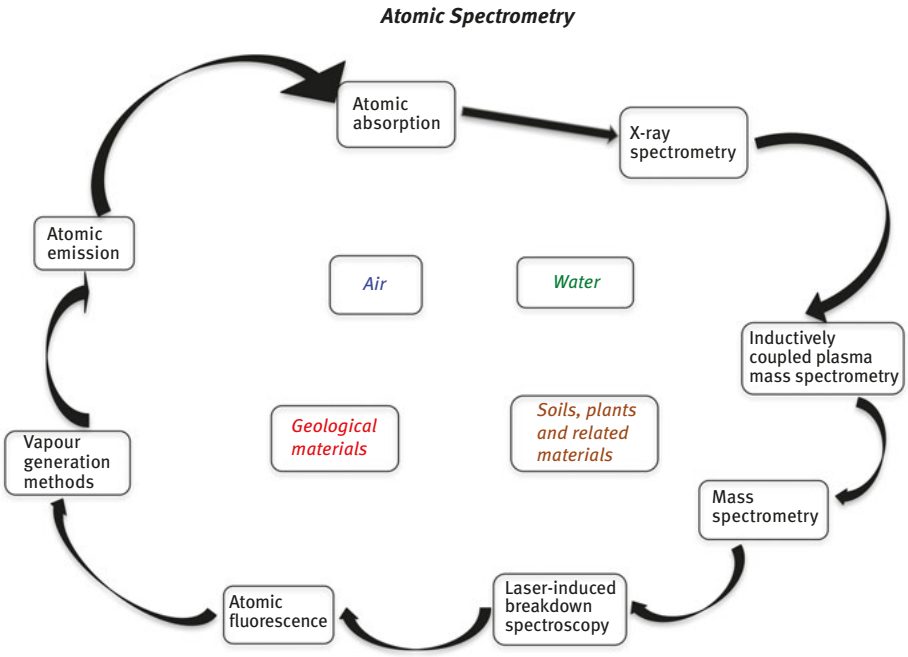

Figure 9.5: Different techniques that are in use nowadays for environmental samples.

plants, and these elements tend to get concentrated as they progress through the food chain. Imbalances or excessive amounts of a metal species along this route lead to toxicity symptoms, disorders in the cellular functions, long-term debilitating disabilities in humans, and eventually death.

Due to the different routes of metals in the environment, the samples to be analyzed are also very different and so are the methods of analysis. Figure 9.5 gives a coarse idea of the different techniques in use nowadays depending on the type of sample and Table 9.1 summarizes the samples related to specific techniques.

9.4 Gas chromatography

There are many research works on GC in different analytical and toxicological fields and the methods are increasing in sophistication to identify and quantify environmental pollutants adequately. They can be distributed and dispersed by different means as we already know: it can be via water, soils, food, industrial activities, air, etc. Due to environmental persistence they remain in nature and are subject to bioaccumulation, which make them very hazardous compounds. This is more difficult because these compounds are present at trace concentrations [2]. Thus, the accurate determination of trace level toxicants is a demanding analytical task. GC is one of the most

Table 9.1: Summary of the techniques in use nowadays regarding the type of sample.

Technique	Sample
Atomic absorption spectrometry	Air; water; soils, plants and related materials; geological materials
Atomic emission spectrometry	Air; soils, plants and related materials; geological materials
Vapour generation methods	Water
Atomic fluorescence spectrometry	Air; soils, plants and related materials
Laser based spectroscopy	Water
Laser-induced breakdown spectroscopy	Soils, plants and related material
Mass spectrometry	Air
Inductively coupled plasma mass spectrometry	Water; soils, plants and related materials; geological materials
Other mass spectrometric techniques	Geological materials
X-ray spectrometry	Air; water; soils, plants and related materials; geological materials

frequently used techniques to analyze volatile and semivolatile organic compounds. In this sense, some major environmental pollutants are: polychlorinated dioxins and dibenzofurans, polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons, polybrominated diphenyl ethers, toxaphenes, organochlorines, pesticides and their major metabolites.

