UNIVERSIDAD DE BUENOS AIRES

Facultad de Ciencias Exactas y Naturales

Departamento de Química Orgánica

Investigación sobre el destino de *Alternaria* y micotoxinas de *Alternaria* en la industria de la manzana

Tesis presentada para optar por el título de Doctora de la Universidad de Buenos Aires en el área Química Orgánica

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Buenos Aires, 21 de diciembre de 2021

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GHENT UNIVERSITY

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Investigation on the fate of *Alternaria* & *Alternaria* mycotoxins in the apple industry

Thesis submitted in fulfilment of the requirements for the degree of Doctor of Universidad de Buenos Aires, Organic Chemistry Area, and Doctor of Pharmaceutical Sciences by Ghent University

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Buenos Aires, 2021

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Resumen en español

La manzana es la tercera fruta de mayor consumo a nivel mundial y uno de los cultivos más importantes en Argentina siendo susceptible a sufrir infecciones fúngicas tanto en el campo como en la etapa postcosecha, causando grandes pérdidas económicas. Dichas infecciones pueden producirse en el exterior o en el interior de la fruta. Algunos de los hongos más frecuentemente hallados como agentes infecciosos pertenecen a géneros toxicogénicos, implicando un riesgo para la salud, especialmente cuando la fruta es destinada a la industria. Sin embargo, actualmente la única micotoxina legislada a nivel mundial en productos de manzana es patulina.

En el presente trabajo de Tesis Doctoral se identificaron los principales géneros fúngicos causantes de enfermedades en manzanas cultivadas en el Alto Valle de Río Negro, Argentina, tanto en el campo como en la etapa postcosecha con destino a comercio en fresco e industrialización, respectivamente. Se analizaron un total de 140 manzanas destinadas a consumo en fresco, de las cuales el 86 % resultaron contaminadas en el exterior y el 34 % en el interior causando corazón mohoso (CM). Del total de 100 manzanas destinadas a industrialización, el 48 % presentaron contaminación externa y el 51 % en el centro de la fruta. Se logró identificar a *Alternaria,* género productor de micotoxinas, como el principal responsable de CM y el segundo género causante de lesiones externas en manzanas destinadas a consumo en fresco (21 %) e industrialización (46 %). Se demostró además que la incidencia de CM aumentó durante el almacenamiento. Dado que esta enfermedad es difícilmente detectada en fruta destinada a la industrialización, este resultado implica el posible procesamiento de fruta contaminada con el consecuente riesgo de la acumulación de micotoxinas de *Alternaria*

en productos a base de manzana. Se identificaron un total de 120 aislamientos de este género según claves taxonómicas, resultando *A. tenuissima* el grupo-especie predominante (84 %), seguido por *A. alternata* (3 %), *A. arborescens* (1 %) y *A. gaisen* (1 %) y 11 % de los aislamientos presentaron características intermedias y se clasificaron como *Alternaria* sp. Estos grupos-especies pertenecen a la Sección *Alternaria* que comprende especies productoras de micotoxinas. Además, un total de 78 aislamientos se caracterizaron según sus perfiles de producción de metabolitos secundarios *in vitro,* determinado por cromatografía de ultra alta performance acoplada a detección UV y espectrometría de masa de alta resolución (HRMS/MS). Se lograron identificar 27 metabolitos secundarios y se observó una mayor capacidad metabólica de las cepas obtenidas de CM, implicando un mayor riesgo para los productos procesados. Posteriormente se evaluó el potencial toxicogénico *in vivo*, simulando condiciones de comercialización (T=25 °C) y almacenamiento (T=4 °C), incubándose 3 cepas previamente aisladas de manzana en el exterior y en el interior de frutos libres de contaminación. A 25 ºC se encontró una mayor acumulación de micotoxinas, sin embargo, el almacenamiento en frío durante períodos prolongados no evitó la producción de toxinas de *Alternaria* en manzanas, siendo ácido tenuazónico (TeA) la micotoxina producida en mayores concentraciones en todas las condiciones.

Las seis principales micotoxinas producidas por *Alternaria*, a saber alternariol (AOH), alternariol monometil éter (AME), altenueno (ALT), tentoxina (TEN), TeA, altertoxina-I (ATX-I), y cuatro formas modificadas de estas, alternariol-3-glucósido (AOH-3-G), alternariol-3-sulfato (AOH-3-S), alternariol monometil éter-3-glucósido (AME-3-G), y alternariol monometil éter-3-sulfato (AME-3-S), se cuantificaron en seis lotes de manzanas destinados a industrialización por cromatografía de ultra alta performance acoplada a espectrometría de masa y se monitoreó su concentración a lo largo del proceso productivo de jugo concentrado. Se encontró que las manzanas de variedad Granny Smith son menos susceptibles a la contaminación con estas micotoxinas que

las de Red Delicious y que la clarificación es una etapa clave en la reducción de la concentración de las toxinas en el producto final.

A su vez, se detectaron por primera vez en Argentina micotoxinas de *Alternaria* y sus formas modificadas en productos comerciales a base de manzana. Se determinó la presencia de AOH, AME, ALT, TEN, TeA, ATX-I, AOH-3-G, AOH-3-S, AME-3-G, y AME-3-S en jugos de manzana clarificados y sin clarificar, mermeladas y papillas infantiles a base de manzana del mercado local. En los jugos clarificados se encontraron niveles detectables de AME, TEN, TeA, AME-3-S y AOH-3-G, mientras que en los sin clarificar se encontraron las mismas micotoxinas más AOH y en concentraciones mayores. En mermeladas se hallaron AME, TEN, TeA y AOH-3G, y en papillas infantiles AOH, AME, TEN y TeA. Con los resultados de incidencia natural de AOH, AME y TeA y datos de consumo provistos por el Ministerio de Salud, se realizaron análisis de exposición y caracterización del riesgo para los niños de entre 6 meses y 5 años de edad de la Argentina por el consumo de jugos de manzana clarificados y sin clarificar y papillas infantiles a base de manzana. Se encontró que el mayor riesgo de exposición afecta a los niños de entre 6 y 23 meses de edad y está asociado al consumo de papillas infantiles. Los metabolitos que representaron mayor riesgo fueron los pertenecientes al grupo de los alternarioles, con capacidad mutagénica y genotóxica. Estos resultados indican la necesidad de mejores estrategias de control de la contaminación de manzanas destinadas a la industria con cepas toxicogénicas de *Alternaria* y la necesidad de establecer legislación para estas micotoxinas. Por último, se establecieron las bases para un modelo basado en un análisis no dirigido por HRMS para la detección de lotes de manzana contaminados con micotoxinas de *Alternaria* como estrategia de control, lográndose diferenciar manzanas no contaminadas de contaminadas tanto en el exterior como en el interior de la fruta almacenadas a dos temperaturas diferentes.

Palabras clave: manzana, *Alternaria*, micotoxinas, seguridad alimentaria, riesgo sanitario

Investigation on the fate of *Alternaria* & *Alternaria* mycotoxins in the apple industry

English abstract

Apple is the third most consumed fruit worldwide and one of the most important crops in Argentina, being susceptible to fungal infection both in the field and in the postharvest stage, that causes great economic losses. These infections can occur on the outside or the inside of the fruit. Some of the most frequently found fungal infectants belong to toxigenic genera, what poses a health risk for consumers, especially when the fruit is destined for industrial food processing. However, the only mycotoxin currently legislated worldwide in apple by-products is patulin.

In the framework of this PhD thesis, the main fungal genera causing diseases in apples grown in the Alto Valle of Río Negro province in Argentina, both in the field and in the postharvest stage destined for fresh consumption and industrial food processing respectively, were identified. A total of 140 apples destined for fresh consumption were analysed, of which 86 % were infected on the outside and 34 % on the inside, causing mouldy core (MC). Of the total of 100 apples destined for industrial food processing, 48 % presented external infection and 51 % were infected in the centre of the fruit. *Alternaria*, a mycotoxin-producing genus, was identified as the main responsible for MC and the second genus causing external lesions in apples destined for fresh consumption (21 %) and industrial food processing (46 %). It was further shown that the incidence of MC increased during storage. Since this disease is hardly detected in fruit destined for industrial food processing, this result implies the possible processing of infected fruit with the consequent risk of the accumulation of *Alternaria* mycotoxins in apple by-products. A total of 120 isolates of this genus were identified according to taxonomic keys, with *A. tenuissima* being the predominant species-group (84 %), followed by *A. alternata* (3%), *A. arborescens* (1%) and *A. gaisen* (1%) and 11% of the isolates presented intermediate

characteristics and were classified as *Alternaria* sp. These species-groups belong to *Alternaria* section *Alternaria* that includes mycotoxin producing species. In addition, a total of 78 isolates were characterized according to their *in vitro* secondary metabolite production profiles, determined by ultra-high performance chromatography coupled with UV detection and high-resolution mass spectrometry (HRMS/MS). A total of 27 secondary metabolites produced by this genus were identified and a greater metabolic capacity of the strains obtained from MC was observed, implying a greater risk for the apple by-products. Subsequently, the toxigenic potential *in vivo* was evaluated, simulating retail (T=25 °C) and storage (T=4 °C) conditions, incubating 3 *Alternaria* strains previously isolated from apples on the outside and inside of fruits free of fungal infection. The tested isolates were able to produce mycotoxins under retail and long-term cold storage conditions in the interior and the exterior of the fruit. The risk of mycotoxin accumulation was higher at 25 ºC, but the long-term cold storage usually performed by processing industries did not prevent the accumulation of secondary *Alternaria* toxic metabolites in apples, with tenuazonic acid (TeA) being the mycotoxin produced in higher concentrations in all conditions.

The six main mycotoxins produced by *Alternaria*, namely alternariol (AOH), alternariol monomethyl ether (AME), altenuene (ALT), tentoxin (TEN), TeA, altertoxin-I (ATX-I), and four modified forms of these, alternariol-3-glucoside (AOH-3-G), alternariol-3-sulphate (AOH-3-S), alternariol monomethyl ether-3-glucoside (AME-3-G), and alternariol monomethyl ether-3-sulphate (AME-3-S), were quantified in six batches of apples destined for industrial food processing by ultra-high performance chromatography coupled to mass spectrometry and their concentration was monitored throughout the production process of apple concentrate. Granny Smith apples were found to be less susceptible to contamination with these mycotoxins than Red Delicious apples and the clarification step was proven as a key step in reducing the concentration of mycotoxins in the final product.

As well, *Alternaria* mycotoxins and their modified forms were detected for the first time in commercial apple by-products in Argentina. The presence and levels of AOH, AME, ALT, TEN, TeA, ATX-I, AOH-3-G, AOH-3-S, AME-3-G, and AME-3-S were determined in clarified and cloudy apple juices, marmalades, and apple-based infant food from the Argentinean local market. In the clarified juices, detectable levels of AME, TEN, TeA, AME-3-S and AOH-3-G were found, while in the cloudy apple juices the same mycotoxins plus AOH were found and in higher concentrations. AME, TEN, TeA and AOH-3G were found in marmalades, and AOH, AME, TEN and TeA were detected in apple infant food. With the results of the natural occurrence of AOH, AME and TeA and consumption data provided by the Ministry of Health of Argentina, exposure assessment and risk characterization were carried out for children between 6 month and 5 year old in Argentina from the consumption of clarified and cloudy apple juices and apple-based infant food. The highest risk of exposure affected children between 6 and 23 month old from the consumption of apple infant food. The mycotoxins that represented the highest risk were the alternariols, with mutagenic and genotoxic capacity. These results indicate the need for better control strategies for the contamination of apples destined for industrial food processing with toxigenic strains of *Alternaria* and the need to establish legislation for these mycotoxins in Argentina. Finally, the bases for a HRMS model were proposed for the detection of contaminated apple batches to prevent their processing and consequent mycotoxin accumulation in apple by-products as a control strategy. The model was able to differentiate non-contaminated apples from contaminated ones, both on the outside and inside of the fruit at two different temperatures.

Keywords: apple, *Alternaria*, mycotoxins, food safety, health risk

Onderzoek naar het lot van *Alternaria* & *Alternaria*-mycotoxinen in de appelindustrie

Nederlandstalig

De appel is het op twee na meest geconsumeerde fruit ter wereld en één van de belangrijkste gewassen in Argentinië, omdat het vatbaar is voor schimmelinfecties, zowel in het veld als in het stadium na de oogst, wat grote economische verliezen veroorzaakt. Deze infecties kunnen zowel aan de buitenkant als aan de binnenkant van de vrucht voorkomen. Enkele van de meest voorkomende schimmelinfecties behoren tot toxigene geslachten, wat een gezondheidsrisico vormt voor de consument, vooral wanneer het fruit bestemd is voor industriële voedselverwerking. Het enige mycotoxine dat momenteel wereldwijd gereglementeerd is in de bijproducten van appels, is patuline.

In het kader van dit doctoraatsproefschrift werden de belangrijkste schimmelsoorten geïdentificeerd die ziekten veroorzaken bij appels die worden geteeld in Alto Valle van de provincie Río Negro in Argentinië, zowel in het veld als in het stadium na de oogst, bestemd voor respectievelijk verse consumptie en industriële voedselverwerking. In totaal werden 140 appels voor verse consumptie geanalyseerd, waarvan 86 % aan de buitenkant besmet en 34 % aan de binnenkant, waardoor een beschimmelde kern (MC) ontstond. Van de in totaal 100 appels die bestemd waren voor industriële voedselverwerking, vertoonde 48% een uitwendige infectie en 51% was besmet in het midden van het fruit. *Alternaria*, een mycotoxine-producerend geslacht, werd geïdentificeerd als de hoofdverantwoordelijke voor MC en het tweede geslacht dat uitwendige laesies veroorzaakt bij appels die bestemd zijn voor verse consumptie (21 %) en industriële voedselverwerking (46 %). Verder werd aangetoond dat de incidentie van MC toenam tijdens bewaring. Aangezien deze ziekte nauwelijks wordt gedetecteerd in fruit dat bestemd is voor industriële voedselverwerking, impliceert dit resultaat de mogelijke verwerking van geïnfecteerd fruit met het daaruit voortvloeiende risico van

accumulatie van *Alternaria*-mycotoxinen in bijproducten van appel. Een totaal van 120 isolaten van dit geslacht werden geïdentificeerd volgens taxonomische sleutels, waarbij *A. tenuissima* de overheersende soortgroep was (84%), gevolgd door *A. alternata* (3%), *A. arborescens* (1%) en *A. gaisen* (1%) en 11% van de isolaten vertoonden intermediaire kenmerken en werden geclassificeerd als *Alternaria* sp. Deze soortgroepen behoren tot de *Alternaria*-sectie *Alternaria* die mycotoxine producerende soorten omvat. Bovendien werden in totaal 78 isolaten gekarakteriseerd op basis van hun *in vitro* productieprofielen van secundaire metabolieten, bepaald door ultrahoge performantiechromatografie in combinatie met UV-detectie en hoge resolutie massaspectrometrie (HRMS/MS). Een totaal van 27 secundaire metabolieten geproduceerd door dit geslacht werden geïdentificeerd en een grotere metabolische capaciteit van de stammen verkregen uit MC werd waargenomen, wat een groter risico inhoudt voor de bijproducten van de appel. Vervolgens werd het toxigene potentieel *in vivo* geëvalueerd, waarbij retail- (T=25 °C) en opslagomstandigheden (T=4 °C) werden gesimuleerd, waarbij 3 *Alternaria*-stammen werden geïncubeerd die eerder waren geïsoleerd uit appels aan de buiten- en binnenkant van fruit dat vrij was van schimmelinfecties. De geteste isolaten waren in staat om mycotoxinen te produceren onder retail- en langdurige koude opslagcondities, zowel aan de binnenkant als buitenkant van het fruit. Het risico op accumulatie van mycotoxinen was groter bij 25 ºC, maar de langdurige koude opslag die gewoonlijk door de verwerkende industrie wordt uitgevoerd, verhinderde de accumulatie van secundaire toxische metabolieten van *Alternaria* in appels niet, waarbij tenuazonzuur (TeA) het mycotoxine is dat in hogere concentraties wordt geproduceerd onder alle voorwaarden.

De zes belangrijkste mycotoxinen geproduceerd door *Alternaria*, namelijk alternariol (AOH), alternariol monomethylether (AME), altenueen (ALT), tentoxine (TEN), TeA, altertoxin-I (ATX-I), en vier gemodificeerde vormen hiervan, alternariol -3-glucoside (AOH-3-G), alternariol-3-sulfaat (AOH-3-S), alternariol monomethylether-3-glucoside (AME-3-G), en alternariol monomethylether-3-sulfaat (AME -3-S), werden

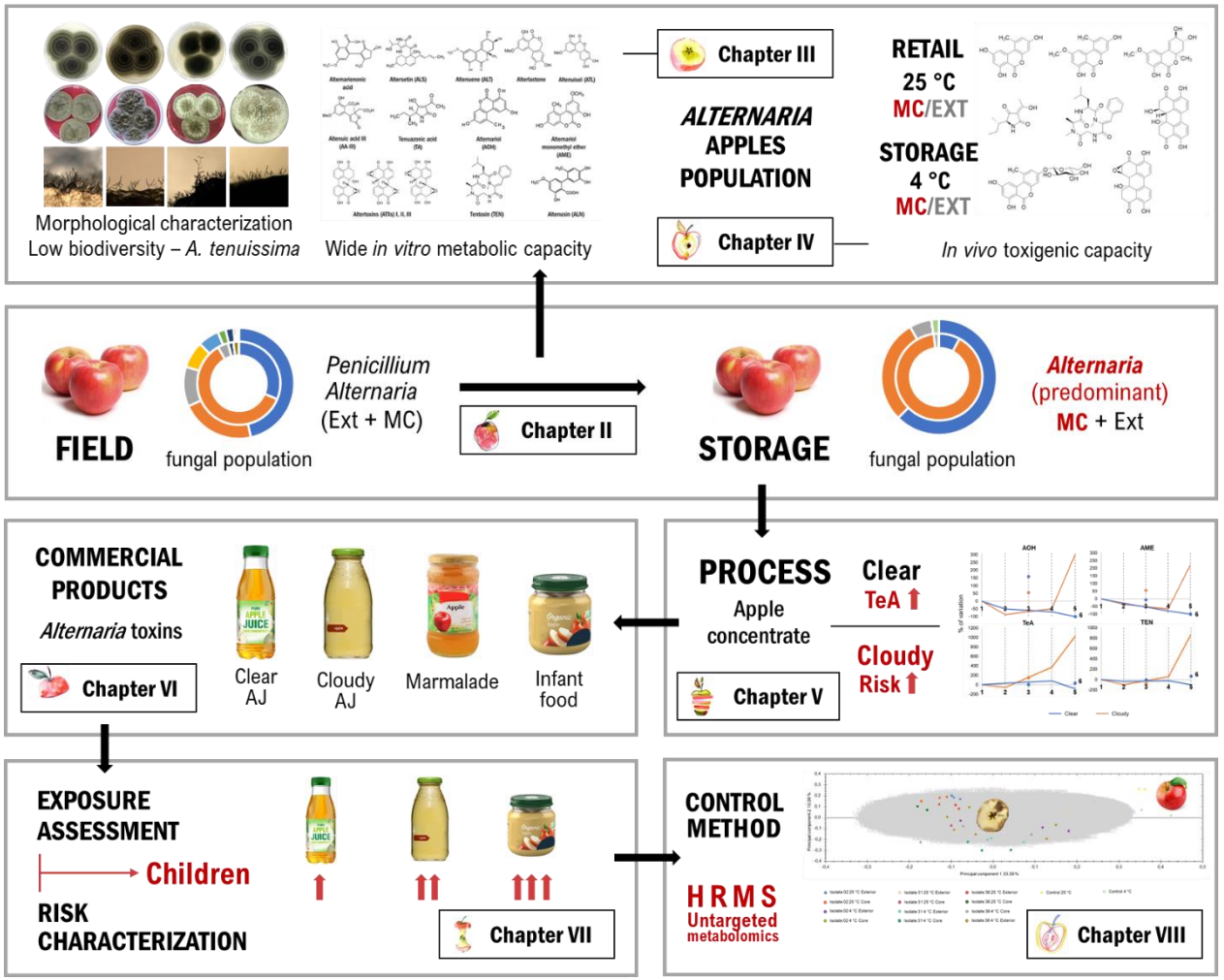
gekwantificeerd in zes partijen appels die bestemd waren voor industriële voedselverwerking door ultrahoge performantiechromatografie gekoppeld aan massaspectrometrie en hun concentratie werd gevolgd tijdens het productieproces van appelconcentraat. Granny Smith-appels bleken minder vatbaar voor besmetting met deze mycotoxinen dan Red Delicious-appels en de klaringsstap bleek een belangrijke stap in het verminderen van de concentratie van mycotoxinen in het eindproduct.

Ook werden voor het eerst *Alternaria*-mycotoxinen en hun gemodificeerde vormen gedetecteerd in commerciële appelbijproducten in Argentinië. De aanwezigheid en niveaus van AOH, AME, ALT, TEN, TeA, ATX-I, AOH-3-G, AOH-3-S, AME-3-G en AME-3-S werden bepaald in heldere en troebele appelsappen, marmelade en babyvoeding op basis van appel van de Argentijnse lokale markt. In de heldere sappen werden detecteerbare niveaus van AME, TEN, TeA, AME-3-S en AOH-3-G gevonden, terwijl in de troebele appelsappen dezelfde mycotoxinen plus AOH werden gevonden en in hogere concentraties. AME, TEN, TeA en AOH-3G werden gevonden in marmelade en AOH, AME, TEN en TeA werden gevonden in babyvoeding van appels. Met de resultaten van het natuurlijk voorkomen van AOH, AME en TeA en de consumptiegegevens verstrekt door het Ministerie van Volksgezondheid van Argentinië, werd een blootstellingsschatting en risicokarakterisatie uitgevoerd in Argentinië voor kinderen tussen 6 maanden en 5 jaar oud in geval van consumptie van helder en troebel appelsap en babyvoeding op basis van appel. Het hoogste risico op blootstelling trof kinderen tussen 6 en 23 maanden oud door de consumptie van appelbabyvoeding. De mycotoxinen die het grootste risico vormden, waren de alternariolen, met een mutagene en genotoxische capaciteit. Deze resultaten geven aan dat er behoefte is aan betere controlestrategieën voor de besmetting van appels die bestemd zijn voor industriële voedselverwerking met toxigene stammen van *Alternaria* en dat er wetgeving moet komen voor deze mycotoxinen in Argentinië. Ten slotte werd de basis voor een HRMS-model voorgesteld voor de detectie van besmette appelpartijen om hun verwerking en

daaruit voortvloeiende mycotoxineaccumulatie in appelbijproducten als controlestrategie te voorkomen. Het model was in staat om niet-gecontamineerde appels te onderscheiden van gecontamineerde appels, zowel aan de buitenkant als aan de binnenkant van het fruit bij twee verschillende temperaturen.

Kernworden: appel, *Alternaria*, mycotoxinen, voedselveiligheid, gezondheidsrisico

Graphical abstract



Acknowledgments

I would like to start by thanking my supervisors for giving me the opportunity to work with them. Thank you for your guidance, wise advice, and most of all, for the freedom, trust, and encouragement.

Andrea, gracias por tu siempre sabio consejo. Gracias por preocuparte por mi bienestar académico y personal, por tus palabras de apoyo, por no dejarme bajar los brazos. Más allá de ser una excelente científica y docente, sos una excelente persona.

Sarah, thank you very much for receiving me at the CEMPH, giving me the opportunity to improve my work, and always having a yes to new ideas.

Marthe, thank you for all your support and trust, and for always pushing my work further.

Virgi, gracias por tus consejos y por abrirme las puertas del labo y permitirme empezar este camino.

Gracias a mis colegas y amigues de la UBA, especialmente a Luli, no solo por hacerme el camino más fácil, sino por acompañarme gran parte del trayecto. A Malu y a Dianita, por ser excelentes compañeras con las que se puede contar para todo. A Nil, por todos los mates con yuyos y pócimas mágicas compartidas, por cuidarnos y hacernos el trabajo incontables veces más fácil. A Pau, por las charlas y las carilinas. A mis vecines Santi, Pupe y Fabri, quienes hicieron el trabajo mucho más divertido. A las chicas del L8, Yani, Dianis, Fio, Vale, Astrid, Ire, por el aguante, las luchas, los festejos y la alimentación. A les becaries del DQO, Mati, Ceci, Lean, Agus, Ale, Nico, Carlos. A les nodocentes, Gaby, Mica, Nancy, Sergio y Betty por todo su trabajo. A todes les docentes a lo largo de mi carrera de grado y de doctorado.

Thanks to my colleagues and friends at CEMPH. To my dearest friend Liesel, thank you for being my confident and for being there for me. To Esther, Celine and Orphelie, for

making me feel at home. To Suzan, for the honest talks. To Pan, Lia, Thanos, Roger and Yuri, for so many good moments shared in my last stay. To Arnau, Moh, Valdet and Karl for their advice with the MS instruments. To Christ´l, Mario, Fre, Annie and Marianne, because our work would not be possible without you. Thanks to all the students at CEMPH, for showing me bits of the world.

Thanks to UBA, MYTOX-South and CESAM for the financial support. Gracias a Gracielita Pose por recibirme en la UNRN y a Facundo Iturmendi por las muestras para esta Tesis.

Gracias a mis amigues por todo el aguante en las buenas y las malas, especialmente a Dani, Chan, El gordo, Yani, y Taly, sin ustedes no sería. A las chicas del colegio por ser un soporte constante desde hace años. A la TCP, amigas conquistadoras de luchas y celebraciones constantes. A Ariel, por invitarme a soplar un panadero y ayudarme a creer que los sueños se hacen realidad.

A mi familia, especialmente a los que ya no están acá. A Dori, por ser un gran compañero. A mi papá, gracias por enseñarme a disfrutar de la vida, ojalá en donde estés, estés orgulloso.

ABBREVIATIONS

AA	acetic acid
AAL toxins	*A. alternata* f. sp. *lycopersici* toxins
ACN	acetonitrile
AJ	apple juice
ALT	altenuene
AME	alternariol monomethyl ether
AME-3-G	alternariol monomethyl ether-3-glucoside
AME-3-S	alternariol monomethyl ether-3-sulphate
AOH	alternariol
AOH-3-G	alternariol-3-glucoside
AOH-3-S	alternariol-3-sulphate
APS	American phytopathological society common names of plant diseases
ATL	altenuisol
ATX-I	altertoxin-I
ATX-II	altertoxin-II
ATX-III	altertoxin-III
a_w	water activity
BW	body weight
BPC	base peak chromatogram
C	apples intended for fresh consumption
CEMPH	Centre of Excellence in Mycotoxicology and Public Health
cvs.	cultivars
DAD	diode array detector
DCMA	Dichloran Cloramphenicol Malt Extract Agar
DDA	automated data-dependent acquisition
DMSO	dimethyl sulphoxide
DRYES	Dichloran Rose Bengal Yeast Extract Sucrose agar
EFSA	European food safety authority
ESI	electrospray ionization
EXT	external inoculation
FAO	food and agriculture organization from the United Nations
HPLC	high-performance liquid chromatography
HRMS	high resolution mass spectrometry
HST	host specific toxins
I	apples were destined to industrial food processing
INTA	national institute of agricultural technology
iso-ALT	isoaltenuene
LB	lower bound
LOD	limit of detection
LOQ	limit of quantification
MC	mouldy core
MeOH	methanol
$MgSO_4$	magnesium sulphate
MMCC	matrix matched calibration curves
MO	months old
MS	mass spectrometry
n.a.	not applicable
NaCl	sodium chloride

PAT	patulin
PCA	Potato Carrot Agar
PCA	principal component analysis
QuEChERS	quick, easy, cheap, effective, rugged and safe
sp.-grp	species-group
STTX-III	stemphyltoxin III
TeA	tenuazonic acid
TEN	tentoxin
TTC	threshold of toxicological concern
UB	upper bound
UPLC-MS/MS	ultra-performance liquid chromatography tandem mass spectrometry
UR-A	urolithin A
YO	years old

TABLE OF CONTENTS

CHAPTER I

GENERAL INTRODUCTION

Ilustration by Tanja Meyer

I.1. Apple fruit

Cultivated apple (*Malus domestica* Borkh.) is a crop that has been domesticated from its ancestor, *Malus siversii,* that originated with the uplift of the Tian Shan mountains in Kazakhstan (Daccord et al., 2017; Duan et al., 2017). Its cultivation expanded through the Silk Road and was introduced to Europe and later, with the European colonization of America, to this continent (Cornille et al., 2019). It belongs to the Rosaceae family, and the tree can grow up to 12 m high and live up to 60 to 90 years. Most of its cultivation is done in temperate zones for the obtention of apple fruit, that originates after cross-pollination of the flowers at the end of spring, and fruit gets harvested mostly by hand at the end of summer (Sheffield et al., 2016). The propagation of the commercial apple tree is performed by either grafting or budding to obtain the uniformity required for profitable production. There are up to 10,000 cultivars, but the global production is dominated by relatively few, like 'Red Delicious', 'Golden Delicious', 'Jonathan', 'McIntosh' among others (Urrestarazu et al., 2016). **Figure I.1** shows a Red Delicious apple tree at full bloom (A, B) and at fruit maturity (C, D).

Apple fruit is the third most popular fruit in the world with over 89 MM tons produced annually worldwide. China is the largest producer representing approximately 40 % of the total world production followed by the USA, Turkey, Poland, Iran, and Italy. It is also an economically important crop in many countries, such as Israel, Australia, France, Greece, South Africa, Canada, Chile, and Argentina, among others. Argentina is one of the main apple-producing countries in the world, with 538 thousand tons of fruit harvested in 2019. In comparison, in Belgium the amount of harvested apples in 2019 was 260 thousand tons (FAOSTAT, 2021).

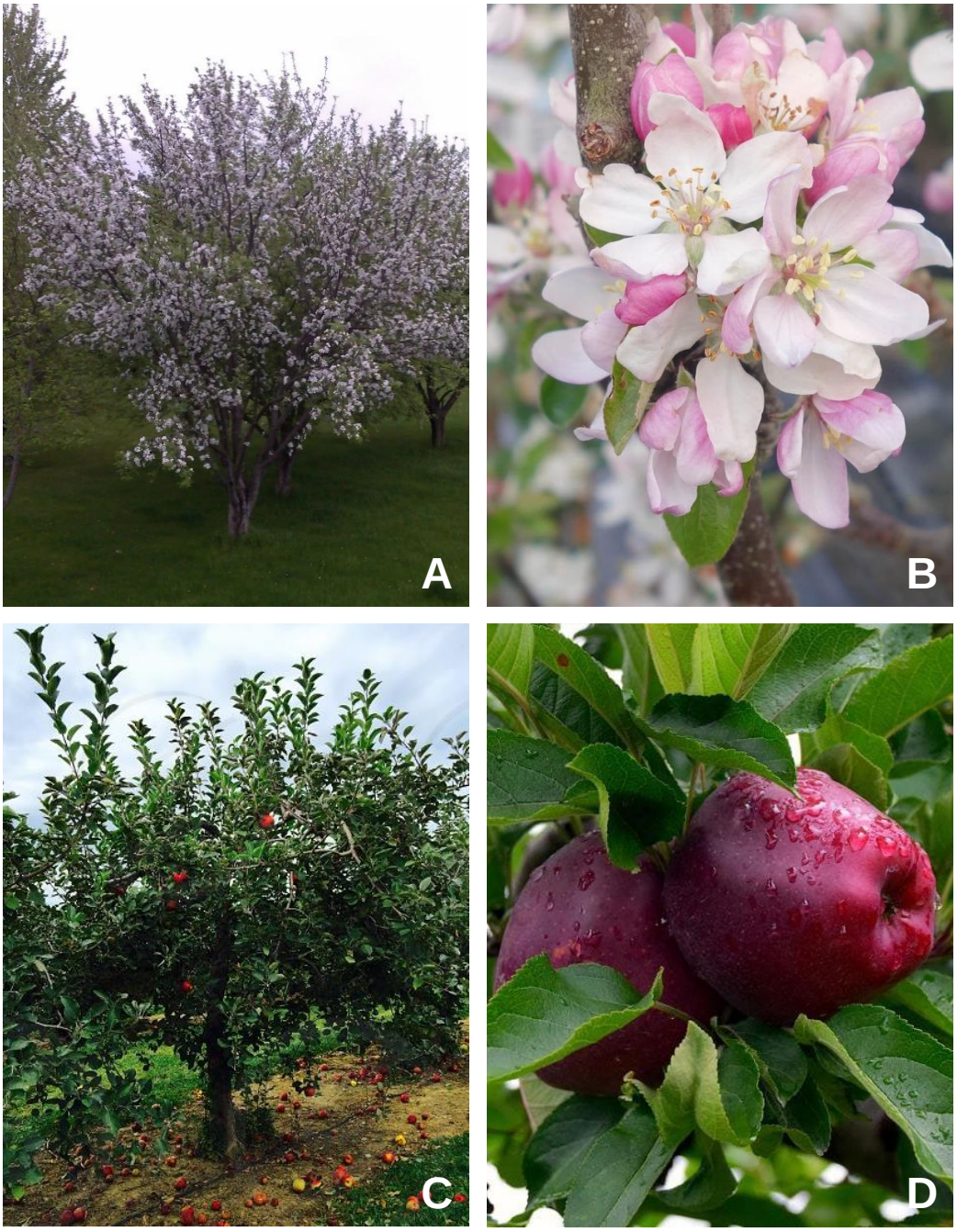


Figure I.1. Different growing stages of the Red Delicious apple tree. A: tree at full bloom; B: close-up of apple blossom; C: apple tree with mature apples; D: close-up of a fully mature Red Delicious apple. Pictures from Google Images.

I.2. Apple processing

Apple fruit is a seasonal crop, and it is consumed either fresh or as by-product. In ancient times, apples were used as medicine and it is believed that the first recipes of apple by-products such as candies, jams and syrups are found in pharmacopoeias, rather than cook-books. In ancient Europe, apples were crushed to make juice and, through a fermentation, produce cider, that was safer to drink than water, and it was believed to promote health. As well, cider was also used in the making of vinegar and apple brandy (Janik, 2011). Nowadays, in addition to cider and its derived products, a wider spectrum of apple by-products is also available in the market. Dry fruit is made by conventional drying or freeze-drying, while jams or marmalades are obtained by mixing the fruit with sugar and submitting it to high temperatures. Apple purees, apple-based infant food, clear and cloudy apple juices are made from fresh fruit or concentrate, and co-products from these process lines are available like apple pomace and aroma among others (Coelho et al., 2021; Joardder and Masud, 2019; Rocha Parra et al., 2019; Rosend et al., 2020).

