

Review

Recent Advances in Mycotoxin Analysis and Detection of Mycotoxigenic Fungi in Grapes and Derived Products

Dimosthenis Kizis ^{1,*}, Aikaterini-Eleni Vichou ¹  and Pantelis I. Natskoulis ^{2,*} 

¹ Laboratory of Mycology, Scientific Directorate of Phytopathology, Benaki Phytopathological Institute, St. Delta 8, 14561 Kifissia, Greece; k.vichou@bpi.gr

² Institute of Technology of Agricultural Products, Hellenic Agricultural Organisation DEMETER, Sofokli Venizelou 1, 14123 Lycovrissi, Greece

* Correspondence: d.kizis@bpi.gr (D.K.); p.natskoulis@gmail.com (P.I.N.)

Abstract: Mycotoxins are secondary metabolites of filamentous fungi that can cause toxic effects in human and animal health. Most of the filamentous fungi that produce these mycotoxins belong to four genera, namely, *Aspergillus*, *Penicillium*, *Fusarium*, and *Alternaria*. Mycotoxigenic fungi, along with mycotoxins, create a constant and serious economic threat for agriculture in many terms, counting product losses due to crop contamination and food spoilage, as well malnutrition when considering nutritional quality degradation. Given the importance of robust and precise diagnostics of mycotoxins and the related producing fungi in the grape food chain, one of the most important agricultural sectors worldwide, the present review initially delivers a comprehensive presentation of mycotoxin reports on grape and derived products, including a wide range of commodities such as fresh grapes, raisins, wine, juices, and other processed products. Next, based on worldwide regulations' requirements for mycotoxins, and referring to the relative literature, this work presents methodological approaches for mycotoxin determination, and stresses major methods for the detection of fungal species responsible for mycotoxin production. The principle of function and basic technical background on the available analytical and molecular biology techniques developed—including chromatography, mass spectrometry, immunochemical-based assays, biosensors, and molecular assays—is briefly given, and references for their application to grape and derived product testing are highlighted.

Keywords: mycotoxins; mycotoxigenic fungi; mycotoxin determination; chromatographic analytical methods; mass spectrometry; immunochemical-based assays; biosensors; polymerase chain reaction



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1. Introduction

Mycotoxins, named from the coupling of the ancient Greek words *mykes* and *toxon*, meaning “mold” and “poisonous arrow,” respectively, are products of the secondary metabolism of filamentous fungi that in low concentrations can cause deleterious effects in animals and humans. The definition of a fungal secondary metabolite as a mycotoxin takes into consideration both the effective concentration of the metabolite and the target organism affected, the type and size of the producing fungus (i.e., mushroom or filamentous contaminating species), and the human intention during food consumption and thus exposure to the toxin(s). However, fungal metabolites that are mainly toxic to bacteria (antibiotics) or plants (phytotoxins), which are poisonous compounds produced by mushrooms (Basidiomycete toxins) that are accidentally ingested via deliberate consumption of the fungal fruiting body, are not considered mycotoxins [1,2].

Mycotoxins comprise a very large and heterogeneous group of substances with diverse chemical structures and biological effects and are produced by a wide number of different fungal species. Their categorization is challenging and varied, following classification norms based either on the fungal species, chemical structure, biosynthetic origin, biological effect, or type of illness they may cause [2,3]. They are considered a serious agriculture,

public health, and economic problem worldwide, in terms of crop contamination, food spoilage, and nutritional quality degradation, and due to their association with causing acute, subacute, and chronic toxicity in animals and/or humans [4–7].

Accounting for the numerous approaches for defining mycotoxins, their heterogeneity, and the presence of many chemically related metabolites, there are more than 400 substances that can be classified as such [2,8,9]. Although only some of them are regularly found in foods and a few of them, including aflatoxins (AFs), ochratoxin A (OTA), fumonisins (FB_{1–4}), certain trichothecenes (deoxynivalenol (DON), nivalenol (NIV), HT-2, T-2), zearalenone (ZEA), patulin (PAT), citrinin (CIT), ergot alkaloids, tenuazonic acid (TeA), and alternariol (AOH), present significant food safety issues [1,8,10–12]. Most of the filamentous fungi that produce these mycotoxins belong to four genera, namely, *Aspergillus*, *Penicillium*, *Fusarium*, and *Alternaria*. These genera include species that are predominant producers and/or common producers of a certain mycotoxin, as well as species being able to produce more than one of the aforementioned mycotoxins [13–16].

The impact mycotoxins generate in the agricultural, food, and public health sectors has led national and international authorities to undertake various preventive and management actions for more than 40 mycotoxin–food combinations, including the formation of mycotoxin regulations and regulatory statutes, establishment of mycotoxin limits in food and feed, implementation of monitoring methods, development of standardized analytical assay protocols, and management recommendations and guidelines [17–23].

Research in the fields of mycotoxins and mycotoxigenic fungi has been a pivotal axis to drive and support the aforementioned actions during the last four decades. There are numerous research and review publications on the development of analytical methods, risk assessment, predictive modeling, and decision support tools, along with management strategies and practices [24–28]. Current needs and emerging issues such as the detection of multiple mycotoxins in samples [29], masked and modified mycotoxins in foods and feed [30–32], and synergistic and additive effects of prolonged low-level exposure to different mycotoxins on human and animal health [33–36] are already new research fields for mycotoxins.

2. Mycotoxins in the Grape Food Chain

The grape food chain comprises the grape berry—the fruit of the grapevine itself—and numerous products derived from grape berry processing, including wine, distillates, vinegar and balsamic vinegar, grape juice, dried grapes, jams, jellies, and *petimezi* or *pekmez* (a must condensed by boiling and used as basic ingredient in some ethnic dessert products in Southeastern Europe and the Eastern Mediterranean). A thorough literature review has revealed that different mycotoxin groups are encountered in grapes and almost every type of grape-related commodity.

Regarding fresh table grapes intended for direct consumption, several review articles can be found in relation to mycotoxigenic fungi and mycotoxin contamination [37,38]. Available are research studies concerning PAT [39,40], aflatoxins B_{1–2} (AFBs) [41,42], FBs [43,44], CIT [39,45], *Alternaria* toxins [46], and OTA [43,47,48], with the latter encountering the majority of mycotoxin research works on grapes.

Fresh grapes often undergo several drying processes for the production of dried grapes, such as the traditional open sun drying and open-air shade drying or with the aid of proper equipment for the reduction of humidity, such as hot-air, freeze, microwave, and vacuum-impulse drying [49]. Dried grapes account for, among others, mainly raisins, currants, and golden raisins, with the difference lying in the grape variety of origin: The first one refers to dried grapes as a generalized term, currants are produced more often from dried Black Corinth grapes, and the latter are made with the Sultanina white grapes from the Eastern Mediterranean, also known as Thompson Seedless in the rest of the world [50]. Numerous reports on dried grapes addressed mycotoxins and the relevant producer fungi, with most of them referring to OTA [51,52]. However, other mycotoxins

were also studied, such as AFBs [53,54], FBs [55,56], *Alternaria* spp. mycotoxins, and others produced by *Penicillium* spp., such as mycophenolic acid [57], PAT [58], and CIT [59].