The toxicants have to be separated from co-extracted materials, which is difficult in case of complex matrices as is the case with most environmental samples: there are many closely related anthropogenic compounds present at orders of magnitude of higher concentrations. Therefore, the analytical methods have to be very sensitive and selective and this is what chromatographic methods have been developed for. Figure 9.6 shows a selection of different GC techniques that have been applied in environmental toxicant analysis.

One-dimensional GC (1-D GC) was the main method for the determination of environmental toxicants and in many laboratories, this is still the preferred or available method. However, for analyzing complex pollutant mixtures this single-column GC technique has remarkable drawbacks such as: lack of resolution, lack of robustness, and uncertainty in the identification. It is almost impossible to separate all components in a single chromatographic run, because the peak capacity of the column in the region where the components must be eluted is exceeded; so, to improve resolution several solutions need to be applied, like the use of a longer or narrower column, or a combination of both. To analyze dioxins a very long column would be required in order to separate quite a big number of compounds, although several critical pairs still exist within this group [3, 4]. On the other hand, the determination, for instance, of individual congeners and atropisomers in PCBs is perhaps one of the

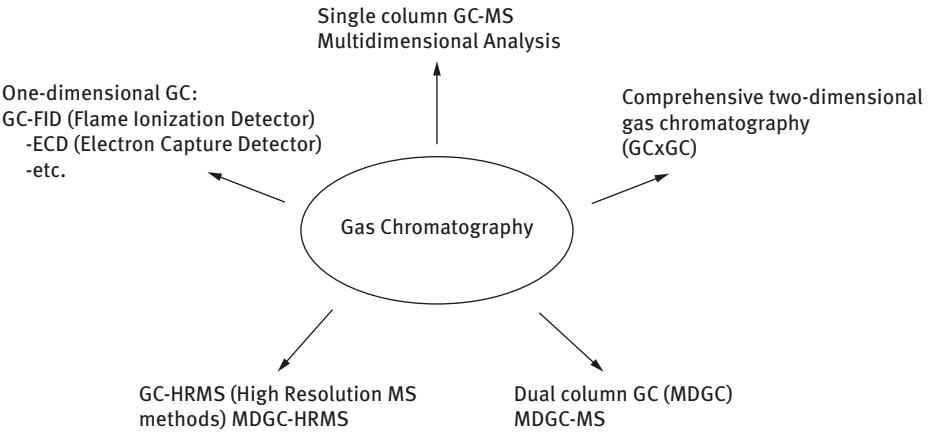

Figure 9.6: Gas chromatographic techniques that are used in the analysis of environmental toxicants.

most difficult applications in environmental analysis of PCBs. One way to improve identification is by using the hyphenated technique of GC-MS: GC coupled to a mass spectrometric detector; but it still has problems as it is not possible to distinguish co-eluting congeners with the same chlorine number. They have the same or very similar mass spectra.

A different way to improve separation and therefore identification is to run the sample in two columns of different polarity to obtain a second set of retention times [5], although in this case it is not still possible to get a complete resolution when different polarities and efficiencies of both columns are optimized.

One alternative to improve separation is by fitting in parallel traps, columns, valves, flow switches, etc., but all this will only lead to longer analysis times and technical difficulties. Another way is by using the corresponding analytical procedure to clean up the sample prior to its separation by GC, which will lead to a cleaner sample and therefore less compounds present to interfere. Although it must be made many times when the samples are complex, it is also convenient to reduce these steps to a minimum as it is time-consuming, and difficult to automate and reproduce. It is also difficult to get good recoveries with these steps and the samples are susceptible to be contaminated when working at trace levels.

