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Extraction and Analysis of Mycotoxins from Whole Wheat Flour - A Methods Efficiency Comparison

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Abstract

Wheat is considered as staple food source for 40% of the population worldwide. Yet, the yield and quality can be compromised by fungal diseases, which are also responsible for mycotoxins presence at wheat seeds and originating foodstuff. In this context, the tackling of this problem by developing regulatory limits and standards have induced the development of various methods for sampling, extraction, identification and quantification of mycotoxins in food samples. This review addresses the comparison of the technical and cost efficiency of methods for the extraction and qualitative- quantitative analysis of mycotoxins from whole wheat flour. Methods of extraction such as the Solvent Extraction method, the Liquid Liquid Extraction, the Solid Liquid Extraction, the Solid Phase Extraction, the Immuno-Affinity Columns, the QuEChERS, and the use of absorbent nanomaterials such as graphene oxide and multi-walled carbon nanotubes in extraction procedures, are described in principle, technical details are presented, and examples of reported use are given. Methods of mycotoxin analysis such as Immunological Assays (LFIA, ELISA, FPIA), the Sensor-based (Surface Plasmon Resonance Sensor, Piezoelectric Sensors, Electrochemical Sensors, Colorimetric Sensors), and Chromatographic Techniques (TLC, GC, HPLC, HPLC-FLD, LC-MS/MS, UPLC-MS/MS, UHPLC-MS/MS, UFLC-MS/MS) are reviewed. To compare their efficiency, main advantages and disadvantages, the ongoing improvements, as well as the validation parameters (linearity, recovery range, RSDr range, RSD_R %, LOQ range, and cut off) are summarized, and pairing of extraction to analysis methods for specific mycotoxins is provided. It was evidenced that none of methods already in use is capable of analyzing all mycotoxin categories at once, because of their chemical characteristics (volatile/non-volatile, co-elution, UV absorption, fluorescence) versus methods restrictions (matrix interferences, cross-reactivity of antibodies, selectivity and reproducibility of data, need for derivatization, etc). Also, depending on the purpose of the analysis (research or screening as part of legal requirements), to date the immunological methods are only suitable for validated matrices, biosensors can be used for routine screening, and that GC-MS and HPLC-based methods fulfill the legal requirements. In conclusion, while the selectivity and accuracy of methods for mycotoxin detection is being improved rapidly (those sensor-based thanks to the use of nanoparticles, nanomaterials, aptasensors, etc., and the chromatographic techniques coupled with mass spectrometry offer a higher selectivity and sensitivity, low detection limits, maintained resolution performance), and the duration of the analysis, the cost, and the need for highly-skilled staff go in favor of rapid methods (immunological and sensors-based), it is the capacity to fulfill legal requirements, which will determine the trend and their future success in the market.

Keywords: mycotoxins, immunological methods, biosensors, chromatography

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Introduction

Wheat is considered as the second most important grain crop globally [1], and as staple food source of 40% of the population [2]. However, the wheat production is threatened seriously by fungal diseases among which the *Fusarium* head blight (FHB) [3] can lead to a reduction in yield (up to 30%) and quality in the form of atrophy, weight loss and discoloration [4]. In addition, *fungal* species can produce various mycotoxins that subsequently appear in the grain [5]. Mycotoxins are low molecular mass (MW ~700 Da) secondary metabolites of filamentous fungi, which are harmful to human and animal health [6]. More than 400 different mycotoxins with various chemical structures and properties produced by a wide variety of fungal species belonging to the genera *Aspergillus*, *Fusarium*, *Penicillium*, *Alternaria*, *Claviceps*, and *Stachybotrys* have been

identified [7][8]. Among the mycotoxigenic fungi the *Fusarium* species, including *F. graminearum* (Schwabe), *F. culmorum* (W.G. Smith) Sacc., *F. avenaceum* (Fries) Sacc., *F. poae* (Peck) Wollenw., and by *Microdochiumnivale* (Fries) Samuels and Hallett [9] are the causal agent of *Fusarium* head blight (FHB). The toxigenic potential varies greatly between species. Thus, *F.culmorum*, *F. graminearum*, *F. langsethiae*, *F. poae*, and *F. sporotrichioides* each produces their own spectrum of trichothecenes, while F. *avenaceum* may produce moniliformin, beauvericin, and enniatins [3].Among the mycotoxins, aflatoxins (AFs), ochratoxin A (OTA), zearalenone (ZEA), patulin (PAT), fumonisins (FUMs), and trichothecenes (TCs) like deoxynivalenol (DON) and T-2 toxin (T-2) are considered as the most concerning [10]. Tricothecenes can be divided into two groups according to their chemical structure: type A have a hydrogen or ester side chain at carbon atom 8—whereas type B have a ketone at C-8. Furthermore, *F. graminearum* and *F. culmorum* produce different trichothecene analogs based on which different chemotypes with marked changes in toxicity and biological activity are described [3]. Several countries have established or proposed regulations for the control of mycotoxins in food and feed. The permitted levels vary depending upon the intended end usage of the commodity. The EU for example has established regulations for the levels of Ochratoxin A (OTA), Deoxyvalenol (DON), Zearalenone (ZON), etc [11], the Food and Agriculture Organization of the United Nations (FAO) has developed regulations for mycotoxins in food and feed [12,13], the US Food and Drug Administration (FDA) has prepared guidance documents [14,13], and the World Health Organization (WHO), have identified potential health hazards to humans and animals associated with food- or feed-borne mycotoxin intoxication and has developed regulatory limits for main mycotoxin classes and selected individual mycotoxins [15, 13]. All of these efforts to establish limits and standards have induced the development of various methods for their sampling [16], extraction, identification and quantification in food and feed samples [15,11,13], which are being reviewed and compared in this paper with aim the comparison of the technical and cost efficiency of use at whole wheat flour, as a raw material of high importance for the food industry. This paper is product of the ongoing research related to the exploitation of the resistance of local wheat cultivars of Albania toward abiotic [88-94], and biotic stresses [93].

2. Methods for Mycotoxins Extraction from whole wheat flour

The determination of mycotoxins in food samples is preceded by several different steps such as sampling and sample preparation. Sampling is significant in determination of mycotoxin levels because mycotoxigenic fungi do not grow even on the substrate, and it is difficult to obtain a representative bulk sample. Moreover, the existing mycotoxin contaminations in natural samples are not homogeneous [17, 13]. In order to standardize the sampling procedures for mycotoxin analysis, Commission Regulation (EC) No. 2782/2023[18] specified the sampling and analysis methods for the official control of the mycotoxins levels in foodstuffs [19]. The example is the cereals and cereal products sampling method for lots <50 tons, where sampling plan shall be used with 10 to 100 incremental samples, depending on the weight, resulting in an aggregate sample of 1 to 10 kg [18]. Inadequate sampling is associated with errors in the evaluation of the mycotoxin level of the lot could easily occur, usually leading to an underestimation. If sampling is performed for monitoring/surveillance purposes, poor sampling could give false information for risk assessors/ managers. For inspection purposes, incorrect sampling can cause problems in litigations [20,13]. In order to accelerate the chemical reaction process of extraction and to increase the chances to detect the mycotoxins, the sample should be ground to the final particle size of approximately 500μm opening size and homogenized to whole wheat flour or powder-like consistency [18,21,13]. Once homogeneity is obtained, the sample should be mixed. Extraction procedure is an important step for the determination of mycotoxins from different matrices, wheat grains included, and the selection of the suitable solvent for this purpose is the critical point, which primarily depends on the type of analyte, and its polarity. After the extraction has released mycotoxins from the matrix, the clean-up process is crucial to reduce matrix effects and eliminate substances, which can interfere with the next mycotoxin detection. The purification of the extract will increase specificity and sensitivity, resulting also in the improvement of quantification accuracy and precision. Generally, extraction of mycotoxins from solid feeds and foods requires organic solvents, and recommended methods are Solvent Extraction also known as Liquid Liquid Extraction (LLE), Solid Phase Extraction (SPE) [22] and Immuno-Affinity Columns (IAC) [13], explained in principle below and in more technical details at Figure 1.