Grapes represent an excellent raw material for juice production. Juices are considered the third largest source of exposure to OTA after cereals and wines and are a commodity often intended for consumption by children, thus presenting an extra required awareness regarding mycotoxin presence. Other products delivered by ecchymosed fresh grapes are pomace, marc, and musts, with the latter term used for freshly crushed grape juice that contains the skins, seeds, and stems of the fruit. The solid portion of the musts is called pomace (or marc) and typically makes up 7–23% of the total weight of the musts, whereas the juice taken after filtration represents commercial grape juice [60,61]. Most studies on grape juices reported on the determination of PAT or OTA levels, either in the final product or intermediate ones such as musts for wine production or pomace intended for distillation. The studies focused on the incidence of OTA in juices being incipient in relation to research on the occurrence of this mycotoxin in wine and grapes, although there are several other works that can be found in the literature [62,63]. To the same extent, works referring to PAT and representative fungal producers for juice, musts, or pomace from grapes also appear [39,64,65]. Some research has been presented regarding CIT [39,66], but existing works on the presence of other mycotoxins such as *Alternaria* toxins, AFBs, and FBs are scarce [67,68].

In contrast to table grapes, where mycotoxin contamination risk may be reduced by visual inspection and moldy grapes would not have any market value, when the issue regards wine grapes this is not always the case. When grapes are meant for production of either a low-quality wine or a wine produced in large quantities, contamination with mycotoxins is often a problem [38,69]. From the first report on OTA in wine [70] and then after, numerous surveys have been conducted on its presence in several types of wines originating from many countries around the globe [71–73], and their findings rank OTA as the most frequently detected mycotoxin in wine. Interestingly, regarding literature on wines, readers can find competent research approaches also investigating the presence of modified forms of OTA, such as its analogs of ochratoxin B, methylochratoxin A, methylochratoxin B, ethylochratoxin A, and ethylochratoxin B in red wines originating from Spain and many other Mediterranean countries [73,74]. To monitor OTA presence in wine, different research works have investigated its fate during winemaking [75–77] and included efforts to remove it from the final product [78,79]. Apart from OTA, the presence of several other mycotoxins in wine has been studied, with the majority concerning AFBs [80,81], but also other mycotoxins such as FBs [82,83], AOH and other *Alternaria* toxins [84,85], PAT, CIT, and other *Penicillium* toxins [39,86,87].

Unfortunately, literature on mycotoxin contamination in vinegar and balsamic vinegar is minimal, with most research works reporting on OTA [88–90], and some on other mycotoxins such as AFBs [91] and CIT [92]. Regarding grape berry jam, jellies, and marmalade products, berry jams with a percentage of grapes in their composition were analyzed to test different QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) extraction methods and found them to be contaminated with AFBs, OTA, and *Alternaria* toxins [93]. Interestingly, there are a considerable number of studies on the presence of mycotoxins, and more precisely of AFBs and OTA, in Turkish *pekmez*, since this kind of product, due to condensation, can represent a potentially serious risk for the consumer [94–96]. Although part of research on mycotoxins involves these dessert products, a lot of work is still pending to ensure their safety.

3. Methods for the Determination of Mycotoxins and Mycotoxigenic Fungi

Current needs for mycotoxin determination are based on issues regarding emerging mycotoxins being discovered day by day and that do not necessarily have a fully characterized toxicity profile, modified compounds due to plant or food commodity metabolic processes that can act as masked or hidden mycotoxins, co-occurrence of several mycotoxins, and the complexity of different food and feed matrices along with difficulties with

the extraction of modified mycotoxins. Thus far, analytical approaches for determining mycotoxins include chromatographic and immunodetection methods covering with high specificity and sensitivity the entire span from a rapid, easy-to-operate analytical test for monitoring quantitative assays. Current demands are for the development of multi-target methods that assure both specificity and sensitivity characteristics and permit the identification of non-target compounds [97,98].

Chromatographic analytical methods for mycotoxin determination include (i) thin-layer chromatography (TLC) and high-performance thin layer chromatography (HPTLC), an improved version to enhance resolution and accuracy; (ii) high-performance liquid chromatography (HPLC) coupled with fluorescence (FLD), ultraviolet (UV), or diode array detectors (DAD); (iii) ultra (high)-performance liquid chromatography with reduced column packing material (UPLC, UHPLC); (iv) liquid chromatography coupled with mass spectrometry (LC–MS) and tandem mass spectrometry (LC–MS/MS); and (v) gas chromatography (GC) coupled with electron capture (ECD), flame ionization (FID), or MS detectors used to identify and quantitate volatile mycotoxins. Mass spectrometry is a technique with the potency to detect and quantify multiple mycotoxins simultaneously (over 100 mycotoxins in a single run) in a wide variety of food matrices. Regarding its use in combination with chromatography, there are different MS interfaces applied, such as atmospheric pressure chemical ionization (APCI), electrospray ionization (ESI), and atmospheric pressure photoionization, and various types of mass analyzers including quadrupole, time-of-flight (TOF), ion-trap, and Fourier transform-ion cyclotron resonance (FT-ICR) [97–100].

Immunochemical-based methods include (i) the enzyme-linked immunosorbent assay (ELISA), with which low-level detection and quantification of mycotoxins is feasible; (ii) immunochromatographic tests (ICT) in diverse formats such as lateral-flow devices (LFDs) and ICT strips for simultaneous multiplex detection, principally used for rapid analysis of mycotoxins and as a portable and easy-to-perform test; (iii) flow injection immunoassay (FIIA); (iv) chemiluminescence immunoassay (CLIA); (v) fluorescence polarization and fluorometric immunoassays; and (vi) biosensors, operating systems that exploit antibody properties to specifically detect mycotoxins coupled usually with portable sensing devices. Their development has focused on features including being easy to use, fastness, and portability, and can be classified as labeled or label-free with the mycotoxin receptor being either a biomolecule (antibody, DNA, enzyme, etc.) or a synthetic chemical (aptamer, mimotope, molecularly imprinted polymer (MIP), etc.) [97,98].

Other novel noninvasive approaches for the detection and analysis of mycotoxins include near infrared (NIR) and Raman spectroscopy, hyperspectral imaging (HSI), capillary electrophoresis, and the use of an electronic nose [8].

Early detection of mycotoxigenic fungi to monitor and predict contamination is crucial for preventing mycotoxins from entering the food chain. Thus far, a considerable number of molecular methods, including (i) conventional end-point and quantitative real-time (using SYBR Green I or TaqMan probe reporter dyes) PCR, either in simplex or multiplex formats; (ii) loop-mediated isothermal amplification (LAMP); (iii) high resolution melting (HRM) analysis; and (iv) to a lesser extent, amplicon-based metabarcoding, have been developed during the last two decades to determine toxin-producing fungal species belonging to the four principal mycotoxin-producing genera, namely, *Aspergillus*, *Penicillium*, *Fusarium*, and *Alternaria*, in different food commodities [101,102].

The aim of this review is to quote the available methods developed and used for the determination of mycotoxins and mycotoxigenic fungi detection in grapes and grape products.