In the nineteenth century, with industrialization, new technology, and advances in transportation, the organization of food production and its distribution worldwide changed. Apples were then stored under cold temperature to keep them fresh by slowing down the production of ethylene (Janik, 2011; Maya-Ambía, 2015). Currently, fresh apples can be stored up to 9 or 12 months in refrigeration chambers, being available in the market all year long and conserving their organoleptic properties. Nevertheless, some fruits do not comply the quality standards for retail due to factors like unappealing appearance, firmness, or sensitivity to bruising and therefore, are used for making apple by-products. The cosmetic quality of fruit destined to apple by-products is usually lower than for fresh consumption, but less attention has been paid to other unwanted characteristics of these fruits, such as latent fungal infections not showing visual signs (Heinmaa, 2020; Varming et al., 2013).

I.3. Fungal spoilage

Apple is susceptible to several fungal diseases which can occur either at preharvest or during postharvest. Some fungi have been indistinctly identified as typical of one or another. In contrast, others can occur in both stages or, more commonly, begin in the field and increase their incidence during the postharvest stage. The Food and Agriculture Organization from the United Nations (FAO) estimated that between 15 % and 50 % of the world production of fruits and vegetables are lost at postharvest stage, mainly due to pathogen spoilage (Gustavsson et al., 2019). In low and middle income countries, the proportion of postharvest loss is even higher, probably due to poor or low-tech availability during storage and transportation (Joardder and Masud, 2019). For apples, fungal diseases can cause significant losses; some are reported to reach up to 70 % yield reduction (Jha et al., 2009) or 80 % of decay in stored fruits (Sánchez-Torres et al., 2018).

I.3.1. Fungal pathogens

Fungal diseases can affect the leaf (leaf blotch) or the fruit (apple rot), and in both cases are responsible for significant economic losses (Harteveld et al., 2013). The identification of the primary fungal pathogens associated with this crop varies with the geographical region, climatic conditions, and agricultural practices of the growing area. Genera as diverse as *Penicillium*, *Botrytis*, *Pleospora*, *Pezicula*, *Colletotrichum*, *Stemphylium*, *Cladosporium*, *Ulocladium*, *Epicoccum*, *Coniothyrium*, *Monilinia*, *Mucor*, *Rhizopus*, *Neofabraea*, *Cadophora*, *Geotrichum*, *Glomerella*, *Sphaeropsis*, *Phoma*, *Trichothecium*, *Fusarium*, and *Alternaria* have been mentioned in the literature as responsible of apple decay (Gao et al., 2013; Konstantinou et al., 2011; Niem et al., 2007; Patriarca et al., 2019; Shtienberg, 2012). Most of them initiate the colonization when wounds or physical damage are present in the skin, either caused by insects or birds in the field, or by inadequate manipulation during postharvest.

Penicillium expansum, the causal agent of the blue rot, is one of the most frequently reported and controlled (Cozzolino et al., 2020; Sarrocco and Vannacci, 2018; Sun et al., 2019), since this fungus produces patulin, a mycotoxin with genotoxic, teratogenic, and immunotoxin properties, with both chronic and acute toxicity manifestation to animals (Ioi et al., 2017).

The mouldy core (MC) is another frequent fungal disease of apple fruit that manifests as a rotten area inside the fruit. Fungal genera like *Penicillium, Cladosporium* and *Fusarium*, but mainly *Alternaria* have been associated with MC (Basson et al., 2019; Elfar et al., 2018; Serdani et al., 1998; van der Walt et al., 2010). Consumers and producers evaluate the quality of apples by their external appearance, but the core is rarely examined, not even when apple concentrate industries select the fruit intended for processing. Additionally, when toxigenic species colonize the fruit, the negative consequences extend to a health risk associated with the accumulation of mycotoxins. The toxicological aspect is particularly relevant in processed food when contaminated apples are used as raw material since many mycotoxins can resist the conventional treatments applied in food processing and then persist in the by-products (Ji et al., 2017; López et al., 2016; Oroian et al., 2014; Zwickel et al., 2016).

I.3.2. Apple diseases caused by Alternaria

I.3.2.1. Fruit spot

On the surface of apple fruit, *Alternaria* spp. cause an infection that manifests as small brown, sunken spots, called “fruit spot” (Harteveld et al., 2014) (**Figure I.2, A**). Significant differences between apple cultivars have been observed concerning the susceptibility of infection with *Alternaria* and the consequent incidence of fruit spot. Gur et al. (2017) studied the resistance of different cultivars (cvs.) to this fungal colonization in Israel. Among Pink Lady, Red Delicious, Golden Delicious, Gala, Granny Smith, Akane, Johnny and Jonathan cultivars (cvs.), they reported the last two as the most resistant ones. Red

Delicious, Gala and Granny Smith showed moderate resistance, while Akane, Golden Delicious and Pink Lady were the most susceptible ones. These results were explained by the presence of inhibitory compounds in the peel of the Jonathan cv. since the resistance was highest close to the skin and decreased towards the seed locule. The fruits of this cultivar were resistant even when stored in cold chambers (4 - 6 °C), which is a standard postharvest handling. When comparing cultivars susceptibility, Harteveld et al. (2014) found that it was higher in Royal Gala than in FB22-47, a breed cultivar. They attributed the resistance to the smoother and shinier skin of the latter, being the cuticle thickness or cuticular wax of the fruit an influencing factor on *Alternaria* spp. infection.

Several studies have postulated that fruit injuries are required for fungal infection. Gur et al. (2017) reported that wounds in the surface and cracks circling the calyx were considered the most conducive factors to the disease. Accordingly, Elfar et al. (2018) found that *Alternaria* spp., inoculated on the epidermis of both immature and mature apples, were not capable of producing necrotic lesions on the surface or the flesh when no wound was present.

Studying the timing of infection, Harteveld et al. (2014) demonstrated that Alternaria fruit spot infection occurs around 100 days after bloom, *e.g.* about a month before harvest. However, the colonization was highly dependent on weather conditions. Rain, relative humidity, and temperature were identified as the most significant climatic factors influencing the disease. In their study, they confirmed that fruit spot mostly occurred when the temperature was below 20 °C. However, previous reports suggested that higher temperatures might induce *Alternaria* infection (Filajdic and Sutton, 1992; Thakur and Nirupma, 2010). Environmental conditions favouring fruit spot in the orchard have not been unequivocally elucidated up to now. As many other factors can interact in the fungus colonization, more studies are necessary on this matter.

I.3.2.2. Mouldy core

Even though other fungal genera have been identified as causal agents of this disease, *Alternaria* has been reported as the main one in several world regions (Gao et al., 2013; Soliman et al., 2015; Elfar et al., 2018). Multiple definitions of the term “mouldy core” coexist in the literature. Some authors refer to mouldy core and core rot as synonyms. Others distinguish between those terms, using mouldy core when the mycelium is only present in the loculus, without invading the apple flesh (mesoderm) (**Figure I.2, B**). When the mesoderm is colonised, the term core rot is preferred (Shtienberg, 2012). The American Phytopathological Society Common Names of Plant Diseases accepts the common name “mouldy core”, including both types of manifestation of the disease (APS, 2021).

Different theories have been postulated on how fungal infection occurs in the centre of apple fruit before harvest. The disease has been described as a core infection that occurs progressively as fungal spores become established on senescing blossoms during and shortly after the full bloom stage of bud development. Afterwards, the spores move through the open calycine tube into the core region of the fruit (Shtienberg, 2012). However, fungal spores may also gain entry to the seed locules via the calycine sinus at any stage of fruit development (Gao et al., 2013). Young fruit may get infected through the open calyx, and mycelia reach the seed and carpel wall in the postharvest stages (Reuveni, 2006). In particular, some varieties such as the Red Delicious have an open calyx, which creates an open channel for fungal colonization, making them more susceptible to this disease (Shtienberg, 2012) (**Figure I.2, C**).

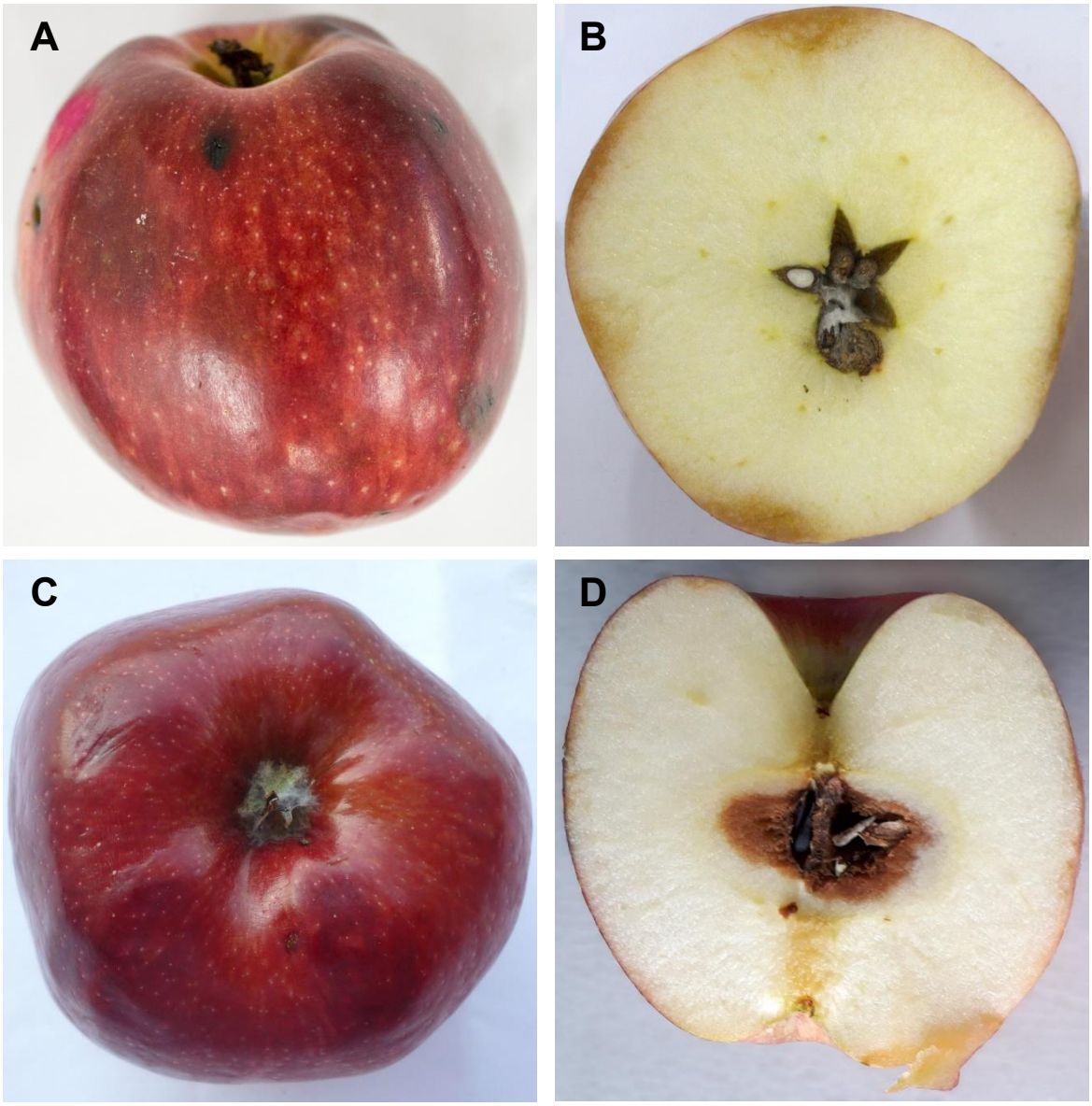


Figure I.2. Apple diseases caused by *Alternaria* spp. A) Fruit spot; B) Mouldy core; C) Calyx infected with *Alternaria* in Red Delicious apple during postharvest; D) Wet core rot. Pictures taken by María Agustina Pavicich.

MC can also be categorised as dry or wet, depending on the aspect of the flesh around the core. The dry type causes a dry appearance of the tissue, which turns dark brown in the loculus and surrounding mesoderm, and it slowly develops (Ntasiou et al., 2015). The wet type (**Figure I.2, D**) can be considered the most relevant since it expands rapidly during the postharvest stage covering the whole flesh of the fruit during storage (van der Walt et al., 2010). The tissue also becomes brown, but the texture is soft and watery. This disease has been observed to progress relatively fast, even under cold storage.

As well as for the external lesions, many factors are necessary for the development of the disease, including a susceptible cultivar, considerable inoculum and favourable environmental conditions. The incidence of *Alternaria* MC in several preharvest instances in two cultivars, Oregon Spur, a susceptible one, and Granny Smith, a resistant one, was studied (Elfar et al., 2019). At flower and fruit stages, the frequency of *Alternaria* spp. was similar in both cvs. Nevertheless, they observed significant differences in the rate of isolation of *Alternaria* spp. in the carpels of immature and mature fruits between the studied cultivars. They relate this fact to differences in the calycine tube between the studied cvs. While in the susceptible apples the tube remained open, it was closed in the resistant ones, preventing invasion.

Another factor related to cultivar susceptibility might be the content of calcium in the loculus wall. As calcium (Ca) may inhibit specific enzymatic activity required by fungi to invade plant tissues, low Ca concentrations would facilitate pathogen invasion, allowing the fungus to penetrate the mesoderm from the loculus. Levin et al. (2019) found that high core rot incidence is associated with low Ca concentration in the endocarp wall of the fruit. Accordingly, Shtienberg (2012) found that the resistant Golden Delicious cultivar had 2.6-fold higher Ca concentration in the endocarp wall than the susceptible Red Delicious.

Regarding the relationship between crop ripeness and the incidence of mouldy core, *Alternaria* spp. showed a high capacity to colonize the carpels of both immature and mature fruit when these were artificially inoculated (Elfar et al., 2018). Thus, cultivar susceptibility exerts a much more significant effect than harvest stage at the moment of infection.

Although environmental conditions have been mentioned as having a significant influence on apple diseases, Shtienberg (2012) found that other factors may prevail on MC development in the orchard. They reported that host physiology seems to be more determinant on the incidence of the disease. Other studies, testing different pathological capacities of *Alternaria* spp., concluded that MC was a necessary combination of opportunistic and favourable conditions in the field rather than specific abilities exhibited by the pathogen (Elfar et al., 2018).

It is worth mentioning that *Alternaria* species are known saprophytes (Thomma, 2003), and therefore, can decompose fallen apple fruit and apport nutrients to the soil. Apple trees planted in soil amended with apple-pomace compost showed potential to grow quickly and were able to support more fruit growth in the first years of cropping (Moran and Schupp, 2003). As well, the use of apple pomace as compost for the soil is a common fertilizing strategy amongst growers, and its application as source of nutrients for different crops together with other wastes from the apple orchard was proposed with good results (Duan et al., 2021; Hanc and Chadimova, 2014; Maldonado et al., 2021). Therefore, a possible symbiotic relationship between apple and *Alternaria*, might have been developing in orchards as an evolutionary process. Further studies are needed to evaluate this theory.

I.4. *P. expansum* and patulin legislation in apple by-products

The risk of mycotoxigenic fungi is particularly relevant when crops are destined to by-products. Apple juices are mostly made by thermal processing which aims to inactivate spoilage microorganisms and enzymes, to increase the shelf-life of the product (Aguilar-Rosas et al., 2007; Wibowo et al., 2019). However, mycotoxins are chemical and thermally stable and, therefore, can persist during storage and food processing (Köppen et al., 2010). *P. expansum* infection has been kept under surveillance by apple processing industries for many years through visual inspection of fruits incorporated to the process, since the blue rot is easily detected. *P. expansum* produces patulin (PAT), a mycotoxin with a maximum of 50 µg/kg established as limit in several apple by-products, such as apple juice, concentrated apple juice and cider, by the European Commission, the United States Food and Drug Administration, the Ministry of Health of the People's Republic of China, and Health Canada (EU Regulation 1881/2006; Zhong et al., 2018). In Brazil, the limit of 50 µg/kg of PAT applies both for apple juice and pulp (Dias et al., 2019). The European Commission established a lower limit of 25 µg/kg for non-clarified apple products such as compote and puree, and of 10 µg/kg for apple based infant food (EU Regulation 1881/2006). In Argentina, a recent limit of 10 µg/kg of PAT in solid apple based infant food was established (Res. Conjunta 09/2021 CAA Art. 156).

The pressure on industries, particularly those exporting or commercializing in high quality standards markets, has led to develop strategies to prevent, control or mitigate the presence of PAT in apple by-products. The achievement of these requirements relies on an integrated management throughout the food processing chain: proper fruit selection and handling at harvest, combined with adequate storage conditions are key factors in PAT mitigation in final products (Ioi et al., 2017). PAT levels can be considerably reduced through processing stages in the apple concentrate production; reductions of up to 75% were reported (Welke et al., 2009). Nevertheless, several studies found PAT levels in apple juices and other apple by-products exceeding the limits established by regulatory

agencies and showed that small children suffered high exposure to this mycotoxin through apple juice and nectar (Baert et al., 2006; Baert et al., 2016; Oteiza et al., 2017; Ünüsan, 2019). Information on other toxigenic genera and their mycotoxins infecting apple fruit and by-products, such as *Alternaria*, is scarce.

I.5. *Alternaria*

As discussed in section 1.3.2, the genus *Alternaria* has been identified as a frequent fungal infectant of apple fruit causing external lesions and MC disease in many regions, including Argentina (Elfar et al., 2018; Gao et al., 2013; Gur et al., 2018; D. O. C. Harteveld et al., 2014; Harteveld et al., 2013; Di Massi, 2007; Niem et al., 2007; Raj et al., 2017).

I.5.1. Alternaria *taxonomy*

Alternaria belongs to the Fungi kingdom, in the family *Pleosporaceae* (*Pleosporales, Dothideomycetes, Ascomycota*). It is a ubiquitous genus that is capable of colonizing seeds, plants, soils, and animals. The species belonging to this genus can provoke allergic reactions and have been associated to human cornea infections (Armitage et al., 2015). The optimal temperature and water activity (a_w) conditions for its growth can vary between species, being 25 °C the optimal for sporulation. Nonetheless, *Alternaria* species can grow between -3 °C and 35 °C (Sommer, 1985), which implies a risk even for food stored at low temperatures (Barkai-Golan, 2008). The best a_w for its growth is 0.99 but it can grow at a_w as low as 0.85, and the pH range for growth goes from 2.5 to 10 (Lee et al., 2015; Magan et al., 1984). *Alternaria* species are characterized for producing melanin, and therefore, have brown to black coloration that is believed to contribute to their pathogenicity (Pappas, 2012; Richardson and Moore, 2017). The conidia are big, multicellular, dark coloured, with longitudinal and transversal septa (phaeodictyospores). Conidia have a club-like shape since they are wider in the base

and gradually tapper into an elongated beak (**Figure I.3**) and are usually produced in chains, but solitary conidia are frequent.

In the past decades, *Alternaria* classification was under discussion (Lawrence et al., 2016). From 1960, E.G. Simmons studied a high number of the available isolates and described approximately 200 species by observing the shape of the conidia, being *A. alternata* the species type (Simmons, 1967). In 1992, he divided the species according to the size of the conidia in large-spored *Alternaria* (600-100 µm conidia) and small-spored *Alternaria* (<60 µm conidia) and organized the genus into species-groups (sp.-grp), gathering the isolates sharing the same sporulation pattern under standardized conditions (Simmons & Roberts, 1993). In 2007, the manual for identification of *Alternaria* was compiled describing 273 taxa organized in 13 sp.-grps (Simmons, 2007). More recently, Woudenberg et al., (2015) organized the genus in 27 sections according to molecular phylogenetic taxonomy, with *Alternaria* section *Alternaria* being the one containing most of the small-spored *Alternaria,* characterized by including toxigenic species.

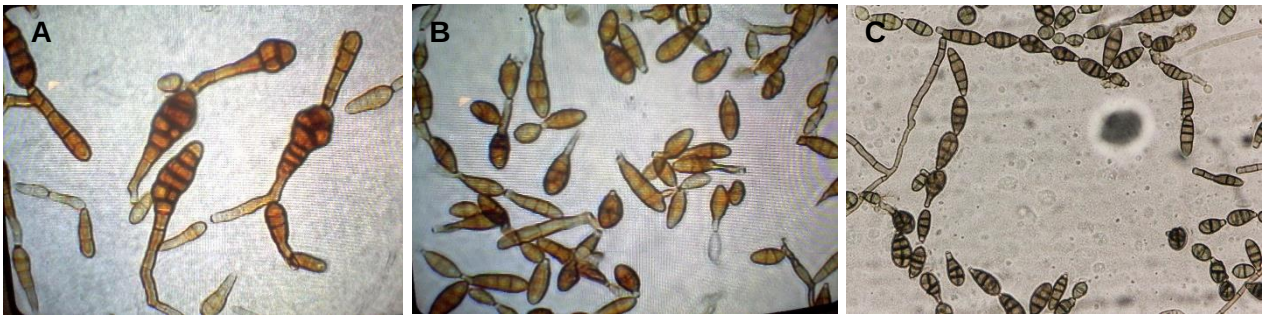


Figure I.3. *Alternaria* conidia under magnifying glass at 40X. A: *A. infectoria* species-group (sp.-grp); B: *A. alternata* sp.-grp; C: *A. tenuissima* sp.-grp. Pictures taken by Andrea Patriarca and María Agustina Pavicich.

I.5.2. Secondary metabolites

Several fungal genera have the ability to produce secondary metabolites, many of which have a beneficial contribution to human health, such as penicillin, a secondary metabolite produced by *Penicillium* species, that has antibiotic activity (Wainwright, 1989). On the contrary, other secondary metabolites of low-molecular weight produced by fungi (150-1000 Da) are known as mycotoxins since they elicit a negative effect on animals or humans (Berthiller et al., 2013; Capriotti et al., 2011). The most frequent toxigenic fungi in food are species of *Penicillium*, *Aspergillus*, *Fusarium* and *Alternaria,* and only a few of the mycotoxins they produce have been regulated (EU regulation 1881/2006, Res. Conjunta 22/2019 CAA Art. 156).

The role that the secondary metabolites play for the producing organism is not always clear. Not all the species of toxigenic genera produce toxins, and those that do usually produce them under specific conditions, such as climatic conditions, insect damage, improper crop breeding and harvesting, inadequate storage, or as a response to oxidative stress (Capriotti et al., 2011; Ünüsan, 2019).

Besides infecting plants in the field or spoiling fruits in postharvest causing economic losses, *Alternaria* species can produce more than 70 different secondary metabolites, many of which have been recognised as mycotoxins (Patriarca et al., 2019). The chemical structure of these secondary metabolites is diverse, complying molecules produced by different metabolic pathways. Some have been proved to serve as colonization factors (Graf et al., 2012). Others are host specific toxins (HST) whose biosynthetic genes are clustered on conditionally dispensable chromosomes, and are associated with pathogenicity on a particular substrate, *e.g.* AAL toxins in tomato produced by *A. alternata* f. sp. *lycopersici*, or AK-toxin I-II produced by *A. gaisen* colonizing Japanese pear. In particular, *A. mali* produces AM toxin in *Alternaria* blotch of apples (Lou et al., 2013; Tsuge et al., 2013).

The chemical structures of only a few *Alternaria* metabolites have been elucidated. Based on their structure, they can be divided in five different groups (Zwickel et al., 2016):

I. Dibenzo-α-pyrone derivatives (**Figure I.4.I**): alternariol (AOH), alternariol monomethyl ether (AME), altenuene (ALT), isoaltenuene (iso-ALT), altenuisol (ATL), among others.

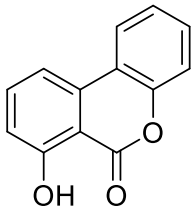
II. Tetramic acid derivatives (**Figure I.4.II**): tenuazonic acid (TeA) and isopropyl tetramic acid.

III. Perylene quinone derivatives (**Figure I.4.II**): altertoxin I, II, III (ATX-I, -II, -III), alterperylenol and stemphyltoxin-III (STTX-III).

IV. Aminopentol esters: HST produced by *Alternaria alternata* f. sp. *lycopersici* and known as the AAL toxins, *e.g.,* AAL TB1 (**Figure I.4.IV**) and TB2.

V. Miscellaneous structures: tentoxin (TEN) (**Figure I.4.V**) a cyclic tripeptide and altenuic acid-III a resorcylic acid substituted with butanolide and a second carboxylic acid in the sidechain.

I



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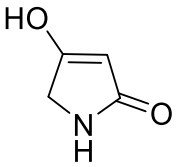
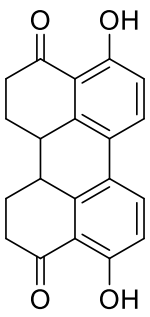
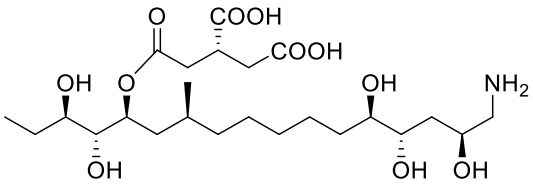


III



IV



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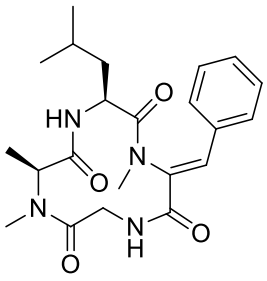

Figure I.4. Chemical structures of I: dibenzo-α-pyrone; II: tetramic acid; III: perylene quinone; IV: aminopentol ester produced by *Alternaria alternata* f. sp. *lycopersici* AAL TB1; V: tentoxin.

As well, some modified forms of AOH and AME such as sulphates and glucosides (AOH-3-S, AME-3-S, AOH-3-G, AME-3-G), that are suspected to be formed during metabolism of contaminated plants, were identified and synthesized for their screening in food commodities (Mikula et al., 2013).

Even though more than 70 metabolites from *Alternaria* are known, information regarding their toxicity is scarce, therefore, they are considered as "emerging mycotoxins". This term is used for mould metabolites which exert toxic effects but are not regulated yet by authorities, due to insufficient data on toxicity and/or occurrence (Aichinger et al., 2020).

I.5.3. Toxicity

The high chemical diversity of *Alternaria* toxins results in a complex toxicological profile. Information about the toxicity of *Alternaria* spp. was first reported by Pero et al. (1973), when some metabolites produced by the genus were shown to exert toxic effects, and several reports have been made ever since, but only on the most relevant *Alternaria* toxins namely, AOH, AME, ALT, ATX-I, -II, and TeA (Aichinger et al., 2018; Crudo et al., 2019; Ostry, 2008; Vejdovszky et al., 2017b). Few reports have been made for the other members of the *Alternaria* metabolome, constituted by the group of metabolites and low molecular weight intermediates present in a cell, tissue or organ (Forcisi et al., 2013). Moreover, most studies have been done *in vitro*; *in vivo* toxicological data are currently scarce and, therefore, not sufficient to define toxicological standard values for the establishment of maximum limits in food and feed (Crudo et al., 2019).

In human cells, the dibenzo-α-pyrones AOH and AME induced DNA strand breaks in the Comet assay (Fehr et al., 2009), were clastogenic and exerted mutagenic potential

(Lehmann et al., 2006; Brugger et al., 2006). AOH influenced inflammatory responses (Kollarova et al., 2018; Solhaug et al., 2016) and had endocrine disruptive potential as an agonist for human oestrogen and androgen receptors (Lehmann et al., 2006; Stypuła-Trębas et al., 2017), as well as the ability to modulate innate immunity (Kollarova et al., 2018). An *in vivo* study on mice did not find AOH to cause systemic DNA damages in liver tissue and bone marrow (Schuchardt et al., 2017). However, toxicity of AOH would probably be limited to the gastrointestinal tract due to poor bioavailability, and more *in vivo* studies, focused on the gastrointestinal tract were recommended (Solfrizzo, 2017). With regards to their potential carcinogenic properties, mice fed for 10 months with 50-100 mg of AME per kg of body weight (BW) developed precancerous changes in their oesophageal mucosa (Yekeler et al., 2001), and their presence has been related to high levels of oesophageal cancer in China (Ostry, 2008). Additionally, it was recently described how lipophilicity of the alternariols led to accumulation into microbial pellets, possibly via interaction with bacterial walls, and affected the growth of the gut microbiota and their ability to produce biofilms, thus influencing the composition and activity of the microbial community inhabiting human intestine (Crudo et al., 2021).

Concerning other dibenzo-α-pyrones, ALT and iso-ALT did not affect topoisomerase activity probably attributed to a more planar structure than the other related compounds (Fehr et al., 2009), and therefore did not present genotoxicity. As well, studies on the toxicity activity of ALT on *Artemia salina* showed a 50% lethal concentration dose superior to that of AOH and TeA (Panigrahi and Dallin, 1994). In the same study, ATX-I from the perylene quinone family was more toxic than ALT and had cytotoxic and genotoxic activity in human cells (Fehr et al., 2009). ATX-II by far exceeded the genotoxic potential of AOH and AME and represented one of the main genotoxic compounds in extracts from *Alternaria alternata* contaminated rice (Schwarz et al., 2012). As well, its structurally related compound, STTX-III, also carrying an epoxide group that might be able to react with different macromolecules, including the DNA

(Dellafiora et al., 2018), was found to be more mutagenic than AOH (Puntscher et al., 2019). Both were reported to induce DNA strand breaks *in vitro* and to act as topoisomerase poisons and inhibitors (Fehr et al., 2009; Fleck et al., 2014).

TeA was phytotoxic and could cause damage to host plants and thus, ease plant infection by *Alternaria* (Kang et al., 2017). On mammalian cells, it exerted mild toxic effects that are mainly attributed to inhibiting the release of proteins from the ribosome (Vejdovszky et al., 2016). Although low toxicity of this mycotoxin has been reported *in vitro* (Schwarz et al., 2012; Zhou and Qiang, 2008), *in vivo* studies carried out on several animal models highlighted more severe effects than the alternariols such as emesis, tachycardia and haemorrhages in mice, dogs and monkeys (Fraeyman et al., 2017). For this reason, it is considered the most acute toxicant among the *Alternaria* mycotoxins (Asam and Rychlik, 2013). A study on chickens found that the application of 1.25 mg/kg BW over 3 weeks caused adverse effects, although it did not increase mortality (Giambrone et al., 1978). In mice and rats, LD_{50} values between 80 and 225 mg/kg of BW were established for TeA (Miller et al., 1963; Smith et al., 1968). In dogs, it caused haemorrhages in several organs at daily doses of 10 mg/kg BW, and in chickens sub-acute toxicity was observed with 10 mg/kg in feed, reducing its efficiency, suppressing weight gain and increasing internal haemorrhaging (Ostry, 2008). Precancerous changes were observed in oesophageal mucosa of mice fed 25 mg/kg BW per day of TeA for 10 months (Yekeler et al., 2001). Moreover, TeA was associated with the human haematological disorder known as Onyalai (Ostry, 2008).

Mycotoxins, as other toxic compounds, can have additive, synergistic or antagonistic effects in the body but data on combined toxic effects are in general limited (Capriotti et al., 2011). An additive effect implies that the final toxicity equals the sum of the individual toxic effects of compounds; a synergistic effect, means that the resulting total toxicity is greater than the sum of individual effects; and an antagonistic effect, when the

combinatory effect is less than the sum (Chou, 2006). Few studies have been made on co-toxicity of *Alternaria* mycotoxins; AOH and AME were found to impact cell viability of colon carcinoma cells in an additive way (Bensassi et al., 2015), and AOH and ATX-II led to cumulative cytotoxic effects in HepG2, HT-29 and HCEC-1CT cells (Vejdovszky et al., 2017). Furthermore, extracts of *Alternaria alternata* cultured on rice were found to exceed the cytotoxic and genotoxic effects of single compounds, pointing to a synergistic toxic activity (Crudo et al., 2019). The combinatory effects of mycotoxins could serve as an explanation for epidemiologically observed biological activities which cannot be linked to effects of a single compound, such as worldwide increases in infertility, early onset of puberty or raising breast cancer rates (Diamanti-Kandarakis et al., 2009).

I.5.4. Alternaria *mycotoxins detection*

Precise quantification of food contaminants is of high relevance, since economic and health related decisions are made upon these results. Therefore, many techniques have been developed to detect and quantify mycotoxins in food. Extraction techniques with organic solvents or a mixture of organic solvents and water, followed by a clean-up step, and combined with chromatography are the most popular ones. For *Alternaria* mycotoxins in apple and apple by-products, several procedures have been developed using techniques that vary from very laborious extractions followed by high-performance liquid chromatography (HPLC) coupled to a UV detector to “dilute and shoot” followed by ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) (Broggi et al., 2013; Gotthardt et al., 2019; Hövelmann et al., 2016; López et al., 2016; Scott and Kanhere, 2001). With a broader access to mass spectrometry and mycotoxin standards, easier and faster techniques have been under development in the past 20 years. Anastassiades et al., 2003 first developed the QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) methodology for pesticides in food, and the first use of QuEChERS for mycotoxins was reported by (Sospedra et al., 2010). In the Centre of

Excellence in Mycotoxicology and Public Health (CEMPH), a validated methodology for apple by-products using QuEChERS, and followed by UPLC-MS/MS with matrix matched calibration curves (MMCC) for the quantification of 10 free and modified *Alternaria* mycotoxins was developed (Walravens et al., 2016), and later, applied in this PhD thesis. The matrix effect influences sensitivity and therefore, MMCC are constructed from non-contaminated matrix and spiked with standards to produce a calibration curve that gives superior results when compared to an external calibration curve generated without matrix (Turner et al., 2015).

Besides quantification of mycotoxins in food, chemotaxonomy mostly based on high resolution mass spectrometry (HRMS) is a valuable tool for species segregation, improving the holistic characterization of the fungal metabolome (Gotthardt et al., 2020). It provides a snapshot of different classes of compounds produced by the fungi. The approach can be either targeted when it is directed to the detection of specific classes of compounds or non-targeted when the aim is to study the widest possible range of compounds (Forcisi et al., 2013).