2.1 Solvent extraction method or also known as liquid-liquid extraction (LLE) is a process, which helps to distinguish compounds based on the different solubility of the toxins in immiscible organic phase and aqueous phase, and to extract the compound into one solvent leaving the rest of the matrix in the other [23]. Factors like polarity, selectivity and reactivity are essential while selecting a solvent. Suitable solvents, such as methanol or acetonitrile should be mixed with water in the presence of salts (NaCl, $MgSO_4$, Na_2SO_4) to reduce the mutual miscibility, allowing in this way the polar analytes to move into the polar organic phase from the aqueous phase [24]. However, this method requires vast amount of solvent, may be accompanied by loss of sample by absorption onto the glassware, and formation of emultions [25, 23, 26, 13].

2.2 Liquid–solid extraction (SLE) is a simple method for mycotoxins extraction from solid matrices of various consistency. The extraction is based on the weighing of homogenized sample, and adding the extraction solvent, followed by agitating it in a shaker [27,13]. It has been confirmed that this method can be used to extract various

mycotoxins from cereals [28,13]. Since mycotoxins tend to be inhomogenously distributed throughout a lot of cereals it becomes expensive to carry out laboratory analysis on the number of samples required to be representative of the particular lot. Therefore simple, rapid, inexpensive methods of extraction, which can be applied outside the laboratory in order to detect the mycotoxins presence in a lot of cereal grain before being analyzed in laboratory is preferable. [11] invented an extraction method, which can replace effectively the use of a high concentration alcohol-in-water solvent, typically 70-30% methanol: water solvent with ethanol: water solvent in 20-40% by weight. Use of ethanol at concentrations much less than that used for methanol produces extraction efficiencies comparable to those of methanol: water solvent when employed in the extraction of mycotoxins from cereal grains. Also, this solvent is less flammable giving this way a solution to safety concerns.

2.3 Solid – phase extraction (SPE) technique offers the possibility for the direct extraction of mycotoxins from liquid samples. It utilizes small disposable cartridges packed with bonded phases or silica gel (stationary phase) necessary to bind impurities or target analytes [29]. The sample passes slowly through the SPE cartridge, making it possible for the analyte and some of the sample matrix compounds to be retained on the SPE material. Depending on the properties of the analyte and the SPE sorbent, a wash solvent can be chosen to selectively remove interfering compounds present in the matrix from the SPE sorbent, while the analyte of interest is eluted in another solvent. For commercial SPE, octadecylsilyl (C18), hydrophilic–lipophilic balance (HLB), amino-propyl (NH2) and silica gel can be used as adsorbents, but the majority of the commercial cartridges are not appropriate for high-throughput screening for multiclass mycotoxins [30,31,13]. SPE is more effective than LLE since they use less amount of organic solvent and are faster. In addition to cleaning the sample, they can also be used to pre-concentrate it, giving better detection results [32]. New SPE methods are developed to meet the requirements for efficient evaluation of mycotoxins co-contamination in food. Nanofibers are considered as a promising new SPE adsorbent, which can reduce the matrix effect giving higher absorption [33], and are easily prepared by electrospinning.

2.4 Immuno-Affinity Columns (IAC) use an antibody-antigen interaction as the principle of extraction. The analyte molecules are bound to the antibodies immobilized on the column after a preconditioning step. As matrix components do not interact with the antibodies, a rinsing step removes most of the possible interferences, and the toxin can be eluted by antibody denaturation [34]. The main advantages of the IAC are the specific interaction between mycotoxins and antibodies, simplicity of use (only denaturing solvent is needed), robust and large volumes can be analyzed, and samples contaminated with different mycotoxins can be purified. On the other hand, there are disadvantages such as the cross-reactivity of antibodies with numerous components in the matrix, high costs, and the fact that columns can be used only once [35,36, 13] as well as the limited ability of columns to absorb mycotoxins. Furthermore, if the contents of mycotoxins in the sample exceed the column binding capacity, the mycotoxin is not effectively capture and bound, resulting in unreliable results [13]. IAC has been successfully applied in simultaneous analysis of OTA, ZEA, and AFs in wheat bran [37,13], OTA, AFs, and *Fusarium* toxins in cereals [38, 13].

2.5 Use of nanomaterials as mycotoxins absorbents in extractions

The use of nanomaterials in the biosensing has been increasing quickly in the last decade. Increased selectivity and sensitivity are the main outcomes of nanoparticle integration, which additionally present a higher surface area, enabling this way the immobilization of more biosensing elements such as antibodies, enzymes, receptors and aptamers [39]. Nanomaterials including carbon nanomaterials and magnetic carbon nanomaterials, have been used for mycotoxin determination, among which the graphene oxide (GO) and multi-walled carbon nanotubes (MWCNTs) are the examples of those used as absorbents. GO was used in pre-concentration of the extraction of aflatoxins. MWCNTs adsorb type A trichothecenes and were therefore used as Solid-phase extraction sorbents in maize, wheat, and rice to purify and enrich mycotoxins [13]. However, many other mycotoxins are awaiting the evaluation of the appropriate absorbent nanomaterials [22].

2.6 QuEChERS extraction method

The QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) is the acronym for a highly beneficial analytical approach, initially developed for pesticide analysis, which is used to facilitate the simultaneous detection of different groups of mycotoxins in various matrices [39,13, 86] by optimizing the extraction and purification processes. This procedure also takes into account cost reduction and automation to enhance the analysis process, while the conventional extraction methods require multiple steps and a considerable amount of solvent and time [86]. Basically, an extraction with acetonitrile water is followed by liquid–liquid partitioning induced by the addition of inorganic salts. As a consequence, some polar components of the matrix remain in the aqueous layer, while mycotoxins are moved into the organic phase. Next, a dispersive solid phase extraction is applied to reduce other matrix compounds from the organic phase [40,13]. QuEChERS has been used among others for the analysis of mycotoxins such as OTA and AFs in cereals [41,13, 86]. Ten mycotoxins were determined in maize and sorghum grains, and extraction solvent, agitation time, salt addition, dilution, and concentration of supernatants in the extraction procedure were all optimized, revealing the fact it is a quick, economical, and environmentally friendly analytical method, and that the optimization of its extraction procedure for different matrices is crucial, as the different stages affect analyte recovery in different ways [41,13, 86]. Magnesium sulfate or sodium sulfate along with NaCl were used to reduce water in

