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Food safety and quality in the tomato processing chain: control and sustainable mitigation strategies for *Alternaria* toxins

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“Life is what happens to you,

while you're busy making other plans”.

- *John Lennon*

List of abbreviations

Abbreviation	Meaning
ACN	Acetonitrile
AFB1	Aflatoxin B1
AFB2	Aflatoxin B2
AFG1	Aflatoxin G1
AFG2	Aflatoxin G2
CECT	Spanish Type Culture Collection
EFSA	European Food Safety Authority
HPLC	High-performance liquid chromatography
IARC	International Agency for Research on Cancer
MeOH	Methanol
MFC	Minimum fungicidal concentration
MgSO4	Magnesium sulfate
PDA	Potato dextrose agar
PDB	Potato dextrose broth
TeA	Tenuazonic acid
AOH	Alternariol
AME	Alternariol monometil ether
WHO	World Health Organization

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ABSTRACT

Mycotoxins produced by *Alternaria* represent a risk across multiple agri-food chains, as they may occur as natural contaminants and contribute to consumer exposure. In the case of tomatoes and tomato-based products, risk assessments by the European Food Safety Authority (EFSA) have highlighted the potential occurrence of *Alternaria* toxins and the need for appropriate analytical and control tools along the processing chain. Within this context, this thesis addresses the risk of natural contamination by *Alternaria* mycotoxins in the industrial tomato processing chain, integrating analytical approaches for process control with a sustainable risk-mitigation perspective aimed at limiting the use of chemical fungicides and supporting operational decision-making in industrial settings.

A first research line focused on the development and validation of a near-infrared (NIR) spectroscopy-based approach for quality control in an industrial environment within the tomato processing chain. Spectra collected from process-chain samples were integrated with reference measurements to build and update multivariate calibrations aimed at the non-destructive estimation of technological and process parameters. The models showed performances that depended on product type and sample size, highlighting the need for structured updating to maintain robustness under operational conditions.
The second study addressed analytical aspects related to *Alternaria* toxins in tomato-derived products, with specific attention to the challenges introduced by extract complexity and non-target co-extraction in QuEChERS-type approaches. The results indicated that extract composition and the co-extraction of non-target components can affect signal reliability and its interpretation, reinforcing the importance of systematically assessing methodological robustness in industrial matrices.

The third study explored plant protein hydrolysates obtained by fermentation with *Bacillus amyloliquefaciens* or *Trichoderma reesei* and formulated as powders by spray drying, assessing their in vitro antifungal activity against *Alternaria* strains and the fungal metabolic response. Antifungal activity, evaluated by agar diffusion and microdilution (MIC/MFC), showed a strongly strain-dependent behaviour and differences between the two fermented products depending on the assay. In parallel, the analysis of non-volatile metabolites (UHPLC–QTOF) and volatile compounds (HS-SPME–GC) showed a time- and treatment-dependent remodulation of metabolic profiles. In the PDA control, signals attributable to toxicologically relevant metabolites, including tenuazonic acid (TeA), were observed, whereas under treated conditions these signals appeared attenuated. In the non-volatile profile, some variables annotated as host-selective toxins were detected selectively in one treated condition, suggesting that the formulation effect may be associated not only with quantitative decreases but also with treatment-specific reorganisations of the fungal response.

Overall, this thesis proposes an integrated framework in which mycotoxicological risk and quality control are addressed through a multi-method approach, including NIR models for rapid monitoring, analytical evaluations focused on extract complexity, and in vitro testing of fermented protein products as mitigation tools. The findings indicate that the assessment of sustainable strategies can benefit from the combined consideration of antifungal efficacy and metabolomic modulation, taking into account not only fungal growth but also changes in secondary metabolism and in the volatile compound profile, with a view to potential applicability in operational settings.

PART I – Scientific context and rationale of the thesis

Chapter 1 – Introduction; the tomato supply chain, Alternaria and mycotoxin risk

1.1 Food safety and food quality in the supply chain: regulatory framework and contaminants

Within agri-food supply chains, the concepts of food safety and food quality constitute indispensable foundations for assessing the overall suitability of a product for consumption.

According to the Codex Alimentarius, food safety refers to the condition whereby a food, if prepared and consumed in accordance with its intended use, does not pose a risk to consumer health (FAO/WHO CXC 1-1969, Rev.2020). In parallel, food quality may be defined, in line with the Food and Agriculture Organization (FAO) and the World Health Organization (WHO), as the set of attributes that influence a product's value and acceptability for the consumer, encompassing sensory, nutritional, technological, and informational aspects (FAO/WHO, 2003).

Within the European framework, the principal regulatory reference in the food sector is Regulation (EC) No 178/2002, known as the General Food Law, which lays down the general principles and requirements of food and feed law and establishes the overarching framework for the protection of public health and consumers' interests. This Regulation establishes the European Food Safety Authority (EFSA), based in Parma, with the task of providing scientific advice, and introduces the principle of risk analysis as a cornerstone of decision-making throughout the entire food chain.

At the international level, the Codex Alimentarius, developed in 1963 by FAO and WHO, represents the main reference in the field of food safety, aiming to support food safety, food quality, and fair practices in the global food trade. In this context, ISO 22000:2018 builds upon the work of the Codex as a voluntary standard that integrates and expands Codex principles—particularly the seven HACCP principles and incorporates them into a management system model aligned with the High Level Structure (HLS) common to ISO standards.

Within the framework of food safety, contaminants represent a category of chemical hazards that requires systematic control throughout the entire production chain.

According to the Codex General Standard for Contaminants and Toxins in Food and Feed, a contaminant is defined as any substance or material extraneous to food that is not intentionally added to food and that may compromise food safety or suitability for consumption. Among naturally occurring contaminants, mycotoxins play a prominent role, as they are secondary metabolites produced by numerous fungal species under specific ecological conditions (Salotti et al., 2024; Pinto et al., 2017; Logrieco et al., 2003).

The term “mycotoxin” derives from the combination of the Greek word mýkēs (fungus) and the Latin toxicum (poison), and it refers to low-molecular-weight compounds with specific toxicological activity. Among the fungal genera most extensively studied for their ability to produce mycotoxins are *Alternaria*, *Fusarium*, *Aspergillus*, and *Penicillium*. Each genus is associated with a characteristic toxicological profile: *Alternaria* synthesizes alternariol (AOH), alternariol monomethyl ether (AME), tenuazonic acid (TeA), altertoxins (ATX I–III), altenuene (ALT), and tentoxin (TEN); *Fusarium* produces type A and B trichothecenes (T-2, HT-2, deoxynivalenol—DON—, nivalenol—NIV), zearalenone (ZEA), and fumonisins (B1–B3); *Aspergillus* is responsible for the production of aflatoxins (B1, B2, G1, G2), ochratoxin A, and sterigmatocystin; and *Penicillium* produces, among others, patulin and ochratoxin A (Moretti et al., 2017; Ostry et al., 2008; Bennett et al., 2003). These compounds are of central concern because they are associated with heterogeneous toxicological effects, including hepatotoxic, nephrotoxic, immunotoxic, and genotoxic outcomes, with implications for both human and animal health (Logrieco et al., 2003; Crudo et al., 2021; Solhaug et al., 2016).

In the industrial tomato sector, the mycotoxins of greatest relevance are those associated with *Alternaria* (Giorni et al., 2025; Escrivá et al., 2017). These compounds are often discussed as emerging mycotoxins because systematic documentation of their occurrence in food has expanded substantially only in relatively recent years, binding maximum limits are still lacking in European legislation, and the available toxicological evidence is insufficient to support consolidated regulatory values (Gruber-Dorninger et al., 2017).

Along the tomato supply chain, the occurrence of mycotoxins in final products is mainly attributable to fungal infection affecting the raw material.

Tomatoes are particularly susceptible to attack by phytopathogenic microorganisms due to their thin and easily damaged epidermis, which can facilitate fungal penetration and subsequent tissue colonization (Fernandes et al., 2023; Adhikari et al., 2017; Zhao et al., 2015).

A further element of practical relevance is that several mycotoxin groups monitored in fruit- and vegetable-derived commodities, including patulin and ochratoxin A alongside *Alternaria* toxins and, in selected crops, trichothecenes, are relatively resistant to heating; therefore, conventional thermal operations may be insufficient to fully remove or degrade these contaminants. In line with this general principle, *Alternaria* mycotoxins are frequently described as chemically stable and may show limited degradation under the thermal treatments commonly used in the canning industry, enabling residual persistence even after processing (Giorni et al., 2025; Puntscher et al., 2019; Estiarte et al., 2017; Alshannaq and Yu, 2017). Conversely, in properly processed and stabilized products, de novo formation of mycotoxins is not expected, because fungal growth and, consequently, the biosynthesis of toxic secondary metabolites are effectively inhibited by the combined action of thermal treatments and the physico-chemical stability conditions achieved in the final product.

The growing body of evidence on the occurrence and potential toxicity of *Alternaria* mycotoxins across different agri-food supply chains has led the European Commission to assign specific risk-assessment mandates to EFSA. In 2011, the CONTAM Panel of the European Food Safety Authority published the first formal European opinion on risks to human and animal health associated with the presence of *Alternaria* toxins in food and feed. Among the compounds assessed, four were identified as being of particular interest: alternariol (AOH), alternariol monomethyl ether (AME), tenuazonic acid (TeA), and tentoxin (TEN). Subsequently, in the 2016 report, based on an expanded set of occurrence and consumption data, EFSA estimated the chronic dietary exposure of the European population to AOH, AME, TeA, and TEN.

Among the four toxins considered in the EFSA dietary exposure assessment, tenuazonic acid (TeA) was associated with the highest exposure estimates.

With respect to dietary contributors, cereal-based foods for infants and young children were identified as the main contributors in the youngest population groups, whereas tomatoes and tomato-based products represented a relevant, and in some age classes predominant, contribution in older groups (EFSA, 2016).

Against this background, and in the absence of Union maximum levels for these compounds, the European Commission adopted Recommendation (EU) 2022/553 of 5 April 2022, inviting Member States, in cooperation with food business operators, to reinforce the monitoring of alternariol (AOH), alternariol monomethyl ether (AME) and TeA in selected food categories, explicitly including processed tomato products, and to report the resulting occurrence data to EFSA. The Recommendation also establishes indicative levels for these toxins: these values are not food safety levels and do not constitute legal limits, but they provide an operational reference above which investigations should be carried out to clarify the factors leading to the observed contamination and to evaluate the effect of processing.

1.2 The tomato (*Solanum lycopersicum* L.)

Tomato (*Solanum lycopersicum* L.) is among the most economically relevant horticultural crops worldwide and is characterized by a dual market destination. On the one hand, the fresh-market segment is closely linked to organoleptic quality attributes and seasonality; on the other, the industrial segment supplies the canning and processing sector through a broad portfolio of products, including pulp, purée, sauces and concentrates, which are traded on both domestic and international markets (Hui and Evranuz, 2015; FAOSTAT).

For 2024, the World Processing Tomato Council (WPTC) reported an estimated global processing volume of approximately 45.8 million tonnes. China accounted for the largest share of processed tomatoes, while California represented another major processing region; within Europe, Italy and Spain remain among the key producing areas, alongside other relevant producing countries such as Turkey (WPTC; WPTC-derived industry reporting).

Within the Italian supply context, Emilia-Romagna represents one of the main hubs for processing tomato cultivation, due to the concentration of cultivated area and the presence of an extensive processing capacity. According to sector reporting for 2025, the national area cultivated with processing tomatoes is close to 77,000 hectares, of which more than 27,000 hectares are located in Emilia-Romagna (Confagricoltura, 2025).

Within this regional framework, the provinces of Piacenza, Ferrara and Parma are consistently indicated among the leading areas in terms of cultivated surface and deliveries to processing plants, reflecting the territorial concentration of primary production in the northern Italian processing tomato basin.

Tomato (*Solanum lycopersicum* L.) and tomato-derived foods are frequently characterised as a plant-based matrix containing multiple bioactive constituents, with particular emphasis on carotenoids, for which lycopene is often used as a reference, and on phenolic compounds (Arballo et al., 2021; Friedman et al., 2023; Hui and Evranuz, 2015; Martí et al., 2016; Srivastava and Srivastava, 2015; Tan et al., 2010). The concentration patterns of these constituents, and the fraction that becomes available during digestion, are not fixed attributes but can shift along the supply chain (Martínez-Huélamo et al., 2015; Capanoglu et al., 2008). Variability is reported in relation to pre-harvest determinants such as genotype and breeding choices, as well as agronomic and environmental conditions (Abushita et al., 2000), and it can be further reshaped by post-harvest handling and by technological processing, with implications for chemical stability and digestive availability (Lima et al., 2022; Cilla et al., 2018). In line with this, work on traditional Spanish tomato lines associated genotype, location and cultivation practices with differences in nutritional and antioxidant-related traits, including measurable variation in selected phenolic acids and in β-carotene (Asensio et al., 2019).

Beyond pre-harvest sources of variability, downstream operations may either increase or reduce the bioaccessible pool of bioactives depending on the specific matrix and processing conditions.

For tomato sauce, the production process has been examined experimentally in relation to the bioaccessibility and bioavailability of phenolic compounds (Martínez-Huélamo et al., 2015), while synthesis evidence based on in vitro digestion indicates that the direction and magnitude of processing effects on carotenoids and polyphenols are not consistent across foods and depend critically on process variables (Cilla et al., 2018). With specific reference to lycopene, industrial treatments are discussed not only in terms of quantitative changes but also in terms of shifts in isomer distribution, which may have implications for bioavailability in the final product (Arballo et al., 2021; Srivastava and Srivastava, 2015).

Current interest extends beyond edible tomato products to include supply-chain by-products, especially pomace (predominantly peels and seeds), which has been proposed as a substrate for recovering phytochemical fractions with antioxidant potential (Farinon et al., 2024). Consistent with a cultivar- and handling-dependent matrix, studies on Italian varieties and post-harvest conditions have reported additional compositional and functional differences (Manzo et al., 2018; Fattore et al., 2016; Hui and Evranuz, 2015).

1.3 The genus *Alternaria*

Alternaria is a genus of filamentous ascomycetous fungi that is frequently reported across both natural ecosystems and human-influenced environments, consistent with a broad ecological plasticity (Thomma, 2003; Ostry, 2008).

Species belonging to this genus have been reported from a broad spectrum of substrates and interfaces, spanning plant-associated surfaces and residues, soils, and agricultural commodities, as well as a range of non-food materials in indoor and outdoor settings (Thomma, 2003; Ostry, 2008). Within agroecosystems, the recurrent detection of *Alternaria* in soils and on crop residues supports the relevance of the soil–residue compartment as an epidemiological reservoir and a contributor to inoculum pressure at the start of the growing season, a conceptual framing that is coherent with syntheses provided for tomato-related pathosystems (Adhikari, 2017; Schmey, 2024).

Beyond its agronomic relevance, *Alternaria* is also discussed in relation to exposure pathways that are not restricted to crop infection. The production and dispersal of conidia contribute to dissemination across new surfaces, and the presence of airborne propagules is considered relevant in allergology.

In this context, Alternaria is framed as a source of aeroallergens associated with respiratory conditions, including severe manifestations, reinforcing the cross-domain relevance of the genus at the interface between environmental mycology and human health (Hernandez-Ramirez, 2021).

From a physiological standpoint, growth, sporulation and secondary metabolism—including mycotoxin biosynthesis—are modulated by microenvironmental parameters such as temperature, water availability and substrate pH (Ostry, 2008).

For *A. alternata*, Ostry (2008) reports growth characteristics with an optimum around 25 °C, while emphasizing that minimum and maximum limits can vary depending on strain and experimental conditions; consistent with this general framework, work focusing on tomato highlights the interaction between temperature and humidity-related variables as determinants of germination and infection onset (Ostry, 2008; Salotti, 2024). In a thesis context, such observations justify treating the occurrence of *Alternaria* and the expression of its secondary metabolism as context-dependent phenomena, shaped by the intersection of strain-specific traits and environmental constraints.

In agronomy, *Alternaria* includes taxa that are widely recognized as economically relevant plant pathogens and are associated with diseases on numerous crops. For solanaceous hosts, syntheses distinguish major disease scenarios on potato and tomato, including syndromes such as early blight and brown spot, underscoring that multiple host–pathogen configurations are encompassed within the genus and that disease labels do not necessarily map to a single species across contexts (Schmey, 2024). Within this broader landscape, *A. alternata*, *A. solani*, *A. tenuissima*, *A. arborescens*, *A. infectoria*, and *A. brassicicola* are among the taxa frequently discussed in relation to distribution, pathogenicity, and agronomic relevance (Thomma, 2003; Habib et al., 2021).

At the same time, the literature has repeatedly highlighted that symptom-based or morphology-driven attribution at species level can be insufficiently robust in isolation, especially when multiple *Alternaria* taxa may coexist on the same host tissue or lesion, a point that becomes particularly salient in complex crop disease settings (Adhikari, 2017; Schmey, 2024).

Historically, the study of phytopathogenic *Alternaria* was driven primarily by agronomic impact, including yield losses and quality deterioration in both pre- and post-harvest phases. More recently, scientific attention has expanded toward food safety, because several Alternaria toxins are recurrently discussed as contaminants of diverse commodities and processed foods (Ostry, 2008; Gruber-Dorninger et al., 2017; Salotti, 2024; EFSA, 2016).

Within this framing, it is methodologically important to distinguish fungal occurrence from mycotoxin contamination: the detection of *Alternaria* does not, per se, constitute evidence that toxins are present, because secondary metabolite production depends on environmental conditions and on the physiological state of the fungus (Gruber-Dorninger et al., 2017). Consequently, the presence of *Alternaria* should be interpreted as an indicator of potential risk that may materialize under conditions conducive to toxin biosynthesis, while the confirmation of contamination requires targeted analytical assessment (EFSA, 2016). This distinction is particularly relevant for supply-chain reasoning, as it supports a preventive logic based on early recognition and management of predisposing conditions, while preserving the analytical rigor needed to substantiate contamination events and to support risk-oriented decision-making (EFSA, 2016; Salotti, 2024). Dealing with environmental parameters, in a synthetic tomato medium, Pose et al. (2010) demonstrated that water activity interacts with temperature to modulate AOH, AME and TeA production by A. alternata, illustrating that toxigenic output is conditional on environmental parameters rather than being an invariant trait (Pose et al., 2010).

1.3.1 *Alternaria* and tomato: species of interest, disease syndromes

Tomato (*Solanum lycopersicum* L.) is a highly perishable fruit in which tissue softness and high moisture content can facilitate the occurrence of small surface injuries during cultivation, harvest and handling, thereby increasing exposure to microbial attack in both field and post-harvest phases (Andersen and Frisvad, 2004).

Within this context, *Alternaria* spp. are repeatedly reported among the fungal groups relevant to tomato diseases and to spoilage, with implications that span yield losses, fruit quality deterioration and, in specific scenarios, contamination risks linked to *Alternaria mycotoxins* (Adhikari et al., 2017; Salotti et al., 2024).

Multiple *Alternaria* taxa have been associated with disease manifestations in tomato, and the literature emphasizes that syndrome-level diagnosis based solely on symptom morphology is often insufficient to define etiology at the species level. This is particularly relevant for foliar blight symptoms, where overlapping lesion phenotypes can be produced by different Alternaria species and may be further influenced by the infection stage and environmental context (Adhikari et al., 2017; Schmey et al., 2024).

Early blight is among the most relevant field syndromes affecting tomato, and A. solani is frequently indicated as a key causal agent. At the same time, several studies and reviews underscore that early blight should be framed as a disease complex, because multiple *Alternaria* species can be involved and may co-occur, while early symptoms can overlap with other leaf spot syndromes, especially when diagnosis relies on visual criteria alone (Adhikari et al., 2017; Schmey et al., 2024). Within this complex, Adhikari et al. (2017) discuss the involvement of multiple *Alternaria* taxa reported in association with early blight, including *A. solani*, *A. tomatophila* and *A. linariae*, and also note that *A. alternata* has been reported in association with early blight-like symptoms. This framing illustrates the practical need for careful attribution when synthesizing disease etiology across studies and when comparing reports based on different identification approaches (Adhikari et al., 2017).

Early blight on foliage is commonly framed in terms of expanding necrotic spots that can show a target-like zonation and surrounding chlorosis. On fruits, lesions are frequently described as dark, depressed areas that may occur near the peduncle insertion site, although the visible phenotype can vary with cultivar, infection pressure and weather conditions (Schmey et al., 2024). Accordingly, the literature emphasizes integrating symptom assessment with isolation and identification workflows, molecular tools and epidemiological context when interpreting disease episodes in tomato (Schmey et al., 2024; Salotti et al., 2024).

In advanced lesions, a superficial dark, velvety appearance can develop as a consequence of abundant *Alternaria* sporulation. This dark coloration is consistent with the presence of DHN-melanin in *Alternaria* conidia, which contributes to their pigmented phenotype (Singh et al., 2015; Thomma, 2003; Carzaniga et al., 2002).