I.5.5. Alternaria *mycotoxins in apple*

Some of the *Alternaria* mycotoxins have already been detected in naturally contaminated apples (López et al., 2016; Puntscher et al., 2020). Hence, these metabolites represent a toxicological threat when the fruit is destined to processing. Moreover, in a recent risk assessment on the European population by EFSA, it was shown that the exposure to *Alternaria* mycotoxins was high (Arcella et al., 2016). However, out of the 400 and increasing mycotoxins known to date, only a few are regulated (Righetti et al., 2016) and in particular, no limits have been established for *Alternaria* toxins in apple products yet. The relevance of these metabolites and their implication to human health have been disregarded by producers and food safety control agencies (Patriarca, 2019). Nonetheless, EFSA requested the generation of more data on the occurrence of

Alternaria toxins in fruit and fruit products, mainly destined for infants and young children. Therefore, a comprehensive study from field to fork on the fungal diversity, toxigenic capacity, natural occurrence, risk assessment and control strategy of *Alternaria* in apples is imperative.

I.6.Objectives

I.6.1. General Objective

The general objective of the present PhD work was to evaluate and provide insights to the evolvement from field to fork of *Alternaria* populations in apple fruit in Argentina, establishing the risk of exposure to its mycotoxins and providing a control strategy.

I.6.2. Specific Objectives

I. Characterize the fungal infection of apple fruit in Argentina evaluating their evolvement from field to process, with main interest on causal agents of mouldy core and *Alternaria* species. (Chapter II)

II. To perform a morphological and chemical characterization of the *Alternaria* population of apples grown in Argentina. (Chapter III)

III. To identify the most common secondary metabolites produced by this population, including mycotoxins and related compounds, using liquid chromatography coupled to high resolution mass spectrometry (LC/HRMS). (Chapter III)

IV. To evaluate the production of known *Alternaria* mycotoxins, namely AOH, AME, ALT, TeA, TEN, ATX-I, and ATX-II, and two modified forms of AOH, alternariol 3-sulphate (AOH-3-S) and alternariol 3-glucoside (AOH-3-G) and two of AME, alternariol monomethyl ether 3-sulphate (AME-3-S) and alternariol monomethyl ether 3-glucoside (AME-3-G) in apple under different environmental conditions simulating harvest and post-harvest conditions. (Chapter IV)

V. To evaluate the effect of the apple concentrate process on the natural contamination levels of 10 key *Alternaria* mycotoxins (AOH, AME, ALT, TeA, TEN, ATX-I, AOH-3-S, AOH-3-G, AME-3-S, AME-3-G). (Chapter V)

VI. To analyse the natural occurrence of free and modified *Alternaria* mycotoxins in clear and cloudy apple by-products from the Argentinean market. (Chapter VI)

VII. To perform a risk assessment from the consumption of these products, based on the most relevant *Alternaria* mycotoxins, for the Argentinean population from 6

months to 5 years old. (Chapter VII)

VIII. To set the bases of a method for a control strategy based on LC/HRMS to detect apples infected with *Alternaria* spp. and their mycotoxins to prevent their incorporation into the process line. (Chapter VIII)

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CHAPTER II

FUNGAL INFECTION OF APPLE FRUIT

Redrafted from: Pavicich, María Agustina; Cárdenas, Paola; Pose, Graciela; Fernández Pinto, Virginia; Patriarca, Andrea. "From field to process: How storage selects toxigenic *Alternaria* spp. causing mouldy core in Red Delicious apples". (2020) *International Journal of food Microbiology*, 322, 108575. https://doi.org/10.1016/j.ijfoodmicro.2020.108575

Ilustration by Tanja Meyer

II.1. Introduction

Argentina produced 539 and 595 thousand tons of apple (*Malus domestica*) and pear (*Pyrus communis*), respectively in 2019. The Argentinean production of pome fruits is concentrated in the region of Alto Valle de Río Negro, in Patagonia. About 85 % of the apple production is located in this area. A 22 % of this harvest is destined to export trade, 28 % to fresh consumption, and the remaining 50 % is industrialized into apple concentrates and other by-products (Bruzone, 2019). For pears, this region concentrates 75 % of the total production, from which 59 % is destined to export trade, 14 % to fresh consumption and 27 % to industry.

The apple concentrate industry works throughout the year to satisfy the local market and export abroad in off-season. Therefore, the fruit incorporated into the process line could be either freshly harvested or stored in refrigerated chambers for up to nine to twelve months. In addition, several concentrate industries process fruit which suffered damage due to unfavourable weather conditions or were discarded for fresh consume, either due to low demand or because they do not fulfil the quality requirement for retailing (Idigoras, 2014). Apple concentrate is the main product of these industries, but they also produce apple-based mix-fruit concentrates destined to different by-products, in which pear is usually incorporated.

Pome fruits are susceptible to fungal infection in the field as well as in the postharvest stage, with the consequent spoilage and severe economic losses. For apple, most decay, such as the blue rot caused by *Penicillium expansum,* is initiated at wound sites, *e.g*., cuts and stem punctures, and is evidenced by lesions in the exterior of the fruit. However, the MC manifests as a rotten area inside the fruit.

Species of several fungal genera have been described as causal agents of MC worldwide, such as *Alternaria, Cladosporium, Botrytis, Epicoccum, Fusarium, Mucor, Penicillium* and *Phoma* (Gao et al., 2013; Serdani et al., 1998; van der Walt et al., 2010).

In particular, *Alternaria* has been reported as the main one in several world regions (Aedo, 2018; Gao et al., 2013; Soliman et al., 2015) including Argentina (Di Masi et al., 2011).

For pears, species of *Penicillium*, *Neofabraea*, *Cadophora*, *Alternaria*, *Cladosporium*, *Botrytis*, *Glomerella*, and *Phytophthora* amongst others, have been reported as fungal infectants of the surface of the fruit (Dobra et al., 2011; Sardella et al., 2016; Wenneker et al., 2016a; Zambounis et al., 2020). In the past recent years, the complex *Alternaria*-*Cladosporium* has been gaining attention since it produced major economic losses in the Northern Patagonia region (Lutz et al., 2017). Unlike MC in apples, scarce information regarding fungal contamination of the interior of pear fruit is available (Kadowaki et al., 2012).

A deep knowledge on fungal infection of apple fruit and the main etiological agents of MC is necessary to implement adequate control strategies to diminish fruit spoilage and the consequent accumulation of toxic metabolites in the fruit and by-products. It can only be achieved through a comprehensive analysis from field to process since storage conditions might influence the mycobiota of the fruit in postharvest stage, selecting those pathogens better adapted to the environment of the storage chambers. Moreover, a full understanding of the fungal population and its distribution in the inner and outer parts of the fruit is relevant given that apple concentrate industries usually perform a visual inspection to prevent decayed fruit entering the process line. External spoilage can be spotted, allowing the removal of infected fruit before processing, while internal spoilage may remain undetected. On the other hand, as pears are usually incorporated into mix-fruit concentrates, together with apples, it is necessary to broaden the information about fungal infectants and MC of pear fruit destined to industrial food processing in Argentina, which so far is scarce.

Additionally, when apples and pears are infected with species of mycotoxigenic fungi such as *Alternaria,* a potential health risk for consumers is implied. Thus, it is necessary to understand the evolution of fungal infectants, specially the mycotoxigenic ones, during the storage period, particularly when they are destined for industrial food processing.

The objectives of this chapter were: 1) to characterize the fungal infection of apple fruit in Argentina evaluating their evolvement from field to process, with main interest on causal agents of MC and *Alternaria* species; 2) to characterize the fungal infection of pear fruit destined to industrial food processing.

II.2. Materials and methods

II.2.1. Samples

A total of 240 apples of the Red Delicious variety, grown in the Alto Valle of Río Negro region, Patagonia, Argentina, were randomly collected and analysed. From this total, 140 were intended for fresh consumption (C), and were obtained immediately after harvest. The remaining 100 apples were destined to industrial food processing (I) and had been previously kept in a refrigeration chamber (0-4 ºC) for 9 months. The first set (C) was provided by the Central Market of Buenos Aires city (Mercado Central de Buenos Aires), where most of the national fruit production is concentrated for its distribution to retail local markets. Sampling of this set of fruit was performed by the Vegetable Safety Laboratory from the Buenos Aires Central Market, including fruit with visible external damage as well as intact fruit. The second set (I) was provided by a fruit processing industry from Patagonia with high export volume, and fruits were taken from the reception point of the process, by random selection. The same processing industry provided a total of 45 pears from the Abate Fetel variety, grown in the Alto Valle region randomly selected. These pears were destined to fruit concentrate and were kept in a refrigeration chamber for 2 months prior to analysis.

II.2.2. Isolations

When external spoilage was evident in apple and pear fruits, a portion of damaged vegetal tissue was cut and transferred to Dichloran Cloramphenicol Malt Extract Agar (DCMA) plates and incubated for 7 days at 25 °C for fungal identification. Afterwards, each fruit was superficially disinfected with ethanol (70 %) and cut in half to evaluate the presence of MC. When the disease was detected, a portion of the centre of the fruit was also placed in DCMA plates and incubated for 7 d at 25 ºC.

II.2.3. Mouldy core grade of apple fruit

When MC was detected in apple fruit, the grade and type of the disease was classified according to guidelines issued by the National Institute of Agricultural Technology (INTA) for producers (**Figure II.1**). According to these guidelines, the MC was classified as wet or dry, and it was graded in a scale from 0 to 4 as follows: 0, symptomless fruit; 1, seed or carpel affected by mycelium or rot; 2, pulp affected by rot up to vascular bundles; 3, rotten pulp, exceeding vascular bundles, but without reaching the exterior; 4, rot extending from core to the exterior of the fruit.

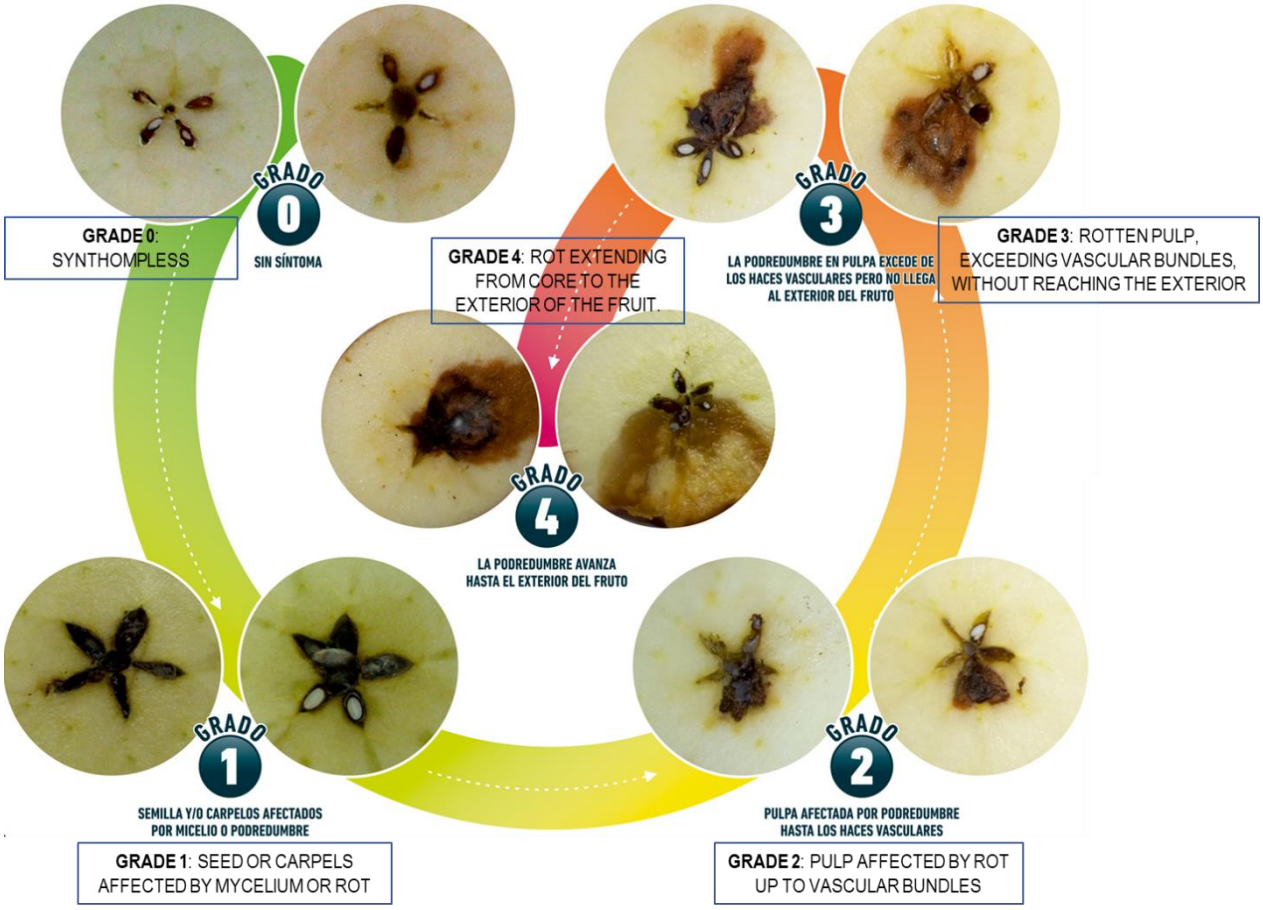

Figure II.1. Guidelines issued by the National Institute of Agricultural Technology (INTA) for mouldy core of apple fruit classification, being 0, symptomless fruit; 1, seed or carpels affected by mycelium or rot; 2, pulp affected by rot up to vascular bundles; 3, rotten pulp, exceeding vascular bundles, without reaching the exterior; 4, rot extending from core to the exterior of the fruit. Adapted picture from INTA: https://inta.gob.ar/documentos/corazon-mohoso-de-la-manzana-0

II.2.4 Identification

The fungal genera were identified based on their morphological characteristics according to Pitt and Hocking (2009) and Samson et al. (2010). Briefly, each isolate obtained from the DCMA plates was incubated in Czapek yeast extract agar (CYA), malt extract agar

(MEA), and 25 % glycerol nitrate agar (G25N) at three points, equidistant from the centre and the edge of the plate and from each other. All the media were incubated at 25 °C for 7 days and CYA plates were also incubated at 35 °C and 5 °C for 7 days. After the incubation period, a microscopic slide preparation from each isolate with a drop of lactic acid was done and observed under 400X magnification and identified following the dichotomous keys. The isolates showing typical *Alternaria* species characteristics such as black, grey, brown, or greenish colour, with cottony or velvety aspect were transferred to V8 agar for further identification to sp.-grp. level.

II.3. Results

II.3.1. Fungal infection of apples for retail and industry

The frequency of infection, type of lesions (external or MC) and MC grade varied with the apple's destination (**Figure II.2**). From the 140 apples for fresh consumption (C), 120 (86 %) showed external fungal lesions, and only 20 (14 %) were undamaged. The incidence of MC was 34 % within this group of apples, and the 48 fruits affected by this disease were also externally contaminated.

On the other hand, the frequency of infection was higher for industrial food processing apples (I); from a total of 100 fruit, only 3 were undamaged, 48 % showed external lesions only, and 51 % were affected by MC, with 2 apples presenting external lesions as well.

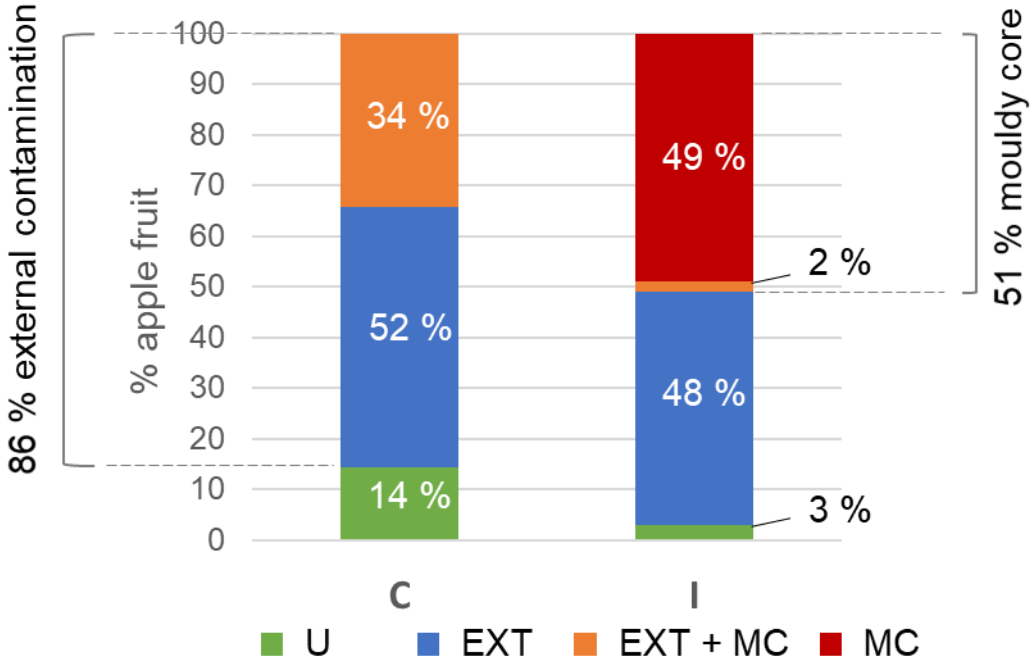

Figure II.2. Type of lesions observed in apple fruit recently harvested and destined for fresh consumption (C) and fruit for industrial food processing (I), previously stored at 0-4°C for 9 months. U: undamaged; EXT: external lesion; MC: mouldy core.

II.3.1.1. Mouldy core classification

The MC grade was 1 for all the C fruit affected by the disease and corresponded to the dry type. Higher grades of MC were observed among the I apples; 39 were classified as grade 1, 11 as grade 2 and one as grade 4. All fruit presented dry MC, except for the grade 4 one, which was of the wet type. **Figure II.3** shows the MC grades detected in the I fruit.

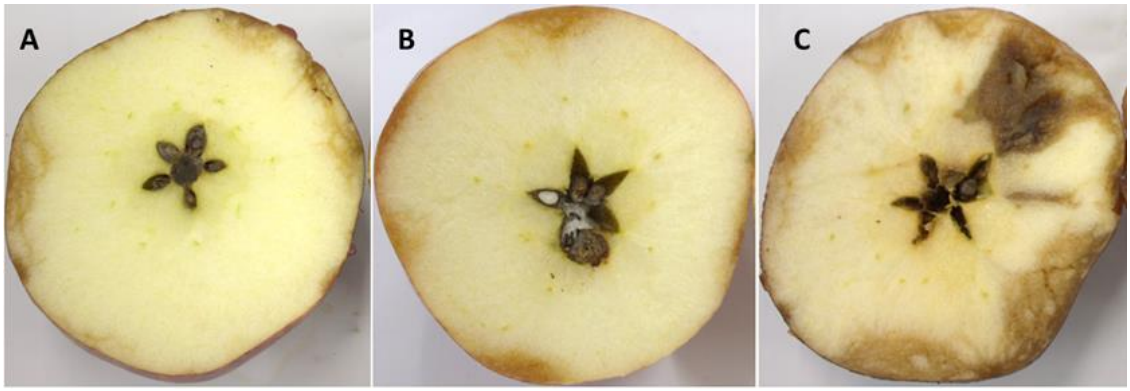

Figure II.3. Mouldy core grades and types observed in apple for industrial food processing (stored at 0-4 °C for 9 months). A) grade 1, dry; B) grade 2, dry, C) grade 4, wet MC. Pictures taken by María Agustina Pavicich.

II.3.1.2. Distribution of apple mycota

The fungal genera isolated from apples for fresh consumption (C) and industry (I), discriminated according to the type of lesion (external or MC), are shown in **Figure II.4**.

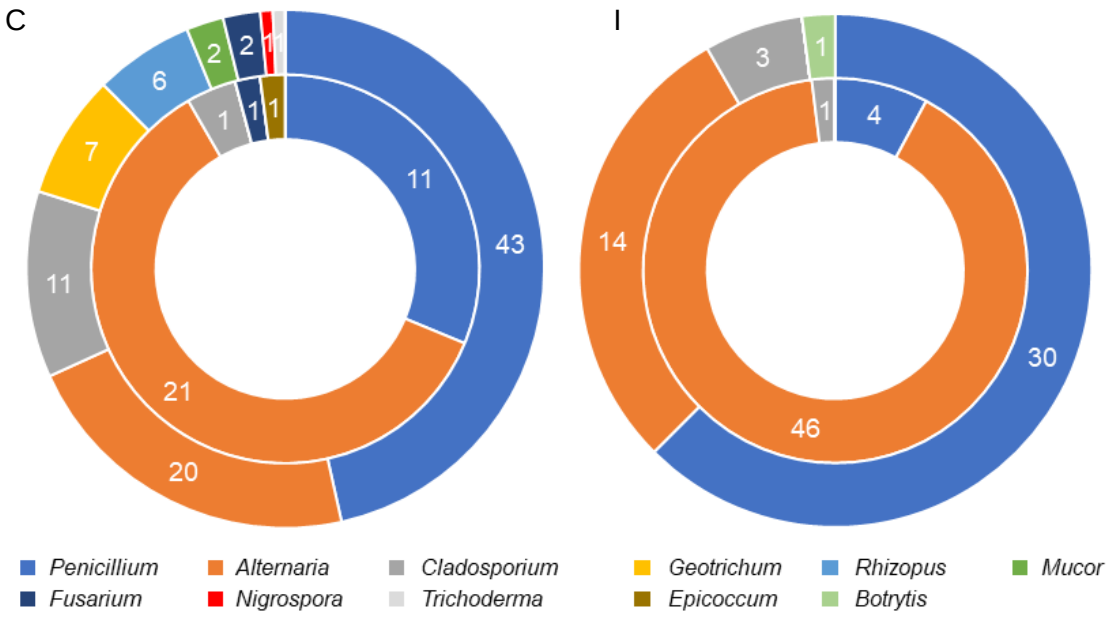

Figure II.4. Percentage of apples infected with each fungal genus. C) recently harvested fruit intended for fresh consumption, I) apples for industrial food processing stored at 0-4 ºC for 9 months. External circle: external infection; internal circle: mouldy core disease.

The predominant genera infecting both types of apples were *Penicillium* and *Alternaria.* *Penicillium* was the most frequent genus infecting C fruit (54 % infection), and it was mainly isolated from external lesions (43 % infected fruit); only 11 % of these apples were affected by MC caused by this genus. *Alternaria*, the second most frequent infectant (41 % infection), was isolated in similar proportions from both external lesions (20 %) and MC (21 %). The remaining genera were found only in external lesions, except for *Cladosporium*, *Fusarium*, and *Epicoccum*, which were isolated from MC in low proportions (1.4, 0.7, and 0.7 % of MC infection, respectively).

A considerable reduction in fungal diversity was observed in apples for industrial food processing (I). The contamination was associated only to 4 genera, *Alternaria*, *Penicillium*, *Cladosporium* and *Botrytis.* **Figure II.5** shows typical external lesions caused by these genera.

Alternaria was the predominant genus (60 % infection), and most of the strains were isolated from MC (46 % infection); only 14 % fruit were externally infected by this genus. On the other hand, *Penicillium* became second in frequency (34 % infection), but most of these isolates originated from external lesions of the fruit (30 %), with considerably lower incidence in MC (4 %).

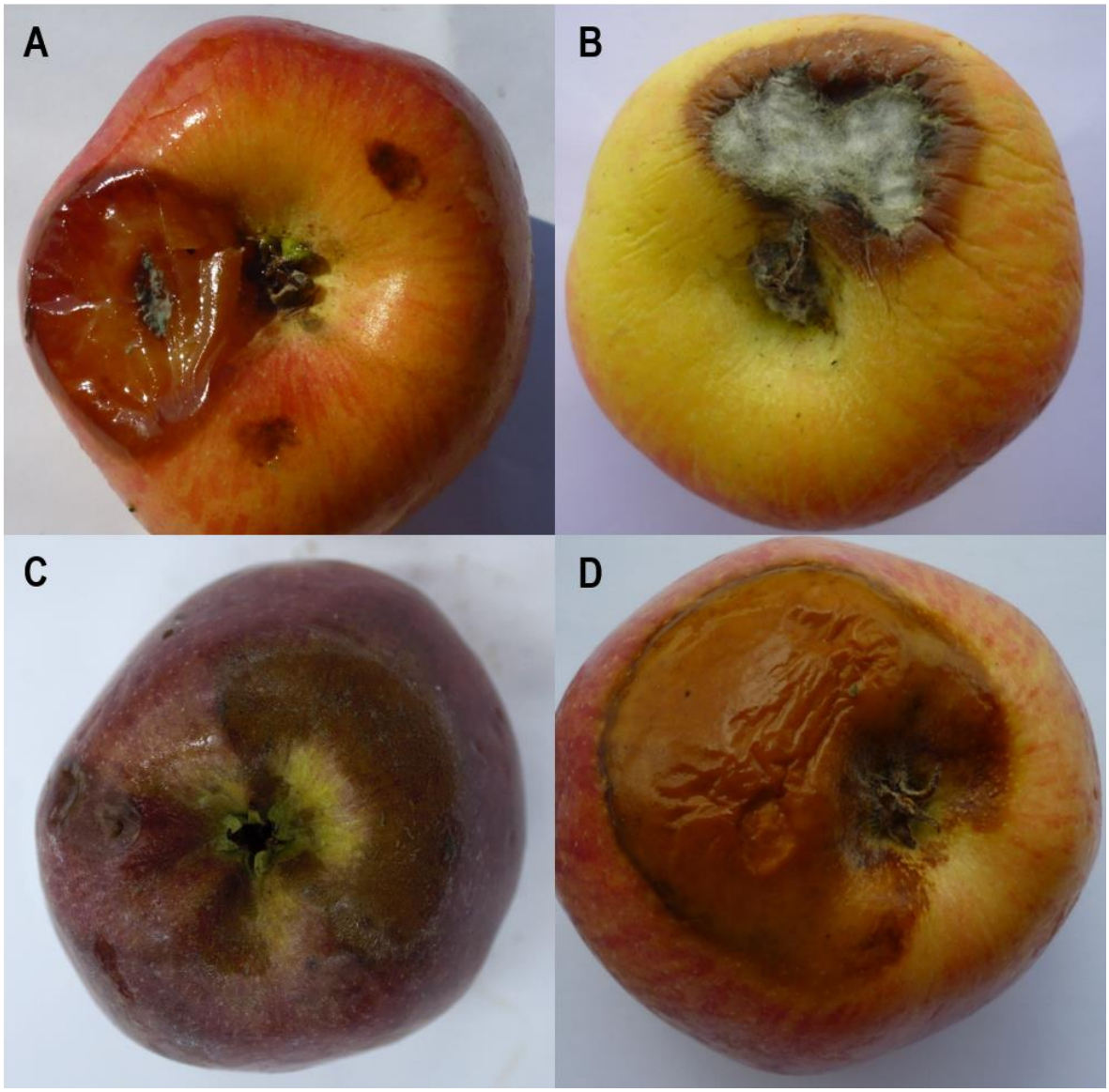

Figure II.5. Apple fruit destined to industrial food processing exhibiting typical lesions of A) *Penicillium* sp., B) *Alternaria* sp., C) *Cladosporium* sp., D) *Botrytis* sp. Pictures taken by María Agustina Pavicich.

II.3.2. Fungal infection of pears for industry

All the 45 analysed pears showed symptoms of external damage such as bruises and punctures. Of the total, 30 (67 %) showed obvious signs of fungal deterioration and a total of 33 mould strains were isolated. **Figure II.6** shows the fungal genera isolated from pears destined to industrial food processing, discriminated according to the type of lesion (external or MC).

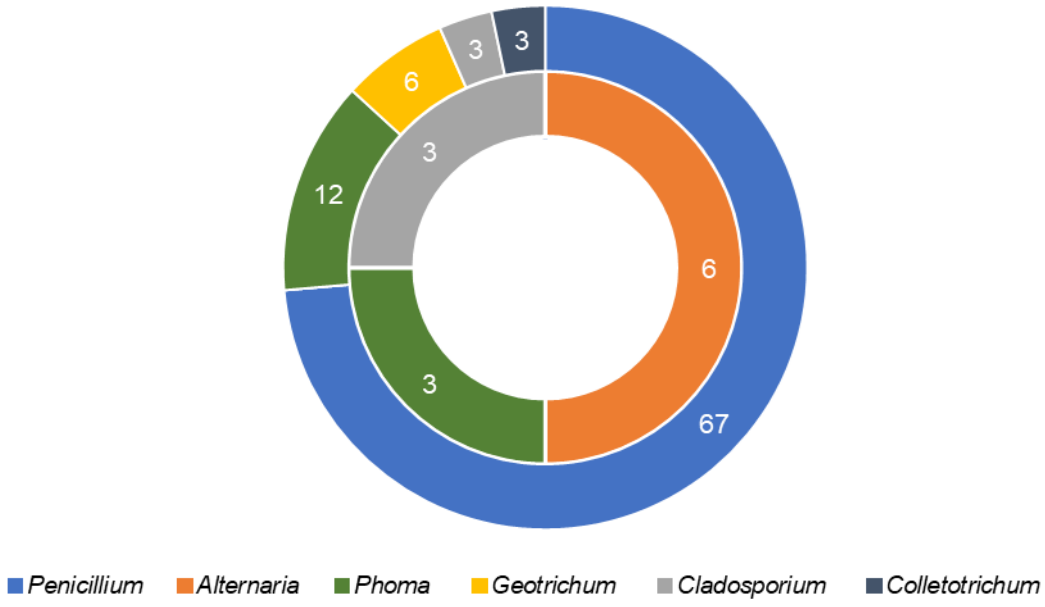

Figure II.6. Percentage of pears destined to industrial food processing infected with each fungal genus. External circle: external infection; internal circle: mouldy core disease.

The genera isolated from the fruit surface were *Penicillium* (67 %), *Phoma* (12 %), *Geotrichum* (6 %), *Cladosporium* (3 %) and *Colletotrichum* (3 %). From the centre of the fruit, *Alternaria* (6 %), *Cladosporium* (3 %) and *Phoma* (3 %) were isolated. **Figure II.7** shows typical infection with some of these genera.

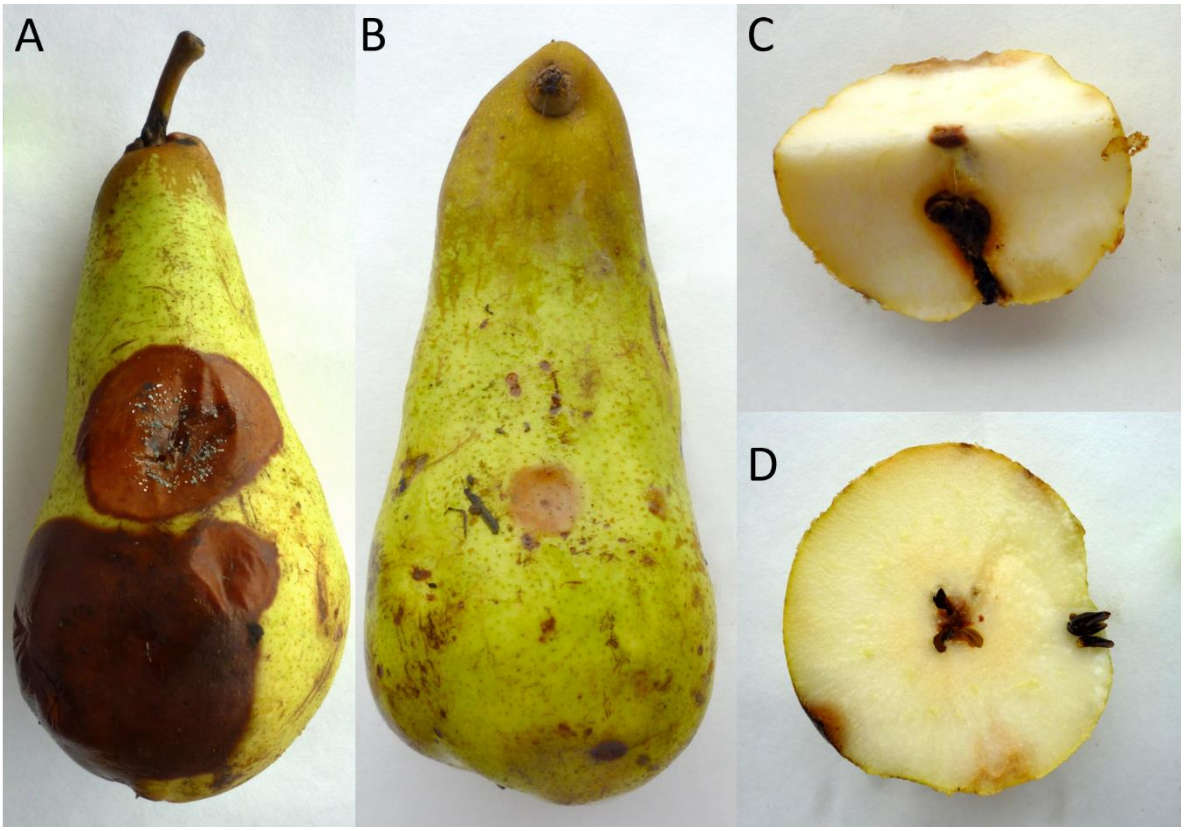

Figure II.7. Pear fruit destined to industrial food processing exhibiting typical lesions of A) *Penicillium* sp., B) *Colletotrichum* sp., C) *Alternaria* sp., D) *Phoma* sp. Pictures taken by María Agustina Pavicich.

II.4. Discussion

Alternaria and *Penicillium* were confirmed as the most frequent fungal genera infecting apples cultivated in the region of Alto Valle de Río Negro, Patagonia. *Penicillium* spp. have been reported as the main cause of postharvest decay in apple fruit worldwide (Andersen et al., 2004; Ballester et al., 2015; Janisiewicz and Korsten, 2002; Logrieco et al., 2003). Nevertheless, *Alternaria* spp. have also been identified among the most relevant causal agents of postharvest rot in apples (Harteveld et al., 2013; Naureen et

al., 2009). Even though these two genera are the most reported ones, several others have been mentioned in relation to apple fruit spoilage in a series of studies from all over the world. A study on 424 samples from 6 cultivars in the United States, pointed to *Alternaria, Cladosporium*, *Penicillium* and *Fusarium,* as the main infectants of this fruit (Tournas and Uppal Memon, 2009). In Greece, Konstantinou et al. (2011) reported *Penicillium, Botrytis* and *Alternaria* as the most common genera infecting Red Delicious variety apples. Wenneker et al. (2016) identified *Neofabraea* as the main pathogen in apples from the Netherlands, although *Botrytis* spp., *Penicillium* spp., *Fusarium* spp., *Alternaria* spp., and *Cladosporium* spp. were also isolated from fruit rot. These differences observed among countries can be attributed to the varieties cultivated in each region, as well as weather conditions and agricultural practices. Despite the discrepancies, *Penicillium* and *Alternaria* are considered important apple pathogens across the globe. These results should be of concern since both genera include species capable of synthesising mycotoxins.