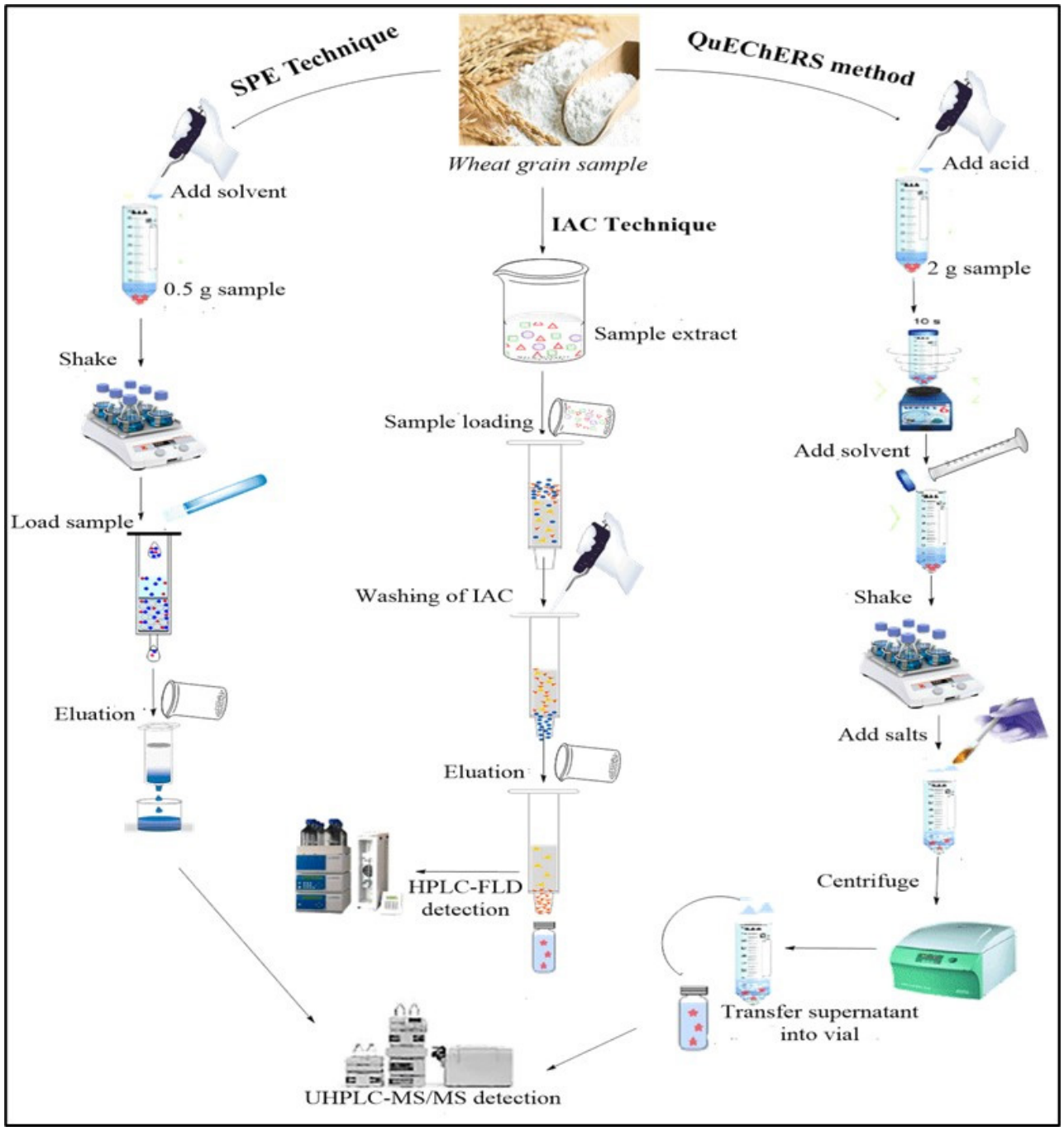

Figure 1. Methods for mycotoxin extraction from whole wheat flour (SPE-Solid-phase extraction, IAC-Immuno-affinity column, QuEChERS-Quick Easy Cheap Effective Rugged Safe).

the sample, while primary secondary amine (PSA) or C18 are adopted as sorbents, and used in the clean-up step [25], since salting induces proper separation of the organic and aqueous phases and reduces the amount of water in the final extract, leading to cleaner extracts and reduced emulsions. Anhydrous $MgSO_4$ and hydrated $MgSO_4$ are the commonly employed drying agents, while NaCl was used to induce phase separation [86]. Due to efficiencies found in solvent extraction, lab spaces and dishwashing processes, this method is considered both environmentally and user friendly [87].

3. Techniques for the Detection and Analysis of mycotoxins from whole wheat flour

Efforts to establish mycotoxins limits and standards have induced the development of various methods for their detection, identification and quantification in food samples [15,11,13]. However, since the scope of this paper is to review methods to be applied on whole wheat flour, Fig 2 summarizes the immunological, chromatographic and sensors-based assays, reported to be successful for this purpose only, while the principle of each method and main relevant findings are described in the following sections.

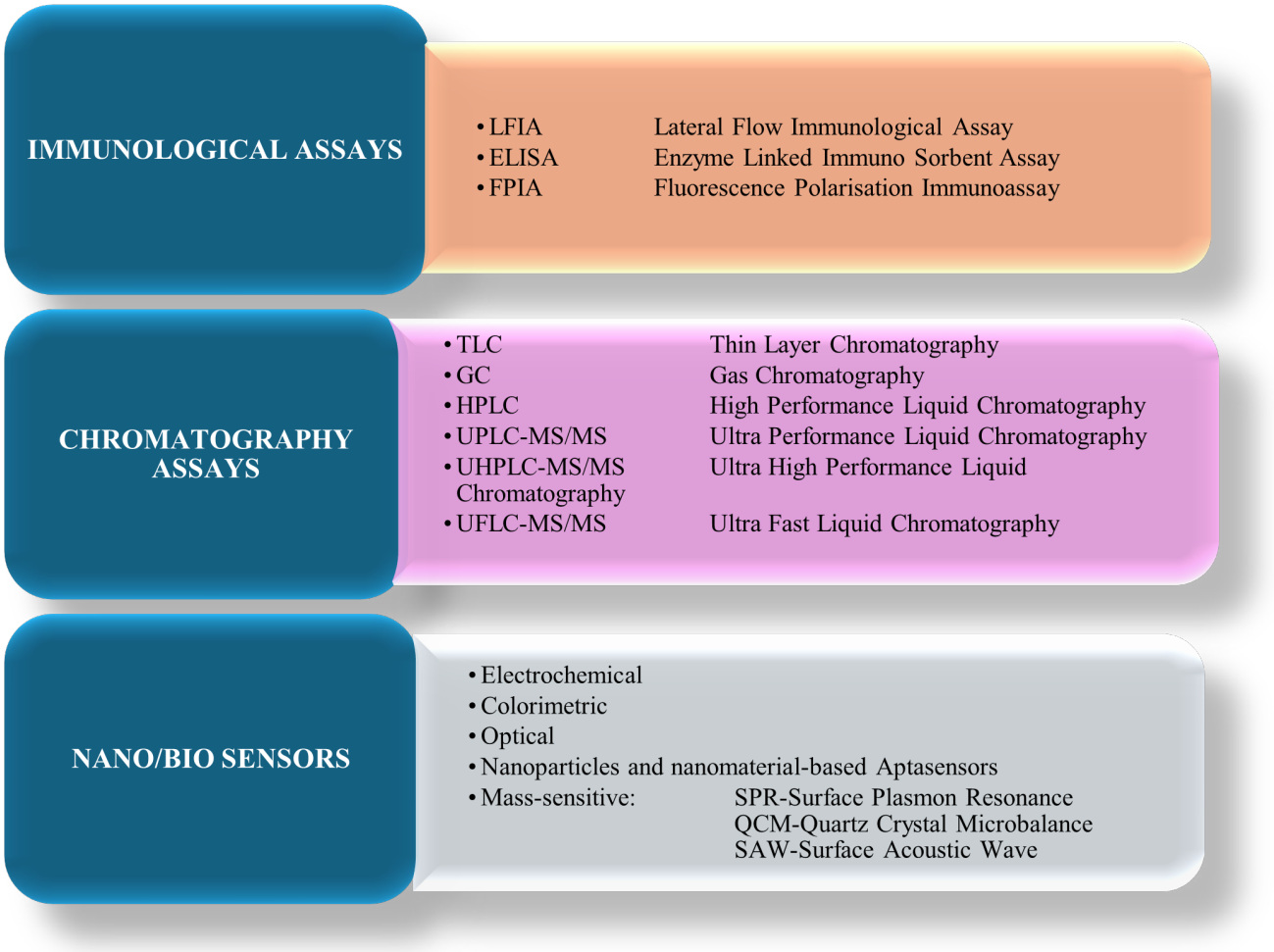

Figure 2. Methods used for the analysis of mycotoxins from whole wheat flour.