In the post-harvest phase, tomato decay is often approached as a mixed-etiology syndrome, and the label “fruit rot” is therefore best treated as syndromic rather than as evidence of a single causal agent.

Within this broad picture, *A. alternata* is commonly reported among isolates obtained from symptomatic fruits. Market-level spoilage surveys illustrate the diversity of associated taxa and, at the same time, highlight that culture-based recovery does not, on its own, establish whether an isolate initiated decay or colonized tissue that was already physiologically compromised (Onuorah and Orji, 2015).

When broad spoilage consortia are discussed, oomycetes may also be mentioned alongside true fungi. For example, Phytophthora spp. are oomycetes rather than fungi, but they are relevant in tomato as aggressive plant pathogens and can appear in mixed-etiology discussions of fruit and tissue rots. Explicitly distinguishing oomycetes from fungi maintains taxonomic clarity while allowing a coherent discussion of disease complexity in the commodity chain.

1.3.2 Pathogenesis and infection cycle in the tomato-*Alternaria* pathosystem

At the supply-chain scale, the onset of *Alternaria*-associated epidemics can be framed as the outcome of coordinated processes that span inoculum sources, dispersal and favorable microclimatic conditions, together with host-pathogen interactions at the tissue level. Articles focused on tomato emphasize that *Alternaria* spp. can persist in crop residues and soil and can be disseminated as conidia, supporting a seasonal carryover of inoculum that contributes to the initiation of infection cycles (Thomma, 2003; Salotti et al., 2024).

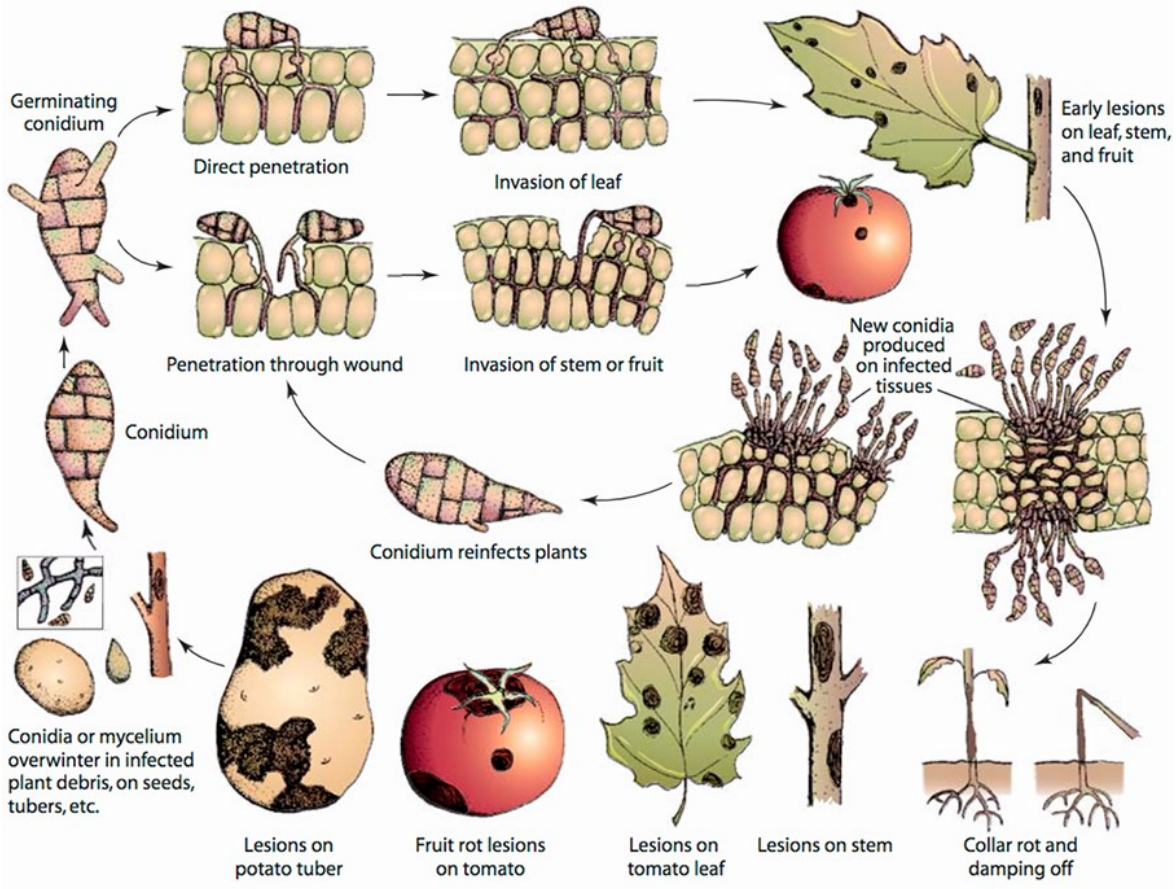


Figure 1. Disease cycle of early blight on tomato caused by *Alternaria solani* (adapted from Agrios, 2005; Adhikari et al., 2017).

From an epidemiological perspective, conidial deposition on aboveground plant organs is followed by germination under permissive moisture and temperature conditions. Disease development is promoted by environmental scenarios that maintain a humid boundary layer and provide leaf wetness periods that enable germ tube emergence and the establishment of infection structures (Schmey et al., 2024; Adhikari et al., 2017).

At the tissue interface, plant surface barriers represent the first obstacle to fungal entry (Fig. 2). The cuticle, composed largely of cutin and waxes, and the epidermal cell wall collectively constrain direct penetration.

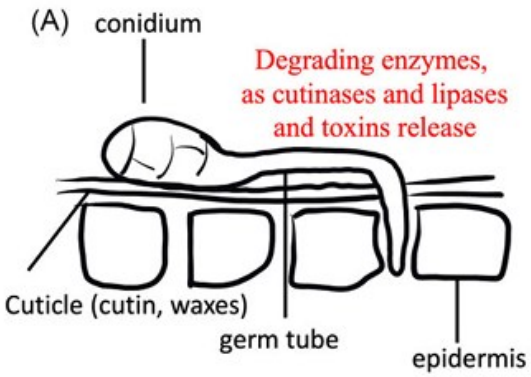


Figure 2. Schematic overview of *Alternaria* pathogenesis mechanisms relevant to plant infection (adapted from Fernandes et al., 2023).

Consistent with general models of necrotrophic fungal infection, *Alternaria* spp. deploy secreted enzymes and surface-associated factors that contribute to adhesion and to the progressive disruption of protective layers, thereby facilitating host entry (Thomma, 2003; Fernandes et al., 2023).

In tomato, penetration is typically discussed as occurring via multiple routes, including direct entry across the surface, invasion through natural openings such as stomata, and exploitation of wounds. The relative contribution of each pathway depends on the infected organ, the surface microenvironment and the presence of pre-existing lesions. After entry, necrotrophic colonization proceeds through the induction of host-cell death coupled to nutrient acquisition from disorganized plant structures (Salotti et al., 2024).

Experimental work across fruit-pathogen systems indicates that pathogens can modulate infection strategies in response to ripening stage, reinforcing the need to explicitly consider maturity when interpreting post-harvest rots and when comparing studies performed at different ripeness levels (Petrasch et al., 2019).

Host defense activation is an integral component of the infection microenvironment. Plant responses to fungal challenge include the induction of signaling cascades and redox-related responses, with reactive oxygen species (ROS) representing both antimicrobial and signaling components (Munir et al., 2025). In necrotrophic interactions, oxidative processes can contribute to lesion expansion because host cell death increases nutrient availability for the pathogen.

At the same time, oxidative stress imposes constraints on the pathogen itself, which must tolerate or detoxify ROS to sustain growth in damaged tissues (Park and Son 2024). Fernandes et al. (2023) discuss stress tolerance and host-defense coping strategies in *Alternaria*, supporting the view that redox homeostasis contributes to fitness during infection and is part of the broader virulence landscape (Fernandes et al., 2023).

Overall, these infection-cycle considerations support a cautious interpretation of presence data, particularly in post-harvest surveys where multiple agents may co-occur and end-point isolation alone may not identify the initial trigger of deterioration (Schmey et al., 2024; Onuorah and Orji, 2015; Andersen and Frisvad, 2004).

Following penetration, tissue colonization can be framed around two complementary functional axes: enzymatic disassembly of structural barriers and the action of phytotoxic secondary metabolites. With respect to the first axis, cell wall-degrading enzymes (CWDEs), including pectinases and other polysaccharide-active activities, are frequently cited as virulence determinants across plant-pathogenic fungi; however, for *Alternaria*, functional redundancy can complicate the attribution of a unique causal role to individual enzymes (Fernandes et al., 2023). This supports an interpretation in which polysaccharide hydrolysis, redox-related stress management and secondary metabolite production jointly shape necrotrophic fitness and tissue exploitation, rather than a model dominated by a single enzyme or a single toxin (Fernandes et al., 2023).

1.3.3 Virulence determinants and phytotoxic metabolites in plant pathogenesis

A recurring feature of *Alternaria* pathogenesis is the deployment of small, diffusible secondary metabolites with phytotoxic activity (Tsuge et al., 2013; Thomma, 2003) A widely used functional distinction separates host-selective toxins (HSTs), whose phytotoxicity is restricted to specific host genotypes and that can act as determinants of pathogenicity in defined pathotypes, from non-host-selective toxins (NHSTs), which exhibit broader phytotoxic activity and may contribute to necrosis and physiological dysfunction across a wider plant range (Wang et al., 2022; Meena and Samal, 2019).

HSTs are described as critical effectors in a limited number of destructive diseases, where they exert severe effects on a relatively narrow host range and are indispensable for disease development in susceptible hosts (Tsuge et al., 2013).

In particular, *Alternaria* stem canker has been associated with the tomato pathotype of *A. alternata* (historically referred to as *A. alternata f. sp. lycopersici*) and is conceptually separated from early blight because it is linked to host-selective toxin activity, cultivar-dependent susceptibility, and the production of AAL-toxins (Tsuge et al., 2013; Yamagishi et al., 2006).

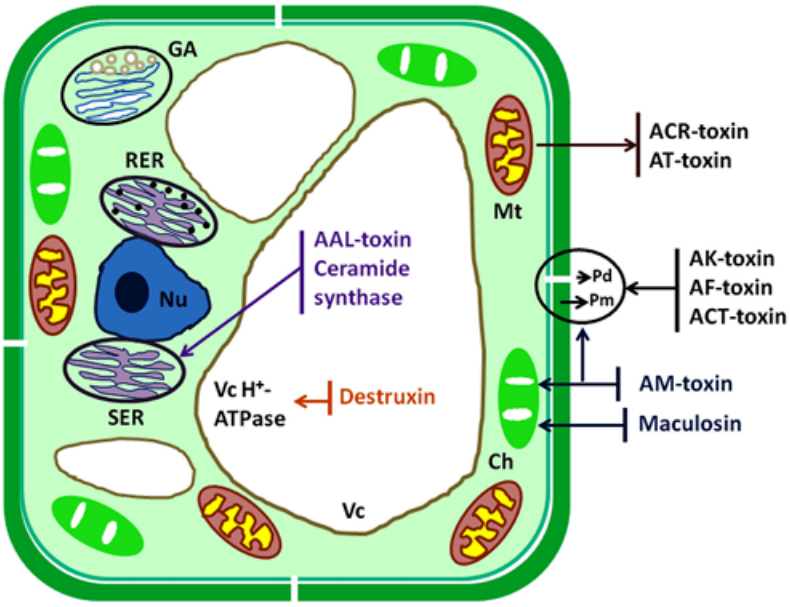


Figure 3. Main intracellular targets of host-selective toxins (HSTs) produced by *Alternaria* species in plant cells (adapted from Meena et al., 2017)

Alongside HST-driven pathosystems, many *Alternaria* toxins are classified as NHSTs. These metabolites can contribute to necrosis and physiological dysfunction without strict host selectivity.

Reviews synthesizing the chemical diversity of *Alternaria* phytotoxins discuss both occurrence and bioactivity and provide examples of NHSTs with documented phytotoxic effects, while also emphasizing that the strength of evidence for a direct virulence role can vary between compounds and pathosystems (Wang et al., 2022; Meena and Samal, 2019).

Tenuazonic acid (TeA) is a particularly informative NHST because it is supported by both mechanistic evidence on plant targets and functional evidence in infection-related experimental models.

TeA has been characterized as a natural inhibitor of photosystem II, with binding at the QB site, providing a mechanistic anchor for phytotoxicity through disruption of photosynthetic electron transport (Chen et al., 2007; Chen et al., 2008). In addition,

Kang et al. (2017) discuss TeA as a virulence-associated factor in an *A. alternata* pathotype–host system, relating toxin production to tissue damage, ROS-associated parameters and infection-associated traits within that experimental framework (Kang et al., 2017). A synthesis oriented toward bioherbicidal perspectives further organizes evidence linking photosystem II disruption to redox imbalance, ROS formation and tissue injury manifesting as chlorosis and necrosis, outcomes compatible with necrotrophic nutrient acquisition (Sotelo-Cerón et al., 2023).

Alternariol (AOH) also has specific consideration because evidence has been provided for a functional contribution to virulence and colonization during plant infection. In A. alternata, Wenderoth et al. (2019) identified the biosynthetic gene cluster for AOH and AME and, within the plant infection context, linked alternariol biosynthesis to colonization-related outcomes, supporting the interpretation of this metabolite as a functional component of pathogenic behavior rather than a mere metabolic by-product (Wenderoth et al., 2019). In parallel, reviews on *Alternaria* phytotoxins place AOH and AME among recurrent metabolites and discuss their occurrence and bioactivity across plant-associated contexts (Wang et al., 2022; Fernández Pinto and Patriarca, 2017).

Additional phytotoxic metabolites reported for *Alternaria* include perylene derivatives and anthraquinone-related compounds. Reviews describe compounds such as altertoxins, macrosporin, and altersolanols among the chemical space of *Alternaria* secondary metabolism and report phytotoxic outcomes in experimental plant assays for selected metabolites (Wang et al., 2022).

Virulence is not limited to toxin production. Enzymes involved in surface establishment and tissue colonization include cutinases, lipases and cell wall-degrading enzymes that contribute to disorganization of cuticular and polysaccharide matrices, improving access to nutrients and facilitating invasion. Proteases are also discussed as key enzymes produced during host-pathogen interaction and have been framed as important contributors to phytopathogenicity across fungi (Thomma, 2003; Chandrasekaran et al., 2016; Fernandes et al., 2023).

Stress tolerance and persistence traits further shape infection success. Moreover melanins derived from 1,8-dihydroxynaphthalene (DHN) are dark, high-molecular-weight pigments produced by many fungi and have been discussed as contributors to resistance against environmental stresses and propagule survival. In *Alternaria*, melanin is a prominent feature of conidia, and immunogold localization studies have mapped melanin within the conidial wall architecture, providing structural evidence for its presence in relevant infection propagules (Thomma, 2003; Carzaniga et al., 2002). At the same time, Thomma (2003) emphasizes the complexity of linking single factors to a unique determinant of pathogenicity, supporting a cautious interpretation in which melanin is framed as a fitness and persistence factor rather than a universally required penetration determinant (Thomma, 2003).

Plant defense responses provide complementary context for framing pathogen virulence. Because fungal cell walls contain chitin, plant chitinases are commonly discussed as defense-related (PR) proteins induced during fungal attack (Dong et al., 2024). Collinge et al. (1993) provide a foundational discussion of plant chitinases as defense proteins, supporting their use as conceptual anchors when describing host-side responses to fungal invasion (Collinge et al., 1993). In applied research, genetic engineering has explored the expression of chitinase genes as a route to enhance resistance to fungal pathogens. In tomato, transgenic expression of a rice chitinase gene has been evaluated in relation to resistance against *Fusarium oxysporum* and *A. solani*, offering an example of how defense-related genes can be leveraged to modulate susceptibility at the crop level (Jabeen et al., 2015).

Finally, the chemical properties of toxigenic metabolites provide a bridge to the broader thesis rationale. Secondary metabolites relevant to plant pathogenesis can also act as contaminants in infected plant materials, linking pathogenicity to food-safety concerns when infected tissues enter processing chains.

For tomato, the integration of disease-cycle knowledge with the occurrence of toxigenic metabolites has been highlighted as a key component for predictive and preventive frameworks, reinforcing the need to treat virulence determinants and mycotoxin risk as connected dimensions of the *Alternaria*-tomato system (Salotti et al., 2024; Fernández Pinto and Patriarca, 2017).

1.4 Toxicology

A major factor that substantially complicates mycotoxin risk management is the non-uniqueness of the relationship between toxins and producing organisms: the same mycotoxin can be biosynthesized by different species and, conversely, a single strain can generate a multi-toxin profile. As a result, contamination patterns become less predictable and analytical surveillance becomes more demanding, because it must be designed to capture variable and non-linear scenarios (Escrivá et al., 2017). This issue is embedded within contamination dynamics that involve heterogeneous matrices, including cereals, fruits, and vegetables, and that arise from the interaction between the presence of the fungus and environmental and technological conditions that favor growth and toxin production. From a causal standpoint, the damage is attributable to toxic molecules: the origin is biological, but the adverse effect is mediated by chemical compounds and therefore does not necessarily coincide with the mere presence of the mould.

Within this framework, toxicological understanding requires linking exposure to the chemical–physical properties that govern bioavailability, distribution, and reactivity. On the one hand, the ability of mycotoxins to cross biological barriers and reach target compartments is modulated by parameters such as lipophilicity (logP), polarity, and topological polar surface area (tPSA), which influence membrane permeation and, consequently, the probability of accessing vulnerable intracellular sites.

On the other hand, chemical reactivity contributes decisively to the damage profile: many mycotoxins display electrophilic functional groups, aromatic systems, and motifs with potential metal-chelating capacity, including tetramic and tetronic acid moieties (Matiadis and Kourounakis 2020).

These features are compatible with interactions with biological macromolecules, redox processes, and the generation of reactive oxygen species, which represent recurring nodes in cellular toxicity (Solhaug et al., 2016).

A substantial fraction of *Alternaria* secondary metabolism is represented by polyketides, a broad class of natural products assembled from simple acyl building blocks and characterized by pronounced structural diversity.

Within this chemical space, pyranones are frequently reported and include dibenzo-α-pyranone derivatives (Li et al., 2024; Zhao et al., 2023; Meena and Samal, 2019). In this context, alternariol (AOH) and alternariol monomethyl ether (AME) can be positioned as polyketide-derived dibenzo-α-pyranones produced by *Alternaria*, providing a coherent biosynthetic and structural basis for the subsequent discussion of their biological activity. This perspective also underscores that *Alternaria* produces toxins with distinct structure–activity trajectories: compared with AOH/AME, tenuazonic acid (TeA) follows a different chemical logic and is therefore discussed separately on the basis of its functional targets.

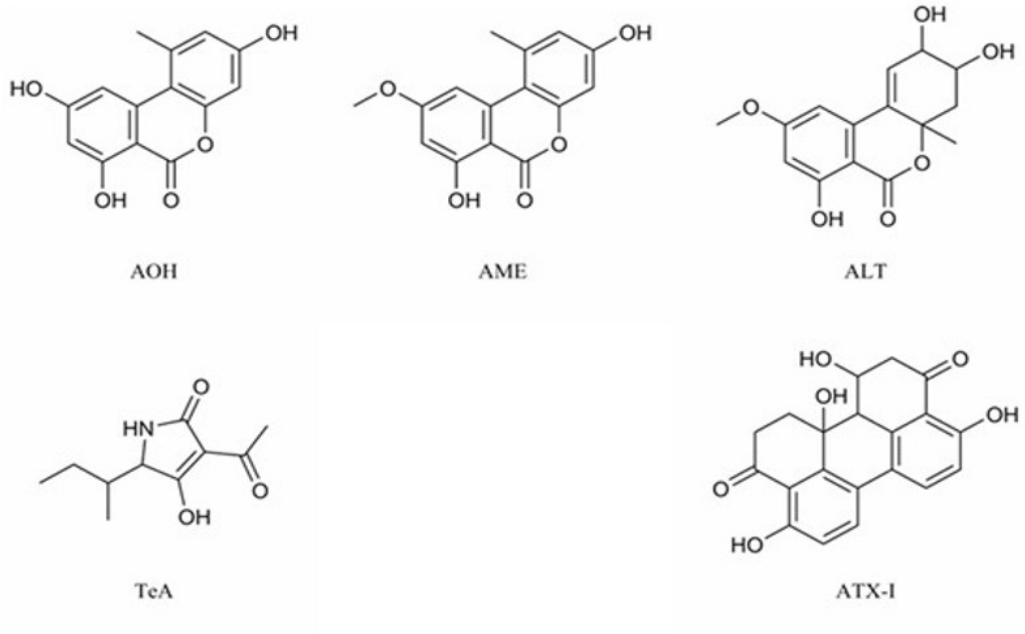


Figure 4. Chemical structures of principal *Alternaria* mycotoxins discussed in this thesis (AOH, AME, TeA, ALT, ATX-I).