A different alternative to reduce the sample handling steps and increase resolution is the use of multidimensional gas chromatography (MDGC). It is based on the separation that takes place in two or more independent separation steps/mechanisms [6] and the components remain separated until the overall analysis is completed; therefore it becomes the only practical alternative to increase resolution. Although GC-MS might be considered a good alternative, there is still isomer co-elution because the separation takes place in one single column so that MDGC will be a valuable tool to improve or completely separate complex mixtures of toxicants [7].

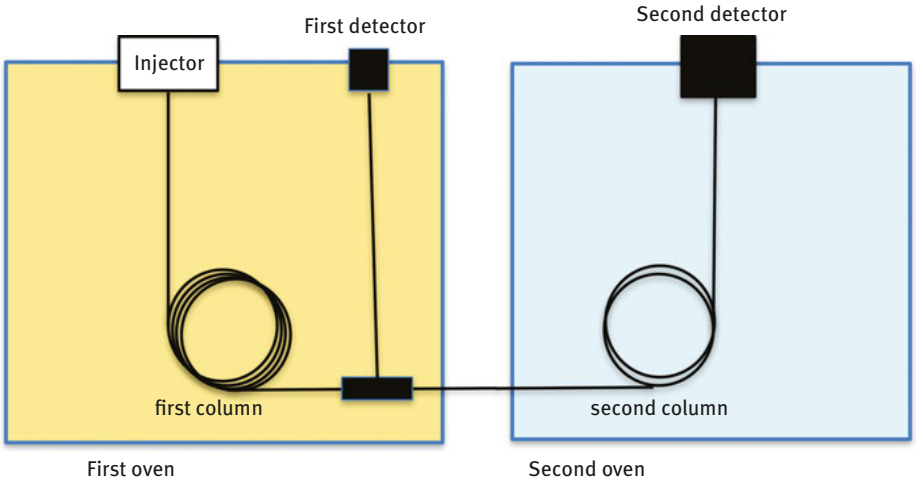

Figure 9.7: Typical schematic diagram of a MDGC setup.

A typical schematic diagram of a MDGC setup is shown in Figure 9.7. As can be seen, it consists of two independent ovens and detectors, a T-piece allows quantitative and reproducible transfer of small, unresolved selected fractions of the eluate from the first column to the second column, where separation takes place.

All types of detectors can be used for two-dimensional GC, like electron capture detector and the MS, operated in the selected/single-ion monitoring (SIM) mode because of their high sensitivity and specificity, for organohalogen analysis.

The resolution of a MDGC system is determined by the column(s)' dimensions and the difference in separation power between the two stationary phases. The longer the columns and the smaller internal diameters, the better the separation. A key aspect is the difference in selectivity for the two columns, which will remarkably affect the final separation; for instance, if polarity is quite different for both stationary phases it may be expected to improve separation.

Some of the applications of MDGC analysis are tabulated in Table 9.2. There are hundreds of papers published on this topic when MDGC technique is used, so this list is just indicative of the need to use it to improve resolution in separation for volatile compounds in environmental analysis. A common goal in all the research is the resolution in specific congener analysis and the isolation of the components of interest for subsequent measurement of chiral ratios [8].

Although MDGC has many attractive points of interest, it is not the only technique used for analysis of volatile and semivolatile contaminants. The lack of robustness has restricted its use in routine laboratories where 1-D GC is still used. On the other hand, it has the relevant limitation that its increased separation power can only be applied to a few regions of the chromatogram, rather than to the whole sample. Thus, in many cases analysis of environmental toxicants is based on GC followed by

Table 9.2: List of analysis of some environmental toxicants employing multidimensional gas chromatography.