When analysing the fungal population of apple fruit from the field (C) to industrial food processing (I), the storage period reduced fungal infectants from a high biodiversity found in recently harvested apples, to a less diverse population consisting mostly of toxigenic fungal genera. Regarding MC, *Alternaria* was found as the main causal agent in the field and during the postharvest stage, but its relative incidence increased during storage. Since the disease is not detected by the visual inspection performed by processing industries, infected fruit are likely to be incorporated into the process line, with a consequent risk of the presence of *Alternaria* toxins in apple by-products.

Regarding pears, all the fruit analysed showed external damage such as bruises and punctures. This is commonly due to this fruit's little turgidity and firmness and is usually observed when poor postharvest handling practices are applied (Garcia, 1995). From the total fruit analysed, 30 (67 %) were infected with fungi and 24 (53 %) presented

infection with at least one strain corresponding to the mycotoxin-producing genera *Penicillium* and *Alternaria*. These have been also reported as pear infectants in Greece (Zambounis et al., 2020) and Malta (Muscat et al., 2020). The genus *Colletotrichum* was isolated only from pear fruit, and is commonly reported as causal agent of the bitter rot disease in pear at the beginning of ripening (Sardella et al., 2016). Despite this, the mycota from apple and pear fruits for industrial food processing were similar, suggesting that either the fungal community in the apple and pear orchards are related, or that these genera get selected in the refrigeration chambers over non-cold-tolerant fungi. The latter has been observed in pears stored for 9 months in refrigeration chambers with *Botrytis cinerea* and *Phytophthora* spp.; even though the pathogens were present in low frequencies in the orchard, the infection remained latent until the conditions were favourable for their development during storage (Sosa et al., 2016).

A comprehensive review of fungal diseases affecting pear fruit has been made (Sardella et al., 2016), but the information regarding infection in the interior of the fruit is scarce. In the present PhD thesis, even though *Alternaria* was isolated only from the centre of the fruit, the incidence of MC in pear destined to industrial food processing was low. A possible explanation would be the low incidence of *Alternaria* in pears (6%) and the fact that MC caused by *Alternaria* seems to worsen by long periods of storage, and these fruits were stored in chambers for a shorter period. In addition, as opposed to apples from the Red Delicious variety which have an open sinus, pears from Abate Fetel variety have a closed one, which could prevent the infection in postharvest stages. Nevertheless, fungal infection in fruit is a dynamic scenario that should be monitored, especially for mycotoxigenic genera when fruit is destined to industrial food processing, to prevent mycotoxin contamination in fruit by-products.

Several quality standards, shared among industries, are applied at different stages along the distribution chain from growers to retailers (Jaeger et al., 2016). In line with this

purpose, industries perform quality controls to prevent that fruit with fungal spoilage are incorporated into the process line (Kadowaki et al., 2012). Despite the widespread development of automated methods for measuring the quality of fruit (Brosnan and Sun, 2004; Hu et al., 2019; Opara et al., 2007; Zhao et al., 2021), most fruit concentrate industries in Argentina still rely on visual inspection for the detection of fungal diseases in the fruit. Blue rot, caused by *P. expansum*, is believed to be mostly controlled in apples for industrial food processing; as the lesion develops most frequently in the exterior of the fruit, rotten apples are easily detected and removed from processing. Besides, the exporting industries need to fulfil the requirements imposed by destination countries, which establish a limit for PAT in apple concentrate and other by-products. A correct control of the disease and avoiding processing rotten fruit enables the fulfilment of this standard. Nevertheless, a study indicated the presence of this mycotoxin in a high percentage of fruit juices and pulp commercialised in Argentina, revealing that 16 % of apple products had patulin levels higher than permitted by EU regulation (Oteiza et al., 2017).

The results from this study showed that the incidence of infection with *Penicillium* spp. was higher in the field (C apples) than after storage in cold chambers (I). However, it was one of the four genera that remained as main infectants after storage in apples destined to industrial food processing. Both in pre- and postharvest stages, it was mainly isolated from external lesions, a fact that reduces the risk of incorporating the decayed fruit into process. A different scenario is the one related to *Alternaria* infection. From occupying the second place in relevance among the field infectants, this genus became the most frequent after prolonged refrigerated storage of apple fruit. Moreover, from a relatively balanced internal/external infection relationship in recently harvested apples, it shifted to a predominantly MC infection when the fruit had been stored for several months in cold chambers. Coincidentally, MC increased in I apples, with respect to C ones, suggesting that the disease progressed in the chambers. It is known that if spores colonize the calyx

in the field, growth might follow during storage, allowing mycelia to reach the seed and carpel (Reuveni, 2006). Once the fungus is inside the fruit, it is protected against contact fungicides, improving the conditions for its development (Reuveni et al., 2002). Moreover, fungicides application to reduce other fungi have seemed to rise the incidence of the disease, as they eliminate competitors for MC causal agents (Snowdon, 1991).

II.5. Conclusions

The main fungal infectants of apple fruit from the Alto Valle of Rio Negro region intended for fresh consumption (C) as well as for industrial food processing (I) were identified. Also, the mycota of pear fruit destined to industrial food processing, that is used to make pear by-products and introduced in combination with apple in some apple by-products was characterized. Both C and I apples were highly contaminated in the exterior and interior of the fruit, indicating the susceptibility of this crop to fungal infectants. As well, the high incidence of MC in apple fruit in Argentina was demonstrated. The storage period selected the toxicogenic fungal genera in apples over the whole mycobiota and *Alternaria* was found as the main causal agent of MC in the field and during the postharvest stage. Even though MC started in the field, the incidence and severity of the disease increased during storage. In this scenario, contaminated fruits are likely to be incorporated into the process line, with a consequent risk of the presence of *Alternaria* toxins in apple by-products. Since the rate of infection with *Alternaria* spp. was much smaller for pears, the focus will be made on apple fruit for the remaining sections of the PhD dissertation.

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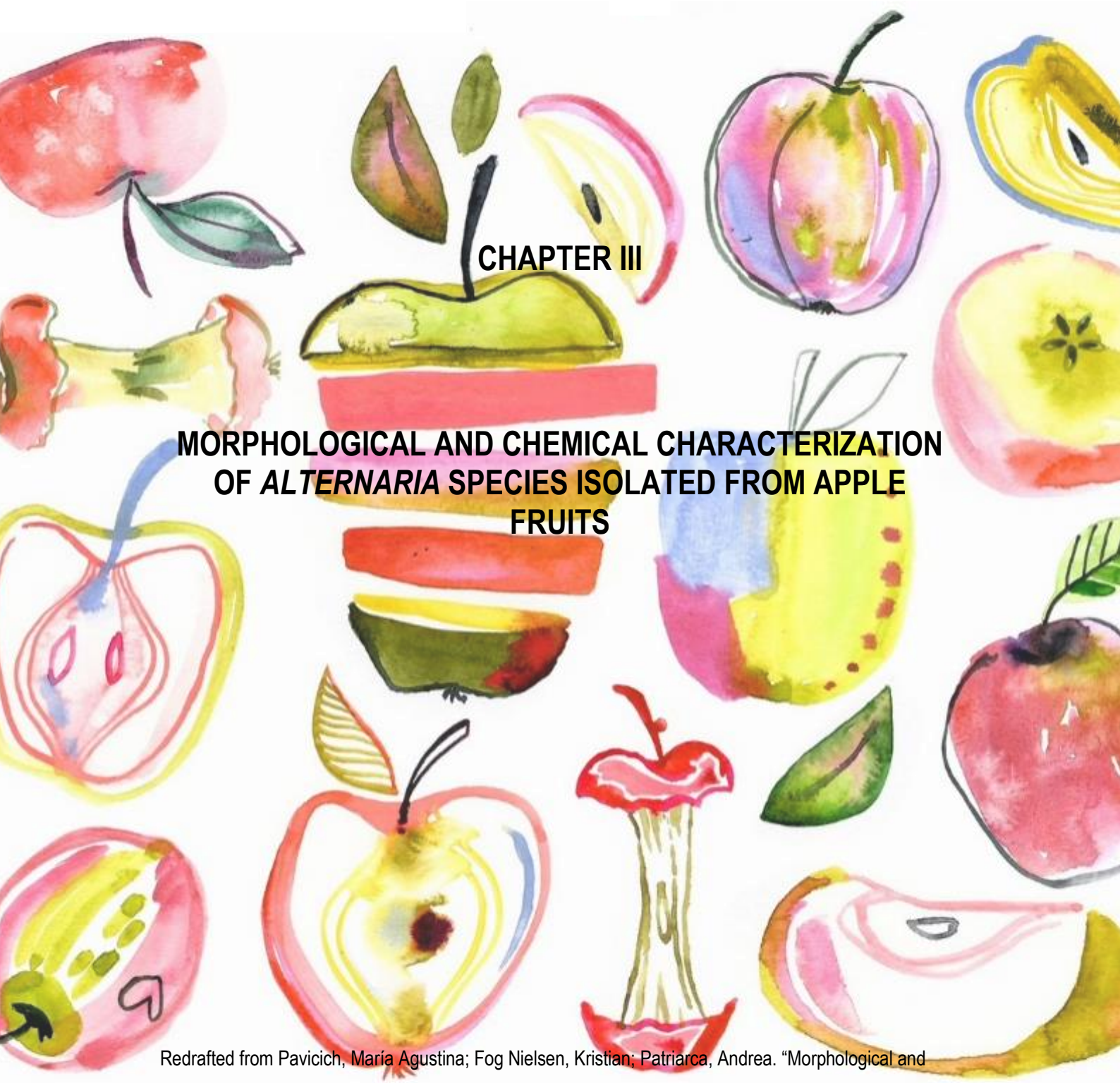

CHAPTER III

MORPHOLOGICAL AND CHEMICAL CHARACTERIZATION OF *ALTERNARIA* SPECIES ISOLATED FROM APPLE FRUITS

Redrafted from Pavicich, María Agustina; Fog Nielsen, Kristian; Patriarca, Andrea. "Morphological and chemical characterization of *Alternaria* species isolated from apple fruits". Manuscript in preparation.

Ilustration by Tanja Meyer

III.1. Introduction

In chapter II, it was demonstrated that *Alternaria* was one of the main fungal agents of apple fruit causing external lesions and the most prevalent causal agent of MC. A deeper taxonomical and chemical insight is necessary to better characterize the etiological agent of apple diseases and to prevent fruit spoilage and the consequent mycotoxin accumulation. Because of the complexity of the taxonomy in the genus *Alternaria*, several approaches have been applied to complement morphological identification. A reorganization of the genus has been proposed through phylogenetic studies (Lawrence et al., 2016; Woudenberg et al., 2015), in which the morphological species described by Simmons (2007) were clustered in sections. Chemotaxonomy, which proved useful to discriminate at species level in other fungal genera, did not produce the same results in *Alternaria,* where the common small-spored food-borne species showed no resolution (Andersen et al., 2015; Kelman et al., 2020; Patriarca et al., 2019a; Zwickel et al., 2018). Nonetheless, the results of chemical analyses agreed with the proposed phylogenetic sections given that *Alternaria* section *Alternaria* had a secondary metabolite profile different from that of section *Infectoriae*.

Other than for taxonomic purposes, a chemical characterization of a crop population provides an insight into its biodiversity, allows the distinction of the most frequent chemotypes present in the food, and constitutes a background knowledge to evaluate the toxicological risk associated with the pathogen. Small-spored *Alternaria* species are able to produce more than 70 secondary metabolites with diverse chemical structures that can accumulate in the edible parts of plants and are resistant to traditional food processing (López et al., 2016, Puntscher et al., 2019). The most relevant and known by their adverse effects on health are the dibenzopyrone derivatives, such as alternariol (AOH), alternariol monomethyl ether (AME) and altenuene (ALT), derivatives from tetramic acid, such as tenuazonic acid (TeA), perylenquinone derivatives, as altertoxins (ATX), and miscellaneous structures from the non-ribosomal peptide pathway, like

tentoxin (TEN) amongst others. Moreover, the small-spored *Alternaria* species can produce not only mycotoxins, but also chemically related compounds for which no toxicological data is available so far.

The group of the small-spored *Alternaria* has been previously reported as the main responsible for apple fruit diseases, particularly the species belonging to *Alternaria* section *Alternaria* (Gao et al., 2013a; Ntasiou et al., 2015; Serdani et al., 2002). Given the wide metabolic capacity of this group, a deeper knowledge on apple fruit populations is necessary to assess the risk associated with these fungi. Additionally, no full chemical characterization is available on the strains capable of causing mouldy core and those only able to colonize through puncture or lesions on the exterior of the fruit. They could either constitute a unique population, or the former might be especially adapted, biosynthesizing chemical compounds that facilitate the fruit invasion. Alternariol (AOH) has been suggested as a colonization factor in tomatoes (Graf et al., 2012), but the role of the wide spectrum of secondary metabolites from the *Alternaria* Section *Alternaria* remains to be investigated.

The objectives of the present chapter were: i) to perform a morphological and chemical characterization of the *Alternaria* population of apples grown in Argentina, including mycotoxins and related compounds, ii) to identify the most common secondary metabolites produced by this population, iii) to evaluate the existence of differences related to the type of apple disease.

III.2. Materials and methods

II.2.1. Morphological Identification

The *Alternaria* species isolated from apple fruit in chapter II and kept in V8 agar were transferred to Potato Carrot Agar (PCA) plates and incubated in cycles of alternating cool white fluorescent daylight consisting of 8 h of light and 16 h of darkness at 23 ºC for 7 d.

On day 5 of incubation, a block of agar and mycelium of about 0.5 x 2 cm was removed from the edge of one of the colonies and after that, incubation continued until day 7. Identification of each isolate to species-group level was performed according to Simmons (2007). The morphological macroscopic characteristics of the strains (colony diameter, colour, texture, growth, and sporulation rings) were observed after the incubation period. The three-dimensional sporulation pattern of the cultures was examined directly from the plates on the cut surface using a stereomicroscope (×80). Further examination (length of primary and secondary conidiophores, secondary conidiophores shape, conidial shapes, sizes, colours, and ornamentation) was done at ×400 magnification on slide preparations made by collecting spores from the colony surface with transparent adhesive tape mounted in lactic acid as shown in **Figure III.1.**

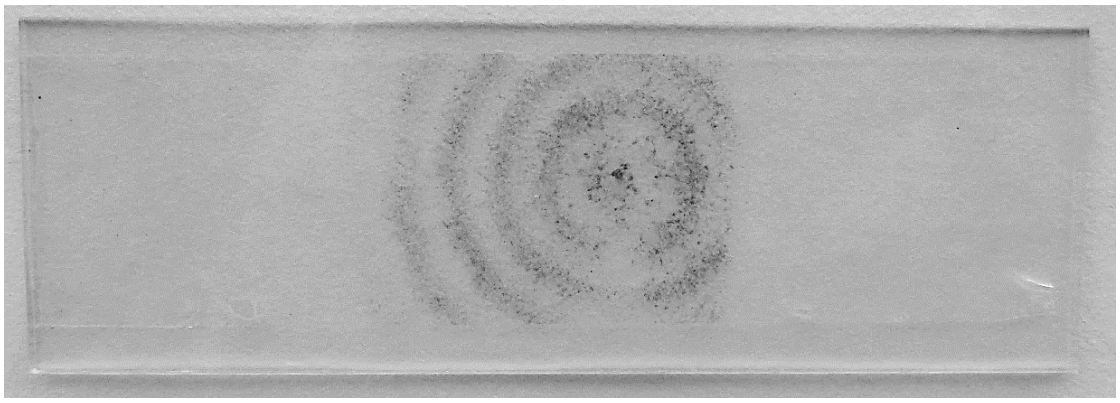

Figure III.1. Slide preparation of *Alternaria* sp. made collecting spores from a 7-day old colony with transparent adhesive tape mounted in lactic acid for observation under the microscope. Picture taken by María Agustina Pavicich.

III.2.2. Secondary metabolite production in vitro & sample preparation for LC-MS analysis

For secondary metabolite production *in vitro*, a total of 74 *Alternaria* strains previously isolated from apple fruits were inoculated individually at 3 points on Dichloran Rose Bengal Yeast Extract Sucrose agar (DRYES) (Samson et al., 2010), and incubated 14 days in the dark at 25 °C. As outgroup, 3 *Alternaria* isolates obtained from pears, 1 from

a bell pepper fruit (*Capsicum annuum*), and 1 from a tomato (*Solanum lycopersicum*) were used. The metabolite profiling was done using a micro-scale extraction method modified for *Alternaria* metabolites (Andersen et al., 2015). Three agar plugs (6 mm diameter) were cut from the centre of each colony and placed in a 2 ml vial; 1 ml ethyl acetate containing 1 % formic acid (vol/vol) was added to each vial, and then sonicated for 60 min. Each extract was transferred to a new vial and evaporated under a gentle stream of N_2 at room temperature. Then, 400 µl of methanol HPLC grade were added to resuspend the extract. Each extract was dissolved under sonication and filtered through a 0.45 µm PTFE filter. The vials were kept at -18 ºC until analysis.

III.2.3. UHPLC–DAD-HRMS analyses for secondary metabolites in vitro

This analysis was carried out at Denmark Technical University (DTU) with the collaboration of Dr. Kristan Fog Nielsen. Injection of samples and operation of the MS instrument was done by Prof. Dr. Andrea Patriarca and Dr. Kristian Fog Nielsen, all the other experimental work was done by María Agustina Pavicich. For metabolite identification, an Agilent Infinity 1290 UPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a diode array detector (DAD) was used. An aliquot of 1 µl of each sample was injected on an Agilent Poroshell 120 phenyl-hexyl column (2.7 µm, 2.1 × 150 mm) that was kept at 60°C. A linear gradient at 0.35 mL/min of water-acetonitrile, both adjusted with 20 mM formic acid was used to obtain chromatographic separation. The gradient started at 10 % acetonitrile and after 15 minutes it was increased to 100 %, maintained for 2 min, then returned to 10 % in 0.1 min and kept there for 3 min before the following run. Water, acetonitrile and formic acid were LC-MS grade (Sigma-Aldrich). The UV/VIS spectra were collected at wavelengths from 200 to 700 nm.

MS detection was made on an Agilent 6545 QTOF MS system equipped with Agilent dual jet stream electrospray ion source. Mass spectra in the range of m/z 85-1700 were recorded in separate runs of ESI^+ and ESI^-, with an acquisition rate of 10 spectra/s. The

lock mass used was Hexakis (2,2,3,3-tetrafluoropropoxy) phosphazene (Apollo Scientific Ltd.). Automated data-dependent acquisition (DDA) MS/HRMS analysis was performed for ions detected in the full scan applying fixed CID energies of 10, 20, and 40 eV with a maximum of three selected precursor ions per cycle.

HRMS data analysis was performed according to Nielsen and Larsen (2015) in three separate ways. Full-scan HRMS data were analysed by aggressive dereplication for the $[M+H]^+$, $[M+Na]^+$, $[M+NH_4]^+$, $[M+H-H_2O]^+$, $[M-H]^-$, and $[M+HCOO]^-$ adducts of all known elemental compositions described from *Alternaria* and related genera. The elemental composition was verified by mass accuracy, isotopic ratios, and isotopic spacing. The UV-Vis data obtained were used for confirmation. Then, the MS spectra were matched against the Denmark Technical University in-house library (approx. 2000 compounds) using the Agilent MassHunter PCDL manager. Finally, all peaks identified were mapped in the samples using Agilent MassHunter Quantitative Analysis. **Figure III.2** depicts the workflow employed for this analysis.

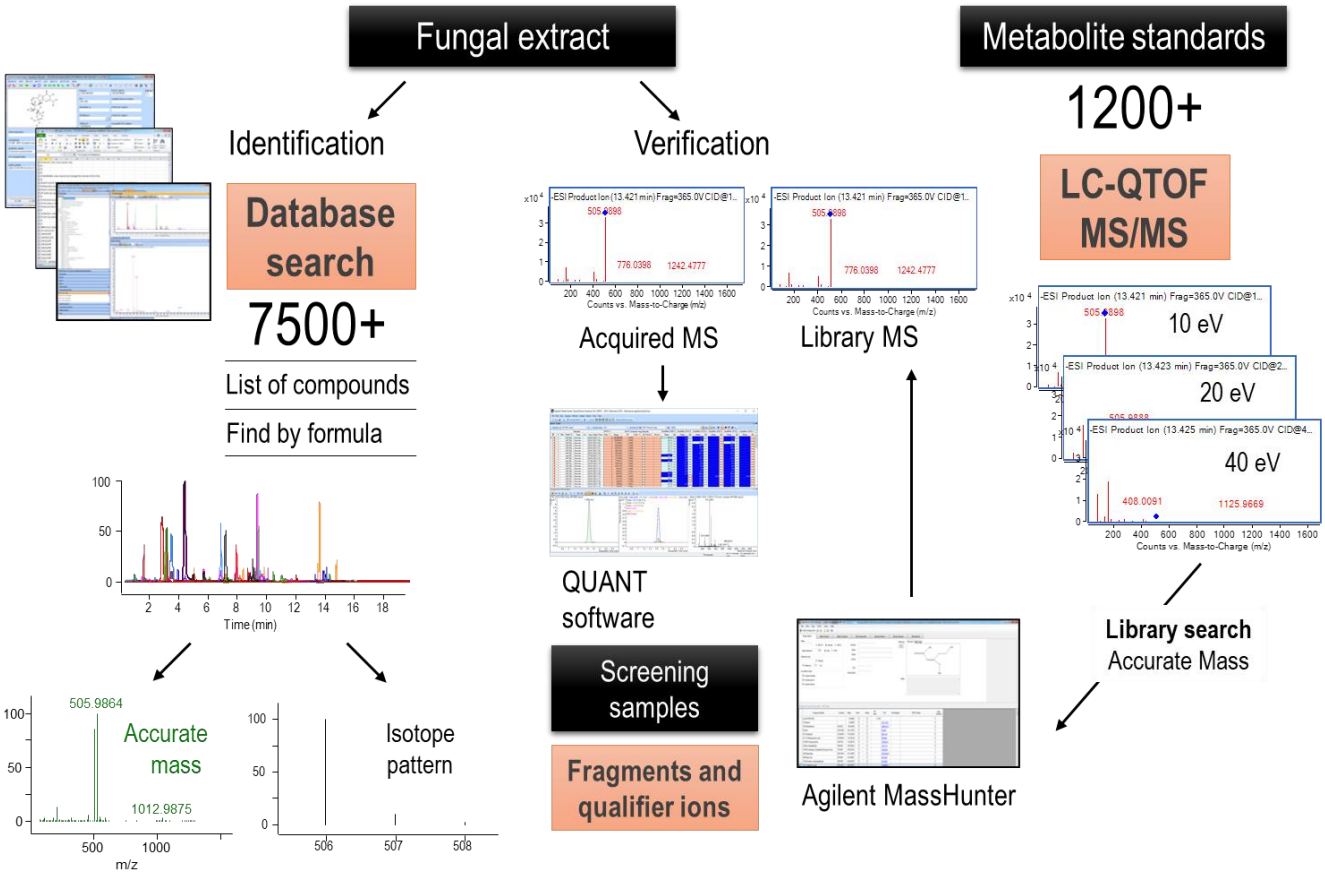

Figure III.2. Aggressive dereplication workflow employed in the analysis of the *Alternaria* strains.

III.2.4. Data treatment

The areas under each chromatographic peak corresponding to the 27 identified metabolites for each strain were transferred into a Microsoft Excel data sheet, and their decimal logarithm was calculated. Principal component analysis (PCA) was performed on this dataset and a Heat Map with clustering and dendrogram options was constructed using RStudio 4.0.5.

III.3. Results

III.3.1. Morphological identification of Alternaria species-groups from apples

All the 120 *Alternaria* isolates obtained from apples corresponded to the section *Alternaria* and belonged to only four sp.-grp, *A. tenuissima* (101), *A. alternata* (4), *A.*

arborescens (1) and *A. gaisen* (1). A total of 13 isolates presented intermediate morphological characteristics among *A. tenuissima*, *A. arborescens* and *A. alternata,* and were classified as *Alternaria* sp. **Figure III.3** shows the distribution of the *Alternaria* sp.-grps. in both types of apples (C: fresh consumption and I: industrial food processing) according to the lesion from which they were isolated.

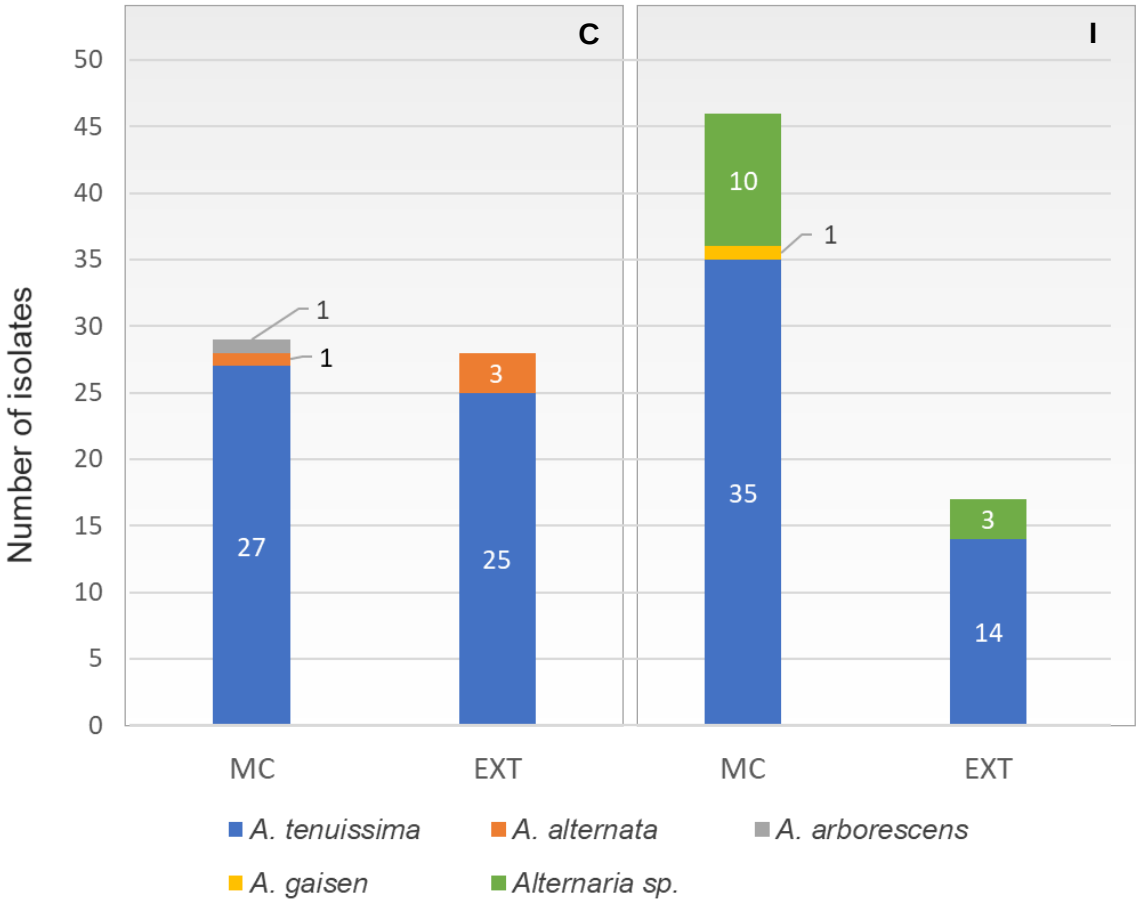

Figure III.3. Number of isolates (n=120) from each *Alternaria* species-group infecting apples for fresh consumption (C) and industrial food processing (I), obtained from mouldy core (MC) or external lesions (EXT).

Figure III.4 shows cultures from a representative of each species-group isolated from apple fruit on PCA, their sporulation pattern visualised on the transversal cut performed on 5-day-old cultures, and colonies on DRYES. Most of the isolates presented a sporulation pattern correspondent to the *A. tenuissima* sp.-grp. (101) (**Figure III.4.A, E, I**), consisting of 8-10 conidia, long, unbranched chains with occasional lateral branches borne from primary conidiophores of varying length, generally short. Secondary conidiophores were infrequent, but when present, they mainly originated from the conidial body, generating short perpendicular branches. Most conidia were ellipsoidal with a greyish to light brown or tan colour, and smooth walls. Colonies on PCA were grey to greyish brown of 6 cm diameter with 5 pairs of concentric circles. On DRYES, colonies were greyish green to light green, with cottony to velutinous appearance.

The isolates corresponding to the *A. alternata* sp.-grp (4) (**Figure III.4.B, F, J**) presented short primary conidiophores with multi-branched chains of 4-10 conidia, frequently with lateral secondary conidiophores. Conidia shapes showed a wide diversity, ranging from ellipsoidal to ovoid; the former were grey to tan and presented smooth walls, while the latter were dark brown to black with rough walls. Colonies in PCA were dark, usually with well-defined areas of growth and sporulation, and of granulated aspect, while colonies in DRYES were greyish green to light green and dark green, with cottony, sulcate and velutinous appearance.

Only one isolate was identified as *A. arborescens* sp.-grp (1) (**Figure III.4.C, G, K**) and was characterized by the presence of long primary conidiophores with a terminal cluster of branching conidial chains. Secondary conidiophores originating mostly from conidial apex were regularly observed. Conidia were darker than those from the other sp.-grp. isolated from apple, most commonly ovoid and with rough walls. Colonies on PCA were dark brown to black, with 4 to 6 well-defined concentric rings of growth and sporulation. Colonies on DRYES were dark green, sulcate and velutinous.

One strain presented characteristics according to *A. gaisen* sp.-grp. (1) (**Figure III.4.D, H, L**), consisting of usually unbranched chains of 5-9 spores, shorter than the previously described isolates. The conidia were short to long ovoid with several transversal septa and 0 to 2 longitudinal septa. Colonies on PCA were of 6-7 cm with 4-5 pairs of black to grey concentring rings. On DRYES, colonies were > 7 cm, light olive khaki to off-white colour with granular clumps.

Three of the strains, with a sporulation pattern resembling that of *A. tenuissima* sp.-grp., developed light grey to white colonies on DRYES, which is not a typical characteristic from this sp.-grp. (**Figure III.5)**.

The isolates that exhibited intermediate characteristics between the sp.-grps. mentioned before were identified as *Alternaria* sp.

The outgroup strains isolated from pear and pepper belonged to *A. tenuissima* sp.-grp. and presented the same characteristics of isolates from this sp.-grp. obtained from apples. The tomato isolate was the only outgroup belonging to *A. arborescens* sp.-grp.

The isolates from *A. tenuissima* sp.-grp. far outnumbered the other species-groups; they were predominant in both type of apples and lesions. *A. alternata* sp.-grp. was isolated both from MC and external lesions, while *A. arborescens* and *A. gaisen* sp.-grp. were only present in MC. Even though the total number of *Alternaria* spp. isolated from both types of fruit was similar (C apples, 57; I apples, 63), their distribution on the apples varied. While C apples showed similar contamination both external and MC, for the I apples, most of the *Alternaria* sp. originated from MC disease. The highest frequency of contamination with *A. tenuissima* sp.-grp. isolates corresponded to MC in I apples (35 isolates)

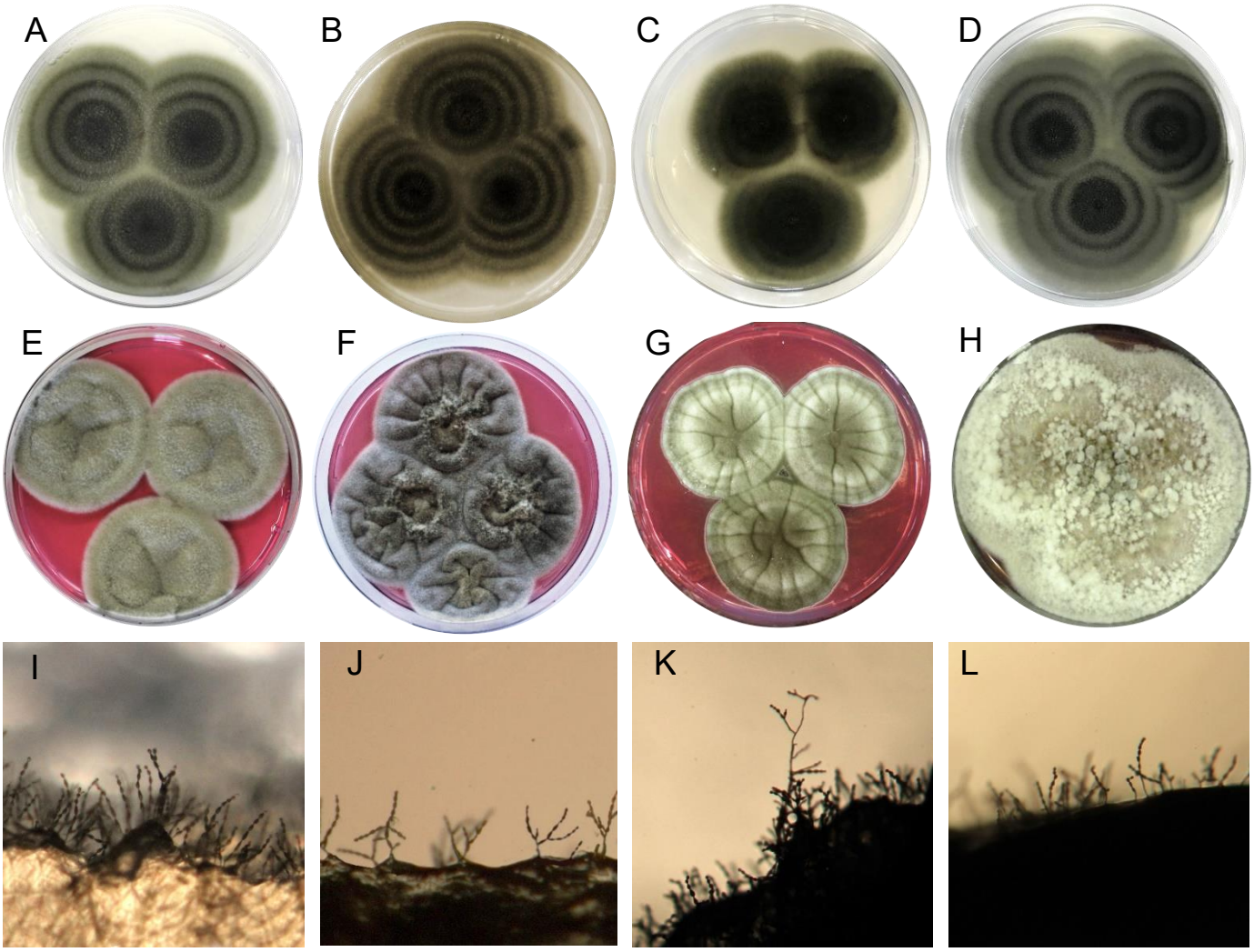

Figure III.4 Representative cultures of each species-group isolated from apple fruit. Above: colonies on Potato carrot agar (PCA) after 7 days of incubation at 23 ºC under alternating light cycles. Middle: colonies on Dichloran Rose Bengal Yeast Extract Sucrose agar (DRYES) after 7 days of incubation at 25 °C in darkness. Below: sporulation pattern on the transversal cut performed on the 5th day of incubation. A, E, I: *Alternaria tenuissima* sp.-grp; B, F, J: *A. alternata* sp.-grp.; C, G, K: *A. arborescens* sp.-grp.; D, H, L: *A. gaisen* sp.-grp. Pictures taken by María Agustina Pavicich.