4. Chromatographic Analytical Methods for Mycotoxin Determination

4.1. Sample Preparation

Chromatographic mycotoxin analysis involves a multistage operation including sample preparation, the extraction of mycotoxins, and finally identification and quantification. However, a considerable number of factors such as mycotoxigenic fungi heterogeneity, my-

cotoxin chemical structure variability, distribution in food-chain products, and the latter's matrix complexity result in difficulties not only for identification and quantification but also for the development and adaptation of a common analytical approach [10,98]. An advised sampling plan and sample preparation are prerequisites for reliable analytical results of mycotoxin quantification, especially when grapes are perceived as raw and intermediate materials that will undergo further processing, such as in the cases of juices, wine, and dried grape production. As a rule, mycotoxins are unevenly distributed in the raw material, and could present extremely high concentrations in sites where fungal proliferation has taken place. Finally, a proper homogenization of the sample is also required before any further preparation of the final sub-sample targeting analysis [103,104]. Sample preparation involves extraction to release the toxin from the product, extract purification to remove impurities, and occasionally concentration of the targeted mycotoxin when high sensitivity in detection is demanded.

4.2. Extraction and Purification

The extraction step aims to disengage the mycotoxin from the sample matrix, and often is followed by a subsequent clean-up step of the delivered extract to reduce matrix effects and kindred substances extracted together with the mycotoxin [10,105]. Some commonly used techniques are liquid-liquid extraction (LLE), solid-liquid extraction (SLE), and supercritical fluid extraction (SFE), but the most frequently applied extraction techniques for mycotoxins are the several types of solid phase extraction (SPE), with some of them presenting the advantage of both purification and pre-concentration at the same time. Nevertheless, the use of immunoaffinity columns (IAC) represents undoubtedly the most common purification technique following the extraction of mycotoxins [98,106]. The choice of suitable solvents and their combination for a successful extraction depends on the mycotoxin structure as well as on the matrix from which the extraction is required. Generally, the extraction of mycotoxins from solid matrices requires organic solvents, and sometimes, usually for liquid matrices, requires the combination of water with an organic solvent. Regarding mycotoxin structure, in cases of polar metabolites such as FBs, the presence of water is necessary, whereas for hydrophobic moieties such as AFs, the application of organic solvents is necessary. Most mycotoxins present high solubility in organic solvents such as acetonitrile, methanol, acetone, ethyl acetate, dichloromethane, among others, and limited solubility in water, with the exception of FBs and PAT, which have fairly good solubility in water [106,107]. Moreover, the addition of water can accelerate matrix penetration by organic solvents, whereas acidic solvents help the breakage of bonds between mycotoxin and food components, enhancing the overall procedure efficiency [108].

In the case of mycotoxin detection, for liquid products derived from grapes such as wine, vinegar, grape juice, etc., an LLE followed by a clean-up step with SPE or IAC is the most common approach for sample treatment, independent of the mycotoxin of interest, whereas for solid samples such as dried grapes, the SLE procedure is best applied before the clean-up step [98,109]. Modern extraction and purification techniques such as the so-called QuEChERS, initially applied for the determination of pesticide residues, as well as dispersive liquid-liquid micro-extraction (DLLME) have become very popular in recent years, with successful applications in OTA from wine [8,108].

Traditional chromatographic mycotoxin determination techniques employed either single mycotoxin or ideally a closely related mycotoxin group quantification. Current trends in mycotoxin determination have moved over a true multi-mycotoxin quantification, based on the powerful instrumentation tools that have emerged during the past decade. Modern analytical developments, such as coupling classic chromatographic detectors with mass spectrometry (MS) and tandem MS, and new sophisticated equipment such as UPLC (or UHPLC), are representing the cutting edge of mycotoxin determination [98].

4.3. Thin-Layer Chromatography (TLC)

A large number of different chromatographic methods have been used and most of them are still in use for the quantification of mycotoxins. TLC has been employed as an approved reference method for AF detection for more than half a century, but is considered effective mostly for mycotoxin screening purposes. Its significance is due to its low cost and simple instrumentation, as well as the easy fluorescent spots appearance under UV, which are diminished by its poor accuracy and low sensitivity, making mycotoxin quantification a difficult task. Nevertheless, TLC was further developed into high-performance TLC (HPTLC) by the reduction of both layer thickness and particle size, achieving more precise and accurate results for certain mycotoxin determination (e.g., AFs and OTA) [10,106,109].

4.4. Gas Chromatography (GC)

GC technology presents many advantages for mycotoxin detection and quantification when mycotoxins are sufficiently volatile at the column temperature. Nevertheless, GC employment for mycotoxin analysis is not widespread due to the fact that most mycotoxin substances are non-volatile and of high polarity, and thus require a previous derivatization step, which could, in addition, encounter degradation problems [8]. GC coupled with electron capture (ECD), flame ionization (FID), or MS detectors are still being applied to the determination of some volatile mycotoxins such as patulin, or after the relative derivatization, such as in the case of OTA in wine and beer and its derivative, O-methyl-OTA, or the derivatives of different trichothecenes [107,109]. Coupling with MS is strongly recommended for peak confirmation, and more specifically, multistage tandem MS analysis such as triple quadrupole (QqQ), and time-of-flight (ToF) detectors are suggested as more accurate for quantification purposes. Finally, the limitations of prior derivatization analysis have displaced GC-MS methods with LC-MS ones [110].

4.5. Liquid Chromatography (LC)

LC presents an exceptional development in mycotoxin detection, providing the potential for the simultaneous determination of several mycotoxins in a single run despite their chemical structure. The separation of analytes and matrix is accomplished in an analytical column in the mobile phase, and can be applied to high polarity, non-volatile, and thermally inconstant mycotoxins [107]. LC with fluorescence detection (LC-FLD) is one of the most widely used techniques for this kind of analysis, replacing the out-of-date photodiode array (PDA) detectors that were in use until the last decade [109]. Mass spectrometry (MS) technology sorts the ions of analytes depending on their mass-to-charge ratio after the ionization of chemical species, and tandem MS (MS/MS) has the additional advantage of chromatographic peak detection, which can improve precision for this separation technique. In recent years MS/MS detection systems have offered high sensitivity and selectivity, and thus their application has appeared to be by far the most accepted methodology in mycotoxin determination and quantification [107,111]. Additionally, MS/MS has been preferred over the fluorescence detection method since it can identify various non-fluorescent and fluorescent toxins and is cost-effective. LC-MS/MS includes relatively simple and non-laborious sample treatment allowing a high throughput of samples and a more precise, accurate, and sensitive method in comparison with simple MS or FLD. Apart from simple MS systems, LC with time-of-flight MS (TOF-MS) analyzers use electromagnetic force to push the ion accelerate down a flight tube, where light ions pass faster to the detector, permitting their identification according to their mass-to-charge ratio and receiving time [106,109]. In addition, rapid surface methods such as direct analysis in real time (DART) and desorption electro-spray ionization (d-ESI) offers new, more reliable, and faster determination of mycotoxins to LC with the advantage of analyzing many subsamples of the same batch, reducing the problem of non-homogeneity in large samples [112].