At the clinical level, mycotoxicosis is defined as a syndrome resulting from ingestion, dermal contact, or inhalation of mycotoxins, with severity modulated by intrinsic toxicity, dose, and exposure duration, as well as host-related factors such as age and nutritional status (Gallo et al., 2015). Acute manifestations, typically associated with high exposures concentrated in a single intake or in repeated doses within 24 hours, often include gastrointestinal symptoms such as vomiting and diarrhea (Bhat et al., 2010).

In settings characterized by high hygienic and sanitary control, attention often shifts toward chronic low-dose exposures, which are difficult to identify diagnostically and have therefore been described as “hidden killers”, with consequent uncertainty regarding the true distribution of exposure within the population (Galvano et al., 2005).

Risk assessment becomes further complicated when multiple mycotoxins coexist within the same matrix. Co-contamination, besides being frequent, raises the possibility of antagonistic, additive, or synergistic interactions, with combined-toxicity profiles that are not necessarily predictable from a simple summation of individual effects (Crudo et al., 2019; Malachová et a., 2018; Arnau et al., 2013; Ibáñez-Vea et al., 2012; Puel, 2010).

Within this broader context, mycotoxins produced by *Alternaria* represent a group of particular interest because of their widespread occurrence and the plurality of their modes of action. Alternariol (AOH), alternariol monomethyl ether (AME), altertoxin II (ATX-II), tenuazonic acid (TeA), and tentoxin (TEN) have been reported in several matrices, including cereals and plant-derived products. Although they belong to different structural classes, they share relatively low molecular size and moderate lipophilicity, features consistent with non-negligible permeability and with some degree of persistence in food matrices (Gruber-Dorninger et al., 2017; Aichinger et al., 2021).

From a risk-assessment perspective, EFSA adopted an approach based on the Threshold of Toxicological Concern (TTC), applying reference values of 2.5 ng/kg body weight/day for AOH and AME (considered genotoxic) and 1500 ng/kg body weight/day for TeA and TEN (considered non-genotoxic). EFSA also highlighted that, in specific vulnerable groups, estimated exposure to some of these toxins may exceed the corresponding TTC values, indicating the need to strengthen the evidence base on exposure and toxicokinetics (EFSA, 2011).

Table 1. Indicative levels (µg/kg) for AOH, AME and TeA in processed tomato products according to Recommendation (EU) 2022/553.

Mycotoxin	AOH	AME	TeA
Indicative levels (µg/kg)	10	5	500

Note: AOH, alternariol; AME, alternariol monomethyl ether; TeA, tenuazonic acid.

AOH and AME, as dibenzo-α-pyrone derivatives with a planar aromatic scaffold, can support interactions with nuclear targets, including topoisomerases, and have been described with a mode of action consistent with topoisomerase poisons (Fehr et al., 2009; Tiessen et al., 2013).

In line with toxicity frameworks centred on oxidative stress and DNA damage, DNA strand breaks, cell-cycle perturbations, and the formation of reactive oxygen species have been reported (Solhaug et al., 2016; Saleh et al., 2024).

For ATX-II, the toxicological profile is tightly linked to structural determinants, most notably the presence of an electrophilic epoxide moiety. This feature can promote direct reactions with biomolecules, particularly DNA, and stable adduct formation with nucleobases has been reported as a mechanistic element consistent with the high genotoxic potency of ATX-II (Soukup et al., 2020).

For TeA and TEN, structure–activity relationships follow different trajectories. TeA, a 3-acyl tetramic acid, has been described as displaying moderate lipophilicity and the ability to complex metal cations, features that are compatible with the involvement of chelating motifs and redox-related reactivity in shaping specific toxicity profiles (Athanasellis et al., 2010; Zaghouani and Nay, 2016). TeA has been reported as an inhibitor of eukaryotic protein synthesis (Wang et al., 2022) and, as a phytotoxin, as interfering with photosystem II functionality (Chen et al., 2007).

An additional critical aspect concerns modified forms: beyond the free toxins, some *Alternaria* mycotoxins may occur as sulfate or glucoside conjugates produced by the fungus or by the host plant.

Such derivatives may escape non-targeted protocols and, in vivo, may undergo hydrolysis with the release of the parent toxin (Rychlik et al., 2014; Soukup et al., 2016; Puntscher et al., 2018). Their identification in processed foods, including tomato sauces, supports the inclusion of modified forms in monitoring programmes and exposure assessments in order to reduce the risk of underestimation (Puntscher et al., 2019).

1.5 Control strategies and risk mitigation along the supply chain: traditional practices and biotechnological approaches

Within crop protection, chemical control based on synthetic fungicides has historically represented one of the most immediate tools to reduce disease severity, particularly under high epidemic pressure. For a long time, phytosanitary protection was often centred on broad-spectrum synthetic products, including preventive applications. However, repeated and not always rationalised use of the same modes of action can impose substantial selection pressure; over the medium to long term, this may promote reduced sensitivity and the emergence of resistance phenomena, with a progressive erosion of treatment efficacy. These criticalities have been associated with collateral effects on the soil microbiota and with environmental concerns. Subsequently, the development of more specific molecules with systemic activity marked an evolution of chemical control; in parallel, advances in biological and chemical knowledge and increasing attention to human health and the environment contributed to a reassessment of the traditional model, favouring the gradual adoption of more sustainable protection strategies (Di Natale, 2016).

1.5.1 Agronomic prevention, monitoring, inoculum reduction, and integrated disease management

From an operational standpoint, disease management can be framed as a combination of preventive measures and control measures. Preventive measures primarily act on the host and the environment, reducing available inoculum and the susceptibility of the cropping system; this category includes agronomic and hygienic–organisational choices, as well as genetic tools when available. Control measures, by contrast, aim to limit the pathogen’s capacity to cause damage during risk phases and include chemical, biological, and, in some cases, biotechnological means.

Within this framework, for the complex of diseases associated with *Alternaria* spp. on tomato, it has been proposed that a robust approach should integrate prevention, monitoring, and targeted interventions, reducing exclusive reliance on chemical control and incorporating biological or biotechnological tools when supported by evidence (Schmey et al., 2024).

The sequencing of measures is of practical relevance: agronomic and hygienic practices aim to reduce inoculum and host susceptibility, whereas control interventions are rationalised in relation to risk windows and the cropping context (Adhikari, 2017). In the pre-harvest phase, crop practices are often presented as a foundational component of risk mitigation because they act on factors governing epidemic initiation and development. In general terms, this domain includes management of inoculum sources and crop residues, the adoption of rotations, and practices that limit the duration of leaf wetness and improve canopy aeration; these are complemented by hygienic–organisational measures intended to reduce the presence of infected tissues and local contamination (Schmey et al., 2024). The literature nonetheless indicates that, under conditions favourable to disease or in scenarios of high pressure, crop measures alone may be insufficient; accordingly, prevention should not be framed as an alternative to interventions, but rather as a prerequisite that conditions their effectiveness and sustainability (Adhikari, 2017).

The evolution of phytosanitary protection has been described as a transition from rigidly scheduled intervention schemes towards approaches based on more informed decision-making. An initial phase, often referred to as “calendar-based control”, involved repeated treatments at fixed intervals aligned with phenological stages, without systematic verification of the pathogen’s actual presence; in this context, the reiterated use of active substances contributed, among other effects, to increased selection pressure and to the development of resistance phenomena. Subsequently, “guided chemical control” became established, grounded in the concept of the economic threshold: an intervention is considered justified when the risk of expected loss equals or exceeds the cost of treatment (Auteri et al., 2004).

Within the contemporary framework, Integrated Pest Management (IPM) has been proposed as an operational model in which the use of chemical products is not excluded, but is subordinated to criteria of necessity and integrated with agronomic, biological, and, where relevant, biotechnological measures, with the aim of reducing chemical inputs while maintaining the long-term functionality of the available options (Schmey et al., 2024).

When applied to the complex of infections attributed to *Alternaria* spp., the integrated approach is oriented both towards reducing the probability of infection and epidemic pressure and towards preserving the sustainability of control strategies, including the management of the risk of selecting less sensitive strains (Adhikari, 2017; Schmey et al., 2024).

Consistent with these principles, experimental evidence obtained under field conditions on early blight has evaluated the combined use of chemical means and biocontrol agents, supporting the view that different tools may be incorporated into integrated programmes as an alternative to exclusively chemical schemes (Sarkar et al., 2016). In the phytosanitary domain, this evolution has also been associated with the progressive withdrawal or restriction of certain active substances and with a push towards solutions considered more eco-compatible (Prieto et al., 2022).

1.5.2 Biocontrol: definitions and operational applications

Within this framework, biological control is positioned as a relevant component. As early as 1974, Baker and Cook defined biological control as the reduction of inoculum or pathogenic activity through antagonistic organisms, achieved naturally or through manipulation of the environment and the host and/or through the mass introduction of antagonists (Baker and Cook, 1974). For fungal pathogens, an established line of application is the use of antagonistic microorganisms, both fungal and bacterial; in Europe, products based on genera such as *Trichoderma*, *Pseudomonas*, *Streptomyces*, and *Bacillus* are commercially available (Ongena et al., 2008). In particular, *Trichoderma* spp., due to their variability and adaptability, are frequently applied through different modalities, including soil treatments in different formulations, seed coating, and application to seedlings at transplanting (Prieto et al., 2022).

1.5.3 Post-harvest risk mitigation strategies: storage, processing, and containment

In the post-harvest phase, risk mitigation can be traced back to two interconnected operational objectives: containing fungal development while preserving quality and shelf-life, and limiting the likelihood that mycotoxin contamination increases along the harvest–storage–processing sequence. Occurrence studies in processed products document the presence of *Alternaria* toxins across different categories of tomato-derived products and motivate a supply-chain approach. Storage represents a critical step: if appropriately managed, it primarily contributes to preventing the onset and growth of fungi and, consequently, to reducing the opportunity for further toxin production in the interval between harvest and processing. In other words, proper storage does not necessarily imply a reduction of toxins already present, but may limit the probability of increases associated with fungal growth and toxinogenic activity (Qin et al., 2022).

1.5.4 Processing and stability

During processing, the available evidence indicates that the effect of technological steps is not uniform across *Alternaria* toxins. In a study simulating process stages, Estiarte et al. (2018) evaluated AOH and AME, observing variations dependent on the specific step and the conditions considered; any reductions observed under specific conditions cannot be interpreted as complete or guaranteed removal (Estiarte et al., 2018). In a critical review, Puntscher et al. (2019) emphasised the variability of responses across compounds and technological conditions and suggested caution in generalising a “detoxifying” effect of processing (Puntscher et al., 2019). More recently, Giorni et al. (2025) assessed the distribution between pulp and peel and the stability under thermal treatments of multiple toxins, reporting differentiated trends among TeA, AOH, and TEN; overall, these results are consistent with a compound-specific interpretation of stability and reinforce the view that prevention of contamination remains a priority lever relative to the expectation of complete detoxification during processing (Giorni et al., 2025).

1.5.5 Biopreservation and post-harvest containment

Alongside logistics management and storage conditions (Conte et al., 2020), biopreservation is discussed as an option to contain moulds and quality decay and, indirectly, to reduce mycotoxicological risk by limiting fungal growth. For lactic acid bacteria (LAB), mechanisms based on acidification and on the production of low-molecular-weight antifungal metabolites have been described; an experimental reference concerns the identification and characterisation of antifungal compounds produced by Lactobacillus plantarum in fermentative contexts (Lavermicocca et al., 2000). Subsequent reviews also discuss the anti-mycotoxigenic potential of LAB, distinguishing between a reduction of fungal growth (with an indirect reduction in toxin production) and possible more direct interactions with mycotoxins or biosynthetic processes, in a strain- and matrix-dependent manner (Dalié et al., 2010; Sadiq et al., 2019). Consistently with this framing, in tomato matrices the use of LAB-fermented media has been reported within biopreservation strategies, supporting the practical feasibility of an approach based on fermentation and metabolites (Luz et al., 2020).

PART II – Methodological framework

Chapter 2 – Extraction of Alternaria mycotoxins from tomato-derived products

2.1 QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) :

origin and physicochemical principles

QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) was originally introduced as a multiresidue approach for fruit and vegetable matrices, based on acetonitrile extraction followed by salt-induced phase partitioning, with the option of extract clean-up by dispersive-SPE (Anastassiades et al., 2003). Over time, buffered variants (acetate- or citrate-based) were developed to control the pH of the aqueous phase during extraction and, consequently, to better manage analytes sensitive to pH-dependent speciation (AOAC, 2007; CEN, 2018).

QuEChERS subsequently became a flexible methodology that has been adapted to different analyte classes as bioactive molecules (Sarraf et al., 2020), and matrices, including contaminants and mycotoxins (González-Curbelo et al., 2015; Perestrelo et al., 2019; Santana-Mayor et al., 2023). From a physicochemical perspective, QuEChERS combines (i) an initial extraction of the matrix with acetonitrile and (ii) a salting-out partitioning step achieved through the addition of salts. Increasing ionic strength in the aqueous phase reduces water–acetonitrile miscibility and promotes the formation of two phases: an acetonitrile-rich organic phase, into which many analytes preferentially distribute, and an aqueous phase (Anastassiades et al., 2003; AOAC, 2007) preferably under cold conditions (Shao et al., 2017). Mixing promotes the approach to partitioning equilibrium, whereas centrifugation plays a primarily physical role by sharpening phase separation and enabling reproducible sampling of the organic layer (AOAC, 2007).

In mycotoxin applications, acetonitrile is frequently used as the extraction solvent because it is compatible with LC–MS/MS and because partitioning in the presence of salts is generally efficient. QuEChERS protocols adapted to mycotoxins in pigmented matrices often employ acidified acetonitrile and may modulate, or in some cases omit, clean-up when the quantification strategy and matrix-effect management allow it (Yogendrarajah et al., 2013). For *Alternaria* toxins, QuEChERS-based methods have been described for several matrices, including apples (Tang et al., 2020). Wolfberry (Xing et al., 2020) fruit purées (Xing et al., 2021), tomato (Sanzani et al., 2019; De Berardis et al., 2018) , in spices (Yogendrarajah et al., 2013), pomegranate (Myresiotis et al., 2015) and cereals (Niaz et al., 2025; Malachová et al., 2018; Puvača et al., 2022).

The salt system and, where applied, buffering conditions regulate the salting-out process and influence the effective composition of the organic phase, with potential consequences for recovery and repeatability across analytes with different polarity and acid–base behaviour. In the context of *Alternaria* toxins, AOH and AME are often reported with relatively stable recoveries under established QuEChERS set-ups, whereas TeA tends to be more method-dependent, both because of its sensitivity to acid–base conditions during extraction and because of the chromatographic challenges reported for LC–MS/MS determination (De Berardis et al., 2018; Tölgyesi et al., 2015; Tölgyesi et al., 2023).

Mild acidification of the extraction solvent has been used in QuEChERS protocols for mycotoxins and can be relevant for modulating the speciation of acidic analytes.

2.1.1 Tomato-derived matrices: co-extraction, matrix-effect management, and clean-up selection

In tomato-based products, sample preparation is influenced by the presence of chromophores and co-extractives (e.g., carotenoids and oxidation products, organic acids, sugars) and, in many cases, by high viscosity (purées, pulps, and semi-concentrates). These features can increase co-extraction and amplify electrospray matrix effects (Walravens et al., 2016; De Berardis et al., 2018). Under these conditions, the extent and type of clean-up should be balanced against the risk of analyte loss: graphitised sorbents used to remove pigments may also interact with planar aromatic compounds, and the literature therefore emphasises the need to experimentally evaluate the trade-off between interferent removal and recovery when optimising QuEChERS for complex plant matrices (Perestrelo et al., 2019; Zhao et al., 2022). In parallel, alternative or comparative workflows based on SPE, or comparisons between SPE and QuEChERS clean-up, have been described for mycotoxin determination in fruits and vegetables, supporting the notion that the optimal purification strategy is strongly matrix- and analyte-dependent (Dong et al., 2019). Additional approaches documented in UHPLC–MS/MS multiresidue analysis on tomato include reducing or omitting clean-up in favour of extract dilution, which under appropriate conditions can improve matrix effects and peak shape; although such evidence derives from pesticide multiresidue contexts, it is methodologically relevant for discussing how matrix complexity impacts ESI performance (Stringhini et al., 2021).

Chapter 3 – Instrumental determination

Food analysis is not merely a technical step; it is a basic prerequisite for supporting quality, authenticity, and safety along the supply chain. Alongside the determination of nutritional components and process indicators, analytical strategies aimed at detecting undesirable contaminants have progressively consolidated over time, requiring high sensitivity, selectivity, and reproducibility.

The determination of chemical substances in food matrices remains intrinsically complex, both because of matrix heterogeneity and because of the physicochemical variability of contaminants, which often necessitates operation at trace levels with performance compatible with surveillance and control activities (Turner, 2015; Zöllner and Mayer-Helm, 2006; Tittlemier et al., 2021). In this context, mycotoxin control plays a priority role in risk management and in the protection of food safety.

From an operational standpoint, mycotoxin analysis is typically configured as a multistage process that includes sampling, sample preparation, extraction with water/organic-solvent mixtures (often polar), possible clean-up steps, and instrumental determination. Consequently, result quality does not depend on a single step, but on the overall consistency between sample treatment and detection technique, with particular attention to matrix interferences and reproducibility (Zöllner and Mayer-Helm, 2006; Turner, 2015).

Within the spectrum of instrumental techniques, thin-layer chromatography (TLC) primarily retains a role as a screening and preliminary control tool: it enables rapid comparisons among samples and a first qualitative reading of profile complexity and relative polarity differences (Geissler et al., 2017). In the mycotoxin field, TLC is often cited as historically relevant for simplicity and cost containment, but it is also described as less suitable when high sensitivity and robust quantification at trace levels are required; for these reasons, it is used mainly as an orientative support, whereas identity confirmation and rigorous quantification are delegated to more selective platforms (Turner, 2015; Agriopoulou, 2020).

Gas chromatography (GC), particularly when coupled to mass spectrometric detection (GC–MS or GC–MS/MS), offers high separation efficiency and strong confirmatory capability. Its use in mycotoxin analysis is nevertheless constrained by the need for analytes to be sufficiently volatile and thermally stable; consequently, for many applications, derivatization is required to obtain species more compatible with GC separation and detection, with an unavoidable increase in operational complexity and potential variability linked to reaction and clean-up. In this sense, GC is described as an important and highly informative technique, but not always the most flexible option for multi-analyte approaches in complex matrices when compared with LC–MS/MS (Turner, 2015; Agriopoulou, 2020).

Capillary electrophoresis (CE) represents an alternative option based on separation in an electric field, appreciated for efficiency, rapidity, and reduced solvent consumption, often aligning with more sustainable analytical chemistry approaches. CE is particularly suited to ionisable species and allows selectivity modulation via pH and buffer composition; however, when trace levels are required in complex matrices, sensitivity can become a practical limitation and may necessitate preconcentration strategies and/or more selective instrumental couplings. As a result, CE is frequently presented as a complementary technique, to be validated case by case depending on matrix and quantitative objective (Turner, 2015; Colombo, 2020).

Finally, high-performance liquid chromatography (HPLC/UHPLC) represents one of the most versatile platforms for the analysis of complex mixtures, given the possibility of tuning separation through stationary-phase choice and mobile-phase conditions (Sforza et al., 2006). Coupling with mass spectrometry is commonly described as an enabling element for trace-level determination and for multi-analyte approaches; overall robustness, however, also depends on sample preparation and chromatographic separation quality, as well as on operational aspects that can influence recoveries, including pre-injection steps and the materials employed (De Girolamo, 2022; Agriopoulou, 2020; Kovač Tomas, 2023).

3.1 LC-MS/MS

In LC–MS, chromatographic separation arises from the different partitioning of analytes between a stationary phase contained in the column and a mobile phase flowing at controlled rate under high-pressure conditions (Niessen et al., 2020; Gross et al., 2017). Selectivity depends on multiple factors, including stationary-phase chemistry, mobile-phase composition, pH and buffer composition, temperature, and elution programme (Songsermsakul et al., 2008). From an instrumental perspective, an HPLC system typically includes a pumping module, a sample introduction system, a chromatographic column (often housed in a thermostatted compartment), and a detector. The pump must provide a stable and reproducible flow; in many modern configurations, solutions that enable composition gradients are adopted.

The injector introduces defined sample volumes; upstream of the analytical column, a prefiltration element or a protective guard column may be present, useful to retain particulates and reduce contamination that would compromise pressure stability and repeatability. The column, defined by length, internal diameter, and particle size, is the core of separation; temperature control contributes to improving retention-time reproducibility. Detection can rely on different principles (e.g., UV/Vis, fluorescence, refractive index), but for analytes present at low levels in complex matrices, coupling with mass spectrometry is particularly advantageous.

Mass spectrometry provides information on the mass-to-charge ratio (m/z) of ionic species and, through controlled fragmentation strategies, can yield elements useful for structural identification.