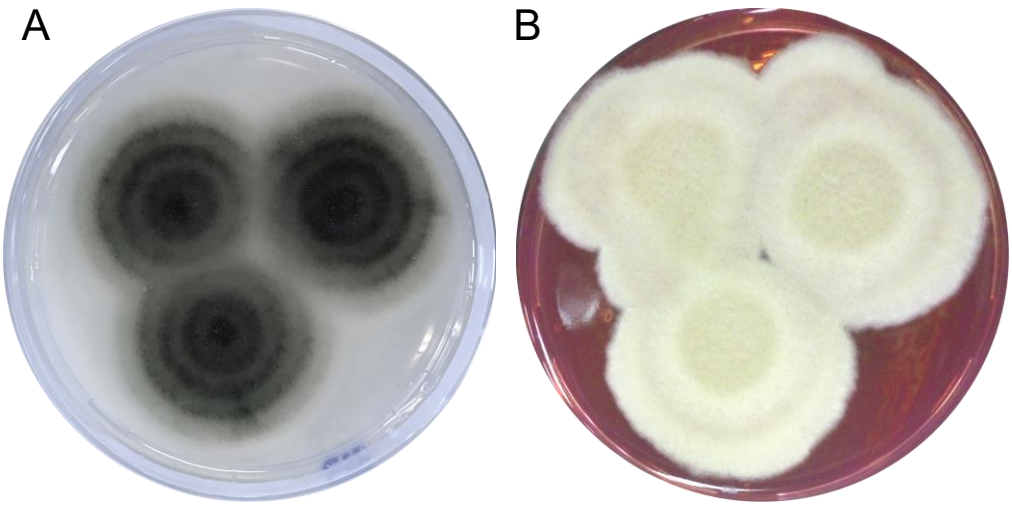

Figure III.5. Atypical strain with a sporulation pattern resembling that of *A. tenuissima* sp.-grp., but developing white colonies on Dichloran Rose Bengal Yeast Extract Sucrose agar (DRYES). A: Potato carrot agar (PCA) after 7 days of incubation at 23 ºC under alternating light cycles; B: DRYES after 7 days of incubation at 25 ºC in darkness. Pictures taken by María Agustina Pavicich.

III.3.2. Secondary metabolite profiles

The 74 *Alternaria* isolates from apple fruit and the 5 strains used as outgroups for the metabolic characterization, their isolate ID, species group, substrate of origin and type of lesion are listed in **Table III.1**. The strains that showed a sporulation pattern resembling that of *A. tenuissima* sp.-grp. and developed light grey to white colonies on DRYES were noted with an (*) in **Table III.1**.

The isolates from apple fruit produced a total of 27 secondary metabolites, 26 of which were of known chemical structure and one had an analogue structure to altertoxins (ATXs) (**Table III.2**). These were confirmed by MS/MS and UV-Vis data, and identical retention index to standards when available (Annex Table III.1). **Figure III.6** shows base

peak chromatograms (BPC) in ESI^+ and ESI^- of isolate 02. The maximum number of compounds produced by a single strain was 25 and the median for secondary metabolite production by the apple isolates was 11 metabolites. Only 3/74 isolates were not able to produce any of the known compounds *in vitro*.

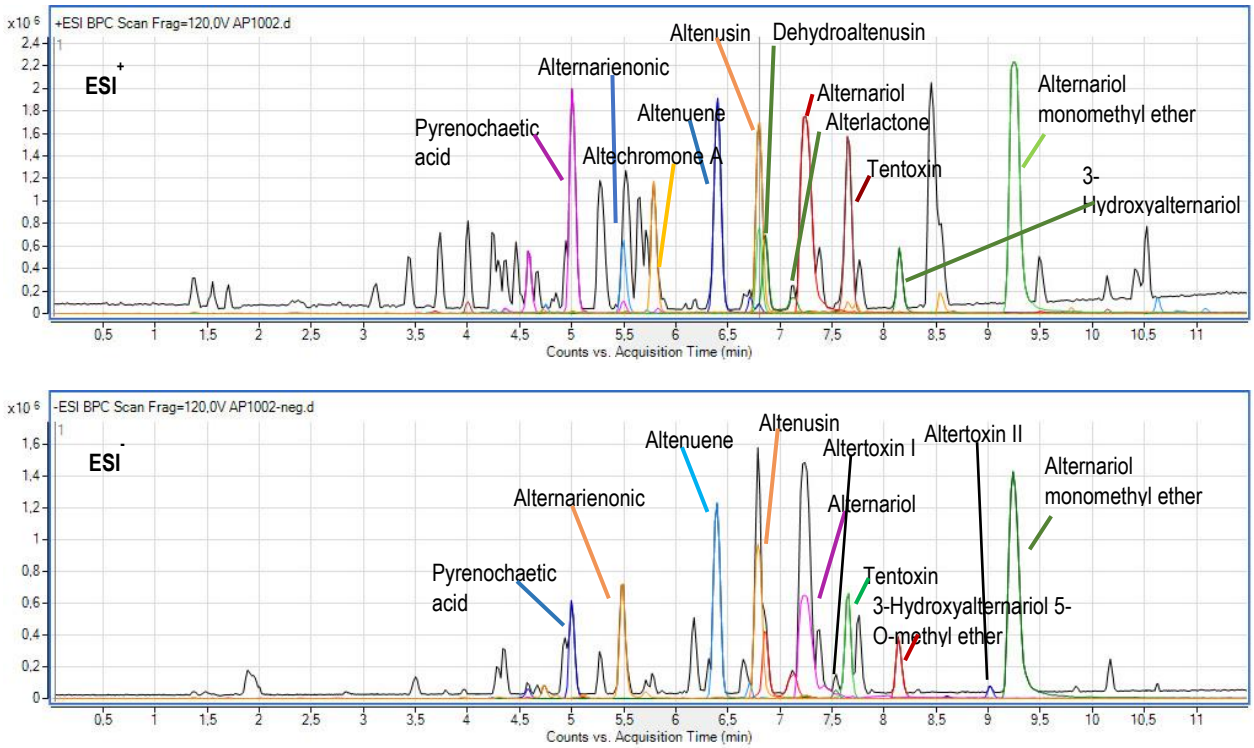

Figure III.6. Base Peak Chromatogram (BPC) of isolate 02 with identified metabolites. Top BPC corresponding to ESI^+ and bottom BPC corresponding to ESI^- mode.

Table III.1. List of isolates analysed for the *in vitro* metabolite production. Isolate identification number, species-group assigned by morphological identification, substrate of origin, and type of lesion.

Isolate ID	Species-group	Substrate	Lesion
1	*A. tenuissima*	pear	External
2	*A. tenuissima*	apple	External
3	*A. tenuissima*	apple	External
4	*A. tenuissima*	apple	External
5	*A. tenuissima*	apple	Mouldy core
6	*A. tenuissima*	apple	Mouldy core
7	*A. tenuissima*	apple	Mouldy core
8	*A. tenuissima*	apple	External
9	*A. tenuissima*	apple	External
10	*A. tenuissima*	apple	Mouldy core
11	*A. tenuissima*	apple	External
12	*A. tenuissima*	apple	Mouldy core
13	*A. tenuissima*	apple	External
14	*A. tenuissima*	apple	Mouldy core
15	*A. tenuissima*	apple	External
16	*A. tenuissima*	apple	External
17	*A. tenuissima*	apple	External
18	*A. tenuissima*	apple	External
19	*A. tenuissima*	apple	Mouldy core
20	*A. tenuissima*	apple	Mouldy core
21	*A. tenuissima*	apple	External
22	*A. tenuissima*	apple	External
23	*A. tenuissima*	apple	External
24	*A. arborescens*	apple	Mouldy core
25	*A. tenuissima*	apple	Mouldy core
26	*Alternaria* sp.	apple	Mouldy core
27	*A. tenuissima*	apple	Mouldy core
28	*A. tenuissima*	apple	Mouldy core
29	*A. tenuissima*	apple	Mouldy core
30	*A. tenuissima*	apple	Mouldy core
31	*A. tenuissima*	apple	Mouldy core
32	*A. tenuissima*	apple	Mouldy core
33	*A. tenuissima*	apple	External
34	*A. tenuissima*	apple	Mouldy core
35	*A. tenuissima*	apple	Mouldy core
36	*A. tenuissima*	apple	Mouldy core
37	*A. tenuissima*	apple	Mouldy core
38	*A. tenuissima*	apple	Mouldy core
39	*A. tenuissima*	apple	Mouldy core
40	*Alternaria* sp.	apple	Mouldy core

Table III.1. List of isolates analysed for the *in vitro* metabolite production. Isolate identification number, species-group assigned by morphological identification, substrate of origin, and type of lesion. (Continuation)

Isolate ID	*Species-group*	substrate	Lesion
41	*A. tenuissima*	apple	Mouldy core
42	*A. tenuissima*	apple	Mouldy core
43	*A. tenuissima*	apple	External
44	*A. tenuissima*	apple	Mouldy core
45	*A. tenuissima*	apple	Mouldy core
46	*A. tenuissima*	apple	Mouldy core
47	*A. tenuissima*	apple	Mouldy core
48	*A. tenuissima**	apple	External
49	*A. tenuissima*	apple	Mouldy core
50	*A. tenuissima*	apple	Mouldy core
51	*A. tenuissima*	apple	Mouldy core
52	*A. tenuissima*	apple	External
53	*A. tenuissima*	apple	Mouldy core
54	*A. gaisen*	apple	Mouldy core
55	*A. tenuissima*	apple	Mouldy core
56	*A. tenuissima*	apple	Mouldy core
57	*A. tenuissima*	apple	Mouldy core
58	*A. tenuissima*	apple	External
59	*A. tenuissima*	apple	Mouldy core
60	*A. tenuissima*	apple	Mouldy core
61	*A. tenuissima*	apple	Mouldy core
62	*A. tenuissima*	apple	Mouldy core
63	*A. tenuissima*	apple	Mouldy core
64	*A. tenuissima*	apple	Mouldy core
65	*A. tenuissima*	apple	Mouldy core
66	*A. tenuissima*	apple	External
67	*A. tenuissima*	apple	Mouldy core
68	*A. tenuissima*	apple	Mouldy core
69	*A. tenuissima*	apple	Mouldy core
70	*A. tenuissima*	apple	External
71	*A. tenuissima*	apple	External
72	*A. tenuissima**	apple	External
73	*A. tenuissima*	apple	External
74	*A. tenuissima**	apple	External
75	*A. tenuissima*	pear	Mouldy core
76	*A. tenuissima*	pear	Mouldy core
77	*A. tenuissima*	pepper	External
78	*A. arborescens*	tomato	External
79	*A. tenuissima*	apple	Mouldy core

(*) strains showing characteristics in DRYES atypical for *A. tenuissima* sp.-grp.

Table III.2. Secondary metabolites produced by *Alternaria* isolates from apples.

Metabolite*	Number of producers (n=74)	Percentage of producers (%)
Altertoxin-I	63	85.1
Altechromone A	56	75.7
Tentoxin	51	68.9
Tenuazonic acid	50	67.6
Isopropyl tetramic acid	49	66.2
Dihydrotentoxin	46	62.2
Pyrenochaetic acid A	44	59.5
Alternariol	43	58.1
Alterperylenol	43	58.1
Alternariol monomethyl ether	42	56.8
Altertoxin-II	38	51.4
Alternarienonic acid	30	40.5
Altersetin	29	39.2
Altenuene	27	36.5
Altenusin	23	31.1
Desmethylatenusin	23	31.1
3-Hydroxyalternariol 5-O-methyl ether	22	29.7
Altertoxin analogue	22	29.7
4-OH-Alternariol monomethyl ether	21	28.4
Altertoxin-III	20	27.0
Dehydroaltenusin	19	25.7
Altenuisol	18	24.3
Stemphyltoxin-III	18	24.3
Altenuic acid-II	17	23.0
Altenuic acid-III	16	21.6
Alterlactone	8	10.8
cis-Dehydrocurvularin	1	1.4

(*) Metabolites in bold are considered mycotoxins

The metabolites exclusive to the *Alternaria* section *Infectoriae* were searched for all the isolates, and none was detected. Altertoxin-I (ATX-I) was the most frequent metabolite, produced by 85.1 % of the strains, while altechromone A was the second most frequent (75.7 %). The compounds that are known as *Alternaria* mycotoxins (Arcella et al., 2016; Solfrizzo, 2017) were produced in high frequency by the apple isolates. TEN, a known phytotoxin, was produced by 68.9 % of them, TeA, considered the most acutely toxic mycotoxin produced by this genus (Asam and Rychlik, 2013), was synthesised by 67.6 % of the strains; its derivative, isopropyl tetramic acid, was found in a similar frequency (66.2 %). Regarding other known mycotoxins from the genus, AOH, AME, and ATX-II were produced by more than 50 % of the isolates. ALT and ATX-III were the less frequent among the *Alternaria* toxins (36.5 and 27.0 % respectively).

III.3.2.1. Type of lesion

A principal component analysis was performed on the metabolite data to evaluate individual differences among the isolates and possible relationships between the metabolite profiles and the type of lesion caused by the isolates in the fruit (external or mouldy core).

The loading plot represented in **Figure III.7** shows the contribution of each secondary metabolite to the principal component analysis. Vectors separated by a small angle (<90º), positively correlate to each other, vectors at 90° do not correlate, and vectors at 180° have a negative correlation. TeA and isopropyl tetramic acid production, both from the same biosynthetic pathway, were positively correlated, and isolates producing these compounds were shifted to the left superior quartile. Similarly, the production of altertoxins, stemphyltoxin-III, and alterperylenol was positively correlated, and located in the upper right quartile. On the other hand, tetramic acids showed no correlation with the production of alternariols. The latter were positively correlated to structurally related compounds synthesised through the same via, such as ALT, altenusin,

dehydroaltenusin, and altenuic acids. TEN, one of the most frequent metabolites, and cis-dehydrocurvularin, produced by only one isolate showed little contribution to the principal components.

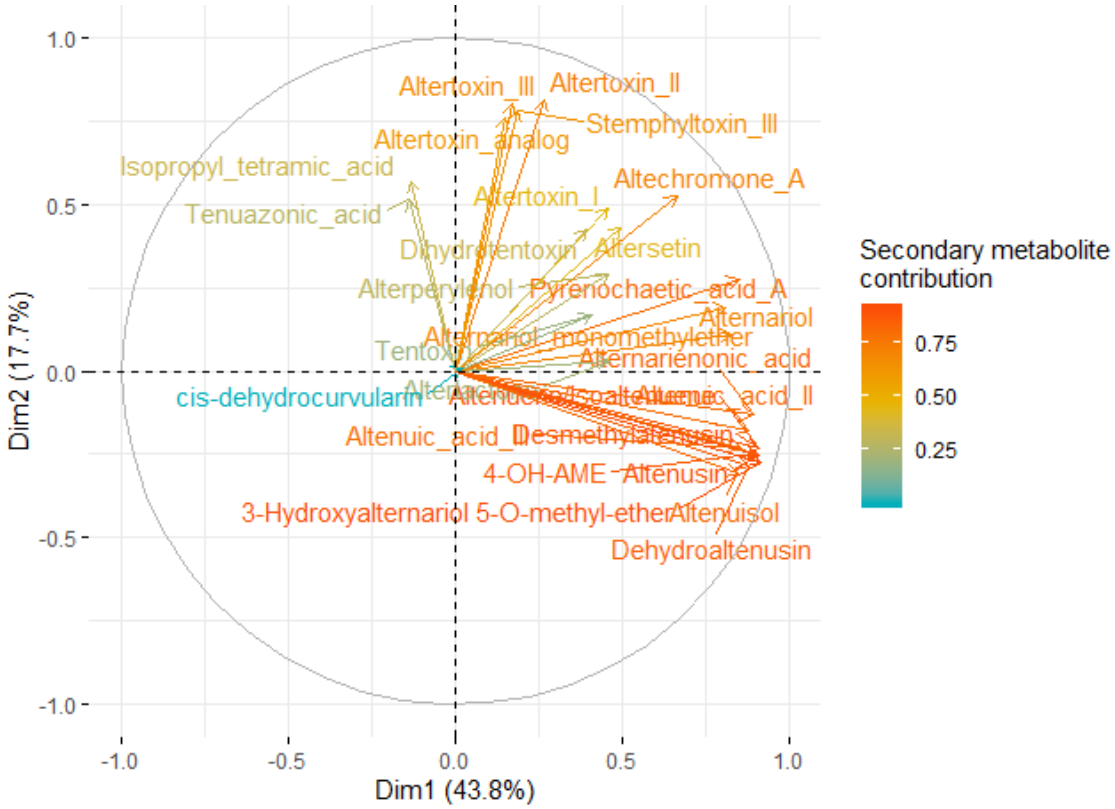

Figure III.7. Loading plot showing the contribution of each secondary metabolite to the principal component analysis, indicated by colour intensity and length of the arrow.

The PCA divided the isolates in two groups based on the production/non-production of secondary metabolites (**Figure III.7**). The two dimensions represented in the loading plot explained 61.5 % of the variance. Group 1, on the left, contained isolates from apple, as well as those from other hosts, such as tomato, pepper, and pear, but no separation was observed between them. The *A. arborescens* sp.-grp. isolate from apple and the three atypical *A. tenuissima* sp.-grp. isolates with whitish colonies in DRYES were also members of this group. Isolates in group 1 were tightly clustered, especially those located

in the lower left quadrant, which produced fewer metabolites than the rest. On the other hand, all the isolates contained in group 2 originated from apple fruit and belonged to the *A. tenuissima* sp.-grp., except from the only *A. gaisen* sp.-grp. strain, which was included in this cluster. These isolates were characterized by producing metabolites from all the chemical families and were more loosely distributed in the cluster than the previous ones.

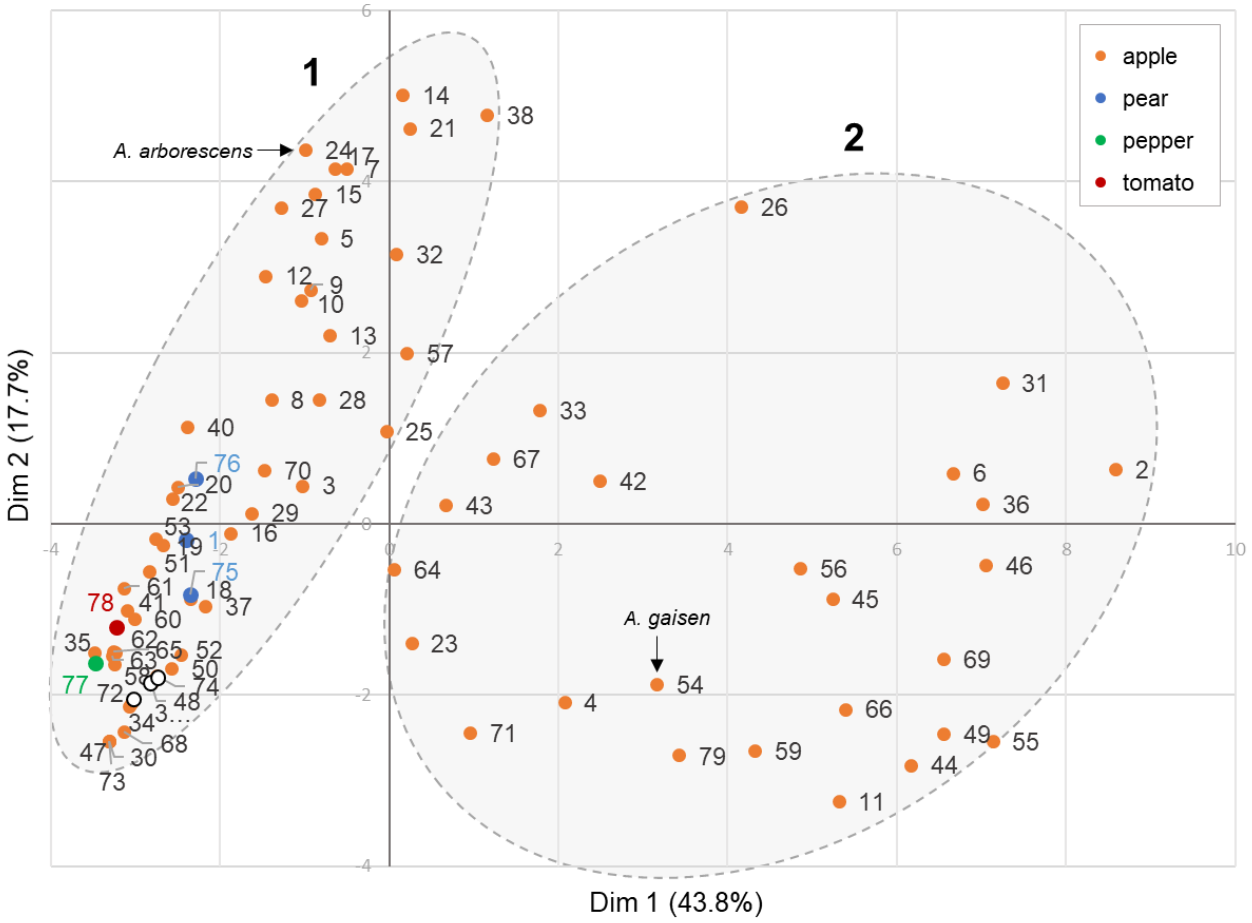

Figure III.8. Principal component analysis of 74 *Alternaria* isolates from apple, 3 from pear, 1 from pepper and 1 from tomato, based on the production of 27 secondary metabolites. (○) *A. tenuissima* isolates developing white/grey colonies in DRYES. Labels correspond to the isolate ID.

When comparing isolates from external lesions with the causal agents of mouldy core (**Figure III.9**), no significant segregation could be attributed to the type of disease. Nevertheless, mouldy core isolates were predominant in group 2, characterized by a wider spectrum of metabolite production (17/25).

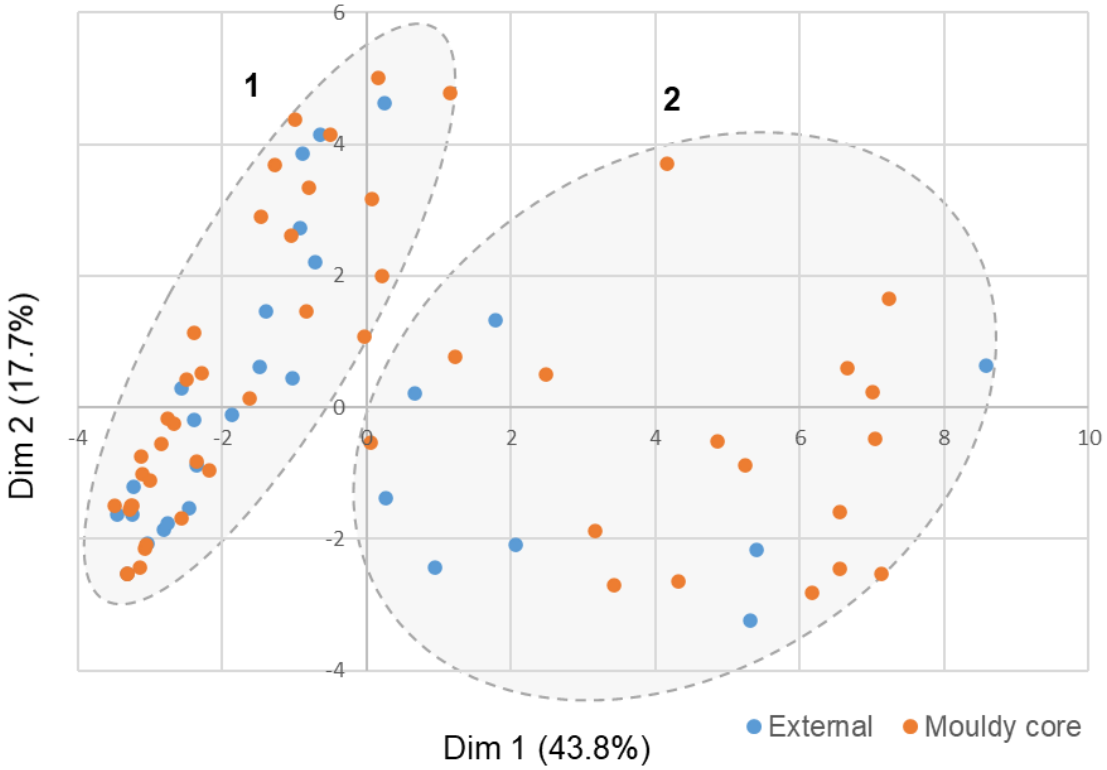

Figure III.9. Principal component analysis of 74 *Alternaria* isolates from apple, 3 from pear, 1 from pepper and 1 from tomato, based on the production of 27 secondary metabolites. Comparisons between isolates from external lesions and mouldy core.

III.3.2.2. Secondary metabolite profiles

Given the existence of differences between metabolite profiles, a semi-quantitative heat map was constructed to obtain a deeper insight into the apple isolates (**Figure III.10**). The colour key indicates the semiquantitative level of production for each metabolite, being yellow for non-production, red the highest level of production, and the colours in

between represent intermediate levels. As quantification was not performed, the parameters are relative, and comparisons are only possible between levels of the same metabolite, and not between different compounds.

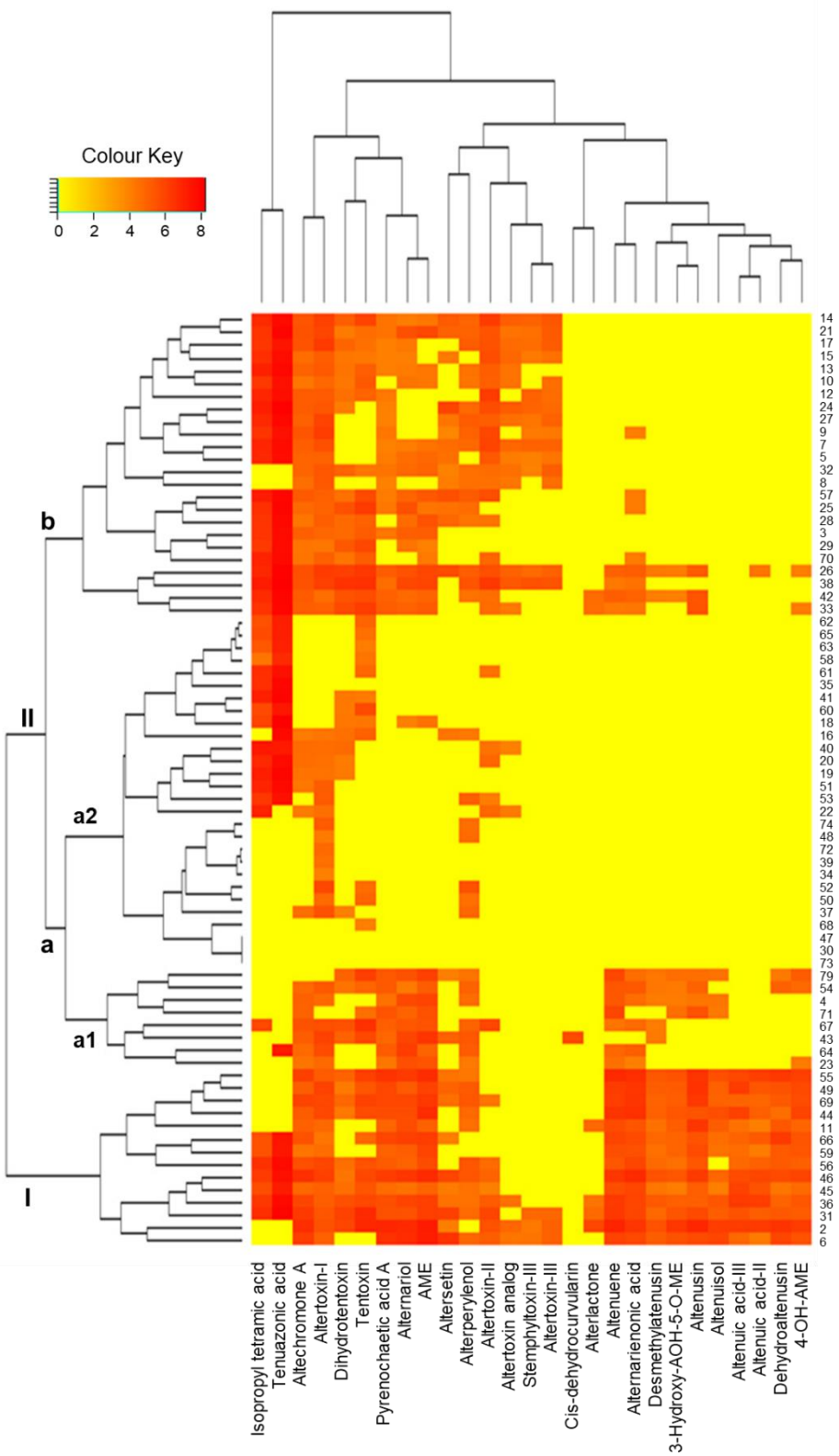

Figure III.10. Semi-quantitative heat map of 74 *Alternaria* isolates from apple fruit. Labels correspond to the isolate ID.

According to the metabolite production, the isolates were divided in 2 major clusters. Cluster I comprised 14 isolates and grouped the strains producing high levels of compounds from all the structural families. The other 60 isolates from apple belonged to Cluster II, which was divided in two subclusters, a and b. Cluster IIa included 36 isolates, distributed in two defined groups. Group a1 was similar to Cluster I in the type of compounds produced, but these isolates accumulated lower quantities of the same metabolites and lacked the ability to synthesise some of them. Besides, none of the strains within this group produced the altertoxin analogue, stemphyltoxin or ATX-III, as well as alterlactone, altenuic acid-II and -III. Group a2 was the largest, gathering 28 isolates, whose common characteristic was a less diverse profile. Some of them produced high levels of tetramic acids, causing thus another subdivision within the group. The most frequent compounds synthesised by these strains were altechromone A, ATX-I, TEN, dihydrotentoxin, and alterperylenol. Only one isolate from this group was able to accumulate alternariols, but not in high amounts compared to the isolates from the other clusters. Most of the compounds structurally related to alternariols or biosynthesised by the same via were not detected in their profiles. Finally, group IIb included 24 isolates, most of which produced the tetramic acids in high amounts, the whole family of ATXs and related compounds, TEN and dihydrotentoxin, and the alternariols. However, most of them did not produce the altenuic acids and other metabolites structurally related to alternariols such as alterlactone, ALT, and altenuisol, among others. Group 2 from the PCA comprised the complete clusters I and cluster II a1, in addition with 3 strains from the first subgroup of IIb, *i.e.* those isolates which showed the capacity of synthesising compounds from all the chemical families.

The metabolite profile of isolates belonging to different sp.-grp. were similar, and no consistent differences could be established among them, neither specific sp.-grp. metabolites could be identified. However, less frequent profiles were detected; 1 strain produced only tentoxin (ID 68), 3 produced only ATX-I (IDs 34, 39, 72) and 2 produced

only ATX-I and alterperylenol (IDs 48 and 74). Among these isolates were those which developed atypical light grey/whitish colonies on DRYES but had a sporulation pattern corresponding to *A. tenuissima* sp.-grp (IDs 48, 72 and 74). Interestingly, the two metabolites produced by the atypical strains are those in common with the secondary metabolite profile of the *Alternaria* Section *Infectoriae*; however, no other compound characteristic from this Section was detected for these isolates.

III.4. Discussion

Alternaria was found as the main causal agent of MC in retail and industry destined apples in chapter II. This fungal genus was also present in the exterior of the fruits. The biodiversity of the *Alternaria* population infecting apple fruit was low in comparison to that previously observed in other crops in Argentina (da Cruz Cabral et al., 2017). *A. tenuissima* was the predominant sp.-grp., and other closely related species were isolated in significantly minor proportion. Strains sharing characteristics between sp.-grps. from *Alternaria* section *Alternaria* had already been seen in several crops (Patriarca et al., 2019b). However, atypical *A. tenuissima* sp.-grp. isolates with colonies resembling those of *A. infectoria* sp.-grp. in DRYES are reported for the first time in the present chapter.

Studies in South Africa also found members of *A. tenuissima* sp.-grp. as major pathogens associated with core rot disease (Serdani et al., 2002, 1998), as well as in Greece (Ntasiou et al., 2015) where its frequency was of 89.3 %, followed by *A. arborescens* in 11.7 %. Similarly, Gao et al. (2013) identified *A. alternata*, *A. tenuissima* and *A. arborescens* as the main species causing MC in China. In Australia, 64 % of isolates from fruit showed an intermediate morphology between *A. alternata* and *A. tenuissima* (Harteveld et al., 2013). In further studies, when fruits were inoculated with different *Alternaria* spp., only those isolates identified as intermediates between *A.*

alternata/A. tenuissima and *A. tenuissima*/*A. mali* caused symptoms in the fruit (Harteveld et al., 2014).

In Chile, the prevalent species were the same, but some less frequently reported ones were also isolated from mouldy core apples. They were, in order of importance, *A. tenuissima*, *A. arborescens*, *A. alternata*, and *A. dumosa* from sect. *Alternaria*, *A. frumenti*, from sect. *Infectoriae*, and *A. kordkuyana* from sect. *Pseudoalternaria* (Elfar et al., 2018). A more recent study from South Africa mentioned *A. tenuissima* and *A. arborescens* as causal agents of apple core rot, but also included *A. infectoria*, *A. dumosa*, and *A. eureka* (Basson et al., 2019). Recently, a new species causing fruit spot in China, *A. malicola* sp. nov., was described. It was isolated from lesions characterized as black dots on apple fruit, and it was also able to produce leaf blotch, a disease of the apple leaves caused by species of *Alternaria*, and mouldy core (Dang et al., 2018). However, this species clustered in sect. *Ulocladioides* and is not related to the previously described ones. Differences in populations composition might be attributed to the susceptibility of the variety of crops grown in each region, climatic conditions associated to geographic location, or agricultural practices exerting a selective effect on the pathogens (Abdelfattah et al., 2016; Niem et al., 2007).