3.1 Immunological Assays

Three immunological methods, Lateral Flow Test (LFT), Enzyme Linked Immuno Sorbent Assay (ELISA), and Fluorescence Polarization Immunoassay (FPIA) were found to be applied for the analysis of mycotoxins at wheat flour. The principles of each of them are described in the following sections, and technical protocol details are given at Figure 3.

3.1.1 Lateral Flow Test (LFIA) - The principle of this assay was based on the interaction between the antibody and antigen detected via color development. This method has been widely employed to identify a variety of molecules, including pesticides, microbes, mycotoxins, heavy metals, and cancer indicators [42]. Usually, the lateral flow strip consists of 3 parts: conjugate pad, porous membrane and absorbent pad. After application on the conjugate pad, the liquid components of the assay move along the membrane by capillary flow to the absorbent pad [43]. As more fluid passes over the strips, particles accumulate and the strips change color [44].

3.1.2 Enzyme-Linked Immuno-Sorbent Assay (ELISA)

The basic process of ELISA includes a competitive assay format that uses antibodies, enzymes, and a target molecule (i.e., the mycotoxin). The direct competitive ELISA is commonly used for screening as a semiquantitative or quantitative approach for the detection of controlled mycotoxins in specific ingredients [45]. A portion of the sample extract (supernatant) and a conjugate of an enzyme-coupled mycotoxin are mixed and then added to the antibody-coated microtiter wells. After washing (this step helps to move unbound molecules) an enzyme substrate is added and blue color develops. The intensity of the color is inversely proportional to the concentration of mycotoxin in the sample or standard. A stop solution containing an acid (sulfuric acid) is added, which terminates the reaction and the color changes to yellow. The intensity of the solution color is measured optically using an ELISA reader with an absorbance filter of 450nm [26]. Generally, ELISA kits give accurate and reproducible results, but the similarity of mycotoxin structures is a challenge, and these antibody-based techniques are prone to cross-reactivity [28], which can vary between commercial testing kits.

3.1.3 Fluorescence Polarization Immunoassay (FPIA) - In the case of mycotoxin detection, FPIA depends on a competition between the mycotoxin and a mycotoxin–fluorophore conjugate (tracer) to capture anti-mycotoxin antibody [46].

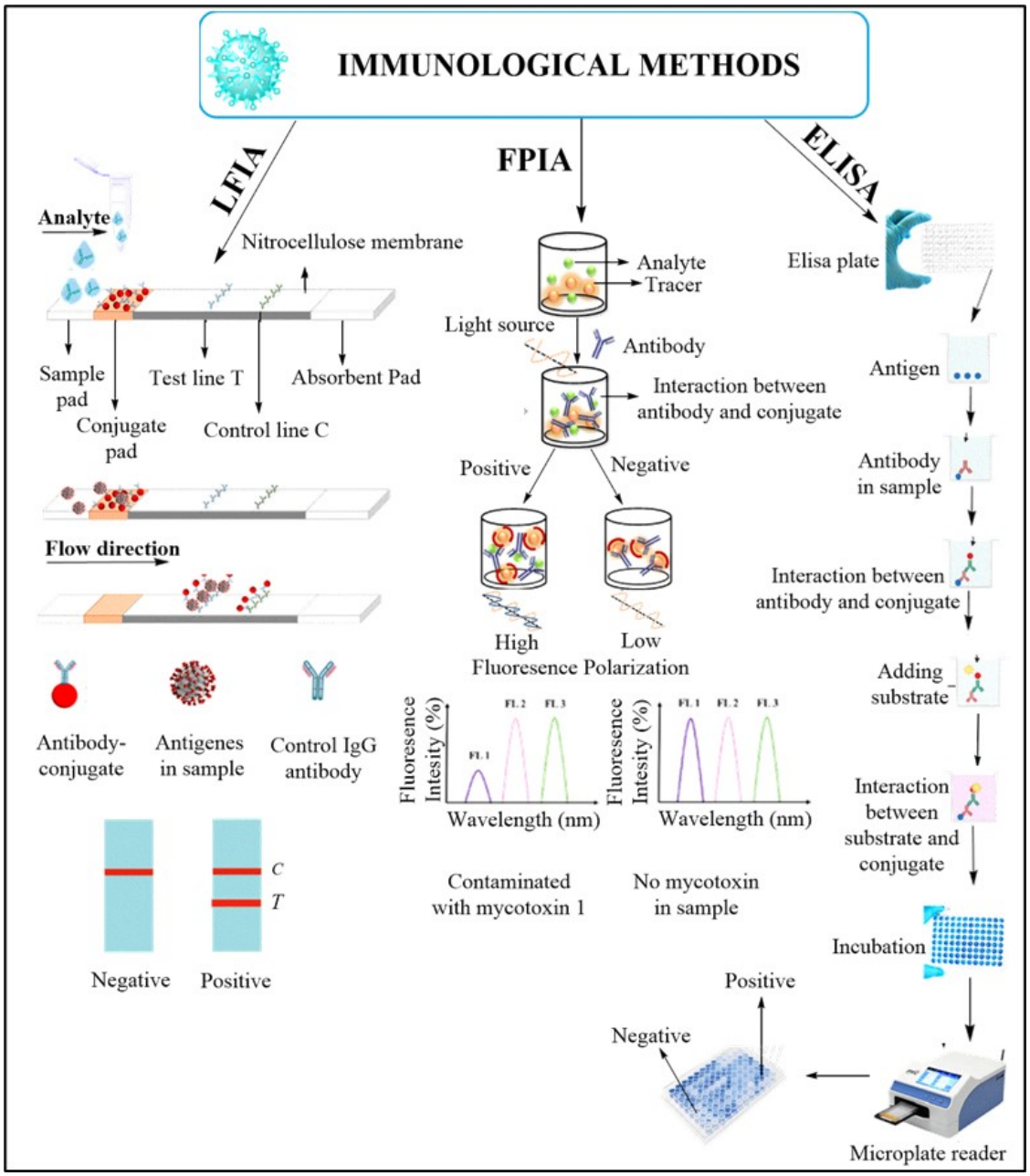

Figure 3. Immunological methods for the analysis of mycotoxins at whole wheat flour (LFIA – Lateral flow test, FPIA – Fluorescence Polarization Immunoassay, ELISA – Enzyme-Linked Immunosorbent Assay, FP – Fluorescence Polarization).

Based on fluorescence polarization, binding of anti-mycotoxin antibody and the tracer is measured by the orientation of the fluorescence emission from both horizontal and vertical directions, which depends on the rotation rate and the fluorophore size. In absence of mycotoxin, the anti-mycotoxin antibody is available to bind to the tracer resulting in increased polarization. In presence of free mycotoxin, the antibody is not available for the trace resulting in decreased polarization [47]. This technique has been used to detect numerous mycotoxins, including aflatoxins (AFs), fumonisin (FBs), deoxynivalenol (DON), T-2 toxin, ochratoxin A (OTA), and zearalenone (ZEA) [48].