In schematic terms, a mass spectrometer comprises an ionisation source, an analyser, and a detector; the system operates under vacuum conditions adequate for ion transmission and manipulation. Sample introduction can occur in the gas phase or, more frequently in LC–MS, through interfaces that convert a nebulised liquid flow into gas-phase ions. Ionisation modes differ in “hardness” and therefore in the extent of fragmentation. Electron ionisation often produces extensive fragmentation and is typically associated with GC–MS; chemical ionisation, being “softer”, can generate more informative molecular ions in several cases. For LC–MS, the most common techniques include electrospray ionisation (ESI) and atmospheric-pressure chemical ionisation (APCI), whereas MALDI is more typical of other applications and is not the standard choice for HPLC eluates (Alshannaq and Yu., 2017; Antignac et al., 2005).

In ESI, the sample dissolved in the mobile phase is nebulised through a capillary subjected to a high potential difference. Formation of a Taylor cone and charged droplets initiates the process; solvent evaporation, assisted by gas and/or heating, progressively increases charge density until droplets undergo instabilities and successive fissions (“Coulomb fission”), ultimately generating gas-phase ions. Ions are then transferred from atmospheric pressure toward progressively lower-pressure regions (for example through a capillary and skimmer) to reach the analyser.

This mechanism makes ESI particularly suitable for polar or semi-polar analytes and for complex matrices, often preserving high informativity on the molecular or pseudo-molecular ion (protonated or deprotonated). In addition, ESI is particularly suitable for coupling with LC because it generates ions directly from a nebulised liquid stream; in many set-ups, more favourable performance is observed at reduced flow rates, and overall efficiency can be improved through column and interface choices compatible with these flow regimes (Niessen et al., 2020).

Analysers can be of different types; among those frequently adopted in LC–MS/MS are ion-trap instruments and quadrupole configurations. In an ion trap, ions are confined via electric fields and manipulated for selection and fragmentation; scanning is achieved by varying conditions governing ion trajectory stability. Quadrupoles can act as selective mass filters, and tandem MS platforms enable selection of a precursor ion and generation of product ions by collision-induced dissociation (CID), thereby acquiring MS/MS spectra useful both for confirmation and identification.

Interfacing HPLC with mass spectrometry requires reconciling two very different operational requirements. On the one hand, liquid chromatography operates with a continuous eluent flow (typically hundreds of µL/min up to mL/min), which may contain salts or mobile-phase additives and passes through the column under high pressure; at the column outlet, however, the flow is at atmospheric pressure. On the other hand, the mass spectrometer must convert that liquid flow into gas-phase ions and transfer them efficiently into high-vacuum regions, a condition indispensable for guiding and separating ions without undesired collisions.

The widespread adoption of LC–MS derives from the possibility of combining the separating power of chromatography (reducing co-elutions and interferences) with the selectivity of MS (discriminating analytes by m/z and, in MS/MS, by fragmentation patterns). This combination helps mitigate interferences and improve identification and quantification; nevertheless, matrix effects linked to ionisation (signal suppression or enhancement) may persist and must be assessed and controlled during validation.

Tandem mass spectrometry (MS/MS) is a key step when the objective is to distinguish isobaric compounds or confirm identity in complex matrices. The principle consists of selecting a precursor ion, fragmenting it in a collision cell, and measuring product ions. This logic can be extended to multiple stages (MS^n) in instruments that support it, obtaining additional structural information; in practice, the number of stages used depends on instrument type and the analytical objective.

3.1.1 Practical workflow for LC–MS(/MS): standards, infusion, tuning, and mass calibration

In day-to-day method development, reference standards are the anchor point: they allow the expected precursor ion to be verified (typically protonated or deprotonated depending on the ionisation mode), the response to be maximised, and operating conditions to be made reproducible. Direct infusion, performed by bypassing the column, is particularly useful when a chromatographic method is not yet consolidated or when the immediate priority is to characterise source behaviour and identify the settings that yield the highest and most stable signal. Automated and manual tuning pursue the same goal with different levels of operator control; manual intervention becomes especially valuable at low concentrations or when ionisation efficiency is intrinsically limited for a given analyte. Finally, mass calibration should be implemented progressively, ensuring that the calibrant signals are sufficiently intense and maintaining traceability of the final settings transferred to the acquisition method.

3.1.2 LC–MS/MS acquisition logic: targeted quantification versus structural confirmation

In LC–MS/MS, acquisition strategy is dictated by the analytical objective. A targeted, quantitative workflow focuses on known analytes monitored through selective transitions and calibration.

Conversely, a qualitative or elucidation-oriented workflow is triggered when an unassigned peak emerges and the task becomes to propose a plausible identity: in that case, full-scan and MS/MS experiments are acquired, selecting isolation windows and collision energies to generate informative product ions. Importantly, product-ion intensities are not arbitrary; they reflect ionisation efficiency, precursor stability, available fragmentation pathways, collision energy, and source/ion-optics settings. Whether fragmentation is limited to MS/MS or extended to MS^n depends on instrument capabilities and on the trade-off between duty cycle, sensitivity, and the level of confidence required: not all platforms support MS^n, and even when available it is not always necessary for a robust identification.

For trace-level targeted analysis, triple-quadrupole LC–MS/MS operated in multiple reaction monitoring (MRM) is widely adopted because it combines chromatographic separation with highly selective precursor-to-product transitions, reducing the impact of matrix interferences and improving the reliability of multi-analyte quantification (De Girolamo, 2022). Within this framework, identification is not reduced to a single signal; rather, it relies on concordance between retention time and the response pattern of specific transitions. Confidence is strengthened by monitoring more than one product ion and by checking whether transition ratios (ion ratios) are compatible with predefined criteria, as formalised for mycotoxins and plant toxins in dedicated European documents and, more broadly, within the confirmation logic developed in EU frameworks for MS/MS-based methods (European Commission, 2023; European Commission, 2002/657/EC).

From a practical standpoint, however, these criteria must be interpreted in light of routine variability. Experimental studies across large sets of analytes and matrices have shown that both retention time and ion ratios can vary appreciably between instruments, laboratories, and fortification levels, which explains why realistic and validated tolerances are needed rather than assuming invariant thresholds (Mol, 2015). In parallel, MRM selectivity should not be treated as an absolute property: modelling approaches based on large databases have proposed expressing the “identification power” of a given combination of transitions and retention information as a probability of random coincidence, making explicit that false positives—although reduced—are not eliminated and depend on matrix complexity and chromatographic choices (Berendsen, 2013).

Consistently, documented cases of false positives attributed to matrix interferences in LC–MS/MS confirm that transitions and ion ratios, while necessary, may not always be sufficient without adequate chromatographic separation and matrix-specific selectivity checks (Kumar, 2014; Kaufmann, 2010; Kaufmann, 2011; Berendsen, 2017).

Chapter 4 – Analytical methodologies currently used for *Alternaria* mycotoxins

For the determination of major *Alternaria* toxins (alternariol, alternariol monomethyl ether, and tenuazonic acid), which may occur in foods at the µg/kg level, chromatographic approaches generally provide the best combination of selectivity and sensitivity. Accordingly, LC–MS/MS is among the most widely adopted configurations for trace-level quantification, both within multi-mycotoxin workflows and in methods specifically targeting *Alternaria* toxins (Ostry, 2008; Tölgyesi et al., 2015; Aichinger et al., 2021; Medina et al., 2021; De Girolamo et al., 2022; Gonçalves et al., 2022).

Within this framework, methodological studies consistently indicate that TeA is often a critical analyte, especially in multi-analyte methods, because chromatographic repeatability and peak shape are particularly sensitive to mobile-phase conditions. In an LC–MS/MS method covering 17 *Alternaria* toxins (including modified forms), Puntscher et al. (2018) reported that a basic eluent system (eluent A: 5 mM ammonium acetate in water, pH 8.7) was decisive for achieving a symmetric TeA peak shape, whereas under acidic eluents TeA was described as markedly broad with pronounced tailing; under the same basic conditions, tailing was also resolved for selected sulfate conjugates. The authors further noted that, in multi-analyte methods, the stability of eluent pH can translate into retention-time variability, which they attributed to the preparation of fresh eluent and found to be particularly evident for TeA (Puntscher et al., 2018). Consistently, De Berardis et al. (2018) described a chromatographic optimisation based on comparing different columns and introducing ammonium carbonate as an alkaline modifier: in their set-up, (NH4)2CO3 (15 mM) yielded a measured pH of 8.8, whereas pH > 9.0 induced co-elution of AME and TEN and shifted TeA retention time, and pH < 8.5 caused shifts and broadening for other analytes.

In the same work, an additional practical aspect related to detection was highlighted, as the [M+H]+ ion of TeA was reported to be unsatisfactory and a methanol adduct was monitored to improve response while retaining additional transitions to support identification (De Berardis et al., 2018).

The difficulty of including multiple *Alternaria* toxins within a single analytical procedure is not solely linked to chromatographic behaviour; it also reflects a pronounced heterogeneity in physicochemical properties, with consequences for extraction, retention, and ionisation. Across this class, a wide range of polarity and pKa values is observed, and these parameters are relevant to anticipate how pH influences ionisation profiles and, consequently, recoveries and retention (Mata et al., 2015; Tölgyesi et al., 2015). As a result, optimisation of the extraction medium must balance a high organic fraction—needed to adequately solubilise apolar species such as AME—with conditions that preserve efficient extraction of TeA, whose extractability is also influenced by its ionisation state (Gonçalves et al., 2022; Rodríguez et al., 2020). In a method proposed as a candidate for standardisation based on SPE + LC–MS/MS with isotope-dilution quantification, Gonçalves (2022) further describes TeA as polar and readily ionisable (pKa 4.28; logKow 0.92) and reports additional complexity related to pH-dependent forms (tautomers/rotamers), which can make chromatographic separation more demanding (Mikula et al., 2013). From an operational standpoint, this implies that selecting mobile-phase pH, injection-solvent composition, and the balance between organic content and acidification during extraction becomes a compromise across analytes: within a constrained experimental space, improving performance for one toxin may reduce recovery or response for another (Gonçalves, 2022).

Chapter 5– The tomato-processing industry and NIR spectroscopy: principles

5.1 Industrial context: product safety and quality control system

Within the industrial setting considered, quality and safety management is implemented as a chain of interconnected activities that accompanies tomato raw material from plant intake through to finished product release and dispatch.

The overarching logic is preventive: the process is organised to minimise the likelihood of deviations and contamination through hygiene- and organisation-related prerequisite programmes, in-line controls of critical parameters and a structured management of non-conformities.

At raw material intake, a reception step is performed that includes weighing and acceptance control, incorporating sampling and conformity assessments. Variability in incoming tomatoes is an intrinsic feature of the supply chain and is managed by collecting information that characterises each lot (for example, qualitative indicators and commodity classification), thereby providing a more complete basis to interpret process behaviour and quality-related parameters throughout processing. This stage also includes decision points: when acceptance outcomes are not compliant, the flow is interrupted and the material is handled as unsuitable, preventing its entry into the production line.

Following acceptance, tomatoes undergo pre-washing and washing with potable water, and then selection steps aimed at removing non-metallic foreign bodies and defective fruit. Selection can be represented as a combination of automated systems and manual checks, with separate handling of plant-based waste and extraneous materials. The purpose is to reduce the burden of undesired material that could interfere with processing and to stabilize the quality of the matrix entering subsequent stages.

Downstream of the preparation operations, the process diverges depending on the product category. For products such as tomato pulp and diced tomatoes, the sequence typically includes mechanical preparation steps (for example, optional peeling, cutting, draining, and further selection) followed by a mixing/conditioning stage in a holding tank, where any technological additions specified in the product requirements can also be managed. In this section of the process, in-line quality control becomes central because several parameters are directly linked both to microbiological safety and product stability, and to commercial compliance.

Safety is ensured through critical control points and process monitoring based on operational and critical thresholds.

Across critical parameters, deviations are handled through a standardised sequence: confirmation of the measurement, definition of the affected time window, physical/documentary segregation, corrective action, and verification of effectiveness. A relevant example is pH control, managed as a sensitive parameter for both safety and product stability. The management logic relies on differentiated limits: a more conservative operational limit and a critical limit, beyond which stricter containment and product segregation measures are triggered. For the product types considered here, reference values may be an upper operational limit of pH ≤ 4.35 and an upper critical limit of pH ≤ 4.40, with the aim of acting already upon exceeding the operational limit in order to avoid reaching the critical threshold. From an operational standpoint, a pH deviation first requires analytical confirmation of the result by recalibrating the instrument with buffer solutions and repeating the measurement, including on an alternative instrument if necessary. If confirmed, the affected time window is defined, product is segregated, and additional sampling is performed on intermediate and finished product, and a stepwise technological correction is implemented (for example, by modulating acidification), accompanied by closely spaced checks until values return within limits. This approach supports operational continuity without compromising safety, because decisions are based on thresholds and verification steps rather than on a single isolated data point.

In addition to pH, quality parameters functional to product conformity, such as °Brix and consistency, are monitored. When values are non-compliant, management follows a comparable logic: repetition of the measurement, repeated sampling on finished and/or intermediate products. If the deviation persists over a meaningful time interval, activation of measures for lot identification and segregation.

For °Brix, management typically distinguishes between marked deviations (leading to classification of the product as non-compliant) and more limited deviations (which may require declaration of the actual value or reassessment of the intended destination), according to criteria that depend on the product category and the expected degree of concentration.

For consistency, interpretation of the measurement is integrated with operational checks because certain process and equipment conditions can influence the liquid-phase content and the test response; consequently, management may include repeated controls over the following hours and targeted corrective actions before concluding that the product is intrinsically out of specification.

A key safeguard concerns the control of physical risk associated with metallic foreign bodies, which is managed as a critical control point through a metal detector placed downstream of the mixing step and upstream of the final treatment and packaging stages. The robustness of this control relies not only on the presence of the equipment itself, but also on the scheduled verification of its performance through routine tests using standardised metallic test pieces representative of different metal classes. These checks are performed at operational moments associated with higher risk, such as start-up and end of shift, line restarts after stops, and situations that may alter line set-up (for example, technical interventions and maintenance). In practical terms, the expected outcome is not limited to detection, but also includes the correct activation of the reject mechanism and assurance that rejected material remains segregated, preventing accidental re-entry into the compliant product flow.

When a critical control or a routine test yields a non-compliant outcome, the workflow immediately shifts from monitoring to containment. This entails stopping, or placing under controlled hold, the affected portion of production; identifying potentially implicated product (in practice, that produced between the last compliant verification and the detection of the anomaly); implementing physical and documentary segregation; and initiating an investigation into root causes. For the metal detector, considered causes include both technical aspects of the instrument and external conditions that may introduce interference or impair test repeatability. Restoration requires repeated verification until compliant performance is re-established, together with documentation of the corrective action and verification of effectiveness, thereby demonstrating that the barrier is again operational and that the risk has been managed in a traceable manner.

Safety assurance does not end with in-process controls, but continues through finished-product checks that are oriented toward stability.

Activities include recording physicochemical parameters and basic sensory evaluations, alongside stability testing under controlled-temperature incubation with checks at defined time points, aimed at capturing alterations that may emerge after packaging. In this context, the combination of pH, observation of anomalies (for example, sensory or physical changes), and process indicators is used as a surveillance tool: a pH drift or an unexpected change can serve as an early signal to trigger further investigation. Where microbiological verification is required, testing is typically structured around acceptance criteria focused on the absence of target pathogens and compliance with limits for hygiene indicators, and is often supported by external certificates of analysis in order to strengthen data reliability and the independence of the assessment.

A further element that contributes substantially to safety is the hygienic–environmental monitoring programme. This is designed by stratifying areas and surfaces according to risk, with particular attention to the most sensitive zones, especially where the product is more exposed and where operations carry a higher probability of indirect contamination. The rationale is consistent with GMP prerequisite programmes: maintaining control over the hygiene of contact and non-contact surfaces, assessing cleanliness of critical equipment components (for example, conveyors and internal machine parts), and, where appropriate, including personnel-related aspects (hands and clothing). Sanitisation effectiveness is then verified through planned checks, which may include surface swabbing and monitoring at a defined frequency throughout the production campaign.

This framework also includes the management of chemicals used for cleaning and sanitisation, an often “invisible” but essential aspect of quality. The use of alkaline and acidic detergents together with oxidising sanitisers requires that cleaning cycles include adequate rinsing and final checks to ensure the absence of residues that could alter the product or introduce undesired sensory notes. Accordingly, beyond correct execution of the cycle, simple but informative controls can be relevant, such as verifying the pH or other indicators of the final rinse, particularly in critical areas such as filling zones and product-contact surfaces. This step directly links hygiene to perceived quality: effective sanitisation must reduce microbiological risk without leaving residues that may affect odour, taste, or stability.

Overall, the system described can be framed as a quality and safety management model built on three integrated elements. The first is the definition of critical parameters and operational limits, with monitoring frequencies aligned to process dynamics and risk severity, as for pH, temperature, and metallic physical contaminants. The second is a structured reaction capacity, which turns a deviation into a controlled event through analytical confirmation, technological or equipment-related correction, segregation of the affected product, and traceability of decisions. The third is verification of effectiveness over time, through hygienic–environmental monitoring programmes, stability tests, and, where required, microbiological monitoring that closes the loop between process and finished product. Under this perspective, robustness does not derive from a single instrument or a single test, but from the interaction among planned monitoring, clearly assigned responsibilities, coherent records, and deviation management oriented toward containment, restoration, and verification of effectiveness.

5.2 Industrial positioning of the project and analytical objectives

The PhD project was carried out in collaboration with ITALTOM (Argenta, Ferrara, Italy), an operator in the tomato-processing sector serving both the food-service and industrial channels, within a production context in which the monitoring of quality parameters on intermediate and finished products represents an essential safeguard to ensure conformity with specifications and, where applicable, to trigger corrective process actions. During the period spent on site, activities included supporting finished-product quality-control checks, managing the operational traceability of samples (lot identification, sampling date and time, and transport conditions), and aligning the sampling plan with the accompanying documentation for samples sent to external laboratories for specific analyses.

Within this framework, a collaboration was established with FOSS, a company specialised in instrumentation for food analysis and quality control, with the objective of evaluating visible and near-infrared spectroscopy (Vis–NIR) as a complementary tool to reference methods, thereby reducing operational time and reagent use while enabling rapid, non-destructive assessment of quality parameters across different categories of processed products.

The analytical objective was the development of predictive models for the main indices used in quality control of tomato-derived products, with particular focus on the core product categories within the company's supply chain (diced tomatoes, tomato pulp, tomato purée, tomato semi-concentrate, and double concentrate) and, where relevant, also including fresh tomato as an additional matrix.

The target parameters were those typically adopted in quality control for processed tomato products: soluble solids expressed as °Brix, pH, colour according to CIELAB coordinates (L*, a*, b* and the a*/b* ratio), and consistency measured using the Bostwick consistometer (Beltrán et al., 2019). Where required by internal specifications, additional parameters were also considered, including dry matter (oven method), titratable acidity (expressed according to the units and endpoint defined by internal procedures), lactic acid (D+L), and reducing sugars .

5.3 Fundamentals of NIR spectroscopy

Near-infrared (NIR) spectroscopy is a vibrational technique that typically operates in the approximate range of 780–2500 nm. In Vis–NIR measurements, data acquisition can be extended into the visible region, thereby increasing sensitivity to chromophore-related phenomena and, consequently, to colour attributes (Reich, 2005; Pasquini, 2018).

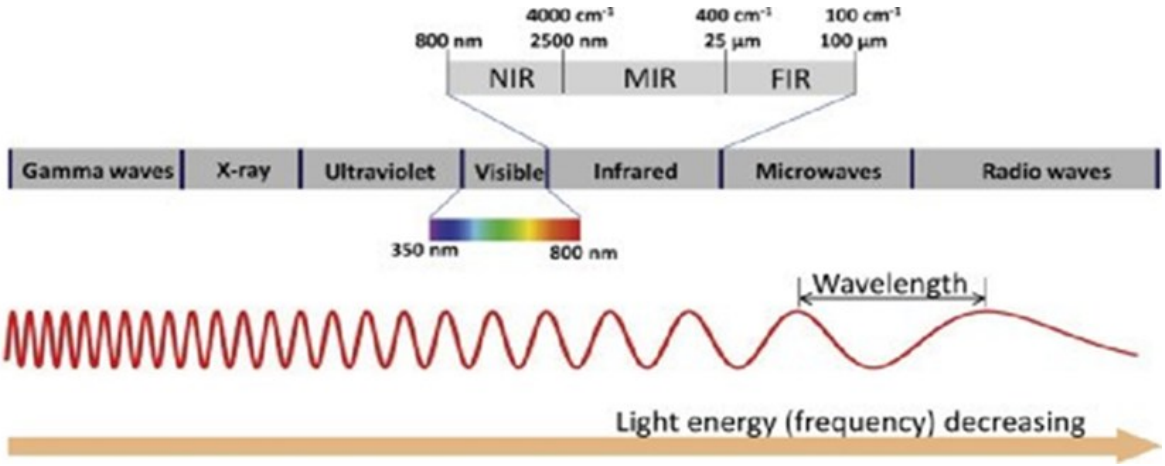


Figure 5. Electromagnetic spectrum highlighting the visible and infrared regions and conceptual representation of molecular vibrational modes (stretching and bending) relevant to Vis–NIR spectroscopy.