The secondary metabolite profiles showed greater diversity than the morphological characteristics. A total of 27 metabolites could be identified, many of which are known mycotoxins, and others are either their derivatives or analogues. When comparing the frequency of production for these compounds, some differences were observed with respect to previous studies. In the chemical analysis of foodborne *Alternaria* from several Argentinean crops, AOH and AME were the most frequent secondary metabolites produced; more than 70 % isolates were capable of their biosynthesis (Andersen et al., 2015; da Cruz Cabral et al., 2017). However, the apple isolates from the present study produced AOH and AME in a proportion of 58 % and 57 %, respectively, and these were

the eighth and tenth more common compounds, respectively. ALT had also been previously detected among the most frequent *Alternaria* metabolites (over 60 %), but only 37 % apple isolates were able to produce ALT. On the other hand, ATX-I, the most abundant in apple isolates (85 %), was synthesised in much lower proportion in former studies. Andersen et al. (2015) reported 62% of producers, and da Cruz Cabral et al. (2017), only 27 %. The proportion of apple producers of TeA and TEN was more similar to previous reports. These results suggest that the *Alternaria* apple population has a specific chemical diversity dissimilar to those from other crops.

Regarding the toxicity of the metabolites constituting the profile of the apple fruit isolates, the compounds recognised as *Alternaria* mycotoxins were detected in high frequency. Apples destined to processing were contaminated with *Alternaria* strains that could produce these metabolites, but whether these toxins were present in the fruit and could persist after processing for the obtention of apple by-products still needs to be further evaluated.

ATX-I, the most frequent toxin produced by apple isolates, has been detected in apple fruit as well as ATX-II (Puntscher et al., 2020a). Moreover, ATX-II can be converted into ATX-I by reduction of the epoxide to the respective alcohol in mammalian and plant metabolism (Fleck et al., 2014; Puntscher et al., 2019), contributing to ATX-I- occurrence in apple products. TEN, the second most recurrent toxin produced *in vitro*, was reported in apple and apple by-products, including infant food (Gotthardt et al., 2019; Puntscher, Marko and Warth, 2020b; Zwickel et al., 2016). TeA was found in different apple products such as apple juice (Fan et al., 2016; Gross et al., 2011; Prelle et al., 2013; Walravens et al., 2016), and apple based infant food (Gotthardt et al., 2019). AOH and AME are widely spread among apple products (Ackermann et al., 2011; Fan et al., 2016; Zwickel et al., 2016). Modified forms of these mycotoxins were produced in vitro, namely 3-hydroxyalternariol 5-O-methyl ether and 4-OH-AME, but there are currently no reports

on their natural occurrence. Nevertheless, the presence of other modified forms of AOH and AME have been detected in apple by-products, such as their glucosides and sulphates (Puntscher et al., 2020b). It has been suggested that AOH glucosides could be products of plant metabolism, which explains their presence in vegetable foods.

Most of the other predominant compounds in the secondary metabolite profiles of apple isolates have not been reported as natural contaminants, but few surveys have included them. This may be due to the unavailability of commercial reference standards for confirmation or the lack of toxicity data justifying their investigation in food. However, given the high proportion of isolates with the ability to synthesise them, their natural occurrence in apple or by-products can be suspected. Their impact on human and animal health remains to be investigated.

The results of the PCA based on secondary metabolite production showed that group 2 gathered isolates with a broader metabolic capacity and strong producers of altertoxins and alternariols (**Figure III.8**). Even though no sharp separation was obtained when isolates form mouldy core were compared with those obtained from external lesions, most of the members of group 2 in the PCA corresponded to mouldy core isolates (**Figure III.9**). This implies that isolates infecting the inner centre of the fruit can synthesise metabolites *in vitro* from all the chemical families and have a wider secondary metabolite profile. If some of these compounds can be produced *in vivo* as well, the associated risk would be greater, given that mouldy core apples usually pass undetected through process line visual inspections in apple concentrate production.

When a deeper insight was taken into the whole apple population, several groups of secondary metabolite profiles were observed based on the semiquantitative data. Even though two major clusters were obtained from the statistical analysis, and different subclusters could be differentiated, the predominant profile was that including several metabolites from different chemical families. Cluster I, and subclusters IIa1 and IIb,

gathered in total 46 isolates with a high metabolic capacity and diversity. The minority of isolates, clustered in group IIa2 (n=28), showed a distinctively less diverse profile, characterized by few families of compounds, mainly altertoxins, tentoxin, tetramic acids, and their respective derivatives and analogues.

The wide chemical diversity and metabolic capacity of *Alternaria* apple population including a high number of toxigenic compounds should be considered when health risks associated with apple products is evaluated. Mixtures of *Alternaria* toxins have been recently evaluated showing genotoxic and anti-estrogenic activity (Aichinger et al., 2019). Some of the genotoxic effect could not be attributed to the compounds present in the mixture, suggesting the contribution of additional unknown *Alternaria* metabolites to the total toxicity. Therefore, a full chemical characterization of foodborne isolates could contribute to a better understanding of the toxicological risk derived from this pathogen.

Since the majority of the toxigenic strains in the present study were obtained from MC apples for industrial food processing, the risk of processing fruit contaminated with *Alternaria* toxins is high.

III.5. Conclusions

The *Alternaria* isolates infecting apple fruit corresponded to *Alternaria* section *Alternaria* and low morphological variability was observed. Nevertheless, the *Alternaria* apple population showed a high metabolic and toxigenic capacity. Most of the secondary metabolites produced *in vitro* were either mycotoxins, or modifications of these. Isolates from MC showed more diverse chemical profiles and the ability to synthesise compounds from different chemical families. Since most of the *Alternaria* isolates from apples destined to industrial food processing originated from MC disease, the presence of mycotoxins in apple and apple by-products is expected. These results serve as a

background for the evaluation of the toxicological risks associated with fungal infestations.

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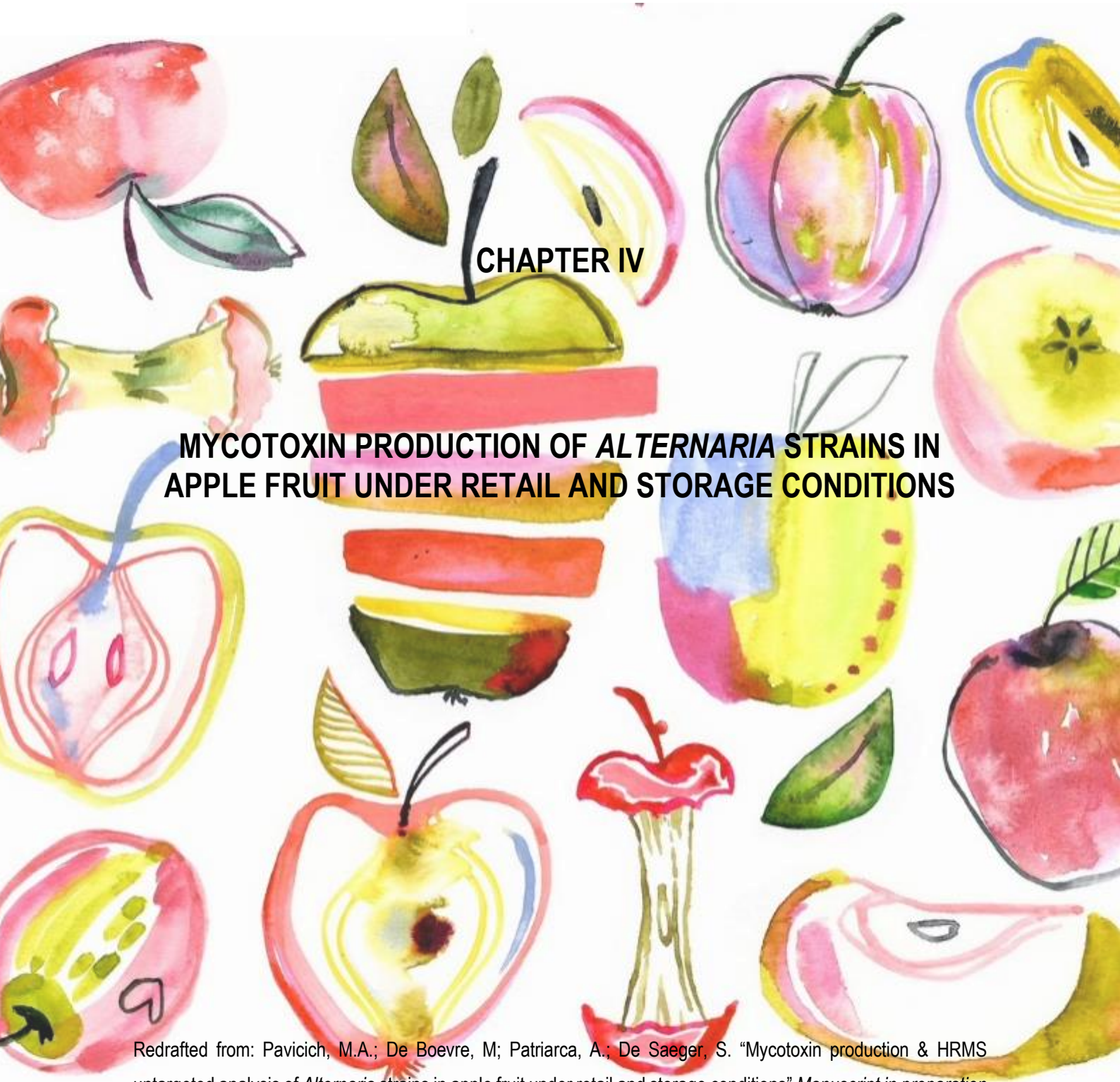

CHAPTER IV

MYCOTOXIN PRODUCTION OF *ALTERNARIA* STRAINS IN APPLE FRUIT UNDER RETAIL AND STORAGE CONDITIONS

Redrafted from: Pavicich, M.A.; De Boevre, M; Patriarca, A.; De Saeger, S. "Mycotoxin production & HRMS untargeted analysis of *Alternaria* strains in apple fruit under retail and storage conditions" *Manuscript in preparation*

Illustration by Tanja Meyer

IV.1. Introduction

The *in vitro* toxigenic potential of the *Alternaria* strains isolated from apple fruit was proved in Chapter III. Metabolites such as AOH, AME, TeA, TEN, ALT, ATX-I, ATX-II, ATX-III, altechromone A, isopropyl tetramic acid, pyrenochaetic acid A, and alterperylenol, among others, can be produced by the apple pathogen and therefore, are likely to accumulate in the fruits. Some of these metabolites are known mycotoxins and have been recently reported in naturally contaminated apple fruit (Puntscher et al., 2020). Nevertheless, the metabolomic capacity of *Alternaria* spp. in apples has not been deeply studied. The production of these mycotoxins can be influenced by several factors such as site of infection in the fruit or storage temperature.

A recent report from the European Food Safety Authority (EFSA) established the need to increase the information on the toxicity, natural occurrence, and distribution of *Alternaria* toxins in the different food chains (Arcella et al., 2016). These toxins are considered as emergent and will be evaluated by legislative authorities in the near future. Their regulation will have a significant impact on international commerce, affecting the economies of both exporter and importer countries. Thus, widening the knowledge on the metabolomic capacity of *Alternaria* strains in apples will represent a relevant contribution to food-producing countries as well as those depending on them by commerce. Therefore, the objective of this chapter was to evaluate the production and accumulation of known *Alternaria* mycotoxins AOH, AME, ALT, TeA, TEN, ATX-I, and ATX-II, and the modified mycotoxins alternariol-3-sulphate (AOH-3-S), alternariol monomethyl ether-3-sulphate (AME-3-S), alternariol-3-glucoside (AOH-3-G), and alternariol monomethyl ether-3-glucoside (AME-3-G) (**Figure IV.1**) in apple fruit simulating retail and post-harvest conditions to determine their toxigenic capacity *in vivo*.

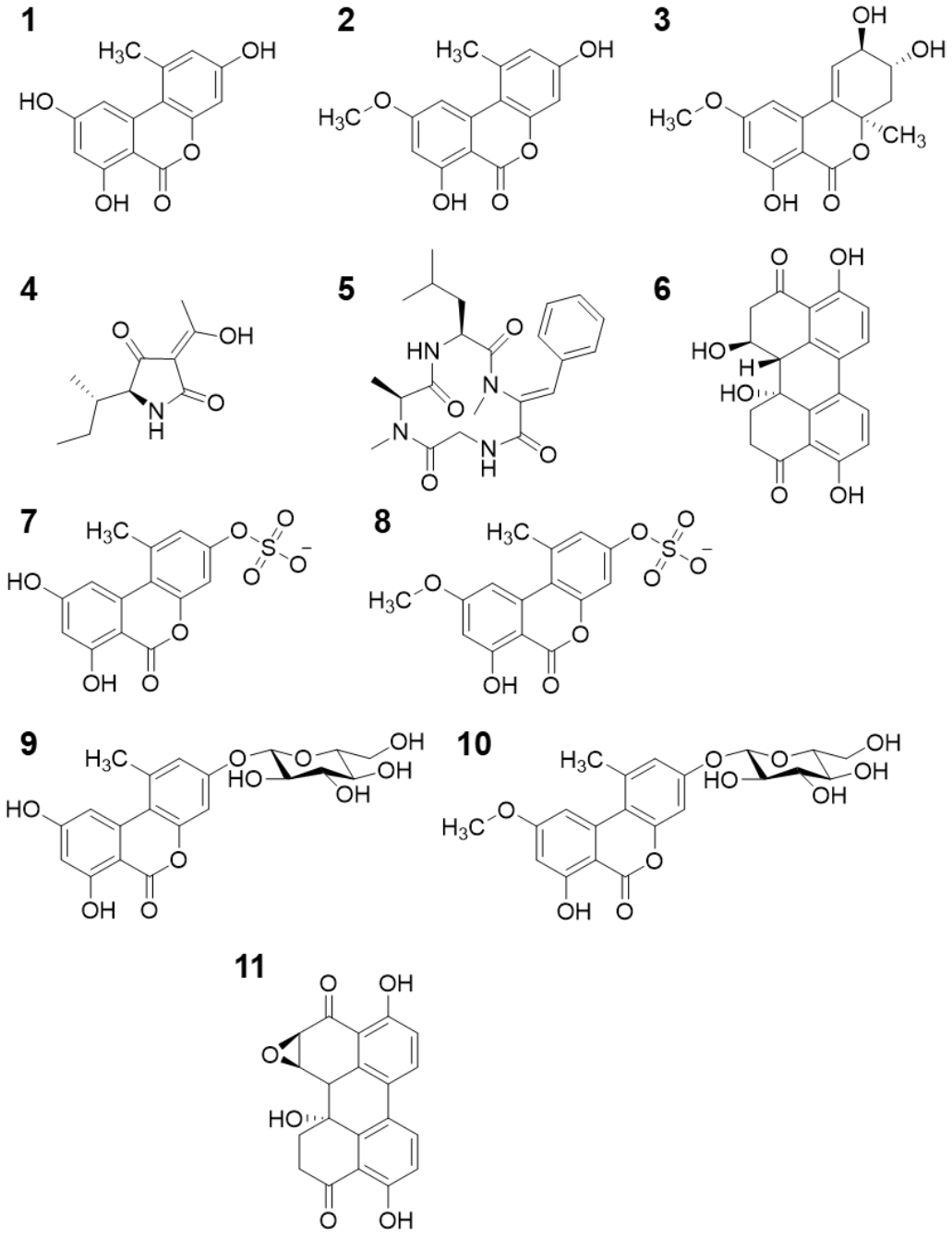

Figure IV.1. Chemical structures of the analysed mycotoxins, 1, alternariol (AOH); 2, alternariol monomethyl ether (AME); 3, altenuene (ALT); 4, tenuazonic acid (TeA); 5, tentoxin (TEN); 6, altertoxin-I (ATX-I); 7, alternariol-3-sulphate (AOH-3-S); 8, alternariol monomethyl ether-3-sulphate (AME-3-S); 9, alternariol-3-glucoside (AOH-3-G); 10, alternariol monomethyl ether-3-glucoside (AME-3-G); 11, altertoxin-II (ATX-II).

IV.2. Materials and methods

IV.2.1. Standards and Reagents

AOH, AME (1 mg standard each), ATX-I and ALT (0.1 mg standard each) were obtained from Fermentek (Jerusalem, Israel) and dissolved in 1 ml of methanol (MeOH). Certified reference standards of TeA and TEN (101.3 and 100.5 mg respectively, dried down) were obtained from Romer Laboratories Diagnostic GmbH (Tulln, Austria) and dissolved in 1 ml of acetonitrile (ACN). ATX-II standard was kindly provided by Prof. Warth (TU Wien). Reference standards of conjugated *Alternaria* toxins (AOH-3-S, AOH-3-G, AME-3-S, AME-3-G) were synthesized as described by Mikula et al. (2013) and stock solutions were prepared at a concentration of 10 µg/ml in MeOH. The internal standard urolithin A (UR-A) (5mg) was purchased from Sigma-Aldrich (Bornem, Belgium) and dissolved in 5 ml of dimethyl sulphoxide (DMSO). Internal standard tenuazonic acid ^{2}H-13 (1 mg) was bought from Toronto Research Chemical (Toronto, Canada) and dissolved in MeOH. Ultra-pure water was obtained from an Arium® pro system (Sartorius, Goettingen, Germany). ACN (absolute, LC-MS grade) and acetic acid (UPLC/MS) were obtained from BioSolve BV (Valkenswaard, The Netherlands), and ACN (HiPerSolv Chromanorm HPLC grade) was acquired from VWR International (Leuven, Belgium). Sodium chloride (NaCl) was purchased from Merck (Darmstadt, Germany), whereas magnesium sulphate ($MgSO_4$, anhydrous) from Sigma-Aldrich (Bornem, Belgium).

IV.2.2. Strains and inoculation

Based on their secondary metabolite production, 3 *Alternaria* strains isolated from apples in chapter II and identified as *A. tenuissima* in chapter III, were selected and inoculated in non-contaminated Red Delicious fruits (isolate ID: 02, 31 and 36). Isolate 02 was obtained from the exterior of the fruit, while 31 and 36 from MC. Healthy apple fruits were obtained from a local market and disinfected with ethanol (70 %). On one half of the fruits, a superficial wound of 1x1 cm was made with a sterile scalpel for exterior inoculation, and the other half was cut transversally for core inoculation. Suspensions of each strain in an aqueous solution of Tween 80 (0.05 %) at a concentration of 10^5 spores/ml were made. One microliter (µl) of suspension

was inoculated with a sterile calibrated loop in the interior and the exterior of the fruits, separately, and incubated at 25 °C and 4 °C for 1 and 9 months, respectively, to simulate retail and storage conditions. Each assay was done in triplicate and each apple was incubated in a separate sterile bag. Control fruits without inoculation were also incubated for the obtention of blank samples.

IV.2.3. Extraction

The extraction procedure was made using a validated method developed at the Centre of Excellence in Mycotoxicology and Public Health (CEMPH) for the detection and quantification of AOH, AME, ALT, TeA, TEN, ATX-I, AOH-3-S, AOH-3-G, AME-3-S, AME-3-G in apple products with some modifications (Walravens et al., 2016). Additionaly, ATX-II was also detected and quantified. Samples consisting of the entire contaminated fruit and blanks were grinded in individual sterile bags and an aliquot of 2.0000 ± 0.0020 g was weighed in an extraction tube. Five blanks were fortified with the studied mycotoxins at concentration levels ranging from 5 to 100 µg/kg and soaked for 15 min. Internal standards UR-A, a dibenzopyrone from the transformation of ellegitannins by the gut bacteria (Garcia-Muñoz & Vaillant, 2014), and TeA D-13 were added in concentrations of 10 µg/kg. After 10 s of vortex-mixing, samples were kept in the dark for 15 min. Subsequently, 10 ml of ACN (HPLC grade) were added and the tubes were shaken in an overhead shaker for 30 min. Sample extracts were briefly centrifuged (1 min, 3,200 g), and $MgSO_4$ anhydrous salt (2.00 ± 0.05g) and NaCl (0.50 ± 0.05g) were added. Afterwards, the tubes were vigorously shaken for 30 s, placed in an overhead shaker for 15 min, and centrifuged (10 min, 3,200 g). Six (6.00) ml of the supernatant were transferred to a tube and evaporated to dryness using a Turbovap LV module (Biotage AB, Uppsala, Sweden) maintained at 40 °C. Finally, the residue was redissolved in 100 µl of injection solvent (ultrapure water/ACN (LCMS grade), 70/30, v/v), vortex-mixed for 30 s, and centrifuged (Ultrafree-MC PTFE centrifugal filter units, 0.22 µm; Merck Millipore, Darmstadt, Germany) for 10 min at 10,000 g prior to analysis.

IV.2.4 Determination and quantification: LC-MS/MS analysis

Determination and quantification of the studied mycotoxins was done on a Waters Acquity UPLC coupled to a XEVO TQ-S mass spectrometer (Waters, Milford, MA, USA). To achieve compound separation, an Acquity UPLC High Strength Silica trifunctional C18 Alkyl phase (HSS T3, 1.8 µm, 2.1 x 100 mm) (Waters, Milford, MA) column was used. The instrument was used in the negative electrospray ionisation (ESI^-) mode. The temperature of the column was 35 °C, and the mobile phases were A: ultra-pure water/acetic acid (AA) (99/1, v/v) and B: ACN/AA (99/1, v/v). The flow rate was 0.4 ml/min and the total run time 7 min following the gradient described by Walravens et al. (2014). The capillary voltage was 30 kV, and nitrogen was applied as spray gas. The source and desolvation temperatures were set at 150 °C and 200 °C, respectively. The argon collision gas pressure was 9×10^{-6} bar, the cone gas flow 50 l/h and the desolvation gas flow 500 l/h. Two selected reaction monitoring (SRM) transitions with a specific dwell-time were optimised for each analyte, in order to increase the sensitivity and the selectivity of the mass spectrometric condition. These are detailed in **Table IV.1** as well as the optimized conditions for each analyte. The validation details of this method are described in detail by Walravens et al. (2014). The limits of detection (LOD) and quantification (LOQ) are informed in **Table IV.2**. For data acquisition and processing, the MassLynx and QuanLynx® version 4.1. software (Micromass, Manchester, UK) were used. After data processing, samples with mycotoxin concentrations outside the working range of the corresponding MMCC were diluted appropriately and reanalysed.

Table IV.1. Optimized MS/MS instrumental parameters of each mycotoxin; AOH: alternariol, AME: alternariol monomethyl ether, ALT: altenuene, TeA: tenuazonic acid, TEN: tentoxin, ATX-I: altertoxin-I, ATX-II: altertoxin-II, AOH-3-S: alternariol-3-sulphate, AME-3-S: alternariol monomethyl ether-3-sulphate, AOH-3-G: alternariol-3-glucoside, AME-3-G: alternariol monomethyl ether-3-glucoside.

Analyte	Precursor ion (*m/z*)	Molecular ion	Cone voltage (V)	Quantifier Product ion (m/z)	Collision energy (eV)	Qualifier Product ion (m/z)	Collision energy (eV)
AOH	257.1	$[M-H]^-$	75	213.1	35	215.1	28
AME	271.2	$[M-H]^-$	60	256.2	21	228.2	28
ALT	291.2	$[M-H]^-$	40	203.1	33	248.2	27
TeA	196.2	$[M-H]^-$	60	139.1	20	112.0	23
TEN	413.4	$[M-H]^-$	45	141.1	20	271.2	16
ATX-I	351.2	$[M-H]^-$	35	315.1	15	333.2	12
ATX-II	349.1	$[M-H]^-$	88	285.1	35	332.0	13
AOH-3-S	337.1	$[M-H]^-$	35	257.2	21	213.1	38
AME-3-S	351.1	$[M-H]^-$	35	271.2	22	256.1	35
AOH-3-G	419.2	$[M-H]^-$	70	228.1	35	256.1	28
AME-3-G	433.2	$[M-H]^-$	65	270.1	31	227.1	45

Table IV.2. Limits of detection (LOD) and limits of quantification (LOQ) for each *Alternaria* metabolite in µg/kg. AOH: alternariol, AME: alternariol monomethyl ether, ALT: altenuene, TeA: tenuazonic acid, TEN: tentoxin, ATX-I: altertoxin-I, ATX-II: altertoxin-II, AOH-3-S: alternariol-3-sulphate, AME-3-S: alternariol monomethyl ether-3-sulphate, AOH-3-G: alternariol-3-glucoside, AME-3-G: alternariol monomethyl ether-3-glucoside.

	LOD (µg/kg)	LOQ (µg/kg)
AOH	1.3	4.3
AME	0.3	1.1
ALT	1.1	3.6
TeA	1.3	4.4
TEN	1.0	3.4
ATX-I	1.5	5.0
ATX-II	1.7	3.4
AOH-3-S	0.4	1.4
AME-3-S	1.5	4.8
AOH-3-G	0.7	2.2
AME-3-G	1.5	4.8

IV.2.5. Statistical analysis

RStudio 4.1.1 was used to perform multifactorial ANOVA analysis to evaluate significant differences of mycotoxins concentrations under the different conditions evaluated and the influence of strain, temperature, inoculation site and their interactions on mycotoxin production.

IV.3. Results

At 25 °C the fungal growth of *Alternaria* isolates 02, 31 and 36 on the apples was faster than at 4 °C. After one month of incubation at 25 °C, fungal growth was evident and abundant in the exterior and interior of the fruits. For the apples incubated at 4 °C, after 9 months of

incubation, contamination was visible in the interior and exterior of the fruit (**Figure IV.2**). Pathogenicity tests could not be performed due to the Corona virus crisis and the impediment to access the research laboratory to take daily measures of the lesions. Control samples did not develop fungal spoilage.

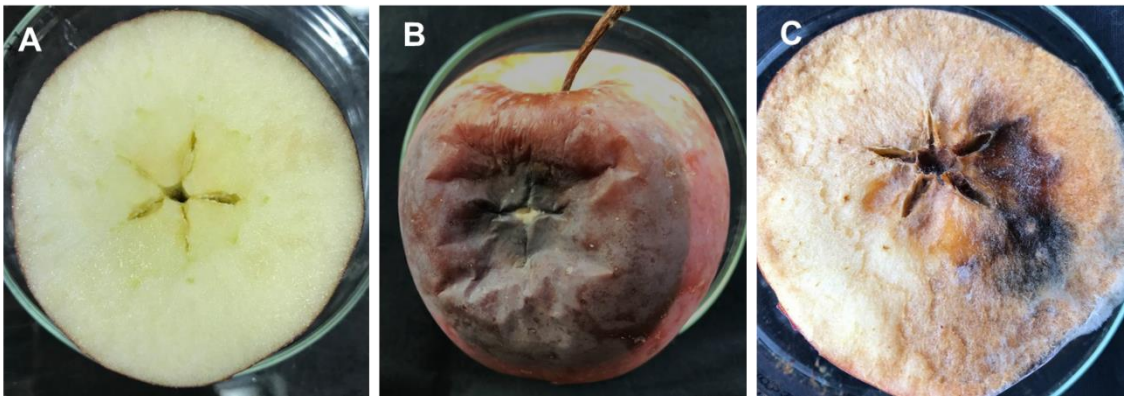

Figure IV.2. View of apples after incubation at 4 °C for 9 months. A: control sample without fungal growth; B: external lesion infested by isolate 02; C: core growth of isolate 02 on one half of the apple. Pictures taken by María Agustina Pavicich.

All the isolates were able to produce mycotoxins under the studied conditions. **Tables IV.3**, **IV.4** and **IV.5** show the concentration of each mycotoxin under the tested conditions for each of the triplicates. AOH, AME, ALT, TeA, TEN, ATX-I, ATX-II and AOH-3-G were detected at certain conditions. AOH-3-S, AME-3-S and AME-3-G were not produced under any of the tested conditions by any of the strains. TeA was produced in the highest concentrations by all the strains. As it had an atypically large residue in the homoscedasticity test, it was excluded from the multifactorial ANOVA analysis.

Significant differences were found between the mycotoxin production by the different strains ($p<0.05$) (Annex Table IV.1). **Figure IV.3** and **IV.4** depict mycotoxin concentrations for strain 02, **Figures IV.5** and **IV.6** for isolate 31 and **Figures IV.7** and **IV.8** for isolate 36. TeA is shown separately from the other toxins due to the different scale required for its concentration.

Table IV.3. Mycotoxin concentration produced by isolate 02 inoculated in the exterior or interior of apple fruit in µg/kg. Incubation period was one month at 25 °C and 9 months at 4 °C. AOH: alternariol, AME: alternariol monomethyl ether, ALT: altenuene, TeA: tenuazonic acid, TEN: tentoxin, ATX-I: altertoxin-I, ATX-II: altertoxin-II, AOH-3-S: alternariol-3-sulphate, AME-3-S: alternariol monomethyl ether-3-sulphate, AOH-3-G: alternariol-3-glucoside, AME-3-G: alternariol monomethyl ether-3-glucoside. n.d.: not detected

Isolate ID	Inoculation site	Temperature (°C)	Metabolite concentration (µg/kg)										
			AOH	AME	ALT	TeA	TEN	ATX-I	ATX-II	AOH-3-S	AME-3-S	AOH-3-G	AME-3-G
02	Exterior	25	1001.5	571.1	407.8	31.3	30.9	7.3	n.d.	n.d.	n.d.	n.d.	n.d.
	Exterior	25	69.9	11.0	20.6	n.d.	156.1	15.4	n.d.	n.d.	n.d.	n.d.	n.d.
	Exterior	25	401.1	13.8	1.8	55.5	3.5	5.3	n.d.	n.d.	n.d.	n.d.	n.d.
	Core	25	530.7	93.7	1022.5	37269.4	163.5	42.5	n.d.	n.d.	n.d.	n.d.	n.d.
	Core	25	1213.2	534.0	1570.6	232.8	75.1	43.4	30.5	n.d.	n.d.	n.d.	n.d.
	Core	25	595.0	691.8	192.8	n.d.	131.6	9.2	n.d.	n.d.	n.d.	n.d.	n.d.
	Exterior	4	2.2	n.d.	n.d.	1416.7	110.0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Exterior	4	n.d.	n.d.	1.2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Exterior	4	427.5	8.6	n.d.	18299.3	4.0	3.0	n.d.	n.d.	n.d.	69.8	n.d.
	Core	4	131.7	8.7	11.7	n.d.	n.d.	2.5	n.d.	n.d.	n.d.	n.d.	n.d.
	Core	4	161.7	10.7	12.0	114.5	n.d.	3.9	n.d.	n.d.	n.d.	n.d.	n.d.
	Core	4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Table IV.4. Mycotoxin concentration produced by isolate 31 inoculated in the exterior or interior of apple fruit in µg/kg. Incubation period was one month at 25 °C and 9 months at 4 °C. AOH: alternariol, AME: alternariol monomethyl ether, ALT: altenuene, TeA: tenuazonic acid, TEN: tentoxin, ATX-I: altertoxin-I, ATX-II: altertoxin-II, AOH-3-S: alternariol-3-sulphate, AME-3-S: alternariol monomethyl ether-3-sulphate, AOH-3-G: alternariol-3-glucoside, AME-3-G: alternariol monomethyl ether-3-glucoside. n.d.: not detected.

Isolate ID	Inoculation site	Temperature (°C)	Metabolite concentration (µg/kg)										
			AOH	AME	ALT	TeA	TEN	ATX-I	ATX-II	AOH-3-S	AME-3-S	AOH-3-G	AME-3-G
31	Exterior	25	1.9	n.d.	n.d.	491.6	1.8	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Exterior	25	1.8	1.6	n.d.	11.3	5.0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Exterior	25	30.8	n.d.	n.d.	19494.7	52.1	45.7	69.8	n.d.	n.d.	n.d.	n.d.
	Core	25	n.d.	n.d.	n.d.	n.d.	24.2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Core	25	2.2	n.d.	n.d.	47108.3	75.9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Core	25	57.1	28.8	n.d.	2100.2	1.6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Exterior	4	2.0	n.d.	n.d.	n.d.	2.8	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Exterior	4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Exterior	4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Core	4	195.3	193.0	n.d.	13597.5	n.d.	n.d.	2.3	n.d.	n.d.	8.4	n.d.
	Core	4	37.3	10.0	n.d.	142900.3	9.3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Core	4	n.d.	n.d.	n.d.	178.8	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Table IV.5. Mycotoxin concentration produced by isolate 36 inoculated in the exterior or interior of apple fruit in µg/kg. Incubation period was one month at 25 °C and 9 months at 4 °C. AOH: alternariol, AME: alternariol monomethyl ether, ALT: altenuene, TeA: tenuazonic acid, TEN: tentoxin, ATX-I: altertoxin-I, ATX-II: altertoxin-II, AOH-3-S: alternariol-3-sulphate, AME-3-S: alternariol monomethyl ether-3-sulphate, AOH-3-G: alternariol-3-glucoside, AME-3-G: alternariol monomethyl ether-3-glucoside. n.d.: not detected.