3.2 Sensors (Nanosensors & Biosensors)

The application of sensors that are capable of sensing changes in plant health and their morphological and physiological traits, has shown to be useful to boost agricultural yields [49, 50]. Among the categories of nanosensors used to detect diverse molecules are: Carbon Nanotube-based sensors, Nanoparticle-based sensors, Nanowire-based sensors, Nanoporous-based sensors, Graphene-based and 2Dsensors, and Plasmonic Nanosensors. They have an immense range of applications from household to industry, which includes detection of a wide variety of fertilizers, pesticides, fungicides, pathogens, heavy metal content, and quality of soil such as pH, temperature, moisture content, soil water content and overall growth

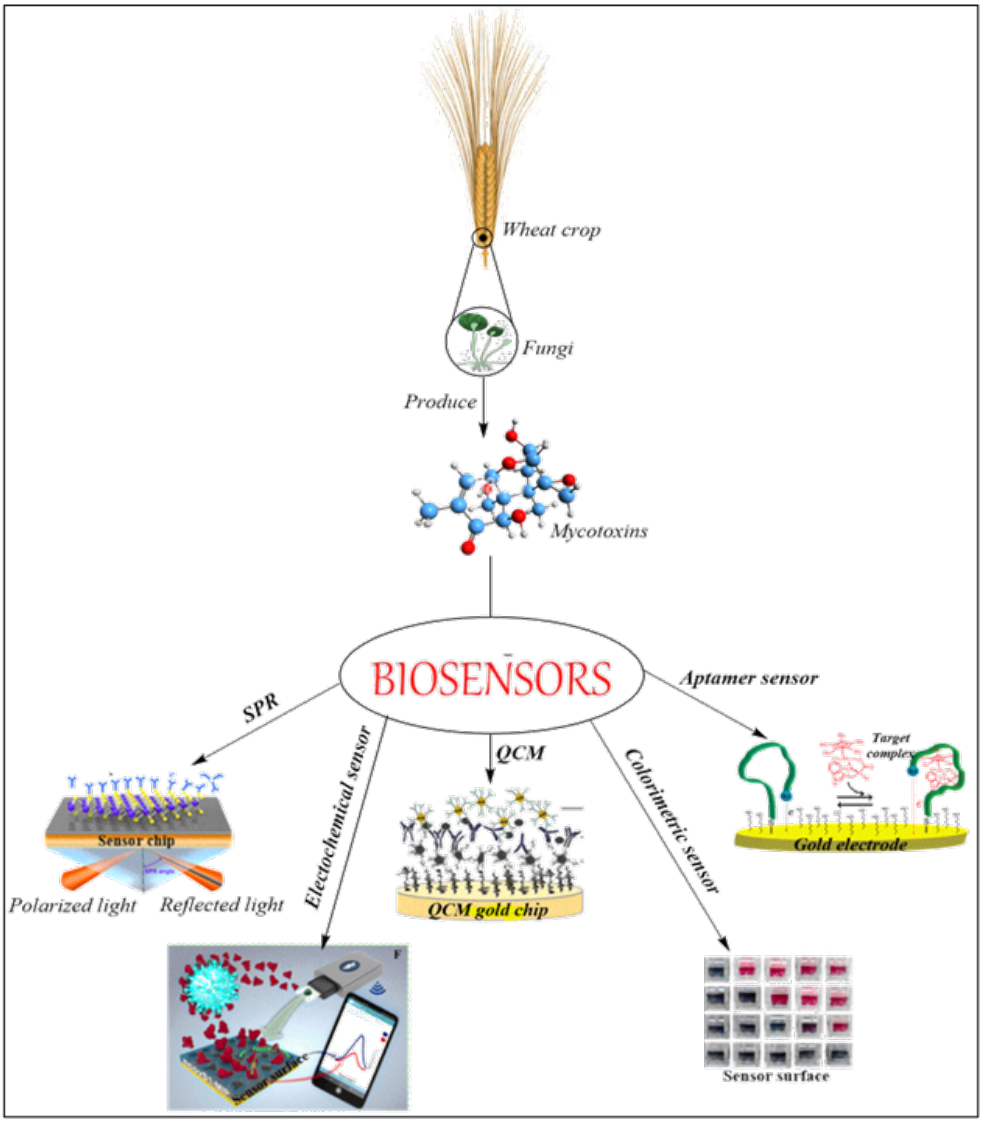

Figure 4. Biosensors used for the analysis of mycotoxins at whole wheat flour (SPR– Surface Plasmon Resonance sensor, Electrochemical Sensors, QCM – Quartz Crystal Microbalance, Colorimetric sensors, Aptamer sensors).

hormone level. Also, there are reports on the use of nanosensors to detect fungal pathogens mainly by detecting mycotoxins. For example, the biosensor 4mycosensor detects different types of fungal mycotoxins in different crops such as oat, corn, barley and wheat [51]. This nano-based diagnostic kit can assist farmers in preventing disease epidemics in the field. Another noteworthy detection method is based on surface-enhanced Raman spectroscopy (SERS), which is capable of recognizing chemical fingerprints. Using silver nanoparticles (AgNPs), this approach quickly detected *Alternaria* mycotoxins in pear fruit, with a limit of detection (LOD) of 1.30μg/L [52].

The second group of Sensors the Biosensors, which according to the official IUPAC (International Union of Pure and Applied Chemistry) by definition are "a self-contained integrated device, which is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element (biochemical receptor), which is retained in direct spatial contact with a transduction element" are also a valuable tool for the detection of mycotoxins. Related to the physic-chemical properties of mycotoxins and/or the type of transduction, biosensors technology can be divided into three groups according to [53]:

3.2.1 Surface Plasmon Resonance sensor (SPR)

Biosensors based on SPR use a thin metal (usually gold or silver) film between two transparent media with different refractive indices, like, a glass prism and the sample solution. The principle of the technique is based on the detection of a change of the refractive index of the medium when an analyte binds to an immobilized partner molecule (antibody) [19]. That means that the changes in the mass concentration of material on the surface are crucial to the sensitivity of SPR sensors. The change in the refractive index produced by the capture of biomolecules depends on the concentration of analyte molecules at the sensor surface and the properties of the molecules [21].