Environmental toxicants studied	Analysis goals
Organophosphorus pesticides	Identification of pesticides in food
Halogenated and organophosphorus pesticides	Identification of pesticide residues in food samples employing two columns of different polarity
PCBs	Analysis of PCBs congeners using column switching
Non-*ortho*-chlorinated CBs	Showed analysis of samples without preseparation will underestimate planar CB concentration
TCDD isomers	Detection of complex isomeric mixtures of TCDDs
Polychlorinated dibenzo-*p*-dioxins toxaphene	Study of gas-phase photodegradation of PCDDs; MDGC interfaced to photoreactor analysis of technical toxaphene mixture containing many congeners and biological samples
Polycyclic xenobiotics	Detection of the enantiomer ratios of polycyclic xenobiotics
Dioxins, PCBs	Determination of dioxins and PCBs

(high-resolution) mass spectrometric detection [9]. The mass spectrometer is often the detector of choice in MDGC as well because it can operate in a universal detection mode (similar to a flame ionization detector) and in a specific ion mode, the latter property being of particular importance for the analysis of a specific target compound.

9.5 Liquid chromatography (LC)

It is a fact that there is an overwhelming number of chemicals in use in the world, therefore they are affecting all environmental areas and the ecosystems where they enter. As it has already been mentioned at the beginning of this chapter, the transformation products of these chemicals or their metabolites can alter even more the environmental balance. The number of organic micropollutants increases and their eco-toxicity can be comparable or even higher than that of the original compound [10, 11]. The problem at this stage is that, when this happens, their chemical structures and toxicological effects are not completely known; so, it is important to develop analytical strategies to monitor as many chemicals as possible and trace their fate in the environment.

The first list of priority pollutants created by the EPA (US Environmental Protection Agency) contained analyzable compounds by GC-MS; however, since the late 80s the hyphenated technique LC-MS has grown very quickly to determine and control environmental toxicants. It offers a series of advantages with regard to the chemical nature of those compounds, because most of them are polar organic contaminants: compared to GC-MS, it is possible to avoid the step of derivatization for those nonvolatile compounds, it increases the number of pollutants that is possible to analyze and reduces the total analysis time [12].

A total of 700 substances have been categorized into 20 classes (NORMAN network) in the European surface waters. The most relevant classes are: pesticides, pharmaceuticals, disinfection by-products, wood preservation, and industrial chemicals. In general, there are several groups of compounds that emerged as particularly relevant:

- Algal and cyanobacterial toxins
- Brominated flame retardants
- Disinfection by-products
- Hormones and other endocrine disrupting compounds
- Drugs of abuse and their metabolites
- Organometallics
- Organophosphate flame retardants and plasticizers
- Nanomaterials (nanoparticles)
- Perfluorinated compounds
- Pharmaceuticals and personal care products
- Polar pesticides and their degradation/transformation products
- Surfactants and their metabolites

However, the different physicochemical properties of those compounds make impossible to develop one method to screen and determine all, as well as their degradation products. The scientific community needs to take up the challenge to develop more advanced instrumentation, to search for new chromatographic materials and to make more effective analytical approaches. Since the impact on aquatic life and human health can be dramatic, a rigorous evaluation of analytical methodology is crucial for contaminants that can compromise flora, fauna, and public health integrity.

Simple and fast sample treatments have been developed to extract and analyze as many compounds as possible simultaneously, in the last years: the main objective has been to save time, expense, and labor. As different pollutants have different physicochemical properties, a balance must be found between handling "dirty" extracts and accepting low recoveries; on the other hand, in case of samples like aqueous ones containing pesticides and drugs, which are very polar and hydrophilic, it has been necessary to lyophilize and evaporate under reduced pressure to enrich them. However, conventional offline SPE on discs and cartridges has still been the most common and used technique due to its high simplicity and flexibility for many different samples. The classical materials for SPE include: C18, graphitized carbon black, N-vinylpyrrolidone-divinylbenzene copolymers, mixed mode cation-exchange cartridges, mixed mode anion-exchange cartridges, weak anion-exchange cartridges and many others. Therefore, depending on the compound a different sorbent is chosen which allows to obtain high recovery percentages and enrichment factors. They vary between 20 and 1,000.