From a molecular perspective, in NIR spectroscopy absorption is primarily associated with overtone and combination bands of vibrational transitions, mainly involving X–H bonds (in particular O–H, C–H, and N–H). These bands are intrinsically broad and frequently overlapped compared with the fundamental bands typical of the mid-infrared region; consequently, Vis–NIR spectra convey information in a markedly multivariate manner rather than through isolated peaks that can be directly interpreted (Burns and Ciurczak, 2007).

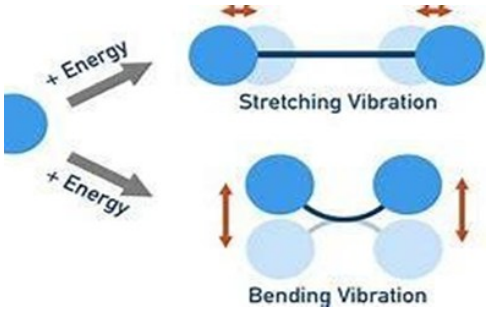


Figure 6. Conceptual representation of molecular vibrational modes (stretching and bending) relevant to infrared spectroscopy.

In industrial contexts and at-line measurements, Vis–NIR spectroscopy is often implemented using different optical geometries, most commonly in reflectance (diffuse reflectance) or transmission mode. The choice of geometry affects the effective optical path length, the relative contribution of absorption and scattering, and sensitivity to sample presentation and physical structure, aspects that become relevant when the objective is quantitative prediction in heterogeneous food matrices.

Within this framework, the spectroscopic measurement does not directly yield the property of interest. The instrument records a spectrum (intensity expressed as reflectance or transmittance as a function of wavelength), whereas the quantitative estimate of a given parameter (for example, °Brix or a colour coordinate) is obtained from a chemometric model calibrated on a set of samples for which reference values are available from conventional methods. In this context, reflectance spectra are often transformed into “absorbance-like” units (for example, log(1/R)) to facilitate modelling and improve linearity with respect to concentration-related effects, while still retaining contributions associated with physical scattering (Reich, 2005; Kharbach et al., 2023).

The spectrum–property relationship is therefore influenced by both chemical composition and matrix-related physical contributions (for example, scattering associated with particle size, dispersion state, and structural heterogeneity), which can introduce non-informative variability and require appropriate statistical treatment of the data (Reich, 2005; Kharbach et al., 2023). In high-water matrices, environmental and operational factors, particularly temperature, can affect the spectral response and consequently the reliability of predictions if they are not adequately represented and controlled across calibration and application conditions (Sheng et al., 2019; Zhang and Yang, 2025). For this reason, in industrial contexts the operational alignment between spectral acquisition and reference measurements is important, both in terms of sample handling and measurement conditions.

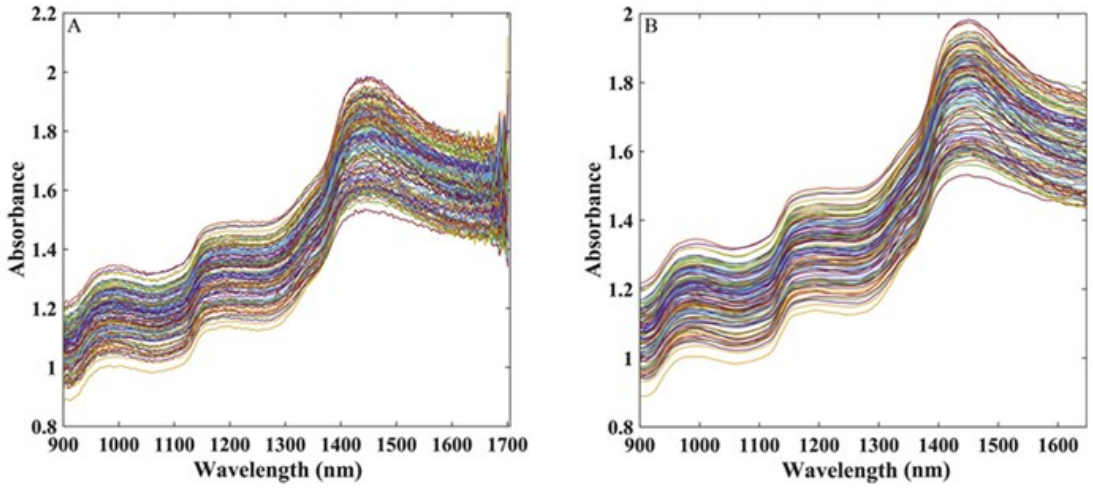


Figure 7. Illustrative comparison between raw spectra and spectra after Savitzky–Golay-based preprocessing (second-derivative smoothing), used to visualise the effect of pretreatment on baseline and fine structure.

Because chemometric modelling depends on the representativeness of the calibration dataset, the identification of anomalous samples is a methodologically relevant step. In practice, outliers may reflect genuine process variability that can be appropriate to include within the calibration domain, or they may arise from measurement artefacts, improper sample handling, or traceability errors. Even when the concept is introduced using univariate normal-distribution thresholds (for example, ±1, ±2, ±3 standard deviations), outlier handling in spectroscopy is typically conducted within a multivariate framework, assessing deviation relative to the model space and the calibration domain rather than with respect to a single variable.

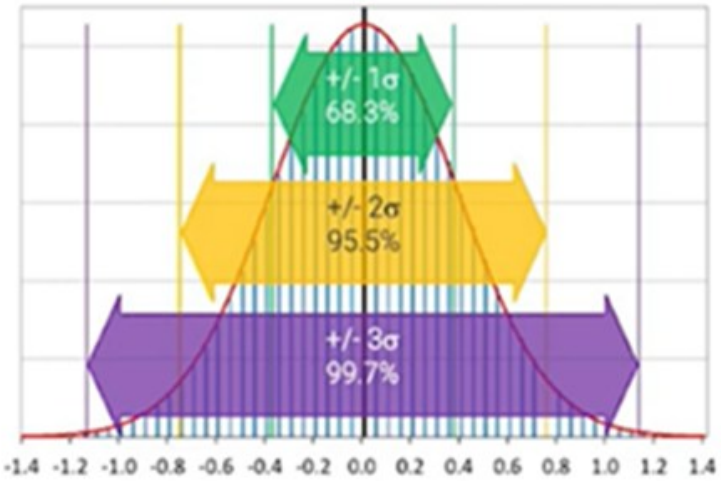


Figure 8. Graphical summary of the empirical 68–95–99.7 rule for a normal distribution, used as an introductory analogy for statistical deviation and outlier screening (Moore et al., 2021).

Part III - Experimental studies: Materials and Methods

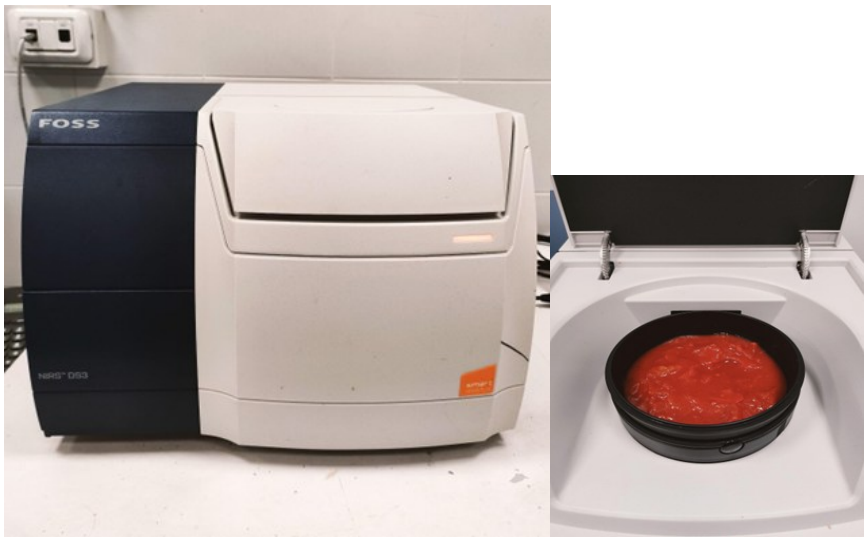
Chapter 6 – Study 1 NIR for quality control in the tomato supply chain

6.1 Use of Vis–NIR in the tomato supply chain and dataset rationale

The use of Vis–NIR along the tomato supply chain is consistent with the need for rapid analytical tools capable of supporting quality-related decisions throughout processing, from the assessment of incoming raw material to the control of intermediate streams and finished products. In processed tomato products, variability in composition and structure across product categories is particularly relevant. Matrices such as diced tomatoes, tomato pulp, and tomato purée exhibit different degrees of dispersion, different distributions of the aqueous phase, and distinct rheological characteristics, whereas tomato semi-concentrate and double concentrate introduce an additional level of variability associated with the degree of concentration.

The dataset evolved progressively within the industrial workflow and was subsequently organised into more homogeneous product groups in order to improve the stability of the spectrum–reference relationship.

Fresh tomato was included as an additional exploratory matrix, mainly linked to incoming raw-material assessment, whereas the core calibration effort was focused on processed tomato products representative of the company workflow. For processed matrices, the variability space was progressively widened through samples collected during the monitored campaign and, where available, through historical plant material from previous campaigns. Only samples associated with corresponding reference values were considered as quantitative calibration points.



(a)

(b)

Figure 9. Vis–NIR instrumentation and sample presentation: (a) FOSS NIRS DS3 instrument and cup-based measurement set-up used in the industrial environment; (b) representative variability in fresh tomato appearance supporting dataset diversity.

From a modelling perspective, heterogeneity across matrices can be addressed through two strategies that are not mutually exclusive. In an initial phase, the joint inclusion of multiple product types within a single calibration makes it possible to cover a wide variability range and to develop first-implementation models oriented to industrial application. Subsequently, as the number of samples increases and the experimental domain is expanded, it is often more effective to organise the dataset into more homogeneous groups based on product characteristics, thereby reducing non-informative structural variability and improving the stability of the spectrum–reference relationship.

In the present work, the evolution of the dataset led to a grouping strategy, based on the variability covered and the stability of the resulting calibrations: one group comprising fresh tomato, diced tomatoes, and tomato pulp; one group comprising tomato purée and tomato semi-concentrate; and one group dedicated to double concentrate.

This approach makes it possible to maintain coherence with operational quality-control workflows, preserving measurement traceability and comparability with reference methods, without assuming a priori that the same calibration structure is optimal for all matrices.

6.2 Instrumentation and spectral acquisition

Vis–NIR acquisitions were carried out using a FOSS NIRS DS3 spectrometer over the 400–2500 nm range, in a cup-based diffuse reflectance configuration, under industrial quality-control conditions. Samples were transferred into the measurement cup and distributed as uniformly as possible, with slight settling when necessary and without compaction, in order to minimise non-informative variability related to sample presentation. Under routine operating conditions, one spectral acquisition per sample was generally performed, whereas reacquisition was carried out only when sample presentation was clearly unsuitable or anomalous. Spectroscopic measurements and reference determinations were performed on the same sample, or on a representative aliquot of the same sample, within the same operational time window of a few minutes, while maintaining conditions as comparable as possible, including for temperature-sensitive parameters.

Spectral processing and equation updating were conducted within the chemometric environment used in the DS3 workflow, using the acquired spectra as input for calibration, internal validation, and iterative equation updating.

6.3 Sampling, sample presentation, and operational conditions

The matrices considered included fresh tomato and the main processed tomato products routinely subjected to quality control in the plant, namely diced tomatoes, pulp, purée, semi-concentrate, and double concentrate. For each sample, operational traceability was maintained in order to preserve consistency between spectral acquisition and the corresponding reference values.

Fresh tomato was included as an additional exploratory matrix, mainly in relation to incoming raw-material assessment and soluble-solids evaluation, whereas the main analytical effort was directed toward processed products. In the case of fresh tomato, the material introduced into the measurement cup consisted of homogenised sample rather than intact fruit.

In general, the sample was transferred into the measurement cup with a spoon and distributed as homogeneously as possible. When necessary, slight settling was applied to minimise macroscopic voids, without pressure compaction. The surface was arranged by distributing the product in order to obtain homogeneous filling, typically slightly below the reference mark, so as to reduce variability related to sample presentation and macroscopic inhomogeneity. As far as operationally feasible, a comparable sample volume was maintained across measurements.

Vis–NIR acquisition and the corresponding reference determination were performed on the same sample, or on a representative aliquot of the same sample, within the same operational time window, typically within a few minutes. This approach was adopted in order to preserve traceability and minimise non-informative variability associated with handling and measurement conditions, while associating each spectrum with a reference value representative of the same sample and of comparable operational conditions.

6.4 Reference methods for quality parameters

Reference values were obtained using routine quality-control methods. Soluble solids were determined by refractometry and expressed as degrees Brix; pH was measured using an electrode. Colour was measured according to the CIELAB scale, reporting L*, a*, b*, and the a*/b* ratio. Consistency was evaluated using a Bostwick consistometer, recording the flow distance in centimetres at a fixed time (30 s) according to an internal procedure. Where required by internal specifications, additional reference determinations included dry matter by oven drying, titratable acidity by automated titration according to internal procedures, lactic acid (D+L) by kit, and reducing sugars by a titrimetric method based on the Fehling reaction, in accordance with in-plant quality-control practice. Reference determinations were used as the basis for calibration and for the internal evaluation of Vis–NIR models.

6.5 Calibration development and evaluation criteria

Predictive model development was initiated from an initial operational baseline available within the instrumental workflow and was subsequently updated through the progressive inclusion of company samples, whenever accompanied by the corresponding reference values. This approach allowed the variability observed under plant conditions to be progressively incorporated into the equations and enabled the calibrations to be adapted to the actual application requirements of industrial quality control.

Because equation availability and reference-data completeness were not uniform across all parameters, the number of samples did not correspond to a single study-wide value, but varied depending on both the quality parameter considered and the matrix group analysed. In order to increase the representativeness of industrial variability, particularly in the case of processed products, company samples from previous production campaigns were also taken into account. These data could contribute to the quantitative updating of the equations only when associated with the corresponding reference values, whereas historical data available exclusively in spectral form were used only as descriptive support for matrix variability. Within this experimental framework, fresh tomato played a more exploratory role, mainly oriented toward the evaluation of incoming raw material, with particular interest in the °Brix parameter, whereas the main applied focus of the study concerned processed tomato products.

In an initial phase, equation updating was performed by jointly including samples from different product types, with the aim of covering a wide variability range. As the number of samples increased and data density became higher within the individual categories, calibrations progressively more oriented toward product specificity were developed, in line with operational quality-control needs and with the aim of stabilising the spectrum–reference relationship within more homogeneous application domains.

Multivariate regression was performed using PLS-based approaches, including the mPLS variant (Modified Partial Least Squares), that is, an operational form of PLS employed in the development of multivariate NIR calibrations. In the chemometric workflow used for equation development, preprocessing was defined on an equation-specific basis and included mathematical treatments based on derivatives and smoothing, together with spectral-region selection and corrections for non-informative physical variability such as scattering and baseline effects. Operatively, equation-specific math treatments, including first-derivative-based settings such as 1-4-4-1, were used in association with mPLS regression and internal validation in order to stabilise the spectrum–reference relationship within matrices characterised by relevant structural and compositional variability. Model performance was evaluated by internal validation within the software environment used. In the applied reporting outputs, the main indicators considered included the number of samples incorporated into calibration (N), the mean of the entered reference values (MEAN), the standard error of cross-validation (SECV), and the 1-VR index. Within the final workflow described here, performance assessment therefore relied on internal validation rather than on a separately documented external validation set.

The management of potentially anomalous samples was not based on a priori exclusion. During equation building and updating, each sample was evaluated both according to the deviation between reference value and predicted value and through model diagnostics in the multivariate model space. The applied reports indeed included T- and H-type statistics, global model indicators, and actual versus predicted plots distinguishing selected from deselected samples.

Any deselection of a sample therefore occurred only after verification of these diagnostics and not in an automatic or preliminary manner. The procedure was traceable within the software environment and was intended to distinguish actual process variability from possible spectral artefacts or inconsistencies with the reference value, thereby improving equation robustness.

Chapter 7 – Study 2: QuEChERS extract complexity and non-target co-extraction

7.1 Rationale and indicators of extract complexity

An indirect approach to support the investigation of the mycotoxins addressed in this thesis is to evaluate the complexity of extracts obtained by QuEChERS. During extraction, endogenous matrix components may be co-extracted together with the target analytes. Such co-extracted constituents can increase overall extract complexity and, in ionisation-based chromatographic quantification workflows, may interfere with the processes governing ion formation and transfer in the source, particularly under co-elution conditions, leading to signal suppression or enhancement. These phenomena are commonly referred to as matrix effects (Matuszewski et al., 2003; Rogatsky and Stein, 2005).

In the context of mycotoxins produced by *Alternaria*, alternariol (AOH) and alternariol monomethyl ether (AME) possess multiple phenolic hydroxyl groups (Pfeiffer et al., 2009; Soukup et al., 2016). Accordingly, the assessment of extract complexity can also focus on the load of phenolic-type co-extractives, understood as a fraction with chemically related functionalities that may be co-extracted together with contaminants of interest. This structural feature does not imply a matrix effect specific to these compounds, but it provides a rationale for quantifying phenolic components and, more broadly, redox-active species as indicators of extract complexity and of their potential contribution to instrumental-response variability in ionisation-based methods (Matuszewski et al., 2003; Rogatsky and Stein, 2005).

In this study, QuEChERS was selected as the extraction approach, given its wide use in agri-food matrices for the analysis of natural contaminants. QuEChERS is a rapid multiresidue procedure and is not intrinsically selective for target analytes; therefore, the co-extraction of endogenous matrix constituents is an expected outcome and should be considered as part of extract characterization.

The experimental workflow was designed as a comparative scheme. For each matrix, QuEChERS extracts were prepared in parallel under two conditions (Q1 and Q2), differing in salt amount/composition and thus in the operational conditions governing partitioning, together with a reference extract distinct from QuEChERS and obtained using reference techniques.

Although conventional extraction systems ensured higher recovery of polyphenols, antioxidant-related compounds, and carotenoids, the QuEChERS conditions were not intended to maximise the extraction of these classes. Rather, they were evaluated as operational extraction conditions potentially more suitable for reducing matrix complexity in view of contaminant-oriented applications, including the extraction of mycotoxins.

Extract complexity was then described through spectrophotometric determinations used as quantitative indicators of the overall load of co-extracted compounds, including total phenolics (Folin–Ciocalteu), total antioxidant capacity (DPPH), and total carotenoids. The reference extract was intended as a reproducible comparative term, obtained by selecting solvents and repeated extraction steps to promote recovery of the compound class under consideration relative to QuEChERS conditions. This choice does not imply that the reference extraction is exhaustive, but it provides a coherent and standardized basis to calculate the relative retention of QuEChERS.