4.6. High-Performance Liquid Chromatography (HPLC)

HPLC is an accepted and well-established chromatographic method for mycotoxin determination with accurate quantification ability. The HPLC methods can vary in the column type (normal or reverse phase, different packing, particle size, etc.), elution mixtures and gradients, and, of course, detectors. Most HPLC techniques are performed in the form of reversed-phase and in an acidic mobile phase, such as, for example, the C18 columns and acetonitrile and methanol or water and combinations of them, acidified by acetic or phosphoric acid. Commonly used detectors for mycotoxins with HPLC are ultraviolet (UV) and fluorescence detectors (FLD), since several toxins possess natural fluorescence properties (e.g., AFs, OTA) and can be detected directly with this equipment. In addition, electrochemical (ECD), diode array (DAD), and many other detectors apply as sensitive modes for quantitative studies in HPLC. These detectors have been successfully coupled with MS and tandem MS detectors, offering high sample throughput, satisfactory analysis efficiency, high detection sensitivity, and finally, eliminating the need for sample derivatization for fluorescence activity, and thus enhancing them to state-of-the-art technology for mycotoxin determination. HPLC separates the sample into chemical compounds and the mass spectrometer ionizes molecules in order to sort and identify them depending on their mass-to-charge ratio (m/z) [107,109]. The improvement of column particle size from approx. 5 μm to less than 2 μm , has led traditional HPLC technology to ultra-HPLC (UPLC or UHPLC), presenting improved sensitivity, maintaining better and faster separation efficiency, and obtaining narrower peaks. Additionally, online SPE can be applied to advance sample throughput, low solvent consumption, and high pre-concentration [106,113].

5. Spectroscopic and Spectrometric Methods

5.1. Fourier-Transformed Infrared (FTIR) Spectroscopy

The use of FTIR spectroscopy as a rapid screening method to detect mycotoxins relies on the differences between spectra absorption and the resulting peaks observed in mycotoxin production. The result of molecular overtones and the combined vibrations associated with several chemical functional groups, such as C-H, O-H, N-H, etc., drives the collection of IR spectra by the measurement of transmittance and reflectance. However, there are considerable difficulties with confidently stating that the observed differences are the result of the specific mycotoxin production, since mold biomass can also produce other non-target mycotoxins or metabolites not directly related to the mycotoxin of interest. Despite this limitation, IR spectroscopic analysis has several advantages, as it is a rapid, sensitive, and non-destructive method acquiring small sample amounts to measure multiple components in a single analysis. An example is the determination of FB presence due to amine (N-H) and other polar functional groups (C=O, O-H), asymmetrical vibrations, or AFs due to C-O and C=O, apart from the C-H, O-H, and N-H groups [114,115].

5.2. Surface-Enhanced Raman Spectroscopy (SERS)

SERS has been explored as a rapid screening method with the potential for on-site measurement with a portable device. As an advanced Raman spectroscopic technique, it enhances the molecular fingerprint of the mycotoxin of interest in the presence of roughened metal particles and surfaces with nano-scale dimensions. SERS can be used for rapid and simple analysis of OTA in food systems such as wines by applying on-site measurements with portable devices. The only limitation is the need for a facile extraction method in order to achieve acceptable reproducibility [116].

5.3. Direct Analysis in Real Time (DART)

DART coupled with MS detectors (DART-MS) is another spectrometric technique, using atoms from heated metastable gases to desorb and ionize an analyte of interest to a final grid electrode source very close to the inlet of the mass spectrometer. Samples are directly introduced into the open-air sample region where gas flows from the DART source are considerably higher than those found in other systems such as GC-MS. Complex matrices

present challenges due to the lack of chromatographic separation, and to overcome this drawback, a number of sample concentrations and clean-up techniques are needed [117].

Another capability of DART is to be coupled with an ultra-high resolving power Orbitrap MS to quantify major analytes, which has already been successfully applied in cases of trichothecenes B, zearalenone, Alternaria toxins, and sterigmatocystin after a QuEChERS-based extraction. The lowest calibration levels are satisfactory, depending on the particular analyte, and the method demonstrated its suitability for high-throughput control of deoxynivalenol and zearalenone with very good accuracy of measurements achieved [29]. A similar approach has been applied to the detection of several AFs in wines with the aid of high resolution mass spectrometer (HRMS) coupled with DART and resulted in very fast detection and quantification with high sensitivity, allowing for identification down to ppb levels of concentration [113].

Some indicative and representative examples (from an extensive bibliography on methods applied to grapes and derived products) of mycotoxin extraction, purification, and analytical methods for detection are presented in Table 1.

Table 1. Characteristic examples of analytical methods for mycotoxin determination in grape and grape products.

No.	Authors—Reference [No]	Mycotoxin(s)	Commodity	Extraction/Clean-Up Procedure	Analytical Method
1	Apell et al., 2019 [114]	OTA	Grape juice, wine	nanosponge SPE	LC-FLD
2	Beltran et al., 2013 [115]	18 mycotoxins including AFs, AOH, DON, FBs, HT-2, NIV, OTA, T-2, ZEN, etc.	Grape, grape juice, Wine	LLE	UHPLS-MS/MS
3	De Berardis et al., 2018 [111]	AOH, AME, TEN, TeA	Apple, grape, and blueberry juice	QuEChERS/d-SPE	HPLC-ESI-MS/MS
4	Di Stefano et al., 2015 [116]	OTA, OTB, AFs	Sweet wines	LLE/SPE-IAC	HPLC-FLD
5	Fan et al., 2016 [84]	AOH, AME, TeA	Wine	Counter-current Chromatography	HPLC-DAD
6	Guo et al., 2019 [46]	ALS, ALT, AME, AOH, TEN, TeA	Grapes	QuEChERS	UHPLC-MS/MS
7	Heperkan et al., 2016 [55]	FB ₂	Raisins	n/a	FTIR spectroscopy
8	İçelli et al., 2019 [118]	AFs, FB ₁₋₂ , trichothecenes	Grape	n/a	HPLC-MS/MS
9	Kholová et al., 2020 [119]	OTA, OTB	Wines	on-line SPE	HPLC-FLD
10	Mikušová et al., 2013 [44]	Apicidin, beauvericin, enniatins, etc.	Dried grapes	Ultrasound-assisted SLE	HPLC-MS/MS
11	Nistor et al., 2017 [113]	AFs, OTA	Wine (red, sweet, and white)	on-line SPE	UHPLC-FLD
12	Nistor et al., 2019 [80]	AFs	Wine (red and white)	LLE	DART-HRMS
13	Ostry et al., 2018 [39]	CIT, PAT	Must, wine	QuEChERS	HPLC-UV-VIS
14	Pérez-Ortega et al., 2012 [120]	AFs, ergocornine, FB ₁ , OTA, ZEN, 36 mycotoxins including AFs, DAS, DON, FBs, HT-2, NIV, OTA, T-2, etc.	Red wine	SPE (polymeric-type cartridges)	HPLC-ESI-TOF-MS
15	Pizzutti et al., 2014 [121]	OTA, OTB, OTC and methyl-OTA	Wine	QuEChERS	UPLC-MS/MS
16	Remiro et al., 2010 [122]	OTA	Wine	IAC	HPLC-FLD
17	Rojas et al., 2021 [123]	OTA	Wine	LLE	SERS
18	Spanjer et al., 2008 [124]	33 mycotoxins including AFs, AOH, DAS, DON, FBs, HT-2, NIV, OTA, T-2, etc.	Raisins	LLE	HPLC-ESI-MS/MS
19	Tamura et al., 2012 [125]	14 mycotoxins including AFs, AOH, DON, FBs, HT-2, OTA, PAT, T-2, ZEN, etc.	Wine (red and white)	SPE	UHPLS-MS/MS
20	Welke et al., 2010 [38]	OTA	Red wine	LLE	UV-HPTLC-CCD
21	Zhang et al., 2018 [81]	20 mycotoxins including AFs, DAS, DON, FBs, HT-2, OTA, T-2, ZEN, etc.	Grape, wine	QuEChERS/ various d-SPE and sorbent mixtures	UHPLS-MS/MS
22	Zwickel et al., 2016 [126]	<i>Alternaria</i> spp. toxins (AA-III, AAL TB ₁ and TB ₂ , ALS, ALT, isoALT, AME, AOH, ATL, ATX-I, ATX-II, STTX-III, TEN, TeA)	Currant juice, grape juice, wine (red and white)	diatomaceous earth SPE	HPLC-(ESI, APCI, APPI)-MS/MS