In the last years, research has been focused on the development of nanomaterials (carbon nanostructured materials, metallic nanosized structures, and metal organic frameworks) because of their potential as sorbents in SPE operations either on conventional or miniaturized scales. Their properties of chemical stability;

thermal, mechanical, and electronic properties [13–15], as well as their large surface area and durability make them suitable for a broad variety of environmental applications. Fullerenes, nanotubes, nanofibers and graphene are the carbon nanostructured materials where more research is developed. They show a very good affinity for hydrophobic compounds, especially aromatic compounds that strongly interact with their graphitic portion. They are also very good for polar compounds, because a preliminary oxidizing treatment introduces polar functionality (hydroxyl, carboxyl, and carbonyl groups) and makes them suitable to interact with polar compounds and for chemical derivatizations [16, 17]. These materials, oxidized or not, can be used in generic sample treatments to extract a large number of organic micropollutants, and with an adequate functionalization they can change their selectivity in a dramatic way, thus becoming specialized sorbents for specific methods of interest.

As an example of the goals accomplished, here is the description of the analysis of a group of marine toxins that are a serious problem in environmental analysis: STX and analogs. They are commonly known as PSP (paralytic shellfish poisoning) toxins, and are rather common worldwide and the most lethal of marine toxins intoxication. They are a group of more than 21 tetrahydropurines, usually quantified by a semiquantitative **MBA** [18], which is the reference method internationally accepted in monitoring programs. The chemical methods used to determine PSP toxins are fluorimetric assays, HPLC with fluorimetric detection (either pre-column or post-column oxidation), LC-MS, and capillary electrophoresis methods.

The HPLC methods are widely used to quantify PSP toxins present in seafood samples, but they are also useful in providing the PSP profile because in chromatography it is possible to identify each toxin as well. These toxins have only a weak chromophore group and it should be modified before detection: when they are oxidized in an alkaline solution, a purine is formed that becomes fluorescent at acidic pH. This reaction can either be a pre-column or post-column one, and obtained purines are monitorized with a fluorescence detector. Figure 9.8 describes the general outline of these two procedures, where in the post-column method different types of columns have been used (1 and 2 options in the scheme) [19, 20].

Pre- and post-column HPLC methods present as advantages a high sensitivity for low concentrations and low variability for results; but their drawbacks are also important. In the case of hydrophobic analogs, they are retained by C18 resins [21]; therefore, HPLC methods do not allow determining their presence in monitoring programs [22, 23]. LC-MS methods are actually being developed to get a good characterization of these compounds, hence it is recommended that the presence of PSP toxins is confirmed by MS. However, the use of reversed-phase conditions, which generally consist of some organic solvent and nonvolatile salts, is not suitable for LC-MS; mobile phases with phosphate content as well as ion-pair formers are a handicap for an efficient application of the LC-MS technique. Therefore, the application of ionic exchange chromatography with eluents containing only volatile compounds to quantify PSP toxins either with fluorimetric or MS detection has been proposed.