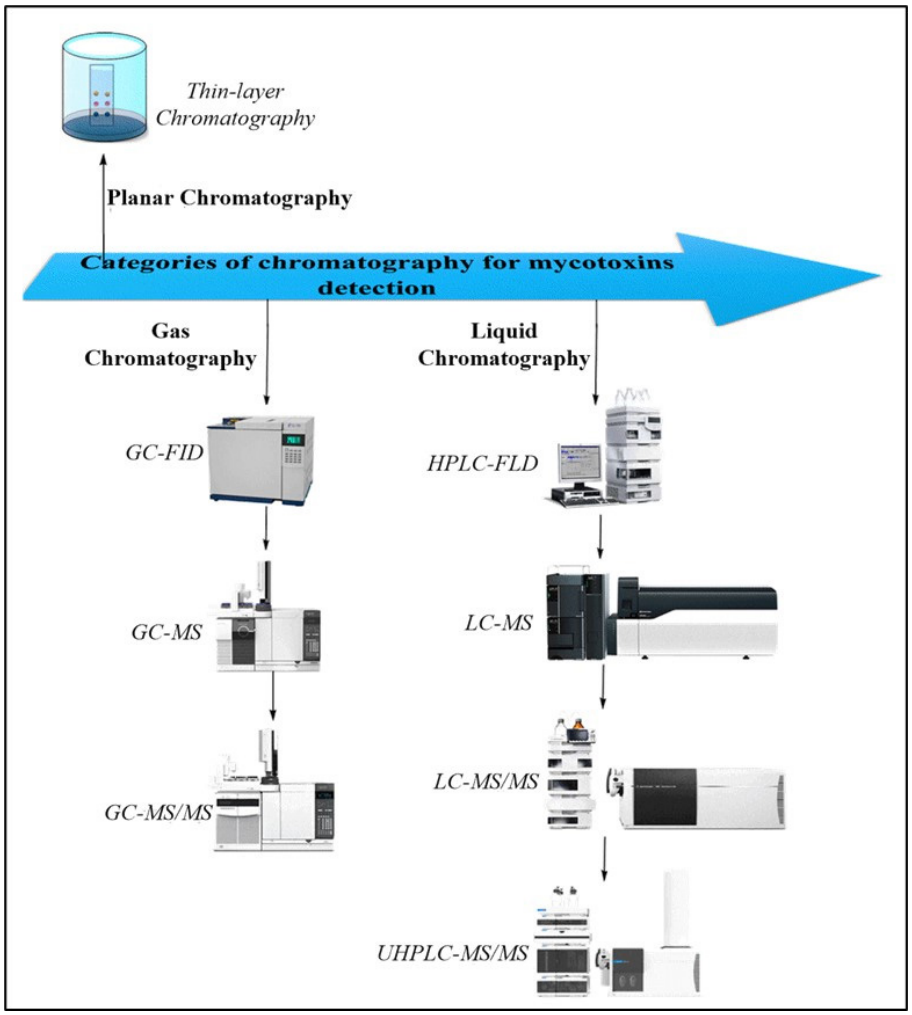

Figure 5. Categories of Chromatography used to analyze mycotoxins at whole wheat flour (GC-FID – Gas Chromatography-Flame Ionization Detector; GC-MS – Gas Chromatography-Mass Spectrometry; GC-MS/MS – Gas Chromatography Tandem Mass Spectrometry; HPLC-FLD – High Performance Liquid Chromatography-Fluorescence detector; LC-MS – Liquid Chromatography-Mass Spectrometry; LC-MS/MS – Liquid Chromatography-Tandem Mass Spectrometry; UHPLC-MS/MS -Ultra High-Performance Liquid Chromatography / Tandem Mass Spectrometry).

3.2.2 Piezoelectric sensors (e.g. QCM)

Another example which has gained importance in the field of biosensors is an acoustic device - quartz crystal microbalance (QCM), introduced in late 1950s' by Sauerbrey [53]. A Quarz Crystal Microbalance (QCM) consists of a thin quartz disk, where are placed the electrodes. The application of an external electrical potential to a piezoelectric material produces internal mechanical stress. QCM-based sensors monitor the shift in the resonance frequency of the quartz crystal in the microbalance device due to mass increase caused by the absorption of analytes onto the sensor [54].

3.2.3 Electrochemical sensors

Chemical sensor is a device that converts chemical data into an analytically usable signal. Their typical part is the presence of a suitable enzyme in the biorecognition layer providing electroactive substances detectable by the physicochemical transducer providing the measurable signal [55]. Electrochemical detection techniques use predominantly enzymes (or antibody, cells), due to their specific binding capabilities and biocatalytic activity. Based on their operating principle, the electrochemical biosensors can generate a measurable current (amperometric), a measurable potential or change accumulation (potentiometric), can generate change in signal for conductance (conductometric) between electrodes [56]. Recently, electrochemical sensors have been applied to monitor mycotoxins on accounts of its high sensitivity, easy to use, low cost and fast detection speed [57]. On the other hand, they have some limitations: electrochemically active interferences in sample, weak long-term stability, selectivity and reproducibility problems. Nevertheless, they offer numerous applications in clinical diagnosis and environmental monitoring [58].

3.2.4 Colorimetric sensors

Colorimetric sensors are based on the visible or UV light transformation into an analytical signal [40], and assays use mostly gold and silver nanoparticles. The aggregation of gold nanoparticles (AuNPs) causes a color change from red to blue,

and for silver nanoparticles (AgNPs) from yellow to brown due to the interparticle coupling effect. This recognizable change of color creates the ability to design colorimetric sensors for target analytes [59]. Due to their excellent sensitivity, selectivity, low cost, ease of fabrication, naked-eye observation, and the fact that they are easy to operate the colorimetric sensors show promising potential for the detection of drugs, pesticides, environmental monitoring, biomolecules, etc [60].

3.2.5 Aptasensors

Aptamers are short DNA or RNA oligonucleotides with the capability to selectively bind specific target molecules and are considered as synthetic antibodies. They can be utilized as receptors for biosensors similar to normal antibodies, however with the advantage of being more robust, affordable, relatively tiny, and unlike the monoclonal antibodies can bind to almost any target of choice, from small molecules to whole cells and microorganisms [61]. Furthermore, they have high-density immobilization, can be separated from targets upon washing with different buffers, and due to their stable chemical structure do not need cold storage. Aptasensors are constructed by a variety of methodologies, such as electrochemical biosensors, optical biosensors and mass sensitive biosensors [62]. Conjugation of aptamers on various nanomaterials such as single-walled carbon nanotubes (SWCNTs), and graphene oxide (GO) have led to highly sensitive and selective aptasensors [62]. In addition to fluorescent aptasensors, colorimetric ones are also widespread techniques for the analysis of mycotoxins because they are simple, rapid to use and have no requirements for complicated instruments [63]. Figure 4 summarizes main biosensors-based methodology for mycotoxins analysis at whole wheat flour to date.

3.3 Chromatographic techniques used for mycotoxin analysis

3.3.1 Thin-Layer Chromatography

Thin-layer chromatography (TLC) is one of the most widely used separation techniques for detection of aflatoxins. It can be used for both quantitative and semi-quantitative purposes [64]. TLC consists of a stationary phase like silica, alumina, and cellulose immobilized on a glass or plastic plate and a solvent as the mobile phase. Extracts from grain samples are spotted onto a plate of glass that is coated on one side with a thin layer of silica gel (which means that the sample extract is deposited on the stationary phase). One edge of the plate is vertically placed in a solvent tank and the solvent moves up the plate by capillary action carrying the mycotoxin [65]. At this point, the substances contained in the mobile phase react with the stationary phase. The plate can be also visualized by UV, fluorescence, MS, or other techniques. Separation depends by the relative solubility of the mycotoxin in the solvent mixture and its interaction with the silica gel [66]. TLC use can provide a simple and low-cost method, allows high reproducibility of the results [67] hence it does not provide identification of the mycotoxin, and sensitive measurements.

3.3.2 Gas Chromatography (GC)

GC is used to identify and quantify the presence of mycotoxins in food samples, but normally is linked with MS, flame ionization detector (FID), or Fourier Transform Infrared Spectroscopy (FTIR) in order to detect the volatile products. Most of the mycotoxins are not volatile, in this way they need to be derivatized for GC analysis [23]. Due to the need for this extra step GC requires more complex sample preparation procedures that could affect throughput in a commercial application. Gas chromatography (GC) separates components by their relative affinity for a stationary column and an inert gas as mobile phase [68]. The stationary phase consists of inert particles coated with a layer of liquid, and is normally confined to a long stainless steel or glass tube called the column, which is maintained at appropriate temperature.

The sample to be analyzed is vaporized into gaseous phase and carried through the stationary phase by a carrier gas. The analytes in the sample mixture with higher affinity for the stationary phase are retarded in their movement through the column, while those of low affinity pass through the column faster [45]. This method of analysis can be quite sensitive for mycotoxin analysis but does require preliminary cleanup of the extracts. Additionally, due to the conditions during the gas phase, only nonpolar, volatile analytes and high-temperature stable compounds can be analyzed and it has been mainly applied to trichothecenes or fumonisins [32].