5.4. Nuclear Magnetic Resonance (NMR) Spectrometry

The metabolomic approach for the characterization of fungal secondary metabolites by applying NMR spectrometry has not been a first choice for the analysis of mycotoxins because of its low sensitivity, lower detection limits, the need for highly skilled and trained personnel to operate, and finally, the limited number of relative software and quantification methods. However, it provides a non-destructive method that requires minimal sample preparation and can elucidate compounds without the need for standards [127]. Some of these limitations have been greatly improved by modern approaches of NMR, such as two-dimensional (2D) and multidimensional (nD) NMR. Nevertheless, there are limited number of studies dedicated to mycotoxin detection, with one noteworthy case of ^1H -NMR and ^{13}C -NMR application for the determination of mycotoxin swainsonine isolated from the locoweed endophytic fungus *Alternaria oxytropis* [128].

6. Immunochemical-Based Methods for Mycotoxin Determination

Immunochemical-based methods rely on the recognition of mycotoxins that act as antigens from specific antibodies. The detection of mycotoxins is typically facilitated by the presence of a marker compound that reacts with an enzyme by non-enzymatic labels conjugated to the antibodies, or without the use of a marker, taking advantage of the natural fluorescence of some mycotoxins. A profound and detailed presentation of the different immunochemical assay principles of function, different formats, advantages, and limitations regarding different assay parameters (specificity, sensitivity, variance, cross reactivity, accuracy, precision, and measurement range), as well as their application and validation for specific mycotoxin determination in different food and feed matrices, has been reviewed in depth in previous publications [129–132]. Here a brief presentation of the different immunochemical methods and of commercially available test kits is made, with reference to their specific application to mycotoxin determination in grapes and derived products. Immunoassay kits are easy-to-use portable rapid tests that can generate results within a short time, suitable for on-site testing and decision making. They include either a customized form of enzyme-linked immuno-sorbent assay (ELISA), ELISA-type adaptations such as a lateral flow immunoassay or a flow-through ELISA, or fluorometric assays such as types of fluorescent polarization immunoassays or immunoaffinity columns coupled with a fluorometric assay.

6.1. Enzyme-Linked Immunosorbent Assay (ELISA)

ELISAs are performed in microtiter plates or well strips and are based on the combination of antibody specificity with the sensitivity of simple enzyme assays for the determination of the analytes of interest. ELISAs are the most common validated immunochemical methods used to monitor mycotoxins, with advantages regarding high specificity, a relatively low limit of detection (LOD), high sample throughput, and certain limitations related to matrix nature complexity [133]. Among the several ELISA types that are currently available, the predominant forms used are the classical competitive and competitive inhibition formats, which perform better when detecting low molecular weight molecules with limited epitope site display, as mycotoxins are [134,135]. There are various ELISAs that have been developed for mycotoxin determination in wine and grape products, with varying perspectives of application in many cases, such as the development of an ELISA per se [136,137] or an ELISA-type variation [138], evaluation of different antibodies for use in other immunodetection assays such as biosensors [139], or for method comparison [140]. Furthermore, there are numerous reports using commercial ELISA kits for mycotoxin detection in various food commodities including wine and grape products.

ELISA kits follow the same assay principle as a homemade protocol, with quantitation possible either via visual color comparison of the test sample and a range of positive control samples or using a conventional ELISA reader, with a number of manufacturers lending or providing custom-made reader apparatuses. Table 2 presents the available commercial kit types for the determination of mycotoxins of principal interest. The majority are

ELISAs and LFDs, with both formats being available for the same mycotoxin in many cases. There are specific kits for the detection or determination of OTA and AFs in wine, vinegar, grape juice, and grape products provided by certain manufacturers as assays validated for the detection of these mycotoxins in the commodities described. Although commercial products other than those presented in Table 2 may also be suitable for the detection/determination of different mycotoxins in wine and grape-derived products, this information has not been declared yet. Thus, kit suitability for these matrices should be defined after further contact with the manufacturers, since a major restriction regarding the selectivity and sensitivity of the test may occur due to the sample matrix, particularly for red wine.

Table 2. Rapid immunodetection assays for mycotoxin detection available by commercial providers (bold abbreviations in table refer to grape products).

[illegible]

Table 2. Cont.

Provider	Assay Type	AFs (Total)	AFB ₁	AFM ₁	CIT	DON	EAL	FUM	OTA	PAT	T2/HT2	ZEN
Neogen	ELISA	CE-GN, CO, CS, FD, GN, PT, RI, SB, WE	-	DR, ML	-	CE	-	CE, DDGS, RI, SB	CE, CF, FR, SB	-	CE	CE, DDGS
	LFD	CO, GN, WE	-	DR, ML	-	GN, VC	RY, WE	GN	CF, GN, VC	-	GN, VC	CO, GN, WE
	FLUO	CO, NU, PT	-	-	-	-	-	-	-	-	-	-
R-Biopharm	ELISA	CE, FD	-	ML	-	BE, CE, FD	-	CE, CO, FD	BE, CE-GN, FD	-	CE, FD	BE, CE, FD
	LFD	CO, FD	FD, VC	-	CE, FD	CE, GN	-	CO	CE, FD, RI, VC	-	CE	CO
Randox	ELISA	-	VC	-	-	-	CE, FD	-	-	-	-	-
	LFD	-	-	-	-	-	-	-	-	-	-	-
Ringbio	ELISA	-	CE, FD	ML	-	CE, FD	-	-	-	-	-	CE, FD
	LFD	-	FD, GN, SD	ML	-	CE, FD, NU	-	-	-	-	-	CE, FD, NU
Romer Labs	ELISA	GN, NU, CE	-	ML, DR	-	GN, NU, CE	-	GN, NU, CE	GN, NU, CE	-	GN, NU, CE	GN, NU, CE
	LFD	GN, NU, CE	-	-	-	GN, NU, CE	-	GN, NU, CE	GN, NU, CE	-	-	GN, NU, CE
Scigiene	ELISA	-	-	ML	-	-	-	-	-	-	-	-
	LFD	-	GN, FD	ML	-	GN, FD	-	-	W (R)	-	GN, FD	GN, FD
Unisensor	ELISA	-	-	-	-	-	-	-	-	-	-	-
	LFD	CO	-	ML	-	-	-	-	-	-	-	-
Vicam	ELISA	-	-	-	-	-	-	-	-	-	-	-
	LFD	CO, GN, PT, NU	-	DR	-	GN, FD	-	GN	CF, WE	-	-	GN
	FLUO	VC	-	ML	-	-	-	VC	VC	-	-	VC