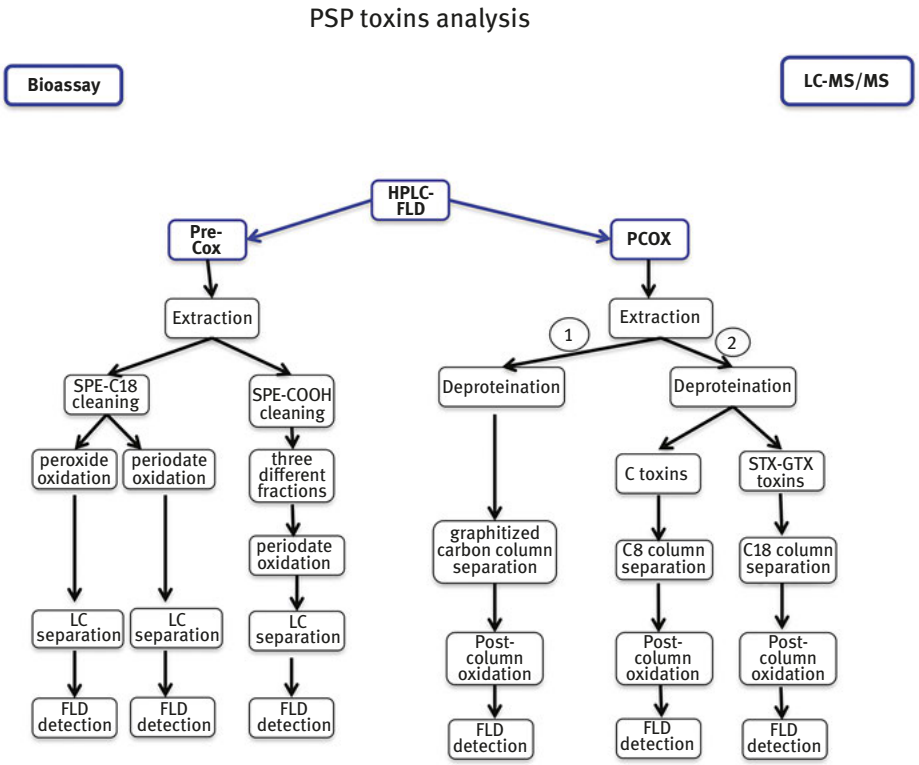

Figure 9.8: General outline of the pre-column and post-column oxidation procedures to determine PSP toxins by HPLC.

Nowadays, environmental analysis is one of the most important application areas of LC-MS, mainly related to the study of occurrence and fate of organic micropollutants, that have been neither considered as a risk nor included in national monitoring plans so far. Figure 9.9 provides a coarse idea of the wide application range of LC-MS and how it is the technique of choice for analysis of most of these compounds.

In the last years, there has been a growing interest in the development of high-throughput, robust, and sensitive chromatographic methods, regardless of the specific research area. Ultra-high performance liquid chromatography (UPLC) technology is one of them, and it only dates back to 2004. It can deliver the mobile phase at pressures up to 1,000 bar, allowing columns packed with very small particles (17–18 μm) to reach their theoretical performance. Stationary phases based on sub-2-mm particles enable elution of analytes in much narrower and more concentrated bands, resulting in better chromatographic efficiency, resolution, and sensitivity with negligible intra-column band dispersion. Compared to HPLC, this extra efficiency occurs at a higher flow rate and can be achieved in a shorter analysis time. UPLC columns with sub-2-μm porous particles have already successfully been employed to speed up the analysis of a large variety of organic micropollutants in environmental