Isolate ID	Inoculation site	Temperature (°C)	Metabolite concentration (µg/kg)										
			AOH	AME	ALT	TeA	TEN	ATX-I	ATX-II	AOH-3-S	AME-3-S	AOH-3-G	AME-3-G
36	Exterior	25	21.5	1.1	n.d.	277508.0	147.0	9.6	n.d.	n.d.	n.d.	n.d.	n.d.
	Exterior	25	10.0	2.4	n.d.	679358.2	92.8	3,00	n.d.	n.d.	n.d.	n.d.	n.d.
	Exterior	25	2.3	n.d.	n.d.	79458.4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Core	25	336.9	171.8	38.3	150427.5	181.5	4.1	n.d.	n.d.	n.d.	n.d.	n.d.
	Core	25	119.4	12.6	16.8	7512.2	45.0	51.2	n.d.	n.d.	n.d.	n.d.	n.d.
	Core	25	1399.1	292.1	402.7	240237.9	3938.9	51.6	98.4	n.d.	n.d.	n.d.	n.d.
	Exterior	4	7.3	n.d.	1.2	884.1	n.d.	7.8	n.d.	n.d.	n.d.	n.d.	n.d.
	Exterior	4	n.d.	n.d.	1.2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Exterior	4	n.d.	n.d.	1.2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Core	4	n.d.	n.d.	n.d.	n.d.	8.9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Core	4	15.3	2.1	1.7	2282.9	n.d.	3.3	n.d.	n.d.	n.d.	n.d.	n.d.
	Core	4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

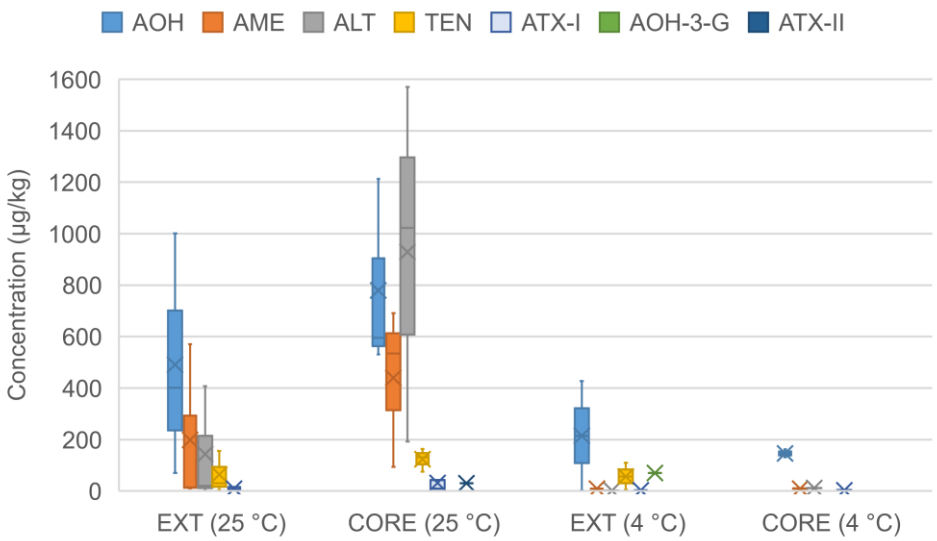

Figure IV.3. Box plot of mycotoxin production by isolate 02 under different conditions. EXT: External inoculation; CORE: Core inoculation. Temperature of incubation indicated between parentheses. Incubation period was one month at 25 °C and 9 months at 4 °C. Only produced mycotoxins were depicted except for tenuazonic acid. AOH: alternariol; AME: alternariol monomethyl ether; ALT: altenuene; TEN: tentoxin; ATX-I: altertoxin-I; ATX-II: altertoxin-II, AOH-3-G: alternariol-3-glucoside.

The ANOVA results showed that temperature and inoculation site had a significant influence on toxin production by isolate 02 ($p<0.05$) (Annex Table IV.2). This strain produced the highest concentration of ALT among the three isolates used for this study. The maximum accumulation of this toxin was observed after of incubation at 25 ºC for 1 month and in MC. High quantities of AOH and AME were also detected at the same conditions. These three toxins were also the ones produced at its highest at 25 ºC in the exterior of the fruit, although in lower levels. At 4 ºC, after 9 months of incubation, AOH was detected in relatively high levels, both in MC and the external lesion. The other toxins

produced at this temperature were AME, ALT, ATX-I, TEN, and AOH-3-G, but at low levels (**Figure IV.3**).

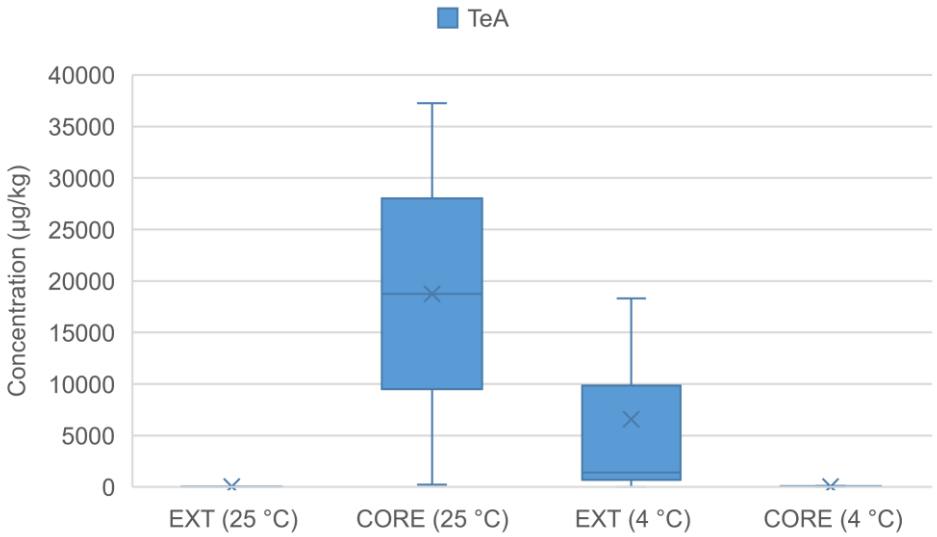

Figure IV.4. Box plot of tenuazonic acid (TeA) production by isolate 02 under different incubation conditions. EXT: External inoculation; CORE: Core inoculation. Temperature of incubation indicated between parentheses. Incubation period was one month at 25 °C and 9 months at 4 °C.

TeA production by isolate 02 showed an interesting pattern (**Figure IV.4**). At 25 ºC, significantly higher amounts were produced in MC. However, the opposite was observed at 4 ºC; the external lesion allowed much higher toxin production than the MC infection.

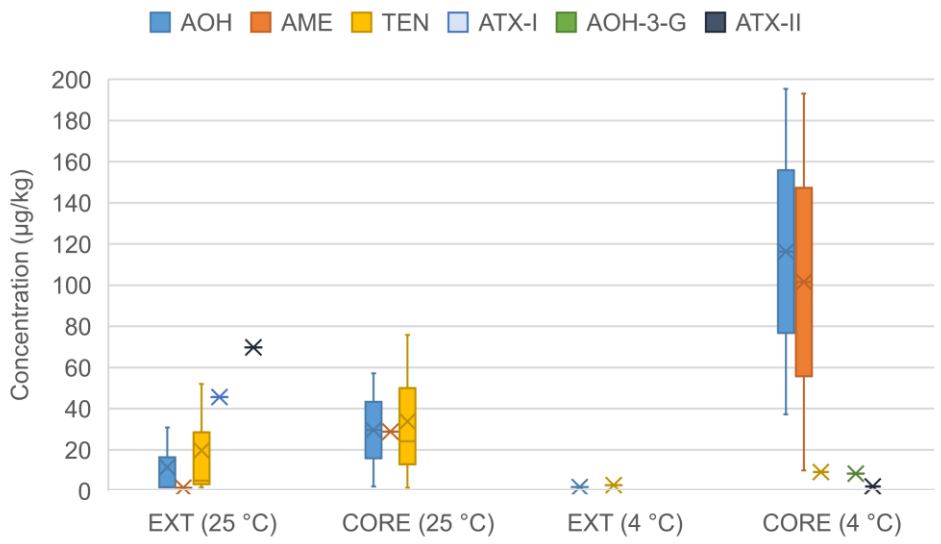

Figure IV.5. Box plot of mycotoxin production by isolate 31 under different conditions. EXT: External inoculation; CORE: Core inoculation. Temperature of incubation indicated between parentheses. Incubation period was one month at 25 °C and 9 months at 4 °C. Only produced mycotoxins were depicted except for tenuazonic acid. AOH: alternariol; AME: alternariol monomethyl ether; ALT: altenuene; TEN: tentoxin; ATX-I: altertoxin-I; ATX-II: altertoxin-II; AOH-3-G: alternariol-3-glucoside.

The ANOVA did not show significant effects of temperature, inoculation site and their interactions on mycotoxin production by isolate 31 (Annex Table IV.3). This can be explained by the fact that this isolate produced low levels of all the analysed toxins. The alternariols and TEN were the toxins accumulated in highest amounts, and were produced in all the conditions, except in the exterior at 4 ºC, where AME was not detected (**Figure IV.5**). TeA production by this strain was higher in MC, although similar levels were accumulated at both temperatures (**Figure IV.6**).

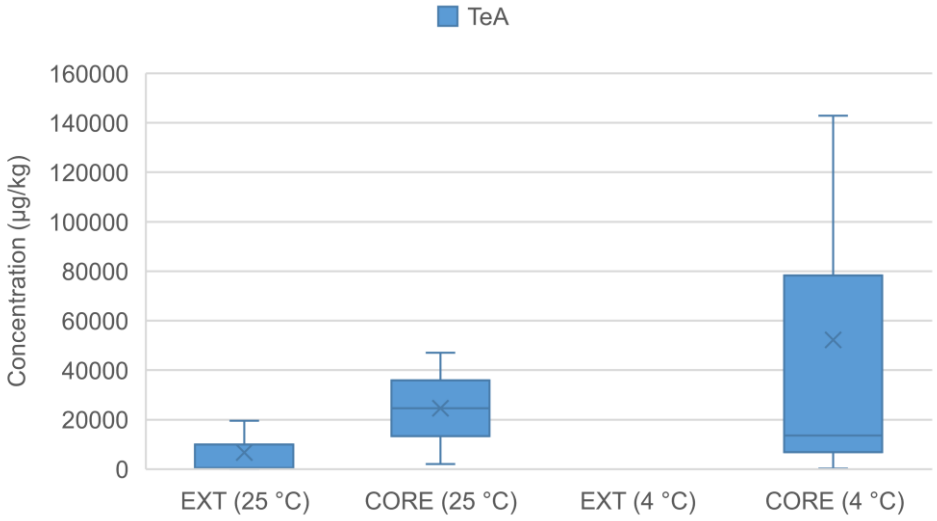

Figure IV.6. Box plot of tenuazonic acid (TeA) production by isolate 31 under different incubation conditions. EXT: External inoculation; CORE: Core inoculation. Temperature of incubation indicated between parentheses. Incubation period was one month at 25 °C and 9 months at 4 °C.

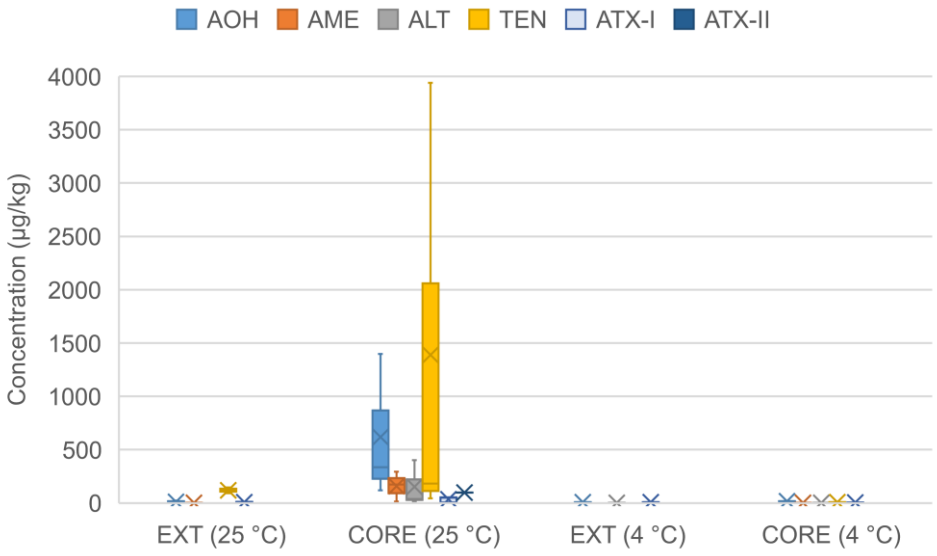

Figure IV.7. Box plot of mycotoxin production by isolate 36 under different conditions. EXT: External inoculation; CORE: Core inoculation. Temperature of incubation indicated

between parentheses. Incubation period was one month at 25 °C and 9 months at 4 °C. Only produced mycotoxins were depicted except for tenuazonic acid. AOH: alternariol; AME: alternariol monomethyl ether; ALT: altenuene; TEN: tentoxin; ATX-I: altertoxin-I; ATX-II: altertoxin-II; AOH-3-G: alternariol-3-glucoside.

Although the ANOVA did not show a significant effect of temperature and inoculation site on mycotoxin production by strain 36 (Annex Table IV.4), MC at 25 ºC was the condition at which higher concentration of toxins were detected. Particularly, TEN and AOH were the toxins with maximum accumulation (**Figure IV.7**). On the other hand, TeA, was highly influenced by temperature, as concentrations observed at 25 ºC were at least 100 times higher than at 4 ºC (**Figure IV.8**)

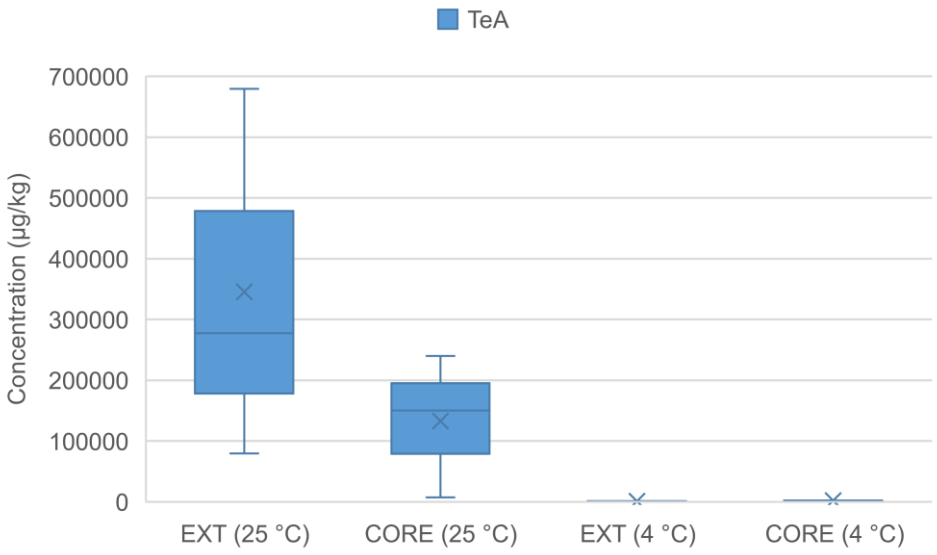

Figure IV.8. Box plot of tenuazonic acid (TeA) production by isolate 36 under different conditions. EXT: External inoculation; CORE: Core inoculation. Temperature of incubation indicated between parentheses. Incubation period was one month at 25 °C and 9 months at 4 °C.

IV.4. Discussion

The metabolic differences of the strains were investigated in chapter III, and a wider *in vitro* metabolic capacity of strains isolated from MC was found. The strains tested *in vivo* were isolated from the exterior (isolate 02) and MC (isolates 31 and 36) of apple fruit, but they all belonged to Cluster I, the one with the ability to synthesise a broader family of compounds (chapter III), therefore, representing the most diverse scenario for mycotoxin production.

When tested *in vitro,* the three isolates selected for this study were able to produce all the tested mycotoxins (**Figure IV.1**) except for the modified forms and isolate 02 did not produce TeA. *In vivo,* these strains could produce the same mycotoxins as *in vitro* at least under one of the tested conditions, except for ALT that was not produced by strain 31 under any condition. Interestingly, isolate 02 that could not synthesise TeA *in vitro* at 25 °C, produced large amounts of this toxic metabolite when colonizing the centre of the fruit at 25 °C for one month or the exterior at 4 °C for 9 months and lower concentrations under the other conditions. Isolates 02 and 31 produced AOH-3-G at 4 °C, a metabolite that was not detected *in vitro*. Glucose conjugates are typically formed by plant metabolism (Puntscher et al., 2019a), therefore, expected in the *in vivo* analysis. Temperature seems to affect the production of this alternariol modified form, but further studies are needed to confirm it. The other modified forms of the alternariols were not produced under any condition in the present study, but there are reports of the natural occurrence of these metabolites (AOH-3-S, AME-3-S) in commercially available tomato products (Puntscher et al., 2019b, 2018; Walravens et al., 2016). Whether they are produced by the tomato plant metabolism or during food processing still needs to be evaluated.

Mycotoxin concentration varied with temperature and inoculation site and was highly dependent on the strain. For strains 02 and 36, mycotoxin accumulation was higher when infecting the fruit in the core at 25 °C for one month (**Figures IV.3, IV.4, IV.7 and IV.8**).

On the other hand, strain 31 accumulated the alternariols in higher concentration when infecting the core at 4 °C than at the other conditions (**Figure IV.5**), but this strain produced lower levels of the other toxins than isolates 02 and 36. Thus, alternariol levels produced by strain 31 at 4 ºC were similar to those produced by the other strains under the same conditions. TeA was the mycotoxin produced at the highest concentrations at all the conditions by the 3 strains *in vivo*. Similarly, a recent report of naturally rotten apples in China also showed that TeA was the toxin with the highest accumulation in rotten fruit in comparison with the other *Alternaria* toxins (Li et al., 2020).

Comparison with literature data is difficult, since there is only one report on the production of AOH, AME, ALT and TeA by an *Alternaria* strain artificially inoculated in the exterior of apple fruit at 25 °C (Ozcelik et al., 1990). In that study, the concentrations of AOH, AME, and ALT when storing fruit for 5 weeks at 25 °C were similar to those detected in the present one after 1 month of incubation at 25 ºC simulating retail conditions. More recently, Puntscher et al. (2020) quantified 17 *Alternaria* toxins in naturally contaminated retail apples and only TEN was reported and in low concentrations. López et al. (2016) analysed retail apples in the Netherlands and from AOH, AME, ALT, TEN and TeA, only low concentrations of AOH were detected. Recently, in China, Li et al. (2020) determined the concentration of AOH, AME, ALT, TEN and TeA on different parts of fresh retail naturally contaminated apples and reported high concentrations of all these toxins in the areas closer to the lesion.

Regarding the other evaluated condition, after a prolonged cold storage period commonly performed by packhouses and apple processing industries, fungal growth was evident and *Alternaria* mycotoxin production occurred. At 4 °C, AOH, AME, ALT, TEN, TeA, ATX-I, ATX-II and AOH-3-G were produced at least by one of the strains either in the exterior or the core of the fruit stored for 9 months. Mycotoxin production at 4 °C was not reported by Ozcelik et al. (1990), but the incubation time at low temperature was shorter (5 weeks) than the one proposed in the current study. Puntscher et al. (2020)

reported AOH, AME, AME-3-S and TEN in lower concentrations than those from the present study in stored apples under professional storage for 6 months. Nonetheless, in a sample with visual fungal growth after storage, they observed high concentrations of AOH, AME, AOH-3-G, AOH-9-G, AOH-3-S, AME-3-S, ALT, its isoform isoALT, ATX-I, ATX-II and alterperylenol. These results suggest that at low temperature, fungal growth and mycotoxin accumulation is rather a slow but steady process.

Overall, incubation at 25 °C favoured, as expected, mycotoxin production and higher levels of AOH, AME, TeA, TEN and ATXs accumulated when *Alternaria* strains from apple colonized the centre of the fruit. On the other hand, storing fruit in refrigeration chambers slowed down the fungal colonization and delayed its grow and mycotoxin accumulation, but it did not prevent it. Consumers eating retail apples, would likely reject a highly infested fruit. However, as long-term cold stored fruit is usually destined to processing, it represents a risk from a food safety point of view, especially when affected with MC. Whether the mixing and grinding of contaminated and non-contaminated fruit results in a significant reduction of the mycotoxin concentration or any other step in processing apple by-products needs to be studied. Overall, with these results, high concentrations of TeA are expected in raw materials of the apple processing industries, as well as AOH, AME and TEN. Lower concentrations of ALT, ATX-I and ATX-II are suspected and the presence of AOH-3G could also be expected in fruit destined to apple concentrate and in apple by-products.

IV.5. Conclusions

The *Alternaria* strains isolated from apple fruit were able to produce mycotoxins under retail and long-term cold storage conditions in the interior and the exterior of the fruit. The risk of mycotoxin accumulation was higher at 25 ºC, but the long-term cold storage usually performed by processing industries did not prevent the accumulation of secondary *Alternaria* toxic metabolites in apples. This is particularly of concern when the

storage period extends for several months. These results imply a risk of the presence of *Alternaria* mycotoxins in commercially available fruit and fruit destined to process and a possible consequent accumulation of toxic metabolites in apple by-products.

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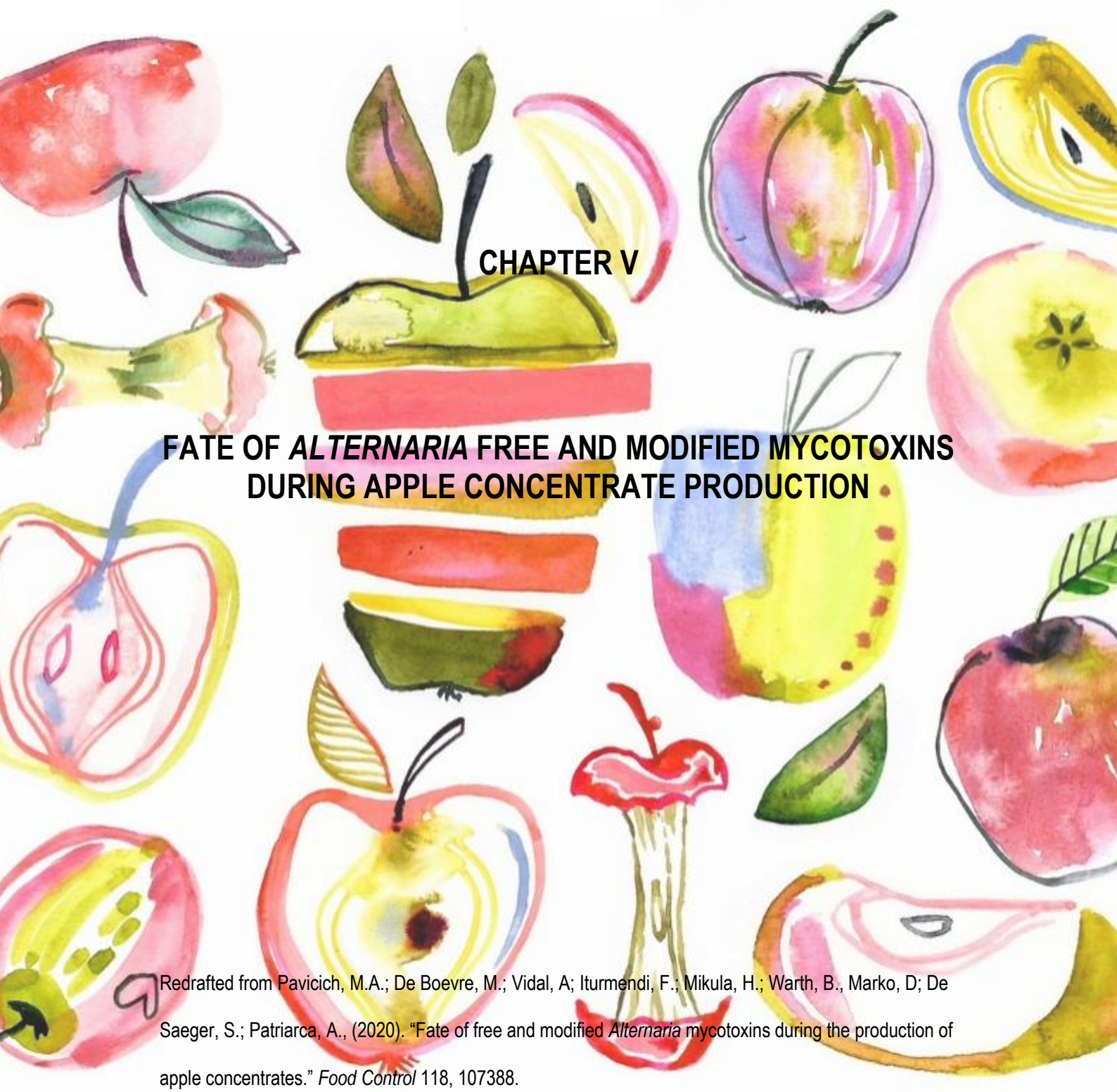

CHAPTER V

FATE OF *ALTERNARIA* FREE AND MODIFIED MYCOTOXINS DURING APPLE CONCENTRATE PRODUCTION

Redrafted from Pavicich, M.A.; De Boevre, M.; Vidal, A; Iturmendi, F.; Mikula, H.; Warth, B., Marko, D; De Saeger, S.; Patriarca, A., (2020). "Fate of free and modified *Alternaria* mycotoxins during the production of apple concentrates." *Food Control* 118, 107388.

Ilustration by Tanja Meyer

V.1. Introduction

Apple juice is the second most consumed fruit juice worldwide (Sulaiman et al., 2017) and can be produced either from the apple concentrate or directly from the fruit (not from concentrate juice) (Gou et al., 2019). There are two types of juices: the “clarified” or “clear” juice, in which a clarification treatment is included, and the so-called “with pulp” or “cloudy” that contains natural colloidal suspensions. In the past, consumers on the global level preferred the clear conventional apple juices; nevertheless, a shift to less processed and organic products was observed as these are considered healthier. Cloudy apple juices, besides being perceived by consumers as a more natural, minimal processed product, have a higher antioxidant activity due to a higher polyphenolic content (Oszmianski et al., 2007; Teleszko et al., 2016). As well, consuming cloudy apple juice produces a reduction on cholesterol levels in serum, while the consumption of clear apple juice has an adverse effect on blood lipids (Ravn-Haren et al., 2013).

In the apple producing area in Northern Patagonia, Argentina, an important apple concentrate industry developed, producing concentrates from conventional and organic crops. Their production supplies both Argentinean industries, as well as the demand from international markets. Apple concentrates are mainly diluted to produce juices, but can also be used as food additives, such as coating for carrot snacks to enhance their properties (Peng et al., 2019), or to treat diseases as acute lunge inflammation (Shaw et al., 2021). From the apple concentrate process, several by-products are also obtained, such as aroma and apple pomace that can be used in sausages, jams, baked goods and animal feed (Coelho et al., 2021; O’Shea et al., 2012).

Fruits that do not comply with quality standards for fresh consume are usually derived to these industries for concentrate production (Idigoras, 2014). The high incidence of MC caused by *Alternaria* in fruit destined to industry and the worsening effect produced by long-term storage of the fruit has been shown in the previous chapters, as well as the ability of infecting strains to produce mycotoxins *in vivo* and *in vitro*. It is therefore likely

that contaminated raw material is incorporated into the process line. Furthermore, these secondary metabolites can be modified by conjugation producing phase-II derivatives that can be reconverted to their native form during the production process of contaminated food commodities or by the human metabolism, contributing to the intake of the native form of the mycotoxin (Puntscher et al., 2019a, 2019b).

The effect of the apple concentrate process on PAT has been studied and it is believed that safe levels of this toxin in the final product are achievable (Pinton et al., 2019; Welke et al., 2009). However, to date no information is available on the fate of *Alternaria* toxins during apple concentrate production.

Therefore, the objective of this chapter was to evaluate the effect of the apple concentrate process on the natural contamination levels of 6 key *Alternaria* mycotoxins, namely AOH, AME, ALT, TeA, TEN, and ATX-I, two modified forms of AOH, alternariol 3-sulphate (AOH-3-S) and alternariol 3-glucoside (AOH-3-G) and two of AME, alternariol monomethyl ether 3-sulphate (AME-3-S) and alternariol monomethyl ether 3-glucoside (AME-3-G).

V.2. Materials and methods

V.2.1. Samples

A total of 34 samples corresponding to five independent apple concentrate processes from an Argentinean company from the Alto Valle of Río Negro, Patagonia, were obtained at different stages and analysed for the mentioned *Alternaria* mycotoxins. The five processes used fruit from the Red Delicious variety, which is the same variety of apples analysed for fungal contaminants during this PhD thesis; 3/5 included a clarification step, yielding a clear final product, while 2/5 omitted this step resulting in a cloudy final product. From the three clear processes, two used conventionally grown fruit and one organic apples. Meanwhile, of the two cloudy processes, one was made with

conventional and the other with organic fruit. Additionally, a sixth process using Granny Smith apples was sampled for comparison; this used conventionally grown fruit and included the clarification step (clear product). Six stages of concentrate production were sampled, namely grinding (1), turbos (2), decanter muds (3), pre-concentration (4), concentrate (5) and rejection (6); the latter was only applied in the clear concentrate process since the cloudy process does not include this step (**Figure V.1**). **Table V.1** provides a description of the different processes and stages sampled. Firstly, fruit arrives either from the field (recently harvested) or from storage chambers and goes through washing and selection. Later, the fruit gets placed in a press house (**Figure V.2, A**) before getting grinded and submitted into a turbo, where skin, seeds and peduncles are withdrawn from the process line. Further, the juice from the turbos is treated with water vapour at 100° C where the aroma is extracted and a maceration with enzymes is performed (**Figure V.2, B**). The resulting product is decanted and pre-concentrated (**Figure V.2, C**), and the decanter muds or pomace is obtained as a sub-product. If the intended final product is a cloudy one, it is further concentrated before cooling and bottling. For the obtention of a clear product, a treatment with activated charcoal and enzymes takes place before an ultrafiltration (**Figure V.2, D**) where pectins are eliminated before cooling and bottling of the clear concentrate (**Figure V.2, E**).

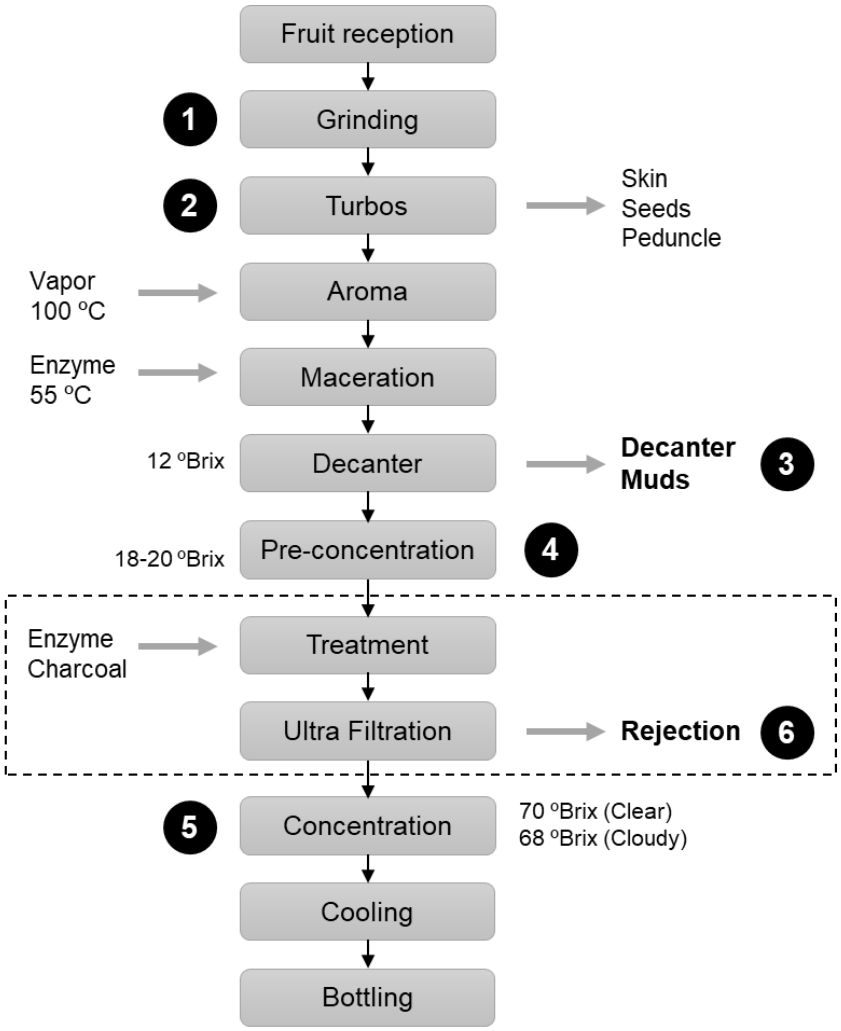

Figure V.1. Flow diagram of the apple concentrate production. The production of the clear apple concentrate includes all steps as shown in the diagram. The cloudy apple concentrate production does not include stages inside the dashed line box. Circles with numbers represent the sampling points.

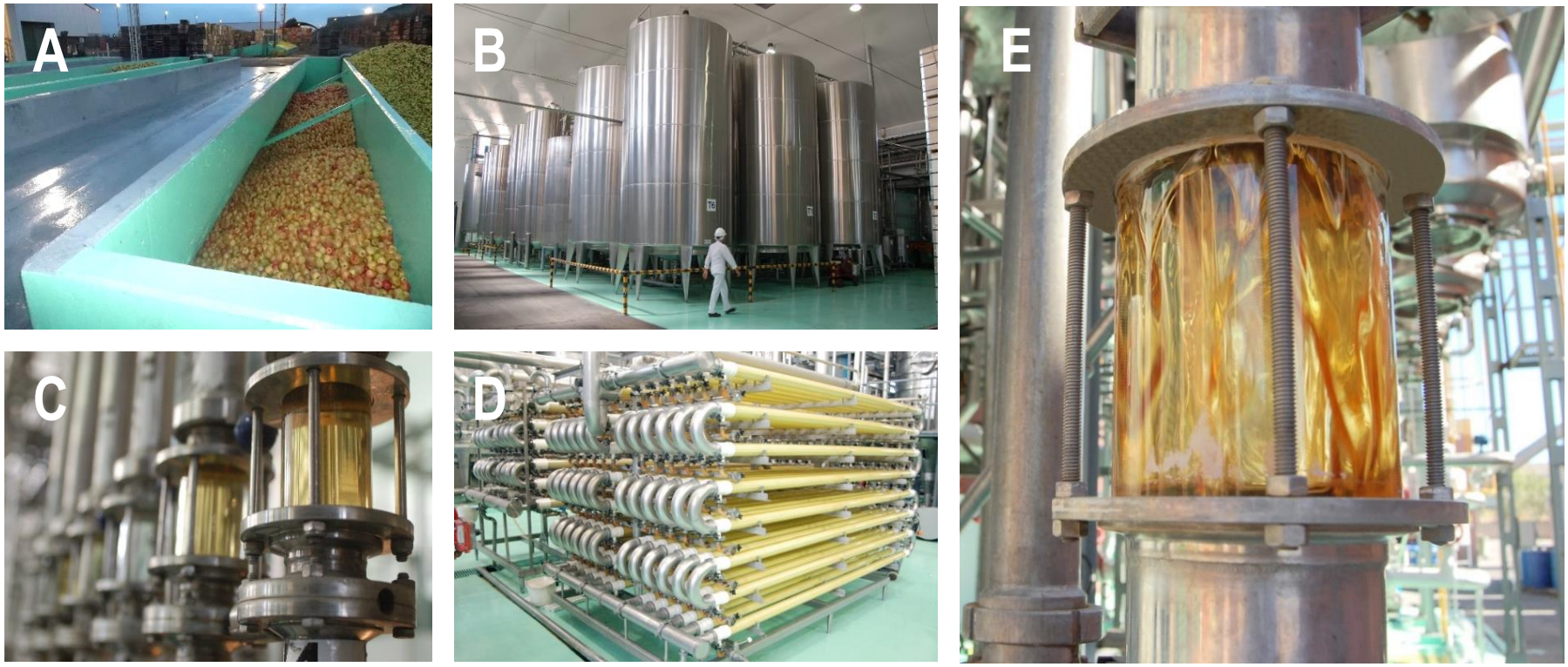

Figure V.2. Overview of production process of apple concentrate. A: press house, B: maceration tanks, C: juice obtained from pre-concentration, D: ultrafiltration, E: final product. Pictures were gently provided by Facundo Iturmendi.