AFs: Total aflatoxins, AFB₁: aflatoxin B1, AFM₁: aflatoxin M1, CIT: citrinin, DON: deoxynivalenol, EAL: ergot alkaloids, FUM: fumonisins, OTA: ochratoxin A, PAT: patulin, T2/HT2: trichothecene group mycotoxins, ZEN: zearalenone, ELISA: enzyme-linked immunosorbent assay, LFD: lateral flow device, FLUO: fluorometric assay. BE: beans, BI: biscuits, BR: beer, CE: cereals, CF: coffee, CO: corn, CP: chili powder, CS: cottonseeds, DDGS: dry distiller grains with solubles, DR: dairy, DS: drinks, FD: feed, FI: fish, FR: dried fruit, GN: grains, GR: grape, IF: infant formulas, JU: juice, ML: milk, MT: meat, NU: nuts, OL: oil, OT: oats, PT: peanut, RI: rice, RY: rye, SB: soybeans, SC: spices, SD: seed, SM: soybean meal, SP: spirits, SS: soy sauce, VC: various commodities, VG: vinegar, W: wine, W (R, Ro, Wh): Wine (red, rosé, white), WE: wheat. Ballya (<https://ballyabio.com/> (accessed on 25 January 2021)), Charm Sciences Inc (<https://www.charm.com/> (accessed on 25 January 2021)), Creative Diagnostics (<https://www.creative-diagnostics.com/> (accessed on 25 January 2021)), Elabscience (<https://www.elabscience.com/> (accessed on 25 January 2021)), Envirologix (<https://www.envirologix.com/> (accessed on 25 January 2021)), Eurofins Tecna (<https://tecna.eurofins-technologies.com/home/> (accessed on 25 January 2021)), Europroxima (<http://europroxima.com/> (accessed on 25 January 2021)), Hygiena (<https://www.hygiena.com/> (accessed on 25 January 2021)), Neogen (<https://www.neogen.com/> (accessed on 25 January 2021)), R-Biopharm (<https://r-biopharm.com/> (accessed on 25 January 2021)), Randox (<https://www.randox.com/> (accessed on 25 January 2021)), Ringbio (<http://www.ringbio.com/> (accessed on 25 January 2021)), Romer Labs (<https://www.romerlabs.com/> (accessed on 25 January 2021)), Scigiene (<https://www.scigiene.com/> (accessed on 25 January 2021)), Unisensor (<https://unisensor.be/en/> (accessed on 25 January 2021)), Vicam (<http://vicam.com/> (accessed on 25 January 2021)).

6.2. Lateral Flow and Flow-through Immunoassays (LFIA, FTIA)

Lateral flow immunoassay is conducted on a piece of nitrocellulose membrane imprinted with specific antibodies usually conjugated with nonenzymatic labels such as gold colloid nanoparticles (AuNPs). It takes advantage of the later flow of the buffer liquid through the membrane to separate the analytes of interest, which are finally bound to the immobilized specific antibodies [141]. LFIA kits, commonly referred to as Lateral Flow Devices (LFDs) (also known as lateral flow, immunochromatographic, or dipstick tests), are most of the time in a competitive ELISA format. Sample preparation and assay are easy to perform and can be used for in situ analysis. The assay can be conducted on complex matrices, but with limitations regarding matrix interferences and sensitivity. The assays provide mainly visual qualitative results, however, semi-quantitative results for any positive samples can be also achieved with dedicated handheld devices. LFDs for multi mycotoxin detection in food and feed are also available [142–144]. A description of different LFDs for mycotoxin detection is presented in Table 2, showing various commercial entities offering LFDs validated for OTA and aflatoxin detection in wine and grape products, among other matrices. As previously reported for the commercial ELISA kits, the suitability of other LFDs for the detection of different mycotoxins in wine and grape-derived products should be determined with the manufacturers.

Flow-through immunoassays are an on-column gel-based approach where the assay buffer liquid flows through a column of microbeads. They are typically in a direct competitive format commonly used in ELISAs. Similarly, as described for LFDs, they can give qualitative and/or semi-quantitative results, are simple, and are capable of generating results within minutes. Flow-through immunoassay kits are comparable to LFDs in terms of rapidity, ease of use, and multiplexing, they generate qualitative results, and can be used for the semi-quantitation of mycotoxins using portable readers. More recently, multiplexing has been shown to be possible with the flow-through approach [145]. As seen in Table 2 there are some flow-through immunoassays for mycotoxins commercially available, but with no specific reference for use in wine and grape product testing.

6.3. Flow Injection Immunoassay (FIIA)

FIIA relies on the different affinity properties that labeled and unlabeled mycotoxins have for specific antibodies immobilized in an immunoaffinity column. The displaced labeled mycotoxin—initially bound to the specific antibody by the incoming unlabeled mycotoxin of the sample—is quantified in a subsequent enzymatic reaction, resulting in the proportional correlation of the removed antigen and the newly bound mycotoxin amounts. There are some assays described for mycotoxins [146–148] but not specifically for wine or grape matrices.

6.4. Chemiluminescence Immunoassay (CLIA)

CLIA is based on the detection of an antigen–antibody (mycotoxin-specific antibody) binding via a catalytically enhanced reaction of a chemical compound (most frequently luminol), which is performed at the final stage of the immunoassay. CLIA has proved to be one of the most sensitive assays used in mycotoxin determination in food and feed testing [149]. There are CLIAs developed for the determination of different mycotoxins [150–152] but have not been tested in wine or other grape products. A chemiluminescence ELISA integrated into a microfluidics system was reported for the detection of OTA in wine [153].

6.5. Fluorescent Polarization Immunoassay (FPIA)

Fluorescence polarization is an example of a homogeneous immunoassay where it is not necessary to separate the bound from the unbound analyte from the immunoassay mixture. Determination of the analyte is achieved due to the different fluorescence polarization signal the analyte bound to the antibody from the unbound analyte generate. FPIA rapid methods have been developed during the last two decades for the determination of many

mycotoxins. They are comparable to ELISAs, but have certain benefits and limitations regarding less intermediate steps, and matrix effect interference and sample pre-treatment, respectively [154,155]. A FPIA was used for OTA determination in red wine [156].

6.6. Fluorometric Assays

Fluorometric assays require the use of specific columns, a solid phase extraction (SPE) or an immunoaffinity (IAC) column, for the purification and enrichment of a preliminary solvent extract prior to analysis with a fluorometer. The purification step results in the reduction or elimination of matrix effects and purification from unspecific fluorescent compounds that could generate false-positive results [157,158]. There are various SPE and IAC products for mycotoxins commercially available to couple with fluorometry readers, which can generate semi-quantitative results within minutes at on-site analysis. SPE and IAC columns are also used for HPLC and LC-MS/MS analysis of mycotoxins [159,160] and have the potential to combine with fluorometric-based biosensors, as described in the following section.