3.3.3. High Performance Liquid Chromatography (HPLC)

HPLC is a chromatographic technique used to split a mixture of compounds in analytical chemistry, biochemistry and industry. This technique is used to identify and quantify the compounds [69]. It uses one stationary phase (chromatographic column), and one mobile phase containing aqueous/organic solvents, which flow through the solid adsorbent. The method relies on pumps that circulate a pressurized liquid solvent (mobile phase) containing the sample mixture through the column. Different components in the sample, e.g. mycotoxins, interact with the adsorbent material in different ways (differences in affinity). The liquid (mobile) phase passes through the column yields separate fractions containing individual components in the sample. In practice, the HPLC technique have a stationary phase such as C-18 chromatography column, a pump that moves the mobile phase(s) through the column, a detector that displays the retention times of each molecule, and mobile phases [45]. HPLC coupled with UV, a diode array detector, or a fluorescence detector can be used as a reliable method for mycotoxin detection [68]. A number of toxins have natural fluorescence (e.g. OTA, AFT, CIT) and can be detected directly with HPLC–FLD, but many do not. These mycotoxins, such as FUMs need to be derivatized prior HPLC detection. For aflatoxins identification can be used trifluoroacetic acid (TFA) (pre-column derivatization) or bromine or iodine (post-column derivatization) [23]. Despite offering good sensitivity and specificity, HPLC techniques are expensive, require qualified staff, co-elution of some compounds is difficult to be

detected, and are time consuming [70].

3.3.4 Mass spectrometry/Tandem mass spectrometry

Liquid Chromatography coupled with Mass Spectrometry or tandem mass spectrometry (LC-MS/MS) has been used for simultaneously screening, identifying, and measuring a large number of mycotoxins, particularly those toxins, which are not fluorescent, like fumonisins [23]. This technique combines the physical separation properties of the HPLC with the mass analysis capabilities of the mass spectrometer (MS). Most of these instruments use atmospheric pressure chemical ionization (APCI) or electrospray ionization (ESI) interfaces coupled with single or triple quadrupole mass spectrometers as a powerful technique for the determination of masked mycotoxins (deoxynivalenol-3-glucoside or zearalenone-4-glucoside, in wheat), which are compound conjugated to more polar substances that are not detected by routine analytical methods [71, 68].

3.3.4.1 Ultra Performance Liquid Chromatography (UPLC-MS/MS)

UPLC system first introduced in 2004 has the same principle as HPLC, with the only difference the fact that this system operates at high pressure (to 15000 psi), using in this way columns with particle size less than 2μm [72]. The basic Instrumentation of UPLC is Sample Injection, UPLC Columns, and Detectors. UPLC system is often organized with: TUV (Tunable-Ultra Violet), ELS (Evaporative Light Scattering), PDA (Photo Diode Array), and FLR (Fluorescence) [73].

3.3.4.2 UHPLC- Ultra High-Performance Liquid Chromatography (UHPLC-MS/MS) system has the same basic principles as HPLC for the separation of components in a matrix. By contrast, UHPLC operates in higher pressures than 600 bar. UHPLC use columns containing particles smaller than the 2.5–5-μm sizes typically used in HPLC [20]. UHPLC system is often organized with: Solvent delivery systems (Pumps), UHPLC has a pump pressure of 100 Mpa; Sample injection; Columns, and Detectors. The most common detectors used in UHPLC analysis are UV/visible-based types. Detection of analytes is conventionally based on absorbance [74].

3.3.4.3 Ultrafast liquid chromatography connected with tandem mass spectrometry (UFLC-MS/MS) UFLC-MS/MS is a derivative of UPLC system and is considered as a new revolution in chromatography. Compared with UPLC, it is ten time faster and three times better separation that UPLC. The UFLC columns are made up of small particles size (2.2μ) leads to highly selective and chemically stable columns with high-speed analysis which results in shorter retention times with reproducible result and highly robust [75].

4. Comparison of the Methods Efficiency

As previously described, different analytical techniques either conventional (Thin-Layer Chromatography-TLC, High-Performance Liquid Chromatography-HPLC, Gas Chromatography-GC, Liquid Chromatography-Mass Spectrometry-LC-MS, etc), or rapid (ELISA and Sensors-based) have been developed to control the levels of mycotoxins in wheat flour. In order to compare their efficiency, Table 1 summarizes main advantages and disadvantages as reported by different authors, while Table 2 describes in details the Validation parameters for each method (linearity, recovery range, RSDr range, RSD_R (%), LOQ range, and cut off), as well as the extraction methods and extraction solvents for categories of mycotoxins detected.

In brief, the immunoassays do suffer from matrix interferences and cross-reactivity of antibodies; the biosensors display problems related to selectivity and reproducibility of data; and chromatography-based assays are expensive, time consuming and require highly-skilled staff. Depending on the purpose of the analysis (for research or routine screening as part of legal requirements), one should consider that immunological methods are easy to use, they do provide quick results, but are only suitable for validated matrices. Meanwhile, the biosensors-based analysis is fast, cheap, portable, and can be used for routine screening, and GC-MS and HPLC based methods offer high sensitivity, specificity, accuracy, and fulfill the legal requirements. However, none of the methods reviewed in this paper can be used to detect and analyze all mycotoxin categories at once, because of limitations such as: LC-MC can be used for non-fluorescent and masked mycotoxins [23]; HPLC requires that compounds must have UV absorption or fluorescence properties, or some compounds is difficult to be detected because of co-elution [70]; GC can be used to analyze only non-polar, volatile analytes and high-temperature stable compounds [32]; and Immunological methods do suffer from cross-reactivity of antibodies [76-78], which may alter the results. More information on the possible pairing of the extraction and detection methods for specific mycotoxins, and technical details on validation parameters for each case, based on previous reports are given at Table 2.

It was evidenced that the chromatographic methods have been greatly improved in time. Thus, after the TLC, which cannot identify single mycotoxins from a group of such [64-67], the GC was developed, which is used to identify volatile mycotoxins only, unless derivatized [23,68], then the HPLC was used to detect fluorescent mycotoxins mainly, unless derivatized [23,68], followed by LC-MS/MS used for non-fluorescent and masked mycotoxins conjugated with more polar substances [23,71,68] further improved by UHPLC-MS/MS, which detects based on the absorbance [74], and to date the UFLC-MS/MS was developed, which constitutes a revolution in chromatographic techniques thanks to the speed and resolution improved many times [75]. Likewise, sensor-based methods have rapidly evolved towards electrochemical, optical, mass sensitive biosensors, and the conjugation with nanomaterials as single-walled carbon nanotubes (SWCNTs), and graphene oxide (GO) have led to highly sensitive and selective aptasensors of the widespread fluorescent and

Table 1. Advantages and disadvantages of several analytical methods for the detection and quantification of mycotoxins at wheat flour.

Nr	Detection Methods	Advantages	Disadvantages	References
1.	Lateral flow strip	Rapid analysis (results are ready in 3-5 min) Don't need special equipment Quantitative results can be obtained using a Lateral Flow Device reader	Matrix interferences	[76]
2.	Enzyme-Linked Immunosorbent Assay (ELISA)	Simple sample extraction Good sensitivity Low limit of detection Easy application Rapid procedure	Matrix interferences (the presence of other substances in the solution that can alter the results) Only suitable for validated matrices Antibodies can react with each-other and can alter the result (cross-reactivity) Possibility of a false positive and false negative result Single use of kits	[77]
3.	Fluorescence polarization immunoassay (FPIA)	Easy to use No need for additional separation steps, washing and incubation Low amount of reagents Fast detection	Sensitive to matrix effect Requires large changes in molecular volume for the generation of maximum signal and assay window Expensive detector	[78]
4.	Biosensors	Cheap Fast Portable Suitable for routine screening	Selectivity and reproducibility problems	[76]
5.	Thin-layer chromatography (TLC)	Simple, cheap, fast Multiple samples can be run simultaneously	Separation may not be precise Does not work for all mycotoxins Does not provide an identification of the mycotoxin	

Table 1. Advantages and disadvantages of several analytical methods for the detection and quantification of mycotoxins at wheat flour.