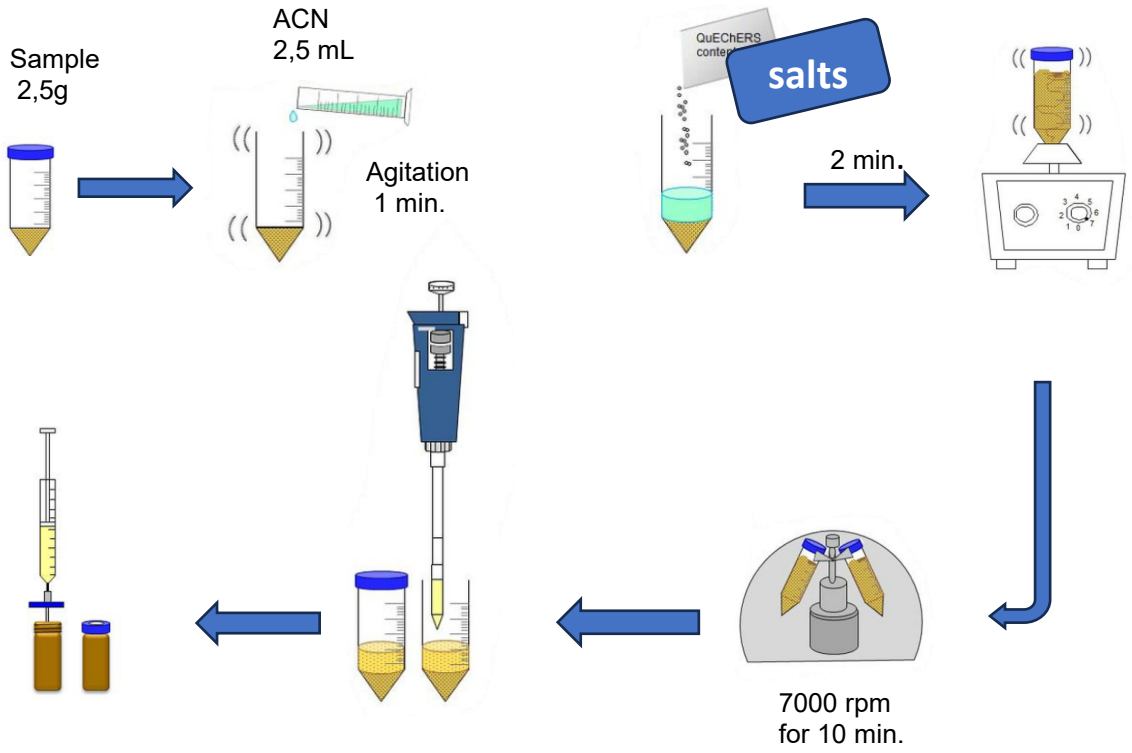
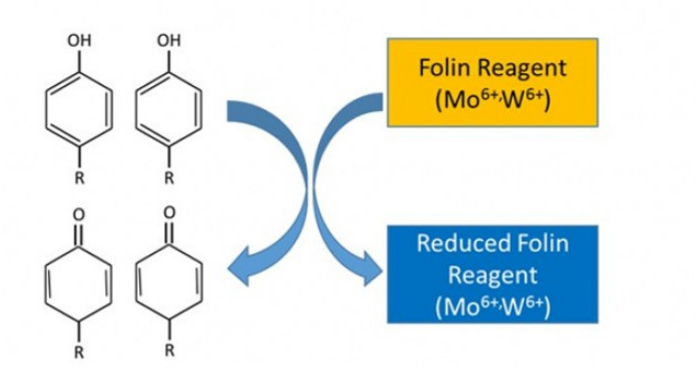
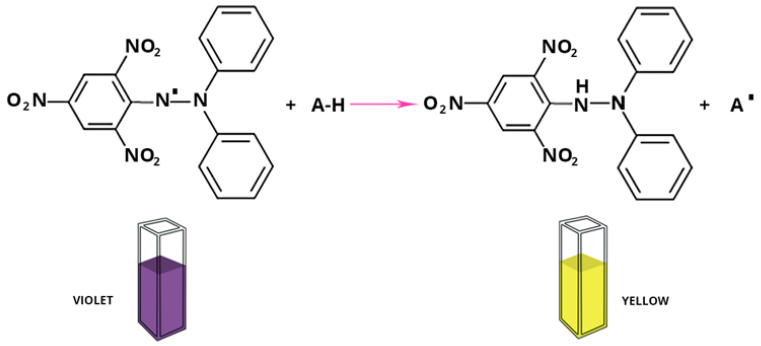


Figure 10. Schematic workflow of the QuEChERS extraction procedure applied in this study (ACN extraction, salt-induced partitioning, and phase separation by centrifugation).



a)



(b)

Figure 11. Representative visual outputs of spectrophotometric indicators used to describe QuEChERS extract complexity , (a) Folin–Ciocalteu reaction (b) DPPH assay

Chapter 8 – Study 3: fermented hydrolysates and the response of Alternaria spp.

This part of the study was carried out during the mobility period at the University of Valencia, in Prof. Giuseppe Meca's laboratory.

The genus *Alternaria* comprises ubiquitous filamentous ascomycetes that can adopt both saprophytic and pathogenic lifestyles across a wide range of plant species. Within the tomato supply chain, interest in *Alternaria* is twofold: on the one hand, certain species are associated with rots and post-harvest deterioration; on the other hand, part of the fungal secondary metabolism includes compounds described as toxins of food-safety relevance, with potential implications for risk and for the need for targeted analytical monitoring (EFSA, 2011; Escrivá et al., 2017; Gruber-Dorninger et al., 2017; Nagda and Meena, 2024).

Within this framework, a key methodological objective is to evaluate control strategies with a more sustainable profile than synthetic fungicides, including fermented hydrolysates, while recognising that the effect of an antifungal agent may not be limited to growth inhibition.

Under sub-inhibitory conditions, modulation of microbial physiology and stress responses may occur, with potential repercussions on the metabolic profile. For this reason, the experimental design combined in vitro antifungal activity assays (agar diffusion and broth microdilution for MIC/MFC) with profiling of non-volatile metabolites by UHPLC–QTOF–MS, in parallel with the characterisation of volatile compounds by HS-SPME–GC, using predefined sampling time points.

8.1 Fungal strains and treatments

The following fungal strains were used: *Alternaria linariae* CECT 2997, *Alternaria alternata* CECT 2662, and an additional *A. alternata* strain (non-CECT). The treatments investigated were pea-protein hydrolysates obtained by fermentation with Bacillus amyloliquefaciens and with Trichoderma reesei.

At the end of fermentation, the broth underwent coarse biomass removal by sieving, followed by centrifugation; the supernatant was collected, supplemented with maltodextrin as a drying aid, and then spray-dried to obtain a powdered product. For in vitro assays on solid media and for metabolomic and volatilomic experiments, the working concentration of the treatments was 10 g/L, unless otherwise specified for MIC/MFC testing.

The selection of the protein matrix and the fermentation microorganisms used in this study was based on preliminary in-house laboratory activities conducted for screening and optimisation purposes. In this phase, several protein matrices of plant and/or animal origin and different candidate biocontrol microorganisms were considered, and comparatively assessed in terms of process reproducibility and performance in preliminary efficacy assays. Based on this exploratory work, Bacillus amyloliquefaciens and *Trichoderma* reesei were selected as fermentation organisms and pea protein isolate as the substrate matrix, adopting operating conditions that included incubation for 5 days at 25 °C under agitation, followed by formulation of the recovered supernatant as a powder by spray-drying.

Alternaria alternata | *Alternaria alternata* CECT 2662 | *Alternaria linariae* CECT 2997

8.1.1 Preparation of the fermentation substrate and production of the powdered fermentate

The fermentation substrate was prepared in distilled water supplemented with glucose (1% w/v) and yeast extract (0.5% w/v; derived from Saccharomyces cerevisiae). A pea protein isolate (declared protein content 80% w/w) was added as the protein matrix for the generation of hydrolysates during fermentation. Fermentation was carried out in large-volume glass bottles (Schott type) closed with aluminium foil and incubated for 5 days at 25 °C on an orbital shaker. At the end of incubation, biomass was subjected to coarse removal by sieving and subsequently separated by centrifugation. The supernatant was recovered by pipetting, after which maltodextrin (2.5% w/v) was added as a drying aid. The liquid product was then spray-dried to obtain a powdered formulation, stored at 4 °C in a closed container (Gotor-Vila et al., 2017; Vassaux et al., 2021; Luft and Mazutti, 2025; Martinez et al., 2023).

Figure 13. Preparation of the fermentate

From a technological perspective, the rationale is to generate a hydrolysate from a plant-based protein matrix (pea protein isolate) through fermentation is that during growth, the microorganisms used as fermentation agents contribute to substrate transformation and to the release of a mixture of soluble compounds, including peptide fractions and other low-molecular-weight metabolites. At the end of incubation, biomass is removed and the supernatant is recovered, which represents the “core” of the treatment because it contains the components released into the medium during fermentation. Subsequent powder formulation by spray-drying, with the use of a carrier, addresses practical needs typical of bioproducts, including stabilisation, dose standardisation, ease of storage, and reproducibility in preparing experimental working concentrations. In this way, the activity observed in in vitro assays and the changes in the *Alternaria* metabolic profile are interpreted as an effect of the fermented formulation added to PDA.

This approach enables a controlled evaluation of an applied objective: assessing whether fermented products obtained from selected microorganisms, produced and formulated according to a technological logic compatible with practical use, can reduce *Alternaria* growth while also modulating, in a treatment- and time-dependent manner, portions of secondary metabolism, including metabolites of relevance to food safety and/or plant pathology.

8.2 In vitro antifungal assessment: agar diffusion assay and broth microdilution (MIC/MFC)

Antifungal activity was assessed using an agar diffusion assay. After preparation of the fungal inoculum and seeding to obtain uniform surface growth, wells were punched into the agar and defined volumes of the treatment solutions were dispensed into the wells. The tested concentrations were 0.1 g/L, 1 g/L, and 10 g/L, in addition to the untreated control (0 g/L). Plates were incubated at 25 °C until sufficient growth was achieved for evaluation, which was based on the presence/absence and the extent of the inhibition zone surrounding the well.

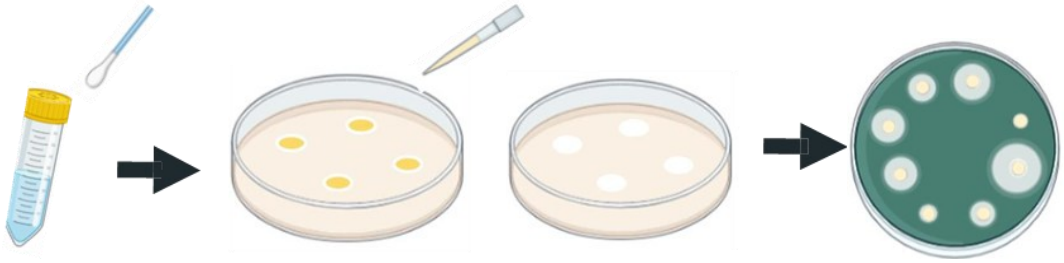


Figure 14. Steps of diffusion agar test

The minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) were determined by broth microdilution in 96-well microplates using potato dextrose broth (PDB). The inoculum was standardised to 10^5 spores/mL. For each treatment, a two-fold serial dilution series was prepared, covering the range 50.00–0.10 g/L.

The plate included medium sterility controls and inoculum growth controls. After incubation at 25 °C for 5 days, the MIC was defined as the lowest concentration showing no visible growth. For MFC determination, aliquots from wells corresponding to the MIC and higher concentrations were plated onto PDA and incubated at 25 °C for 5 days; the MFC was defined as the lowest concentration with no mycelial regrowth. Determinations were performed in four independent replicates for each treatment/strain combination.

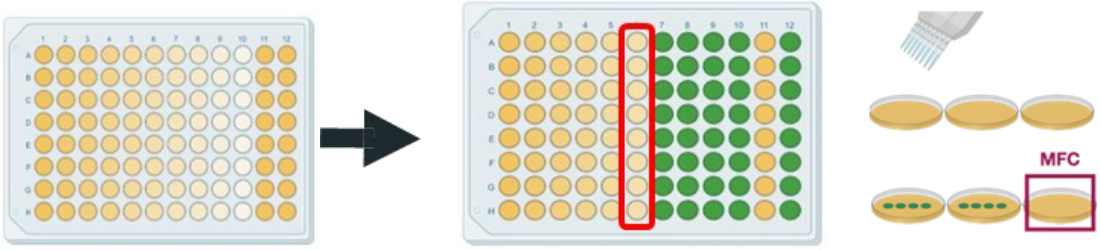


Figure 15. Preparation minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC)

8.3 Non-volatile metabolite profiling (UHPLC–QTOF): extraction and acquisition

For non-volatile metabolite analysis, Alternaria alternata CECT 2662 was grown on Petri dishes containing PDA either in the absence (control) or in the presence of the treatment (10 g/L). Sampling was scheduled at 6, 9, and 16 days post-inoculation, with three biological replicates per condition and time point. Mycelium was collected and extracted with methanol (20 mL) under agitation overnight. Extracts were diluted 1:5 and filtered through a 0.22 µm membrane prior to instrumental analysis.

LC–HRMS analyses were performed using UHPLC coupled to QTOF with electrospray ionisation in both positive and negative polarity. Chromatographic separation was carried out on a ZORBAX RRHD SB-C18 column (2.1 × 50 mm, 1.8 µm) using a gradient of water and acetonitrile, both supplemented with 0.1% formic acid; the flow rate was set to 0.4 mL/min and the injection volume to 5 µL. The gradient started at 2% organic phase and reached 95% in 22 minutes, followed by re-equilibration, for a total run time of 25 minutes. Compound annotation was performed by comparison with an Alternaria-dedicated library, applying a match score ≥95% and a mass accuracy tolerance of 1 ppm, as reported in the draft (Dopazo et al., 2021).

A single commercial standard, namely alternariol, was available for confirmation. No internal standard was used. In the absence of authentic standards for the other annotated compounds, semi-quantification was performed by external calibration using the available reference compound.8.4 Volatile profiling (HS-SPME–GC–MS(/MS)): extraction and acquisition

For volatile compound analysis, Alternaria alternata CECT 2662 was grown in vials on PDA either in the absence (control) or in the presence of the treatment (10 g/L), with sampling at 6, 9, and 16 days. Volatile compounds extraction was performed by headspace solid-phase microextraction (HS-SPME) by exposing the fibre in the headspace for 40 minutes; desorption occurred thermally in the GC injector. Identification was carried out by spectral comparison against the NIST library, applying a similarity criterion of at least 95% and using helium (99.99%) as the carrier gas (Basurto et al., 2017).

In addition to the treated fungal samples, exploratory LC–HRMS analyses were also performed on the fermented hydrolysates alone; however, the results discussed in this thesis are primarily focused on the metabolic response of *Alternaria* grown on PDA in the absence or presence of treatment.

Data processing and statistical analysis

LC–HRMS and GC data were processed using a workflow including pre-processing and normalisation, followed by exploratory multivariate analyses. Heatmap visualisation was used as a descriptive tool; for comparisons among experimental groups, analysis of variance (ANOVA) was planned, maintaining a conservative approach in the interpretation of observed differences.

Part IV Results and discussion

Chapter 9 Results and discussion: performance of NIR models and interpretation in an industrial setting

The NIR results should be interpreted within an applied industrial framework, in which equation updating was performed progressively through traceable association between spectra and routine reference measurements. Consequently, performance should be read jointly in terms of matrix type, parameter-specific sample size, width of the covered variability range, and operational coherence between spectral acquisition and the corresponding reference method.

Over the course of the production season, the development of Vis–NIR calibrations progressed from an initial setup based on standard operational models toward a configuration increasingly representative of the variability observed in the plant. Equation updating was carried out through iterative inclusion of new samples associated with their corresponding reference values, with internal performance evaluation based on the system’s applied reporting outputs.

As sample size increased and data density grew within each commercial category, the strategy was oriented toward more homogeneous application domains, thereby reducing non-informative structural variability and stabilising the spectrum–reference relationship for each matrix group.

A first descriptive indication of the acquired variability is provided by the overlay of spectra, in which an envelope of response is visible that is not uniform across the entire spectral range. This variability is consistent with the coexistence of different matrices and physical states within the supply chain and provides the technical basis for the subsequent organisation of calibrations into product groups.

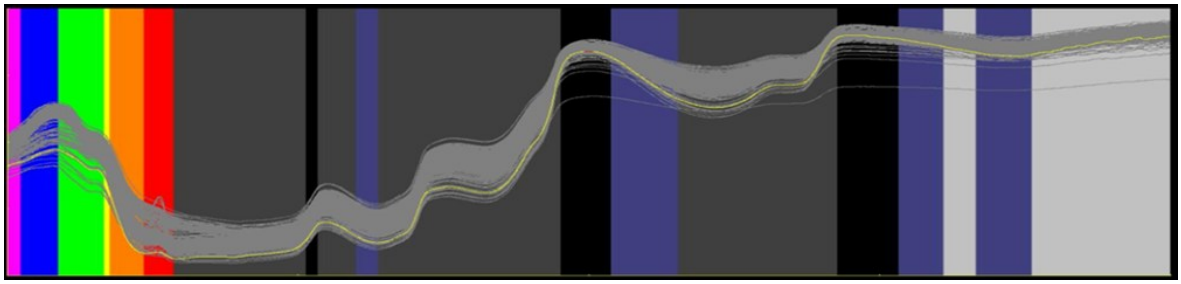


Figure 16. Overlaid Vis–NIR spectra acquired on supply-chain samples, showing the overall spectral variability considered for calibration and iterative model updating. The x-axis represents wavelength (nm); the y-axis reports the spectral response in the export format used by the instrument/software environment.

The final model performance is reported in tabular form for each matrix group. In line with the operational framework of quality control, emphasis was placed on the key parameters °Brix (Ro), pH, consistency (Bostwick), and sugars, while still providing a complete account of all variables included in the available equations.
The tables report the number of samples included in calibration (N), the mean of the reference values entered (MEAN), the internal validation error estimate (SECV), and the 1-VR index as presented in the applied reporting outputs.

Table 4.3. Final model performance for the pulp, fresh tomato, and diced tomato group.

Paramater	N	MEAN	SECV	1-VR
A	284	25.1097	0.6466	0.9812
B	284	13.5737	0.3143	0.9647
L	416	23.6418	0.5862	0.9420
Citric acid	218	0.5322	0.0407	0.4985
pH	439	4.2770	0.0463	0.0572
Ro	440	6.9825	0.1947	0.9518
RS	63	8.8275	0.4364	0.5613
Bostwick	112	6.6522	0.8679	0.5251
Lactic acid	191	0.1808	0.0461	0.5634
Reducing sugars	187	4.0123	0.3996	0.1859
Bostwick l.	55	7.1964	1.0436	0.2602
Bostwick s.	55	6.5618	1.0848	0.2294

Note: A, B, and L correspond to the CIELAB coordinates a*, b*, and L*; Ro is expressed in °Brix; Bostwick is expressed in cm at 30 s according to the internal procedure

Table 4.4. Final model performance for the purée and semi-concentrate group.

Parameter	N	MEAN	SECV	1-VR
A	138	27.8819	0.4380	0.9810
B	141	14.5522	0.2284	0.9697
L	188	24.4319	0.2791	0.9648
Citric acid	109	0.8175	0.0475	0.8044
Lactic acid	90	0.4019	0.1405	0.5151
pH	179	4.3169	0.0457	0.4557
Ro	191	11.2363	0.2302	0.9774
Reducing sugars	89	5.5661	0.5331	0.3635
Dry metter	36	12.3922	0.5190	0.8983
Bostwick	161	3.6607	0.6991	0.8113

Note: A, B, and L correspond to the CIELAB coordinates a*, b*, and L*; Ro is expressed in °Brix; Bostwick is expressed in cm at 30 s according to the internal procedure

Table 4.5. Final model performance for the double-concentrate group.

Parametro	N	MEAN	SECV	1-VR
A	4	25.9225	0.6033	-0.9200
B	4	12.6725	0.2490	-1.0751
L	16	20.7450	0.5759	0.8399
Citric acid	15	1.4747	0.3428	0.8370
Lactic acid	7	0.6114	0.0343	0.8899
pH	33	4.1736	0.0381	0.3238
Ro	34	28.5047	0.2593	0.5229
Dry metter	15	30.5187	1.0561	-0.0457
Reducing sugars	6	13.7633	0.1728	-0.8289

Note: for double concentrate, some determinations (e.g., colour and consistency) were handled through dedicated calibrations in accordance with the operational measurement procedures

Discussion

Overall, the adopted approach makes it possible to traceably link the variability observed in the plant to the construction of progressively more specific calibrations. The figure showing overlaid spectra provides a descriptive indication of relevant spectral variability across the entire Vis–NIR range, which in complex matrices may reflect both compositional differences and physical contributions related to structure and dispersion state. In an applied context, handling such variability is consistent with a strategy that, after an initial more general phase, progressively steers calibrations toward more homogeneous product groups when the available sample size allows it.
Similarly, for consistency (Bostwick), the physical variability of the matrix and the sensitivity of the test to execution conditions can contribute to data dispersion and to the stability of the spectrum–reference relationship; within this framework, more homogeneous application domains may support more stable calibrations.
For the parameters considered, performance interpretation should be viewed jointly in terms of sample size (N), the width of the covered variability range, and the coherence between spectral measurement and the reference method. In particular, °Brix tends to benefit from a more stable spectrum–reference relationship in many food matrices, whereas for pH the Vis–NIR estimate is typically indirect and depends on secondary correlations with the matrix; when the pH range is narrow, relative error can be more sensitive and the 1-VR metric may decrease even when absolute error remains limited.

Direct literature comparisons are not uniformly available for all matrix-group and parameter combinations considered in this industrial workflow, because many published studies focus on more narrowly defined tomato products rather than on grouped company-specific application domains. Within this context, the comparatively stronger behaviour observed for soluble solids is coherent with the broad suitability of NIR-type approaches for solids-related traits in tomato products, whereas pH should be interpreted more cautiously because the NIR response is indirect and strongly dependent on matrix-mediated correlations and on the width of the covered variability range. For matrices characterised by greater physical heterogeneity, and especially for smaller datasets such as double concentrate, a conservative interpretation of applicability remains appropriate.