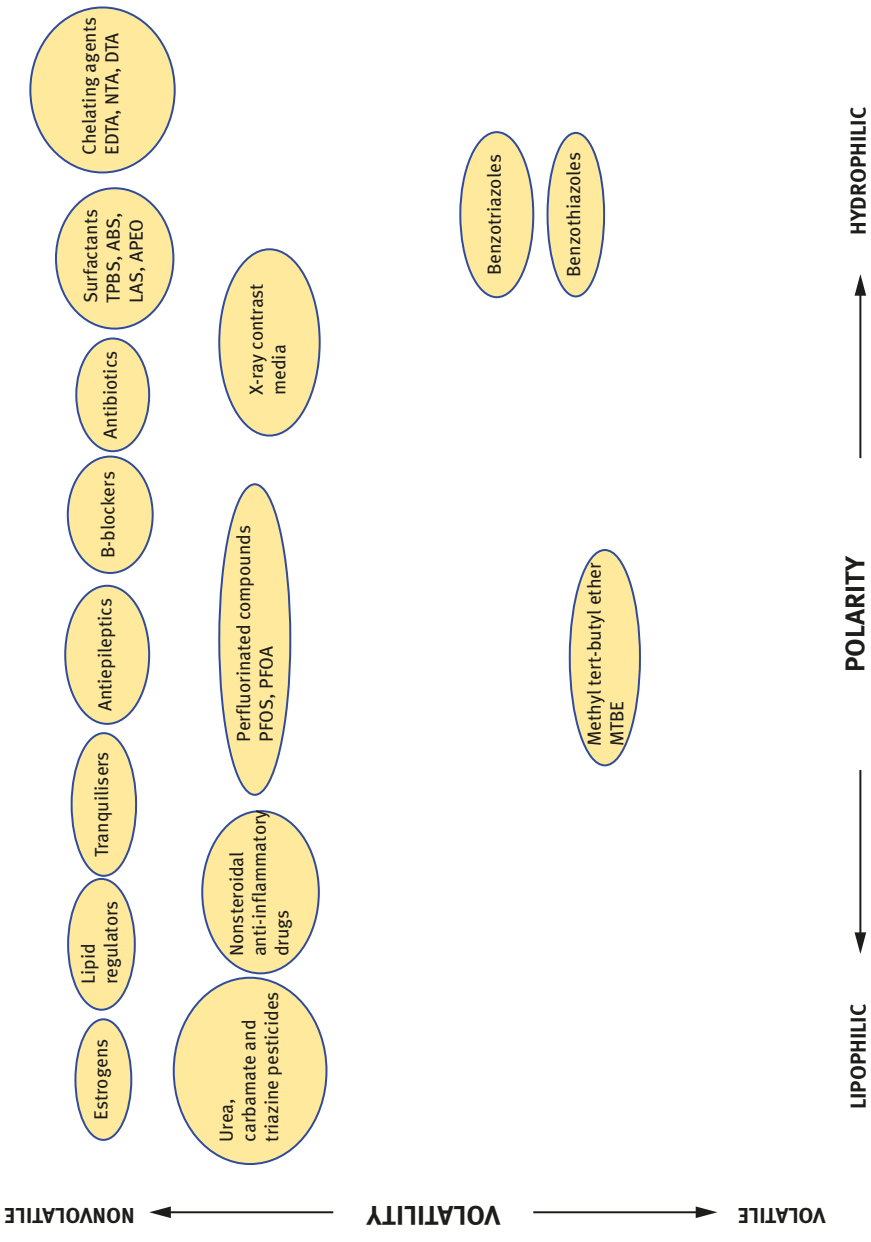

Figure 9.9: LC-MS and some of its applications.

samples: drugs [24, 25–27, 28] personal care products [25], pesticides [11, 25, 29, 30, 31, perfluorinated compounds [32], and endocrine disruptors [33, 34].

Keywords: atomic spectrometry, gas chromatography, liquid chromatography, metals, volatile compounds, non-volatile compounds

Abbreviations: 1-D GC: one-dimensional gas chromatography; CBs: chlorobiphenyls; EPA: Environmental Protection Agency; GC: gas chromatography; GC-ECD: gas chromatography with electron capture detector; GC-FID: gas chromatography with flame ionization detector; GCxGC: comprehensive two-dimensional gas chromatography; GC-HRMS: gas chromatography coupled to high resolution mass spectrometry; GC-MS: gas chromatography-mass spectrometry; HPLC: high-pressure liquid chromatography; LC: liquid chromatography; LC-MS: liquid chromatography-mass spectrometry; MBA: mouse bioassay; MDGC: multidimensional gas chromatography; MDGC-MS: multidimensional gas chromatography-mass spectrometry; PCBs: polychlorinated biphenyls; PCDDs: polychlorinated dibenzodioxins; PSP: paralytic shellfish poisoning; SFE: supercritical fluid extraction; SIM: single-ion monitoring; SPE: solid-phase extraction; SPME: solid-phase microextraction; TCDD: tetrachlorodibenzodioxin

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