Nr	Detection Methods	Advantages	Disadvantages	References
6.	Gas chromatography–mass spectrometry (GC-MS)	High sensitivity High specificity (low interference)	Data analysis is time-consuming Expensive Requires highly-skilled technicians to carry out the analysis	[32]
7.	High-performance liquid chromatography (HPLC)	High sensitivity Small amounts of sample are needed Applicable to complex matrices Great reliability Highly accurate Fulfills legal requirements	Time consuming Compounds must have UV absorption or fluorescence properties Expensive Requiring large quantities of expensive organics. Troubleshooting problems may be difficult due to the presence of different modules, columns, and mobile phases in the instrument	[22]
8.	Liquid chromatography/ Mass spectrometry (LC/ MS)	Low detection limits Qualitative and quantitative results Applicable to complex matrices Minimal sample treatment required Can cover a wide range of analytes	Expensive Highly trained personnel required to carry out the analysis Time consuming compared to rapid tests Sensitivity relies on ionization technique	[76]
9.	Ultra performance liquid chromatography (UPLC-MS/MS)	High resolution performance Selective Sensitive Lower operating cost and shorter run time Reduce solvent consumption	Maintenance and shelf life of columns is short Expensive equipment Highly-trained staff is needed	[79]
10.	Ultra high performance liquid chromatography (UHPLC-MS/MS)	Decreases run time and increases sensitivity and performance Maintains resolution performance Less solvent consumption. Very fast separations with good resolution	Require more maintenance and reduces the life of the columns. Expensive equipment Highly-trained staff is needed	[20]

Table 2. Comparison of the efficiency of methods for the mycotoxin detection at wheat grains based on their validation parameters.

No	Detection Method	Mycotoxins detected	Extraction method	Extraction solvent	Linearity	Recovery range (%)	RSDr range (%)	RSD_R (%)	LOQ range (µg/kg)	Cut off (µg/kg)	Ref
1.	Lateral Flow Strip	DON	LLE	Water	-	-	38 (STC=1750 ug/kg)	-	-	920	[80]
2.	ELISA	Total Aflatoxin	LLE	70% Methanol	0.98	61-93	10.5-29.5	-	3.15	-	[81]
3.	Surface plasmon resonance sensor	DON OTA ZEN AFB1	LLE		0.99			-		-	
4.	TLC	Total Aflatoxin	LLE	Methanol/H_2O (3:1)	0.99	75.4-101.1	0.2-1.77	0.42-1.94	2.22-3.11	-	[41]
5.	GC-MS	DON FUS 15-acetyl DON NIV ZON	QuEChERS	Acetonitrile	0.98	52-103	0.7-21	-	5-40	-	[82]
6.	HPLC	DON OTA AFB1 AFB2 AFG1 AFG2 ZEA	IAC	Phosphate Buffer Saline	0.99	70.2-105.8	1.7-7.5	4.5-12	0.42-38.11	-	[83]
7.	LC-MS/MS	AFB1 AFB2 AFG1 AFG2 FB1 FB2 T2 HT2 OTA ZEA	QuEChERS	Acetonitrile/H_2O (80:20)	0.99	80.77-109.85	5.25-14.11	5.25-13.59	0.53-89.28	-	[84]
8.	UPLC-MS/MS	DON AFB1 AFB2 AFG1 AFG2 AFM1 AFM2 FB1 FB2 OTA ZEA	LLE	Hexan/ Formic acid 3%/ Acetonitrile	0.99	70.5-109.8	0.9-13.4	3.8-16.7	0.2-12.27	-	[18]
9.	UHPLC-MS/MS	AFB1 AFB2 AFG1 AFG2 FB1 FB2 T2 HT2 OTA ZEA	LLE	Acentonitrile/H_2O/Formic acid (79:20:1)	0.99	89.4-115.7	1.4-17.4	1.1-9.2	0.5-200	-	[85]
10.	UFLC-MS/MS	AFB1 AFB2 AFG1 AFG2 FB1 FB2 OTA ZEA	LLE	Methanol/ H_2O/ Formic acid (79:20:1)	0.99	74.96-100.6	0.89-16.5	2.15-6.31	0.15-0.5	-	[17]

colorimetric categories for the analysis of mycotoxins [49-63].

Conclusions

Preserving the yield and quality of wheat from the negative impact of abiotic and biotic environmental factors, as well as human activity (application of herbicides, pesticides, fungicides, etc.) is a priority for the modern agriculture. Fungal diseases are among the phytopathologies with a significant impact on wheat and its products, responsible for the reduction of yield, and for the synthesis of mycotoxins. The latter, being toxic to humans and animals, constitute a potential risk that must be addressed through the legal regulation of measures to limit the causative pathogens, as well as to determine the methods for their extraction, detection and quantitative analysis in seeds (wheat flour) and by-products. The EU, FAO, WHO, and FDA have examined these needs by creating a set of regulatory documents on the quantitative limits allowed in food products for mycotoxins in general or their classes in particular.

However, the chemical complexity of the mycotoxins synthesized by the filamentous fungi in wheat stems from the large number of pathogens responsible, their coexistence in the wheat plant, as well as from the fact that this class of toxins exhibits chemotypes with variable toxicity and biological activity. The above mentioned have encouraged the need for the development and use of different methods for the extraction and qualitative- quantitative analysis.

In this paper are reviewed methods for the extraction and evaluation of mycotoxins in total wheat flour. The first group can be categorized into: Solvent extraction method, the Liquid Liquid Extraction-LLE, the Solid Liquid Extraction-SLE, the Solid Phase Extraction-SPE, the Immuno-Affinity Columns-IAC, the QuEChERS, and the use of absorbent nanomaterials such as graphene oxide (GO) and multi-walled carbon nanotubes (MWCNTs) as innovations in development. Meanwhile, methods reported for the detection and qualitative assessment of mycotoxins can be grouped into: immunological, chromatographic and sensor-based (nano and biosensors). Previous reports prove that sensor-based methods are experiencing a great development thanks to the use of nanoparticles, nanomaterials, aptasensors, etc., and that chromatographic techniques are progressing through the coupling with mass spectrometry, offering a higher selectivity and sensitivity, low detection limits, maintained resolution performance, etc. The evaluation of the advantages and disadvantages of the above-mentioned methods showed that generally the immunoassays do suffer from matrix interferences and cross-reactivity of antibodies; the biosensors display problems related to selectivity and reproducibility of data; and chromatography-based assays despite their excellent sensitivity, specificity, and accuracy are expensive, time consuming and require highly-skilled staff. The possibility of pairing methods of extraction to those for the qualitative-quantitative analysis of mycotoxins for specific classes or types of the last, as well as comparing the effectiveness of the reported methods based on their validation parameters was also reviewed. It was evidenced that none of them can be used to analyze all categories of mycotoxins at once, because of their chemical characteristics (volatile/non-volatile, co-elution, UV absorption, fluorescence properties) versus the screening capacities and restrictions of methods. It can be concluded also, that despite the technical and cost-related efficiency of each method, depending on the purpose of the analysis of mycotoxins (research or screening as part of legal requirements) one should consider that immunological methods are only suitable for validated matrices, biosensors can be used for routine screening, and that GC-MS and HPLC-based methods fulfill the legal requirements. In this context, the further improvement of the existing methods with the aim of reducing the identified disadvantages constitutes the prospective goal. While in general the selectivity and accuracy of methods for mycotoxin detection is being improved, it is the use of biosensors-based methodology, which thanks to the unique capacity to evaluate the toxicity and biological activity of mycotoxins, will most probably be a new near future trend. In conclusion, while the selectivity and accuracy of methods for mycotoxin detection is being improved rapidly (those sensor-based thanks to the use of nanoparticles, nanomaterials, aptasensors, etc., and the chromatographic techniques coupled with mass spectrometry offer a higher selectivity and sensitivity, low detection limits, maintained resolution performance), and the duration of the analysis, the cost, and the need for highly-skilled staff go in favor of rapid methods (immunological and sensors-based), it is the capacity to fulfill legal requirements, which will determine the trend and their future success in the market.

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