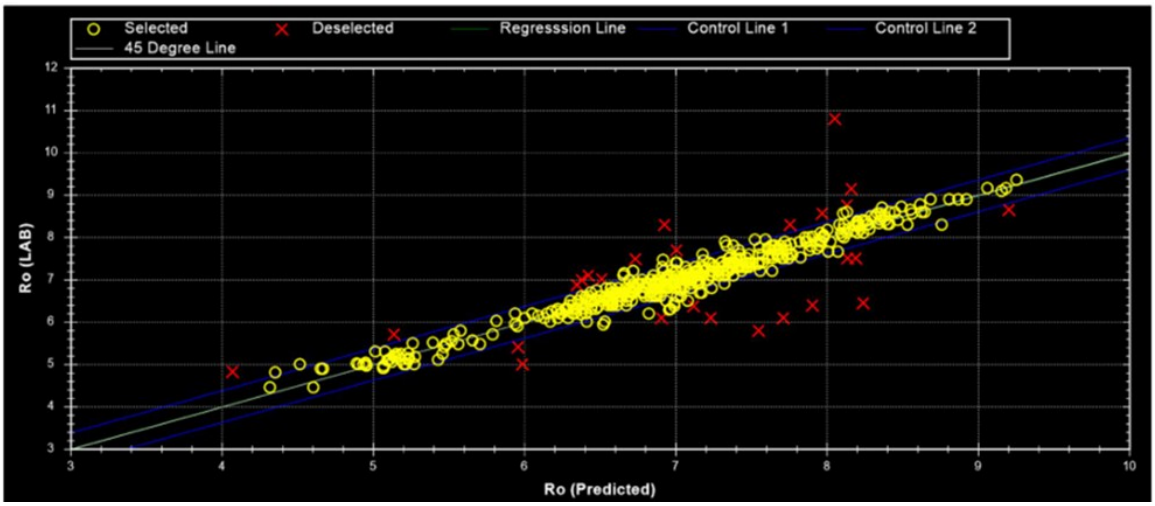


Figure 17. Comparison between soluble-solids reference values (LAB, °Brix; y-axis) and model estimates (Predicted, °Brix; x-axis).

Selected points indicate samples retained in the model, whereas Deselected points indicate samples excluded on the basis of model diagnostics. The 45-degree line represents ideal agreement; the regression line summarises the trend of selected samples.

For double concentrate, the availability of a smaller number of samples for some variables, together with procedural constraints associated with reference determinations, supports a prudent approach based on dedicated calibrations and on a conservative definition of the applicability domain, while maintaining consistency with in-plant procedures.

Overall, the developed approach supports the use of Vis–NIR as a rapid, non-destructive tool for decision support along the processing chain. Practical reliability depends critically on dataset representativeness, operational alignment between spectral acquisition and reference methods, measurement traceability, and controlled handling of anomalous samples within the model space.

Chapter 10 – Results of Studies 1–2: Extraction performance, non-target co-extraction, and analytical implications

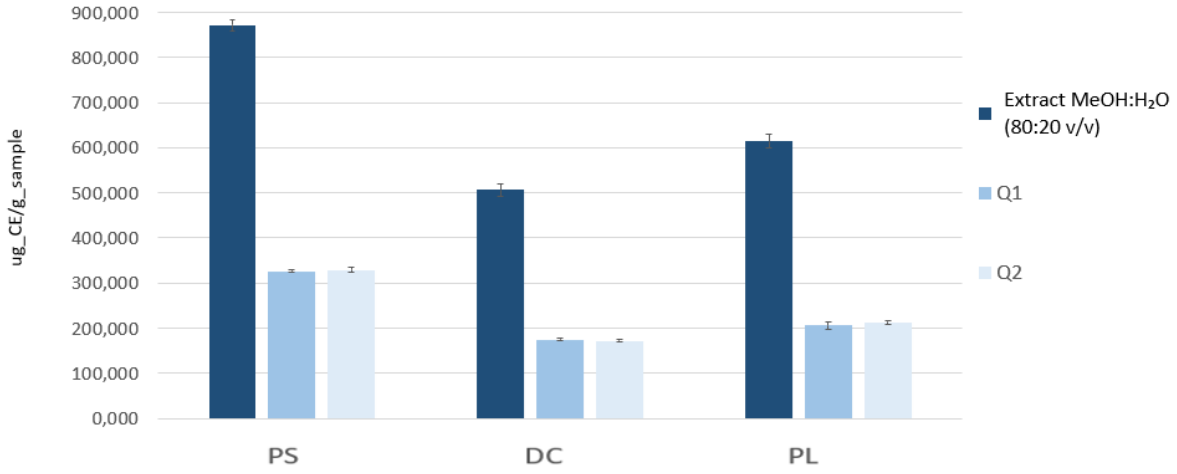
Across all matrices, values measured in QuEChERS extracts were consistently lower than those obtained from the corresponding reference extracts. The magnitude of this reduction depended on the compound class considered and, to a variable extent, on the QuEChERS condition (Q1 vs Q2).

For the Folin–Ciocalteu phenolics index, reference extracts ranged from 506.3 to 872.4 µg CE/g. QuEChERS extracts ranged from 174.2 to 326.0 µg CE/g in Q1 and from 172.0 to 328.8 µg CE/g in Q2. Tomato paste showed the highest values in both reference and QuEChERS extracts. For total antioxidant capacity (DPPH), reference extracts ranged from 8208 to 14553 µg TE/g. QuEChERS extracts ranged from 1756 to 2719 µg TE/g in Q1 and from 1742 to 2540 µg TE/g in Q2.For total carotenoids, reference extracts ranged from 112.3 to 193.6 µg/g. Measured values in QuEChERS extracts were markedly lower, ranging from 8.56 to 9.77 µg/g in Q1 and from 4.27 to 5.85 µg/g in Q2.

Retention percentages, defined as (QuEChERS/reference)×100, are summarised in the tables below for each indicator class.

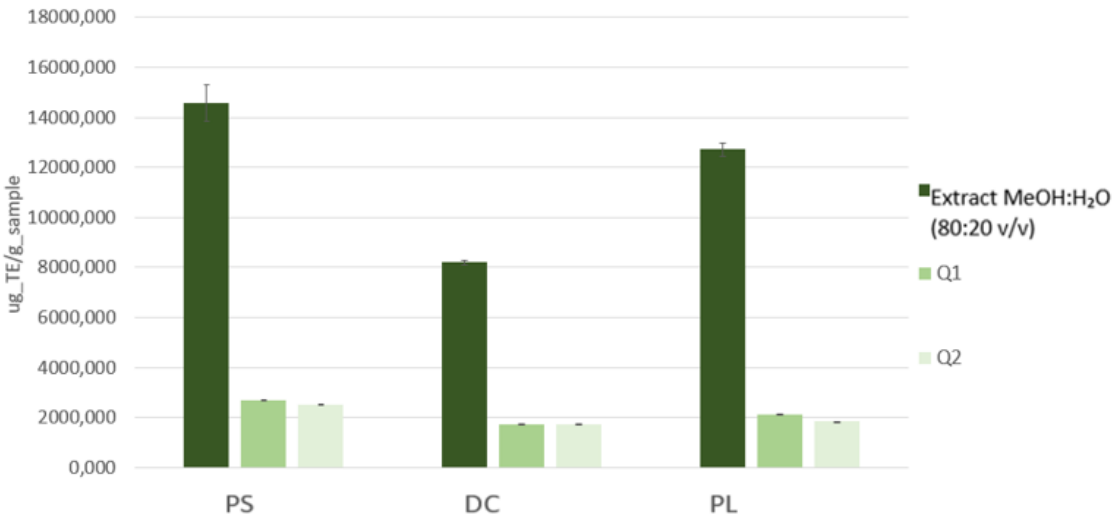
Total phenolics (Folin–Ciocalteu). Retention values are reported as mean ± SD (%).

Matrix	Retention Q1 (%) ± SD	Retention Q2 (%) ± SD
Tomato paste	37.37 ± 1.47	37.69 ± 1.92
Pulp	33.50 ± 3.47	34.60 ± 2.46
Diced tomato	34.41 ± 2.51	33.98 ± 2.31



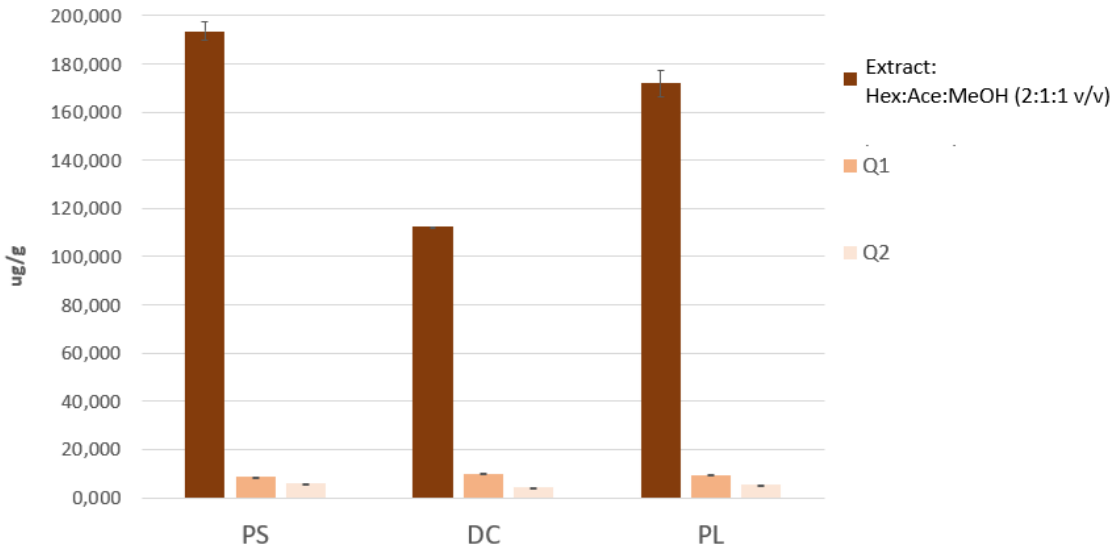
Total antioxidant capacity (DPPH). Retention values are reported as mean ± SD (%).

Matrix	Retention Q1 (%) ± SD	Retention Q2 (%) ± SD
Tomato paste	18.68 ± 2.08	17.45 ± 1.95
Pulp	16.72 ± 0.84	14.55 ± 0.70
Diced tomato	21.40 ± 0.77	21.23 ± 0.52



Total carotenoids. Retention values are reported as mean ± SD (%).

Matrix	Retention Q1 (%) ± SD	Retention Q2 (%) ± SD
Tomato paste	4.42 ± 0.20	3.02 ± 0.13
Pulp	5.33 ± 0.60	3.15 ± 0.24
Diced tomato	8.70 ± 0.74	3.81 ± 0.13



Discussion

Retention patterns differed systematically across indicator classes. For the Folin–Ciocalteu index, retention remained close to one third of the reference level and differences between Q1 and Q2 were limited, indicating relatively stable co-extraction of Folin–Ciocalteu-reactive material under the tested conditions. For the DPPH response, retentions were lower than those observed for the Folin–Ciocalteu index and showed a stronger dependence on the matrix, consistent with the fact that the DPPH assay captures a broad reducing capacity that is not necessarily proportional to the Folin–Ciocalteu index.

For carotenoids, retentions were confined to a few percentage points and were consistently lower in Q2 than in Q1, suggesting that the two QuEChERS conditions differ more clearly with respect to the transfer of the carotenoid-associated fraction.

Conclusions

Overall, QuEChERS extracts retained a substantial fraction of the Folin–Ciocalteu phenolics index (approximately 33–38%) with limited differences between Q1 and Q2.

Total antioxidant capacity showed lower retentions (approximately 14–21%) and greater matrix dependence. Total carotenoid retention was markedly lower (a few percentage points) and consistently reduced in Q2 compared with Q1. Taken together, these findings provide a quantitative description of non-target co-extraction under the investigated QuEChERS conditions and support the use of retention values as an operational descriptor of extract complexity.

Chapter 11 – Results and discussion: agar diffusion assay and MIC/MFC determination for Alternaria strains

In the agar diffusion assay, the antifungal activity of the two fermented products was strain- and concentration-dependent. For Alternaria alternata (non-CECT strain), the Trichoderma reesei fermentate showed a dose-related increase in inhibition, shifting from no inhibition zone at 0.1 g/L to moderate inhibition at 10 g/L (–, +, ++). Under the same conditions, the Bacillus amyloliquefaciens fermentate produced no inhibition zone at 0.1 and 1 g/L and a detectable zone only at 10 g/L (–, –, +). For Alternaria linariae CECT 2997, the T. reesei fermentate exhibited inhibition only at the highest dose (–, –, +), whereas the B. amyloliquefaciens fermentate showed no inhibition zone at any of the tested concentrations (–, –, –). For A. alternata CECT 2662, the T. reesei fermentate was already active at 0.1 g/L and increased progressively up to 10 g/L (+, ++, +++), while the B. amyloliquefaciens fermentate was inactive at 0.1 g/L but active at 1 and 10 g/L (–, +, ++).

The assay was read qualitatively, classifying inhibition levels based on the visibility and extent of the inhibition halo.

MIC and MFC determinations by broth microdilution indicated that, for all combinations for which a value was obtained, MIC and MFC coincided. For A. alternata (non-CECT strain), the B. amyloliquefaciens fermentate showed MIC = MFC of 0.78 g/L, whereas the T. reesei fermentate showed MIC = MFC of 1.56 g/L. For A. linariae CECT 2997, both fermentates showed MIC = MFC of 3.13 g/L. For A. alternata CECT 2662, the B. amyloliquefaciens fermentate showed MIC = MFC of

12.50 g/L, whereas for the T. reesei fermentate no MIC was observed within the tested concentration range and, consequently, no MFC value was determined.
Overall, the results indicate clear strain dependence. In addition, comparison across assays shows that the relative performance of the two fermentates varies with the test used: the agar diffusion assay highlights a marked activity of the T. reesei fermentate against A. alternata CECT 2662, whereas in the quantitative broth assay the threshold determined for the B. amyloliquefaciens fermentate is high for the same strain and the MIC for T. reesei falls outside the adopted experimental range.

The results confirm that the powdered fermentates, when applied in vitro, can exert measurable antifungal activity in a strain-dependent manner. At the same time, they indicate that efficacy estimates can differ between assays because the two methods probe different aspects of the phenomenon. Agar diffusion is influenced not only by the intrinsic potency of the treatment, but also by the diffusibility of bioactive components within the solid medium and their local stability; broth microdilution, in contrast, evaluates fungal response under homogeneous exposure to the treatment across a broader concentration series. Within this framework, the case of *A. linariae* CECT 2997 is illustrative: the absence of an inhibition halo for the *B. amyloliquefaciens* fermentate on agar coexists with a measurable MIC/MFC in broth, consistent with reduced effectiveness in generating an active gradient in solid medium while retaining activity under uniformly ensured contact.

For *A. alternata* (non-CECT strain), MIC/MFC values indicate lower thresholds for the *B. amyloliquefaciens* fermentate than for *T. reesei*, suggesting higher efficacy in the microdilution context.

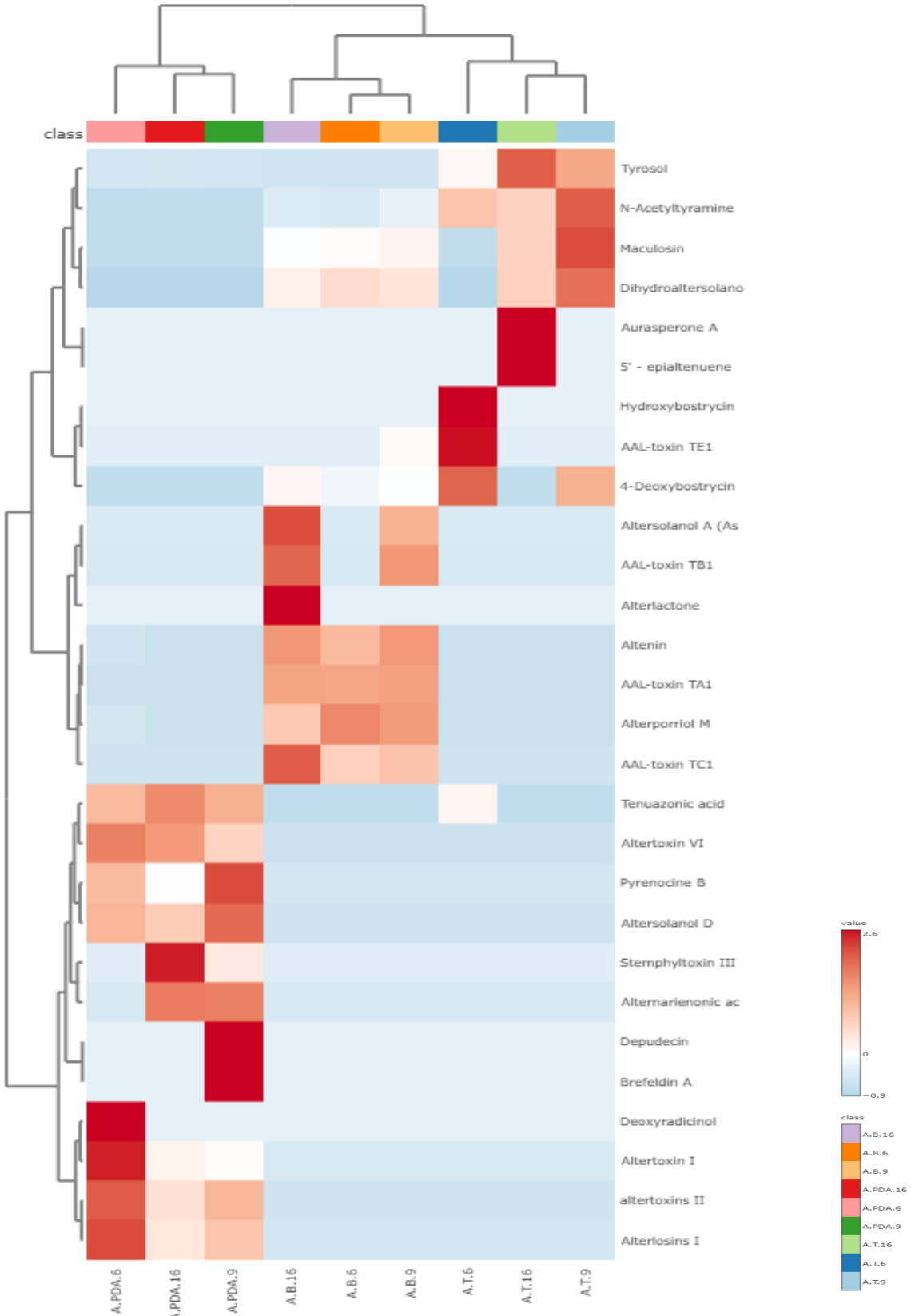
For *A. alternata* CECT 2662, by contrast, the overall pattern suggests greater tolerance: in the quantitative assay, a higher concentration of the *B. amyloliquefaciens* fermentate is required to achieve complete inhibition and fungicidal activity, whereas no MIC was observed for the *T. reesei* fermentate within the tested range, despite clear inhibition halos and a dose-dependent response in agar diffusion. Taken together, these findings can be discussed as indicating that, for certain strains, a treatment may produce a readily detectable local effect under agar diffusion conditions without necessarily translating into a complete-inhibition threshold within the concentration interval explored in broth.

From an applied perspective, this supports the value of considering qualitative and quantitative assays jointly and interpreting the “potency” of a formulation in relation to the experimental context, avoiding the extrapolation of an absolute hierarchy between treatments on the basis of a single test.

Figure 18. Petri-dish cultures of *Alternaria alternata* prepared for extraction and non-volatile metabolite profiling by UHPLC–TOF–MS. From left to right: growth on non-supplemented PDA (control); growth on PDA supplemented with the fermentet from *Bacillus amyloliquefaciens*; growth on PDA supplemented with fermentation filtrate obtained from *Trichoderma reesei*. The plates show macroscopic differences in colony appearance (pigmentation and sporulation pattern) across the three conditions.