6.7. Biosensors

Biosensors are analytical devices composed typically of three components: (a) a detection layer that incorporates a biological element with which a biorecognition event takes place; (b) a transducer, a material that converts the biological element's physical change generated during the biorecognition event into a measurable signal and transmits this for downstream measurement processing; and (c) an output system that processes and displays the signals produced into a readable format. The detection layer and the transducer consist usually of a single unit, with the former integrated into the latter.

The biological elements utilized vary both in nature and in size. They can be small biological molecules, either natural (antibodies, enzymes, nucleic acids, cell receptors, etc.) or biologically derived and synthetic (aptamers, recombinant antibodies, imprinted polymers, biomimetic (peptide) molecules, etc.), or of bigger-sized biological units such as organelles, microorganisms, or tissues [129,161]. Transducers may be optical (based on surface plasmon resonance, fluorescence, optical waveguide light mode spectroscopy, and total internal reflection ellipsometry), electrochemical (impedimetric, potentiometric, amperometric), piezoelectric (quartz crystal microbalance), or thermal signal-calorimetric [162,163].

There are various biosensors of different functions principally developed for the detection of mycotoxins in various food and feed commodities, all presenting certain advantages and limitations regarding sensitivity, selectivity, cost efficiency, and in situ ease of use. Many of them have been also tested on different kinds of wine, principally for the detection of OTA as well as AFB₁ and AFM₁.

Secondary fungal metabolites such as mycotoxins can be determined according to their physicochemical properties with the aid of the electronic nose (e-nose) technique. More specifically, e-nose instrumentation is based on the detection of volatile compounds released by food through specific metal-oxide- and/or metal-ion-based-sensors [164]. These volatile compounds have a unique fingerprint for each food, which in the case of contaminant presence are altered, providing an insight into contamination, and thus a rapid and early determination tool for mycotoxins. When this correlation between mycotoxin presence and the sensors' reaction to the e-nose system is processed with an appropriate and powerful statistical approach, such as support vector machines (SVM) or artificial neural networks (ANN), e-nose system can achieve performances with high accuracies as in the case of AFB₁ and FBs in maize [164]. It is noteworthy that this approach has also been successfully applied in the past for the discrimination of toxigenic and non-toxigenic strains of *A. carbonarius* and *A. niger* isolated from grape samples [165], as well for the detection of OTA contamination derived from *P. nordicum* in hum [166]. An indicative presentation of different biosensors applied to mycotoxin analysis in wine and grape commodities can be found in Table 3.

Table 3. Different types of biosensors for the determination of mycotoxins in wine, grape, and grape products.

No	Authors—Reference	Mycotoxin	Recognition	Transducer Type	LOD/LOQ	Commodity
1	Barthelmebs et al., 2011 [167]	OTA	Aptamer	Colorimetric	1 ng mL ⁻¹	Red wine
2	Castillo et al., 2012 [168]	OTA	Aptamer	Impedimetric	0.12 nM	Red wine
3	Karczmarczyk et al., 2017 [169]	OTA	Antibody	Quartz crystal microbalance	0.16 ng mL ⁻¹	Red wine
4	Karczmarczyk et al., 2017b [170]	OTA/AFM ₁	Antibody	Potentiometric	0.15/3.04 ng mL ⁻¹	Red wine
5	Nan et al., 2019 [171]	OTA	Aptamer	Impedimetric	0.030 ng mL ⁻¹	Grape and products
6	Sheng et al., 2011 [172]	OTA	Aptamer	Fluorescent	21.8 nM	Red wine
7	Tang et al., 2018 [173]	OTA	Aptamer	Amperometric	0.23 pg mL ⁻¹	Red wine
8	Wu et al., 2012 [174]	OTA	Aptamer	Electrochemical	0.095 pg mL ⁻¹	Red wine
9	Zhu et al., 2015 [175]	OTA	Aptamer	Surface plasmon resonance	0.005 ng mL ⁻¹	Red wine

LOD/LOQ: Limit of detection/Limit of quantification.

7. Molecular Methods for the Detection of Mycotoxigenic Fungi

The main causal agents of grape contamination with mycotoxins are saprophytic fungi of the genus *Aspergillus* (particularly species of *Aspergillus* section *Nigri*) and *Penicillium* [176,177]. The *Aspergillus* genus consists of more than 300 species and several of them are of high economic, agronomic, or medical importance. Black aspergilli (*Aspergillus* section *Nigri*) is a group of 26 species encountered mainly in soil and plant sources [178]. *A. carbonarius* and members of the *A. niger* aggregate are main OTA producers, with the former being the predominant species responsible for OTA contamination in grapes and wine, due to the ability of almost all its strains to produce high levels of the toxin [179–181]. Several *Aspergilli*, including *A. flavus*, *A. parasiticus*, and *A. nomius*, are potent producers of aflatoxins in different food commodities, including grapes [182]. Fumonisin (mainly FB₂) are also produced by *Aspergillus niger* strains and occur naturally in grape and must [183,184]. More than 50 species in the *Penicillium* genus are reported to produce various secondary metabolism extrolites, including several mycotoxins, such as ochratoxins, citrinin, patulin, penicillic acid, and verrucosidin [185]. In grapes, various *Penicillium* species including *P. expansum*, *P. chrysogenum*, and *P. nordicum*, along with related mycotoxins, have been determined, with *P. expansum* being one of the most potent producers of OTA and patulin [185,186].

Regarding the identification of mycotoxigenic fungi responsible for grape contamination, various molecular methods based on the principle of the polymerase chain reaction (PCR) have been developed so far. The application of such methods covers two different aims: the characterization and taxonomic classification of known and new or cryptic species among mycotoxigenic fungi, and the necessity for rapid and accurate determination of certain species directly in grape samples.

7.1. Methods for the Characterization of Mycotoxigenic Fungi Isolates

Characterization and classification of mycotoxigenic fungi isolated from grape microflora are performed principally by the application of multilocus sequence analysis (MLSA) and restriction or amplified fragment length polymorphism of PCR amplicons (RFLP, AFLP) [176,187–189]. Similar approximations are based on the methods of random amplification of polymorphic DNA (RAPD-PCR) and the use of microsatellites [190–193]. MLSA uses PCR-amplified DNA of key and barcoding genomic regions, such as the β -tubulin, calmodulin (*CaM*), and elongation factor-1 alpha genes (*efl-a*), along with specific regions of the nuclear ribosomal locus, such as small and large subunits (SSU and LSU) and the internal transcribed region (ITS) for sequencing [187,189]. Mycotoxigenic fungi characterized by morphological criteria can be further analyzed by PCR-RFLP, a method where the restriction patterns of the enzyme digested amplicons—usually of the ITS and *CaM* loci—can be compared with those obtained from reference strains [179,188,194,195]. RAPD-PCR has been applied to characterize fungal isolates from grape. Species-specific primers designed for RAPD allowed for the characterization and detection of many As-

pergillus and Penicillium species [190,191]. Black Aspergilli can be characterized in relation to the taxonomy of *Aspergillus* section *Nigri* using a loop-mediated isothermal amplification (LAMP) reaction [196] and arbitrarily primed PCR (ap-PCR) sequence analysis [197].