Results: non volatiles heatmap description and temporal dynamics

The heatmap shows a clear separation between the control condition, consisting of *Alternaria alternata* grown on non-supplemented PDA, and the conditions in which *A. alternata* was grown on PDA supplemented with spray-dried fermentate powders obtained from protein hydrolysates (a *Bacillus amyloliquefaciens* fermentate and a *Trichoderma reesei* fermentate). The three control time points (6, 9, and 16 days) cluster together, whereas the two treated conditions are positioned on a distinct branch. Within the treated samples, the *T. reesei* fermentate at 9 and 16 days appears more similar to each other than to the 6-day time point, while the *B. amyloliquefaciens* fermentate displays an overall cohesive profile across the time course.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Tyrosol
N-Acetyltyramine
Maculosin
Dihydroaltersolano
Aurasperone A
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Hydroxybostrycin
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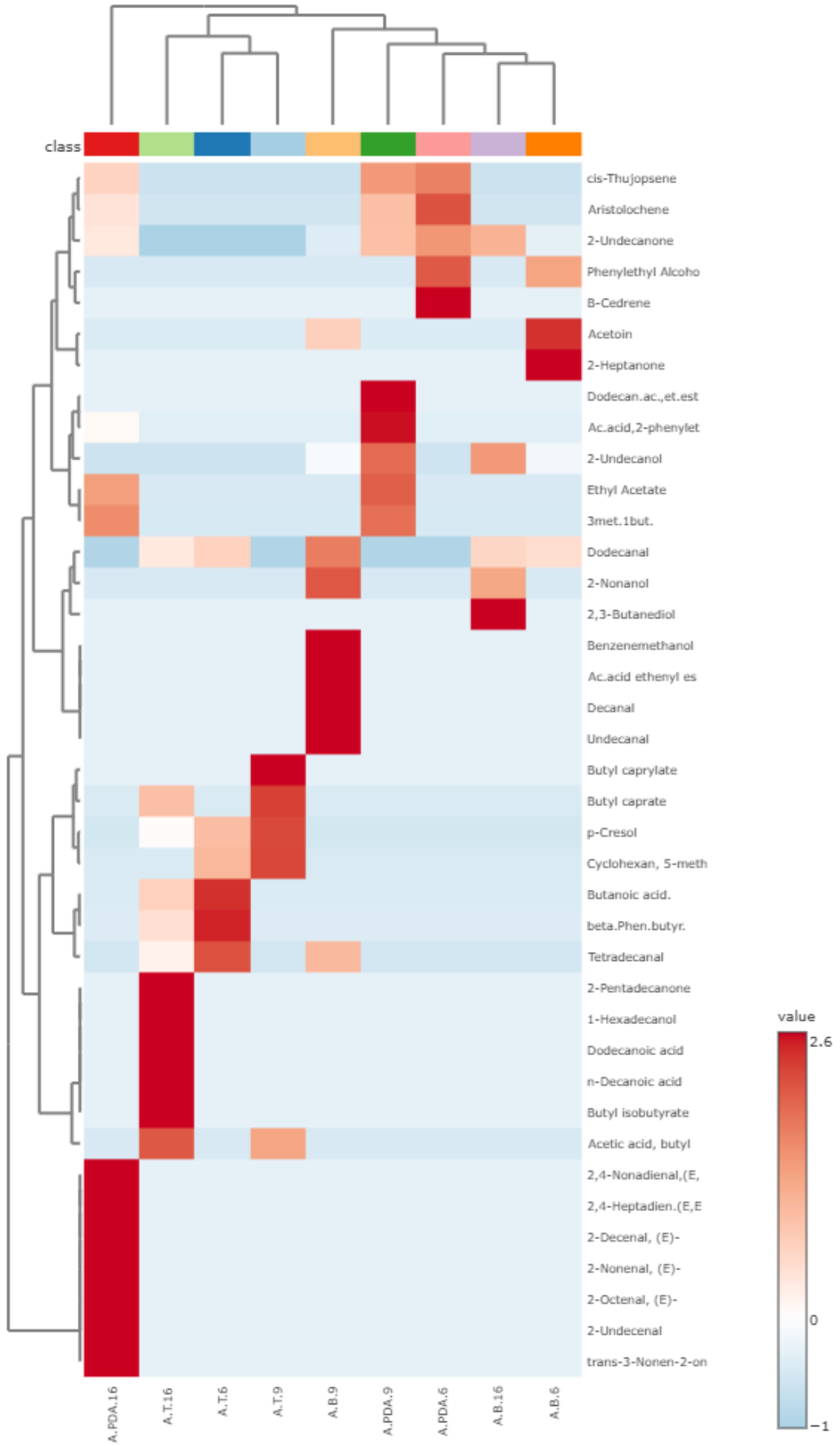
Altersolanol A (As
AAL-toxin TB1
Alterlactone
Altenin
AAL-toxin TA1
Alterporriol M
AAL-toxin TC1
Tenuazonic acid
Altertoxin VI
Pyrenocine B
Altersolanol D
Stemphyltoxin III
Alternarienonic ac
Depudecin
Brefeldin A
Deoxyradicinol
Altertoxin I
altertoxins II
Alterlosins I
A.PDA.6
A.PDA.16
A.PDA.9
A.B.16
A.B.6
A.B.9
A.T.6
A.T.16
A.T.9
value
2.6
0
−0.9
class
A.B.16
A.B.6
A.B.9
A.PDA.16
A.PDA.6
A.PDA.9
A.T.16
A.T.6
A.T.9

At the metabolite level, the heatmap highlights treatment-dependent and time-dependent patterns. A first set of compounds is characterised by high signal intensity in the PDA control and attenuation under treatment conditions.

This set includes tenuazonic acid, which in the control maintains high signal across 6, 9, and 16 days, with fluctuations but without being confined to a single time point. Under treatment conditions, the tenuazonic acid signal is strongly reduced: it is not observed for the *B. amyloliquefaciens* fermentate at any time point and is detected only at day 6 in the *T. reesei* fermentate condition, without persisting at days 9 and 16. The same “control-associated” cluster includes several perylene-quinones (altertoxins/stemphyltoxins), which appear more pronounced in the control especially at earlier times and tend to shift towards a more balanced profile as time progresses; under fermentate conditions, these signals are overall attenuated. Within the control, some compounds show a more time-specific pattern: brefeldin A and depudecin are more evident at day 9 than at the other time points, consistent with a more temporally confined production along the time course.

A second group of compounds is preferentially associated with the *B. amyloliquefaciens* fermentate condition and exhibits internal temporal modulation. Alterporriol M remains elevated across the entire time course, with a more evident maximum at day 6, whereas alterlactone represents an example of a late signal with maximal intensity at day 16. Altenin appears distributed across multiple time points rather than concentrated at a single stage of the kinetics. In this region of the heatmap, features labelled as AAL-toxins are also present; these are more represented under the *B. amyloliquefaciens* fermentate condition and vary across 6, 9, and 16 days.

A third group of compounds is preferentially associated with the *T. reesei* fermentate condition, particularly at days 9 and 16. Tyrosol and N-acetyltyramine are higher than in the control and higher than in the *B. amyloliquefaciens* fermentate condition, with a pronounced increase towards day 16 for tyrosol. Aurasperone A appears predominantly associated with day 16 under the *T. reesei* condition. In addition, some features differentiate the day 6 profile under the *T. reesei* condition from later times: hydroxybostrycin and the feature labelled AAL-toxin TE1 are mainly concentrated at day 6.


class
cis-Thujopsene
Aristolochene
2-Undecanone
Phenylethyl Alcoho
B-Cedrene
Acetoin
2-Heptanone
Dodecan.ac.,et.est
Ac.acid,2-phenylet
2-Undecanol
Ethyl Acetate
3met.1but.
Dodecanal
2-Nonanol
2,3-Butanediol
Benzenemethanol
Ac.acid ethenyl es
Decanal
Undecanal
Butyl caprylate
Butyl caprate
p-Cresol
Cyclohexan, 5-meth
Butanoic acid.
beta.Phen.butyr.
Tetradecanal
2-Pentadecanone
1-Hexadecanol
Dodecanoic acid
n-Decanoic acid
Butyl isobutyrate
Acetic acid, butyl
2,4-Nonadienal,(E,
2,4-Heptadien.(E,E
2-Decenal, (E)-
2-Nonenal, (E)-
2-Octenal, (E)-
2-Undecenal
trans-3-Nonen-2-on
A.PDA.16
A.T.16
A.T.6
A.T.9
A.B.9
A.PDA.9
A.PDA.6
A.B.16
A.B.6
value
2.6
0
−1

In the present experimental system, the measured volatile compounds reflect the overall headspace profile of the vial. However, several compounds included in the panel are explicitly reported in the literature as being emitted by A. alternata in vitro and can therefore be considered, with higher confidence, compatible with a fungal contribution. In particular, thujopsene and β-cedrene are described as dominant VOCs emitted by *A. alternata* across different experimental settings, with relative contributions that vary with culture age (Weikl, 2016).
In vitro volatile compounds profiles of *A. alternata* including 3-methyl-1-butanol and phenylethyl alcohol together with β-cedrene and thujopsene have also been reported (olive endophyte study, 2017). In an additional study on tomato and A. alternata, 3-methyl-1-butanol is among the identified metabolites and is discussed as a time-dependent variable during the host–pathogen interaction; the same study also includes a profile of *A. alternata* grown on PDA (Encinas-Basurto et al., 2017).
For other volatile compounds in the panel (e.g., medium-chain aldehydes and alkenals), their time-specific appearance in the PDA control is compatible with culture physiological changes and/or oxidative processes affecting medium components. However, the consulted literature does not support treating them as specific in vitro markers of *A. alternata*; consequently, they are discussed only descriptively and within general interpretative scenarios.

Descriptive results of the heatmap

The heatmap summarizes headspace volatic compounds across nine experimental groups (three time points for the A. alternata PDA control and three time points for each treatment). Only a limited number of compounds display high and condition-specific intensity, whereas many variables remain low or absent across most conditions; thus, the clustering structure is mainly driven by dominant volatile compounds emerging under specific treatment–time combinations.
In the PDA control, cis-thujopsene and β-cedrene are among the most characteristic signals at early and intermediate stages, whereas at 16 days the profile becomes enriched in aldehydes and alkenals (e.g., 2,4-nonadienal, 2,4-heptadienal, 2-decenal, and related compounds), which are less represented at the other time points. This results in a temporal trend in which the terpenoid fraction is more prominent earlier, whereas the aldehydic fraction becomes more evident at the late stage.

In the treatment with the B. amyloliquefaciens fermentate, medium-to-long chain aliphatic compounds are particularly prominent, with clear signals for 2-undecanone and 2-undecanol and a marked contribution of dodecanal at one of the time points. Some variables are more temporally restricted (e.g., acetoin and 2-heptanone), indicating that the Bacillus condition includes both relatively persistent VOCs and volatile compounds linked to specific phases of the time course.
In the T. reesei fermentate treatment, the profile shows a strong time dependence, with esters and aromatics emerging in distinct temporal windows: beta-phenylethyl butyrate is particularly pronounced at an early time point, whereas butyl caprate and butyl caprylate are more represented at 9 days. p-Cresol increases progressively over time, outlining a variable that changes continuously within this treatment.

Discussion

In the PDA control, the presence of sesquiterpenes such as thujopsene and β-cedrene is consistent with studies describing A. alternata as a strong sesquiterpene emitter and showing that emissions can be higher in younger cultures, while the relative contributions of individual compounds change during growth (Weikl, 2016). From this perspective, the relative decrease of the terpenoid fraction in the late control is compatible with a time-dependent remodeling of the volatilome associated with the physiological state of the culture.
The emergence at 16 days in the control of a set rich in aldehydes and alkenals may indicate a more oxidized profile at advanced stages. In a postharvest pathology context, tomato inoculation with *A. alternata* has been associated with changes in volatiles even before visible symptoms, and some volatile compounds have been proposed to contribute to physiological transitions during the interaction, although additional evidence is required to establish a causal role (Encinas-Basurto et al., 2017).
In the samples treated with the *B. amyloliquefaciens* and *T. reesei* fermentates, two non-mutually exclusive scenarios can account for the observed patterns. One scenario is a remodeling of the A. alternata volatilome in response to the microenvironment generated by the fermentates, with a relative attenuation of sesquiterpenes that dominate the PDA control.

A second scenario is a direct contribution of the fermentate to the headspace profile: in the literature, Bacillus spp. is described as emitting mixtures of aldehydes, ketones, and alcohols in which dodecanal, 2-undecanol, and 2-undecanone can be abundant (Li, 2020), and studies on B. velezensis report acetoin, 2-heptanone, and 2,3-butanediol among the identified volatiles (Calvo, 2020).

From an ecological standpoint, the defensible conclusion is that the volatilome associated with *A. alternata* in vitro is temporally dynamic, and that the addition of fermentates is associated with distinct profiles for Bacillus versus Trichoderma, consistent with a condition-dependent metabolic response and with the possibility that part of the VOC signal derives from the fermented mixture. Any specific ecological role assigned to individual compounds remains hypothetical within the scope of these data and would require dedicated functional evidence.

Discussion

From an applied standpoint, these results indicate that supplementing the growth substrate with spray-dried fermentate powders is not limited to a broad quantitative shift in overall signal abundance, but is associated with a selective reorganisation of the secondary-metabolite profile with a temporal component. This aspect is relevant because, within the genus *Alternaria*, part of secondary metabolism is discussed as pertinent both to food safety and to the biology of fungus–host interactions.

With respect to human health, tenuazonic acid is frequently described among the *Alternaria* toxins of major interest in the context of food contamination (Escrivá et al., 2017; Saleem, 2022).

In the present data, tenuazonic acid is high in the PDA control and markedly reduced under fermentate conditions, with detection only as a transient signal in a single condition and time point. From an applied perspective, this pattern is consistent with a potentially favourable outcome, because it indicates a reduction of a toxicologically relevant analyte relative to growth in the absence of treatment.

In the same direction, the overall attenuation of perylene-quinone signals (altertoxins and stemphyltoxins) under treatment conditions is relevant because these compounds are discussed in the literature as emerging mycotoxins with concerns linked to genotoxic effects in cellular models, with altertoxin II frequently cited as particularly genotoxic and stemphyltoxin III included in studies on DNA damage and repair (Solhaug et al., 2016; Escrivá et al., 2017).

The presence of altertoxin I and stemphyltoxin III in the profiled panel is also consistent with studies reporting them as metabolites isolated from *Alternaria* with bioactivity in cellular assays (Ola et al., 2022). Overall, the heatmap suggests that, under the experimental conditions considered, fermentate supplementation is associated with a relative decrease in signals attributed to compounds of food-safety relevance compared with the control.

From a phytopathological perspective, a distinct category is represented by host-selective toxins of the tomato pathotype, including AAL-toxins, which are discussed for their role in plant damage and for mechanisms linked to perturbation of sphingolipid metabolism; these toxins are also discussed as relevant to mammalian cells, and therefore potentially of interest across both phytopathological and toxicological domains (Fernández Pinto and Patriarca, 2017; Escrivá et al., 2017).

In the dataset, features labelled as AAL-toxins are associated mainly with the *B. amyloliquefaciens* fermentate condition and show time-dependent dynamics. In an applied perspective, this element should be discussed cautiously but not disregarded: the data indicate that the metabolic response of *A. alternata* in the presence of fermentates is not univocally a “suppression” of secondary metabolism, but rather a reorganisation that, under some conditions, includes the appearance or relative increase of metabolites classified in the literature as phytotoxins or as determinants of pathogenicity in specific pathosystems.

For the fermentate associated with *T. reesei*, the literature reports that products or interactions attributable to *Trichoderma* can influence toxin production and modulate the *Alternaria* metabolome in a time-dependent manner and depending on the type of interaction (Tian et al., 2023).

The fact that the *T. reesei* condition at 9 and 16 days clusters separately and displays distinguishing metabolites relative to the control and to the *B. amyloliquefaciens* condition is consistent with the idea that fermentates can induce differentiated metabolic responses over time. Overall, the data support a cautious applied interpretation: fermentate conditions are associated with a relative reduction in signals attributed to metabolites of food-safety interest, but also with a reorganisation of secondary metabolism that, under some conditions, includes metabolites with potential phytopathological implications. This reinforces the importance of considering antifungal efficacy together with secondary-metabolite profiling when evaluating candidate products for field use.

Chapter 12 – Integrated Discussion and conclusions: linking risk, control strategies, and analytical tools

The risk associated with *Alternaria* toxins in the tomato supply chain can be interpreted as a supply-chain problem in a strict sense, i.e., as the outcome of a sequence of events that begins in the field and continues through post-harvest handling, storage, processing, and industrial flow management. From this perspective, mitigation does not coincide with a single intervention, but with a coherent combination of prevention, monitoring, and operational decisions at different critical points, with distinct objectives: limiting pathogen development, reducing the likelihood of toxin increase, and ensuring that control measures remain sustainable over time (Adhikari, 2017; Schmey et al., 2024; Qin et al., 2022).

In the pre-harvest domain, a robust approach to infections attributed to *Alternaria* spp. is typically framed within integrated management strategies, where agronomic measures reduce inoculum and system susceptibility and control measures are rationalised according to risk windows and epidemic pressure (Adhikari, 2017; Schmey et al., 2024).

In post-harvest stages and processing, mitigation takes a different operational meaning: the main objective becomes limiting the opportunity for fungal growth and further toxin production in the interval between harvest and processing, rather than “removing” what is already present (Qin et al., 2022). Consistently, available evidence indicates that the effect of technological steps is not uniform across different *Alternaria* toxins and that reductions observed under specific conditions cannot be generalised as a guaranteed detoxification effect (Estiarte et al., 2018; Puntscher et al., 2019).

An additional layer of complexity relates to modified forms, such as sulfate or glucoside conjugates, which may escape non-targeted protocols and contribute to underestimation if not considered within monitoring programmes (Rychlik et al., 2014; Soukup et al., 2016; Puntscher et al., 2018; Puntscher et al., 2019).

These elements lead to a central point of this integrated discussion: risk management requires not only biological or technological containment measures, but also analytical and decision-making capacity able to operate under variability, uncertainty, and differences among matrices and conditions.

The determination of *Alternaria* toxins in tomato-derived products faces a structural challenge: the extract is a chemical representation of the matrix, and its complexity can interfere with signal reliability in ionisation-based quantitative methods, even in the absence of evident chromatographic issues. Phenomena commonly described as matrix effects, with signal suppression or enhancement as a function of co-eluting endogenous compounds, are a key aspect for interpreting LC–MS data robustness (Matuszewski et al., 2003; Rogatsky and Stein, 2005). In this framework, assessing the complexity of QuEChERS extracts through quantitative indicators of non-target co-extraction makes it possible to convert a frequently “qualitative” problem into an operational descriptor, useful to compare extraction conditions and matrices and to contextualise the interpretation of instrumental results.

The results systematically show that QuEChERS extracts retain only a fraction of the response measured in reference extracts for the considered classes of indicators, and that this fraction depends on both matrix and chemical class, with particularly low retention for the carotenoid fraction and higher retention for the Folin–Ciocalteu index. In an integrated reading, this supports the view that the choice of extraction procedure is not merely a technical step, but a determinant for signal reliability and for the transferability of data across industrial matrices.

Industrial process control requires response times compatible with operational decisions. In this sense, Vis–NIR spectroscopy can be positioned as a complementary tool to reference methods, aimed at rapid, non-destructive estimation of technological and process parameters across different product categories. In this work, the evolution of calibrations indicates that model performance critically depends on dataset representativeness, operational alignment between spectral measurement and reference determination, and controlled handling of outlier samples within the model space. In high-water matrices and in products with high physical variability, environmental and operational factors may affect spectral response and therefore prediction stability if not adequately represented under calibration and application conditions (Sheng et al., 2019; Zhang and Yang, 2025). In an integrated reading, the contribution of NIR is not the “determination of toxins”, but the development of a quality monitoring system that reduces time and resources, increases control frequency, and supports more timely identification of technological deviations, within a quality management framework combining monitoring, reaction capacity, and verification over time.

The in vitro evaluation of protein fermentates formulated as powders introduces an additional discussion level: beyond growth inhibition, a treatment may be associated with a modulation of the fungal secondary metabolism. Antifungal assay outcomes clearly show strain dependence and variability across tests, confirming that efficacy cannot be described as an absolute property of the treatment, but rather as an interaction among strain, dose, and assessment method.

The non-volatile metabolomic profile and the volatile-compound profile indicate that exposure to fermentates is associated with a time-dependent reorganisation of the metabolic profile. In the PDA control, signals associated with toxicologically relevant metabolites, including tenuazonic acid, are evident, whereas in treated conditions these signals are attenuated. In parallel, the selective appearance of variables annotated as host-selective toxins in one treated condition indicates that the effect of the formulation may be associated with a reorganisation of the fungal response rather than a purely global quantitative reduction.

From a phytopathological perspective, host-selective toxins of the tomato pathotype, including AAL-toxins, are discussed in the literature for their role in plant damage and for mechanisms linked to disruption of sphingolipid metabolism, with a relevance that may extend to the toxicological domain for mammalian cells (Fernández Pinto and Patriarca, 2017; Escrivá et al., 2017). A more cautious integrated interpretation is therefore that the evaluation of a candidate sustainable treatment cannot stop at the growth metric: it should also include consideration of the direction and specificity of metabolic reorganisation, particularly when the final application targets a supply chain where food-safety and phytopathological dimensions intersect.

In the volatile-compound profile, the control is dominated by terpenoid compounds, whereas in treated conditions an enrichment in aliphatic compounds and/or esters emerges, with time-dependent dynamics.

Overall, the results support an integrated view of *Alternaria* risk management in the tomato supply chain. Analytical robustness, understood as the ability to interpret signals correctly in the presence of complex extracts and potential modified forms, is a necessary condition to avoid under- or overestimation of risk. Rapid NIR-based quality control contributes to more frequent and timely monitoring of process parameters, improving deviation management and operational traceability.

Fermentates represent a mitigation line potentially consistent with more sustainable strategies, but their evaluation requires considering antifungal efficacy together with pathogen metabolic reorganisation, because outcomes may include both attenuation of signals associated with toxicologically relevant metabolites and the selective appearance of metabolites with potential phytopathological significance.

This conceptual integration enables risk, measurement tools, and control strategies to be linked coherently: risk management does not derive from a single datum or a single test, but from the interaction between rapid monitoring, robust analytical methods, and mitigation strategies evaluated also in terms of their effects on pathogen metabolism, within a cautious interpretation oriented to practical transferability.

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