7.2. Methods Used for the Detection of Mycotoxigenic Fungi in Grapes

Numerous simplex or multiplex endpoint and real-time PCR assays for the detection and quantification of species belonging to the major mycotoxin-producing genera have been reported and are available in the literature. These methods have been developed for the determination of putatively mycotoxigenic species in different food commodities, including grape, with the scope of accurate and specific determination of such species at the initial stages of fungal contamination. Regarding grapes, several PCR assays have been developed, including endpoint, real-time quantitative (rt-qPCR), and TaqMan (indicative references [198–202]. These assays target different loci, including ITS, calmodulin, and genes involved in toxin biosynthesis such as polyketide synthases (*pks*), non-ribosomal peptide synthetases (*nprs*), velvet A (*veA*), O-methyltransferase (*omt-1*), and isoeopoxidon dehydrogenase (*idh*), and have proved efficient for the detection of predominant mycotoxigenic species in grape samples with accuracy and a high degree of sensitivity. Interestingly, a step further has been also made with the application of molecular approximations that distinguish mycotoxigenic from non-mycotoxigenic strains based on the detection of mutations on mycotoxin biosynthetic genes [203,204]. Such a datum could be the basis for the development of molecular assays that would distinguish such strains putatively on the basis of single nucleotide polymorphisms (SNPs). A detailed description of PCR-based methods applied for the detection of different *Aspergillus* and *Penicillium* species in grapes is presented in Table 4, with relevant references within.

Table 4. Molecular methods applied for detection of mycotoxigenic fungi in grape and grape products.

No	Authors—Reference	Species Targeted	Assay Type	Gene Target(s)	Sensitivity
1	Atoui et al., 2007 [198]	<i>A. carbonarius</i>	qPCR	<i>pks</i> Ac12RL3	1 pg
2	Ayoub et al., 2010 [205]	<i>A. carbonarius</i> , <i>A. niger</i>	qPCR	<i>pks</i>	2.37 10 ⁻² ng µL ⁻¹
3	Gil-Serna et al., 2009 [206]	<i>A. carbonarius</i> , <i>A. westerdijkiae</i>	qPCR	ITS1	10 ² spores mL ⁻¹
4	Gonzalez et al., 2009 [199]	<i>A. carbonarius</i>	qPCR (SYBRGreen and TaqMan)	ITS2	0.4 pg g ⁻¹
5	Kizis et al., 2015 [102]	<i>A. carbonarius</i>	qPCR	<i>veA</i>	10 ⁴ conidia g ⁻¹
6	Mule et al., 2006 [200]	<i>A. carbonarius</i>	qPCR (TaqMan)	calmodulin	5 × 10 ⁻⁴ ng
7	Patino et al., 2006 [207]	<i>P. brevicompactum</i>	PCR	ITS1-2	—
8	Rodriguez et al., 2011 [208]	<i>Aspergillus</i> spp., <i>Penicillium</i> spp.	qPCR (SYBRGreen and TaqMan)	<i>otanps</i> PN	1–10 conidia g ⁻¹
9	Rodriguez et al., 2012 [201]	<i>A. carbonarius</i>	multiplex qPCR (TaqMan)	<i>omt-1</i> , <i>otanps</i> PN, <i>idh</i>	1–3 cfu g ⁻¹
10	Sanzani et al., 2016 [87]	AFs, OTA, PAT producing moulds	nested qPCR	β-tubulin	1 fg
11	Selma et al., 2008 [209]	<i>A. carbonarius</i>	qPCR (SYBRGreen and TaqMan)	<i>pks</i> AcKS10	5 × 10 ² conidia g ⁻¹
12	Selma et al., 2009 [202]	<i>A. carbonarius</i> , <i>A. niger</i> aggregate	duplex qPCR (TaqMan)	<i>pks</i>	30 gen. eq./reac.
13	Spadaro et al., 2011 [210]	<i>A. carbonarius</i>	PCR	<i>Acps</i>	—
14	Vogt et al., 2017 [211]	<i>P. oxalicum</i>	LAMP	PDE 07106	100 pg
15	Xanthopoulou et al., 2019 [212]	<i>A. niger</i> aggregate	HRM	ITS2	10 ⁴ conidia g ⁻¹

High-resolution melting (HRM) analysis is a robust closed-tube post-PCR method, the analytical principle of which is based on monitoring the gradual DNA denaturation resulting from incremental heating. The method can detect genetic variation in DNA by measuring changes in the fluorescence level of a melting DNA amplicon. The method generates characteristic melting profiles that can be used to discriminate between species at the level of single-nucleotide polymorphisms (SNPs) and small insertion/deletions through the analysis of the melting behaviors of double-stranded DNA [213]. An HRM assay has been developed that can discriminate with high accuracy main black Aspergilli in grapes [212]. Another advantageous alternative to known PCR assays is LAMP, which can

be used directly on grape samples. LAMP utilizes specific sets of primers and offers rapid, robust, and specific detection of fungal species. A LAMP assay has efficiently detected *P. oxalicum* in grape samples without inhibiting effects due to substances originating from grapes [211]. Furthermore, current needs for real-time determination of fungi promote approximations that can be applied on site, such as LAMP and PCR metabarcoding.

8. Conclusions

The present work gives a brief presentation of a span of chromatographic, immunological, spectrometric, and molecular methods used for mycotoxin analysis and detection of mycotoxigenic fungi, pointing out the most recent technological advances and describing the state as this applies to grapes and derived products by including relative reports. A thorough bibliographic review showed that the majority of reports discussed the determination of different mycotoxins via analytical approximations mainly in grape and wine, whereas publications in which immunochemical based approaches were used focused mainly on the determination of OTA in wine. Unfortunately, regarding many grape product commodities such as *pekmez*, vinegar, jams, jellies, and marmalades, there is a limited number of reports referring to mycotoxin issues. Likewise, reports are minimal on other mycotoxins, such as FBs, AFs, or *Alternaria* toxins, for the majority of grape products.

Mycotoxin determination becomes technically a demanding issue, if not particularly delicate, when one bears in mind that the legislative permitted levels for these substances in grapes and derived products are on the order of a few parts per billion. Taking as an example OTA reports on grape products, there are reports of up to 10 ng/mL in grapes juices and wines [61,72] or even higher than 10 ng/mL values for dried grape products [51], when the EU maximum permitted levels are at 2 and 10 ng/mL for the two commodities, respectively. Furthermore, it should be taken in consideration that the legislative levels are set principally for the mycotoxin most often and abundantly detected in a certain commodity, and neglect minor levels of other mycotoxins that may be present and could create an additional danger to human health due to synergistic effects and an augmented mycotoxin burden. A complete report on current legislative limits of several mycotoxins in grape and derived products can be found in the comprehensive review by Gonçalves et al. [69].

An important perspective through which the mycotoxin problem in grapevine culture worldwide should be dealt is definitely the radical climate change occurring around the globe. Undoubtedly, predictive modeling of both mycotoxin production and the responsible fungi monitoring have evolved along with analytical and detection methods over the past years. Nevertheless, new methodological approaches and tools developed should cover analytical laboratory needs for sample high throughput determination of multiple mycotoxins, if possible with low sample volume requirements and without pretreatment, along with the needs of production sectors, regarding on-site and in-hand multiplex monitoring of mycotoxigenic fungi and produced mycotoxins.

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