Table V.1. Description of the six apple concentrate processes sampled, including the process number, the type of final product, the type of crop used, the apple variety and the stages sampled in each process. Stages 1: grinding; 2: turbos; 3: decanter muds; 4: pre-concentrate; 5: concentrate; 6: rejection.

Process Number	Product Type	Crop	Apple Variety	Stages sampled
1	Cloudy	Conventional	Red Delicious	1-5
2	Cloudy	Organic	Red Delicious	1-5
3	Clear	Organic	Red Delicious	1-6
4	Clear	Conventional	Red Delicious	1-6
5	Clear	Conventional	Red Delicious	1-6
6	Clear	Conventional	Granny Smith	1-6

V.2.2. Extraction

For each step, blank samples were made mimicking the apple concentrate process at laboratory scale with apple fruits free from fungal spoilage for the construction of matrix-matched calibration (MMC) curves. The extraction and quantification procedure were made following the same procedure described in chapter IV with the exclusion of ATX-II because of the lack of standard (Walravens et al., 2016). **Figure V.3** shows an UPLC-MS/MS chromatogram of an apple concentrate spiked at 100 µg/kg. LOD and LOQ for each compound is presented in **Table IV.2**.

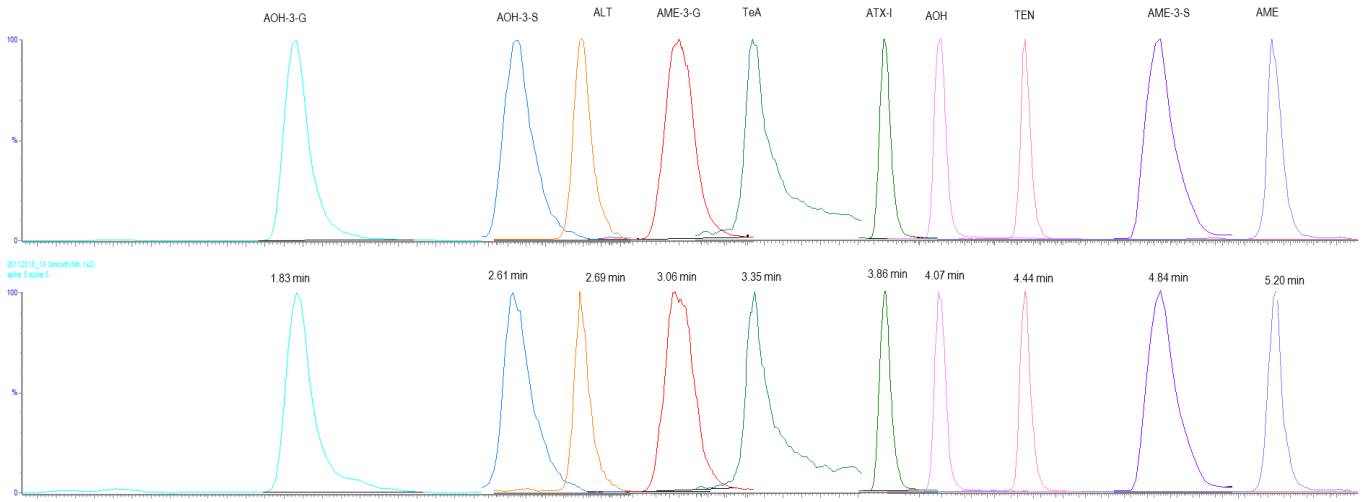

Figure V.3. UPLC-MS/MS chromatogram of an apple concentrate spiked at 100 µg/kg. A: quantifier ions and name of compound, B: qualifier ions and retention time. AOH-3-G: alternariol-3-glucoside; AOH-3-S: alternariol-3-sulphate; ALT: altenuene; AME-3-G: alternariol monomethyl ether-3-glucoside; TeA: tenuazonic acid; ATX-I: altertoxin-I, AOH: alternariol; TEN: tentoxin; AME-3-S: alternariol monomethyl ether-3-sulphate; AME: alternariol monomethyl ether.

V.3. Results and discussion

Table V.4 shows the concentration of each of the six *Alternaria* toxins and their modified forms by step and process. ALT and ATX-I were not detected in any of the processes studied at any stage, as well as AME-3-G, a modified form of AME. The levels of AOH, AME, TeA and TEN and their changes throughout the stages of the six processes studied are represented in **Figure V.4**. The prevalence of each metabolite, average, median, and range for the five Red Delicious processes, detailed by stage, are listed in **Table V.5**.

Table V.4. Concentration of *Alternaria* metabolites in each stage of the six independent apple concentrate processes.

Step		Process Number	Process Type	Crop	Metabolite concentration (µg/kg)									
					AOH	AME	ALT	TeA	TEN	ATX-I	AOH-3-S	AME-3-S	AOH-3-G	AME-3-G
GRINDING	1	1	Cloudy	Conventional	7.6	4.5	n.d.	7.1	8.0	n.d.	n.d.	n.d.	n.d.	n.d.
	1	2	Cloudy	Organic	11.1	8.9	n.d.	30.6	n.d.	n.d.	n.d.	4.8	n.d.	n.d.
	1	3	Clear	Organic	13.6	9.6	n.d.	119.0	18.4	n.d.	n.d.	2.5 *	n.d.	n.d.
	1	4	Clear	Conventional	14.8	6.4	n.d.	50.7	17.7	n.d.	n.d.	5.8	n.d.	n.d.
	1	5	Clear	Conventional	11.4	9.1	n.d.	15.9	7.0	n.d.	n.d.	n.d.	n.d.	n.d.
	1	6**	Clear	Conventional	n.d.	4.0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
TURBOS	2	1	Cloudy	Conventional	n.d.	2.6	n.d.	2.8 *	n.d.	n.d.	n.d.	3.0 *	n.d.	n.d.
	2	2	Cloudy	Organic	2.9 *	6.3	n.d.	13.6	n.d.	n.d.	n.d.	3.9 *	n.d.	n.d.
	2	3	Clear	Organic	8.0	7.9	n.d.	89.6	1.4 *	n.d.	n.d.	3.9 *	n.d.	n.d.
	2	4	Clear	Conventional	7.9	5.2	n.d.	70.5	12.1	n.d.	n.d.	4.4 *	n.d.	n.d.
	2	5	Clear	Conventional	4.0 *	4.6	n.d.	26.9	7.6	n.d.	n.d.	3.6 *	n.d.	n.d.
	2	6**	Clear	Conventional	n.d.	1.8	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
DECANTER MUDS	3	1	Cloudy	Conventional	42.1	8.0	n.d.	31.1	n.d.	n.d.	2.4	7.3	4.0	n.d.
	3	2	Cloudy	Organic	19.9	11.6	n.d.	18.6	n.d.	n.d.	n.d.	4.8	2.6	n.d.
	3	3	Clear	Organic	44.9	10.6	n.d.	29.9	7.4	n.d.	n.d.	9.7	3.6	n.d.
	3	4	Clear	Conventional	28.5	6.8	n.d.	41.8	9.2	n.d.	1.4	6.0	n.d.	n.d.
	3	5	Clear	Conventional	29.1	5.9	n.d.	33.1	13.8	n.d.	n.d	4.3 *	2.2	n.d.
	3	6**	Clear	Conventional	n.d.	2.7	n.d.	n.d.	n.d.	n.d.	n.d.	4.5 *	n.d.	n.d.
PRE-CONCENTRATE	4	1	Cloudy	Conventional	6.5	2.5	n.d.	61.9	n.d.	n.d.	2.3	4.8	n.d.	n.d.
	4	2	Cloudy	Organic	1.5 *	1.1	n.d.	20.9	3.1*	n.d.	1.8	2.4 *	n.d.	n.d.
	4	3	Clear	Organic	9.5	3.5	n.d.	103.8	13.3	n.d.	3.2	5.6	n.d.	n.d.
	4	4	Clear	Conventional	3.4 *	1.2	n.d.	63.0	8.5	n.d.	2.0	2.3 *	n.d.	n.d.
	4	5	Clear	Conventional	n.d.	0.9*	n.d.	52.9	8.8	n.d.	1.5	1.7 *	n.d.	n.d.
	4	6**	Clear	Conventional	n.d.	n.d.	n.d.	1.4*	n.d.	n.d.	1.6	2.0 *	n.d.	n.d.
CONCENTRATE	5	1	Cloudy	Conventional	46.4	18.5	n.d.	135.8	10.7	n.d.	6.0	10.0	n.d.	n.d.
	5	2	Cloudy	Organic	21.1	20.1	n.d.	102.6	18.1	n.d.	4.4	9.7	n.d.	n.d.
	5	3	Clear	Organic	n.d.	n.d.	n.d.	1.9 *	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	5	4	Clear	Conventional	n.d.	n.d.	n.d.	19.5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	5	5	Clear	Conventional	n.d.	n.d.	n.d.	n.d	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	5	6**	Clear	Conventional	n.d.	n.d.	n.d.	n.d	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
REJECTION	6	3	Clear	Organic	n.d.	n.d.	n.d.	30.3	8.3	n.d.	n.d.	n.d.	n.d.	n.d.
	6	4	Clear	Conventional	n.d.	n.d.	n.d.	37.2	14.8	n.d.	n.d.	n.d.	n.d.	n.d.
	6	5	Clear	Conventional	n.d.	n.d.	n.d.	46.2	26.2	n.d.	10.8	n.d.	18.7	n.d.
	6	6**	Clear	Conventional	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

AOH: alternariol, AME: alternariol monomethyl ether, ALT: altenuene, TeA: tenuazonic acid, TEN: tentoxin, ATX-I: altertoxin-I, AOH-3-S: alternariol-3-sulphate, AME-3-S: alternariol monomethyl ether-3-sulphate, AOH-3-G: alternariol-3-glucoside, AME-3-G: alternariol monomethyl ether-3-glucoside. n.d.: not detected.

* Values between LOD and LOQ presented in **Table IV.2**.

** Process using Granny Smith variety apples.

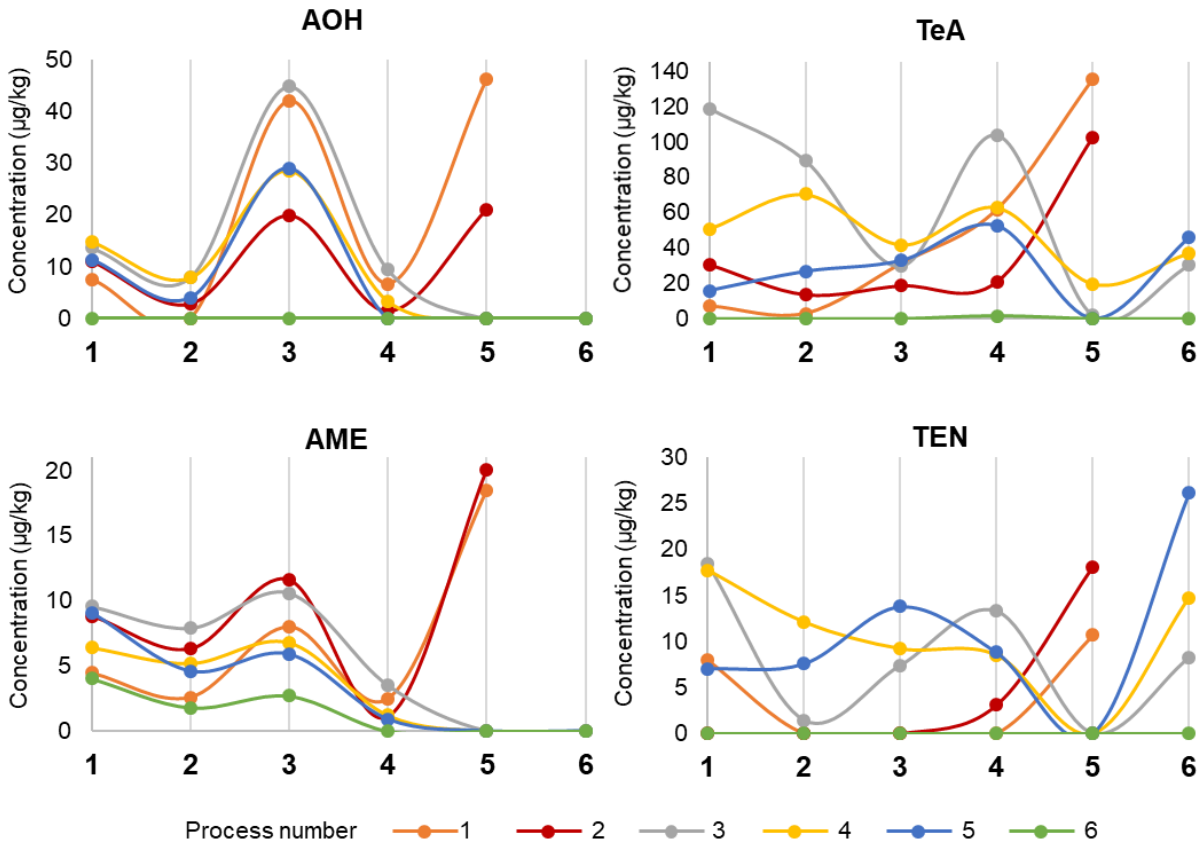

Figure V.4. Concentration of alternariol (AOH), alternariol monomethyl ether (AME), tenuazonic acid (TeA) and tentoxin (TEN) in different stages of six independent apple concentrate processes. Stages 1: grinding; 2: turbos; 3: decanter muds; 4: pre-concentrate; 5: concentrate; 6: rejection (only present in clear processes).

Table V.5. Number of positive samples, average (µg/kg), median (µg/kg), and range (µg/kg) per step of production in five Red Delicious apple concentrate processes.

	AOH	AME	ALT	TeA	TEN	ATX-I	AOH-3-S	AME-3-S	AOH-3-G	AME-3-G
Grinding										
N° of positive samples	5/5	5/5	0/5	5/5	4/5	0/5	0/5	3/5	0/5	0/5
Average (µg/kg)	11.7	7.7	-	44.7	12.8	-	-	4.4 *	-	-
Median (µg/kg)	11.4	8.9	-	30.6	12.9	-	-	4.8	-	-
Range (µg/kg)	7.6-14.8	4.5-9.6	<LOD	7.1-119.0	7.0-18.4	<LOD	<LOD	2.5*-5.8	<LOD	<LOD
Turbos										
N° of positive samples	4/5	5/5	0/5	5/5	3/5	0/5	0/5	5/5	0/5	0/5
Average (µg/kg)	5.7	5.3	-	33.9	7.0	-	-	3.8	-	-
Median (µg/kg)	6.0	5.2	-	23.3	9.2	-	-	3.9	-	-
Range (µg/kg)	2.9*-8.00	2.6-7.9	<LOD	2.8*-89.6	1.4*-12.1	<LOD	<LOD	3.0*-4.4*	<LOD	<LOD
Decanter muds										
N° of positive samples	5/5	5/5	0/5	5/5	3/5	0/5	2/5	5/5	4/5	0/5
Average (µg/kg)	32.9	8.6	-	30.9	10.1	-	1.9	6.4	3.1	-
Median (µg/kg)	29.1	8.0	-	31.1	9.2	-	1.9	6.0	3.1	-
Range (µg/kg)	19.9-44.9	5.9-11.6	<LOD	18.6-41.8	7.4-13.8	<LOD	1.4-2.4	4.3*-9.7	2.2-4.0	<LOD
Pre-concentration										
N° of positive samples	4/5	5/5	0/5	5/5	4/5	0/5	5/5	5/5	0/5	0/5
Average (µg/kg)	5.3	1.8	-	60.5	8.4	-	2.2	3.4 *	-	-
Median (µg/kg)	5.1	1.2	-	61.9	8.7	-	2.0	2.4 *	-	-
Range (µg/kg)	1.5*-9.5	0.9*-3.5	<LOD	20.9-103.8	3.1*-13.3	<LOD	1.5-3.2	1.7*-5.6	<LOD	<LOD
Clear Concentrate										
N° of positive samples	0/3	0/3	0/3	2/3	0/3	0/3	0/3	0/3	0/3	0/3
Average (µg/kg)	-	-	-	10.7	-	-	-	-	-	-
Median (µg/kg)	-	-	-	10.7	-	-	-	-	-	-
Range (µg/kg)	<LOD	<LOD	<LOD	1.9*-19.5	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Cloudy Concentrate										
N° of positive samples	2/2	2/2	0/2	2/2	2/2	0/2	2/2	2/2	0/2	0/2
Average (µg/kg)	33.8	19.3	-	119.2	14.4	-	5.2	9.9	-	-
Median (µg/kg)	33.8	19.3	-	119.2	14.4	-	5.2	9.9	-	-
Range (µg/kg)	21.1-46.4	18.5-20.1	<LOD	102.6-135.8	10.7-18.1	<LOD	4.4-6.0	9.7-10.0	<LOD	<LOD
Rejection										
N° of positive samples	0/3	0/3	0/3	3/3	3/3	0/3	1/3	0/3	1/3	0/3
Average (µg/kg)	-	-	-	37.9	16.4	-	10.8	-	18.7	-
Median (µg/kg)	-	-	-	37.2	14.8	-	10.8	-	18.7	-
Range (µg/kg)	<LOD	<LOD	<LOD	30.3-46.2	8.3-26.1	<LOD	<LOD-10.8	<LOD	<LOD-18.7	<LOD

AOH: alternariol, AME: alternariol monomethyl ether, ALT: altenuene, TeA: tenuazonic acid, TEN: tentoxin, ATX-I: altertoxin-I, AOH-3-S: alternariol-3-sulfate, AME-3-S: alternariol monomethyl ether-3-sulfate, AOH-3-G: alternariol-3-glucoside, AME-3-G: alternariol monomethyl ether-3-glucoside.

Average, median and range do not include negative samples.

*values between LOD and LOQ informed in **Table IV.2**.

V.3.1. Apple variety

The process using Granny Smith apples differed from those based on Red Delicious apples both in qualitative and quantitative terms of *Alternaria* metabolites detected (Table 2). The batch made with Granny Smith apples was contaminated with low levels of AME and its modified form, AME-3-S, at some stages of the production, although these compounds were not detected in the final product. Low levels of AOH-3-S and non-quantifiable but detectable levels of TeA were also observed, but only in the pre-concentrate step. The other five processes showed higher levels of contamination, and most of the studied metabolites were found in any of the stages sampled (**Table V.5**).

Although more samples should be analysed to confirm that Granny Smith apples are less prone to mycotoxin accumulation, a lower susceptibility to *Alternaria* infection has been reported for this variety. In a study in Greece, the frequency of recovery of *A. tenuissima* from Granny Smith apples was lower than from others (Konstantinou et al., 2011). It was attributed to the fact that *Alternaria* species mainly contaminate the centre of this fruit, causing mouldy core, and this variety of apple does not have an open sinus, a factor that enhances the postharvest contamination by this genus. In the same study, significantly lower levels of patulin were synthetized on Granny Smith apples than on other varieties, showing a correlation with the acidity of this cultivar. The lower incidence of *Alternaria* toxins in this variety is in accordance with Tournas & Uppal Memon (2009), who indicated that intact Granny Smith apples are less susceptible to fungal contamination due to their high acidity.

V.3.2. Type of field handling

No significant differences were observed between organic and conventionally grown apples with respect to AOH, AME and TEN contamination ($p>0.1$). Only one of the organic processes used raw material highly contaminated with TeA (process number 3), but the other toxins were in similar levels than those detected in the rest of the raw material sampled. da Cruz Cabral et al. (2019) showed that the application of fungicides

in a synthetic culture media partially reduced the production of TeA by *A. tenuissima*, while it had no impact on the production of the alternariol-derivatives. This could explain the higher levels of TeA found in the organic apples with respect to the other processes and the rest of the metabolites. Another possible explanation is that since stronger fungal competition occurs in the organic grown apples, the biosynthesis of TeA could be used as a virulence factor, favouring competition with other fungal species (Kang et al., 2017).

V.3.3. The effect of processing steps

For a better understanding of the effect of the different process stages on *Alternaria* toxins, the percentage of variation of the concentration of AOH, AME, TeA, and TEN with respect to the initial contamination (grinding step) was calculated for the five Red Delicious processes. The behaviour of the toxins was similar for all processes involving a clarification step (clear process) and differed from those which omitted this step (cloudy process). The average percentage of variation of these toxins for cloudy and clear processes is shown in **Figure V.5**.

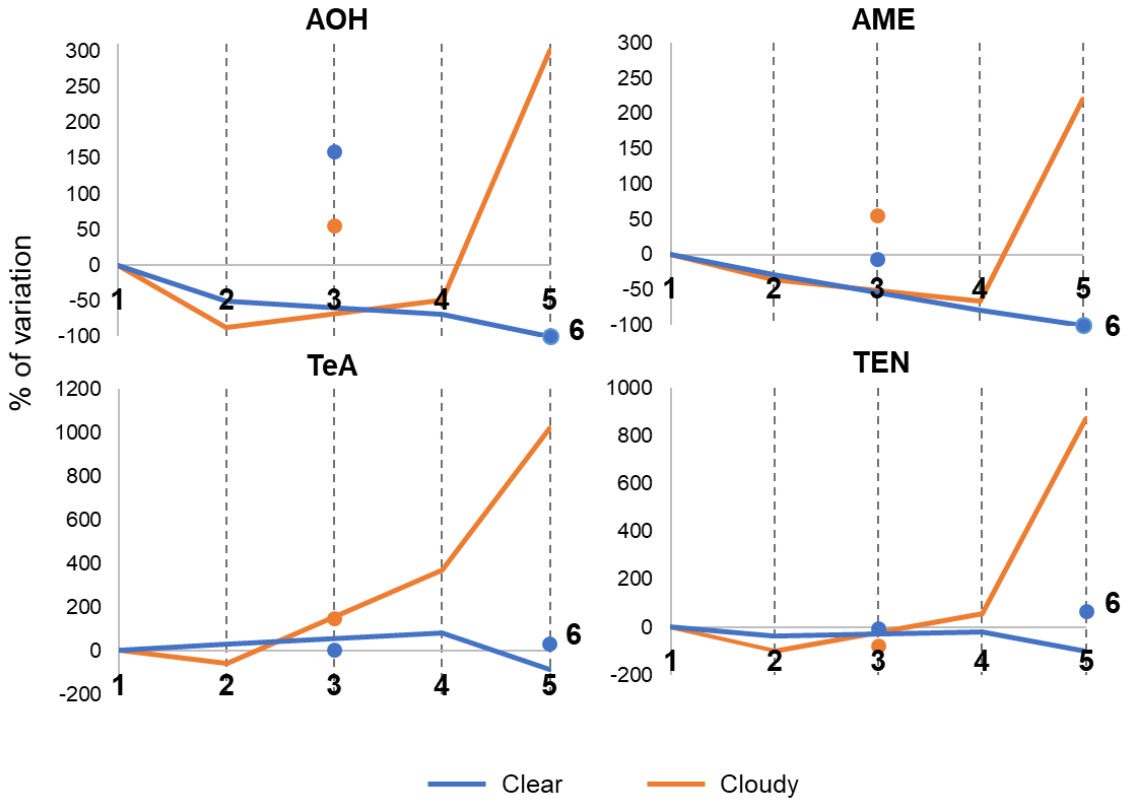

Figure V.5. Percentage of variation of alternariol (AOH), alternariol monomethyl ether (AME), tenuazonic acid (TeA) and tentoxin (TEN) throughout the apple concentrate production process with respect to the initial contamination. 1: grinding; 2: turbos; 3: decanter muds; 4: pre-concentrate; 5: concentrate; 6: rejection (only present in clear process). Circles represent out-of-process steps.

V.3.3.1. Step 1. Grinding

Quantifiable levels of AOH, AME, TeA and TEN, were observed in the ground raw material (step 1) from the five Red Delicious processes, except for process 2, in which TEN levels were <LOD. Regarding the modified forms of these mycotoxins, only AME-3-S was present in quantifiable levels in the raw material of three processes (**Table V.4**).

The natural presence of these toxins in the analysed ground apples indicates that the raw material used in these batches was contaminated with toxigenic species of *Alternaria*. In chapter II it was shown that *Alternaria* was frequently isolated from apples destined for industrial food processing (60 %). Since most of the *Alternaria* spp. were infecting the inner centre of the fruit (46 %), it is expected that their presence remains undetected by apple concentrate industries when they perform visual inspection of raw material. Moreover, when the toxigenic capacity of the strains isolated from apple fruit was tested, 58 % was capable of producing AOH, 57 % AME, 69 % TEN, 68 % TeA, 85 % ATX-I, and 37 % ALT, indicating high toxigenic capability. In chapter IV, AOH, AME, TEN, TeA, ATX-I, and ATX-II were synthesized *in vivo* proposing that these toxins will be present in the raw material for apple by-products. Higher levels of TeA were expected and confirmed in the present chapter. The *Alternaria* mycotoxin concentration in ground apples may vary due to the quality of the fruit destined to industrial food processing or the season in which the fruit is processed. The grinding step does not reduce the mycotoxins concentrations, however, grinding as milling step could redistribute the mycotoxin concentration.

When studying the toxigenic capacity of the *Alternaria* strains isolated from apple fruit *in vitro*, none of the modified forms investigated in the process was found, and AOH-3-G was the only modified mycotoxin that was produced *in vivo*. The finding of AME-3-S in ground raw material could imply that this modified form of AME is present in the fruit itself as a phase-II metabolite. Recent studies reported the presence of this metabolite in tomato products as well as in wheat flour (Puntscher et al., 2019) and contaminated apple fruit stored professionally for 6 months (Puntscher et al., 2020). Consistently, Walravens et al. (2016) observed this metabolite in 50 %, 32 % and 78 % of tomato juice, sauce and concentrate, respectively. Its presence should be considered in surveys of incidence of *Alternaria* mycotoxins in apple or apple-by-products since it might contribute to the total ingestion of its parent form.

V.3.3.2. Step 2. Turbos

After grinding the complete fruit, the resulting paste is centrifuged in a turbo, where peels, seeds and other solid parts of the fruit are eliminated from the flow line. The concentration of the neutral parent toxins (AOH, AME, TEN) decreased in the flow line after solids were eliminated (**Figure V.5**). On the contrary, the acidic toxin TeA, with higher affinity for the aqueous phase, showed a slight average increment (27 %) in the clear processes after the solid removal stage. Presumably, this step did not indicate a mycotoxin degradation, but a redistribution of the mycotoxin content.

The use of the waste, generated at this stage from fruit processing industries, for compost, cookies and other by-products has been suggested (Maldonado et al., 2019; Quiles et al., 2018; Rocha-Parra et al., 2019). However, since the concentration of neutral toxins (AOH, AME, TEN) decreased in the flow line after eliminating solids such as skin, seeds, peduncle, it is likely that these are concentrated in toxins. Thus, this waste should be analysed for their presence prior to its use for food or feed.

V.3.3.3. Step 3. Decanter muds

After the elimination of the solid parts, the product from the turbos is treated with water vapour at 100 °C for pasteurization, enzyme inactivation, protein denaturalization and starch gelatinization. In this part of the process, the aroma is extracted. The resulting paste is subjected to an enzymatic treatment at 55 °C and a maceration occurs to maximize the yield of the process. After these thermal treatments, a separation takes place and decanter muds are obtained as waste (step 3).

The decanter muds showed high contamination with AOH. The concentration detected in this waste was in average 55% and 159% higher than the original contamination for the cloudy and clear processes, respectively. AME and TeA concentrations only were

higher than those in the raw material in cloudy processes; AME in 55% and TeA in 149%, while TEN was in lower amounts than in the ground apples.

The thermal treatment did not seem to have a degradation effect on the analysed mycotoxins since they were detected in further steps in the process line. Similar results are presented by Estiarte et al. (2018) where they concluded that AOH and AME are relatively stable in the food process chain, the latter showing the highest stability. Scott & Kanhere, (2001) also reported that AOH and AME were stable at 80 °C up to 20 minutes and 2 weeks at room temperature.

V.3.3.4. Step 4. Pre-concentrate

Following the separation of decanter muds, a pre-concentration to 18 to 20 °Brix occurs (step 4). Both AOH and AME suffered further reductions in this step, except for AOH in the cloudy processes, where its concentration was slightly higher than in the previous step but still 49 % lower than that from the raw material (**Figure V.5**). The concentration of TEN continued reducing in the clear process, but increased in the cloudy one, reaching an average level of 55 % higher than the initial value, although there were no significant differences between the processes. TeA, on the other hand, increased in both types of process, although the increment was higher in the cloudy one, with average levels reaching 370 % the initial contamination.

Mycotoxin reduction has been previously observed in the flow line of apple concentrate after the enzymatic treatment; Welke et al., (2009) reported that patulin was reduced in 28 % due to the pectinase treatment. Nevertheless, the observed decline in the concentration of the alternariol-derivatives can be attributed to their adsorption in the decanter muds, causing the decanter juice, which has less free water than the initial phases of this process, resulting in a minor average concentration of these toxins. This effect was not observed for TEN, which agrees with its low adsorption in the muds. On the other hand, the average concentration of TeA in the decanter juice increased with respect to previous stages. Given the acidic nature of the molecule, this toxin has a

higher water solubility, thus its retention in the solids is expected to be lower. Even in cloudy processes, in which the average retention of TeA in the decanter muds was higher, the concentration of the toxin rose after the enzymatic treatment. These results showed that while for the alternariol-derivatives the retention in the solids was proportional to the initial contamination, for the TeA the decanter muds seem to saturate with 30 to 40 µg/kg of this fungal metabolite (**Fig V.4**, Step 3). Consequently, if the raw material shows low contamination with this toxin, a big proportion is transferred to the flow line in the muds. On the other hand, with high initial contamination, the muds get saturated and this mycotoxin remains in the flow line causing a concentration in step 4 of the process.

V.3.3.5. Step 5. Concentrate

After the pre-concentration step, the cloudy process continues with further concentration by water evaporation, while the clear one includes a clarification treatment with enzymes and activated charcoal. Then, this product is ultra-filtrated to eliminate fine particles, generating a retentate as a waste (step 6, rejection). The final clear product is obtained by concentration to 68-72 °Brix.

The biggest difference in mycotoxin concentration between both type of processes was observed in this final step. For the clear processes, after enzymatic treatment and ultra-filtration (clarification step) all the mycotoxins analysed underwent a significant reduction to non-detectable levels. Only TeA remained in the final product of 2 out of the 3 clear processes, but, in average, it was reduced with 86 % with respect to the original contamination of the fruit.

On the contrary, cloudy processes showed much higher final levels of the four mycotoxins. The concentration in the final product increased 301 % for AOH and 221 % for AME, with respect to the raw material, TEN was concentrated 872 % and TeA 1,024 %.

This cloudy final product can either be clarified once the market demand grows again, in which case some of the toxins would diminish to non-quantifiable levels, or can be destined to by-products other than juice, in which case the final destination of the toxins should be further investigated in each of them. TeA should be of special concern since it was quantified in both cloudy and clarified products, implying that the clarification is not able to reduce this toxin concentration to non-detectable levels, and moreover, it is considered the most acutely toxic *Alternaria* mycotoxin (Asam & Rychlik, 2013). Based on the findings of chapter IV and the recent report of the presence of ATX-II, AOH-9-G (alternariol-9-glucoside), and alterperylenol in apples contaminated with *Alternaria* fruit spot, the screening of these *Alternaria* toxins should also be evaluated in apple by-products (Puntscher et al., 2020).

V.3.3.6. Step 6. Rejection

The analysed retentates generated in clear processes were contaminated with TEN and TeA, besides the modified forms of AOH, AOH-3-G and AOH-3-S (**Table V.5**). The levels of TeA were similar to those present in the raw material, but TEN was found in higher amounts. Similarly, Kadakal et al. (2002) reported that a percentage of patulin was adsorbed in the charcoal, in the clarification step of the apple concentrate process.

This fact should be taken into account, since many uses have been proposed for this waste for being a source of free sugars, protein, polysaccharides, amino acids, fatty acids, sterols, triglycerides, and procyanidins (Cruz et al. 2018). Nevertheless, the presence of *Alternaria* toxins should be determined if it is intended for food or animal feed.

V.3.4. Effect of the process on modified toxins

The average concentration of AOH-3-S and AME-3-S and their respective parent forms, as well as the ratios modified/free form throughout the cloudy and clear processes is represented in **Figure V.6**. AOH-3-S was not detected in the early stages of the process, while AME-3-S was found in the raw material but in lower levels than its parent form.

Both metabolites increased their concentration throughout the process with respect to the initial contamination. The ratio of modified to parent form showed an increasing transformation of both alternariol-derivatives into their sulphate conjugates, which reached its peak in the pre-concentrate. After this step, a difference was observed between both type of processes; while the ratio of modified to parent form was reduced in the cloudy process for both metabolites, the sulphate conjugates were reduced to non-detectable levels in the clear one. This could be due to the enzymatic treatment applied in the latter, which might be responsible for the reversion of conjugates to free forms. Another possibility is that the modified forms can be more easily adsorbed in the solids that are removed after this step, thus being eliminated from the final clear product. Nevertheless, both metabolites were concentrated to quantifiable levels in the final cloudy product. It is noteworthy that the process using the Granny Smith variety also showed contamination with the sulphate conjugates in the pre-concentrate step, and AME-3-S was detected in the decanter muds obtained from this process (**Table V.4**).

With respect to the alternariol glucosides, even though AOH-3-G was produced by the tested strains in chapter IV, it was not detected in the raw material or consistently along the process, it was present in quantifiable levels in the decanter muds and in one of the retentates. Contrarily, AME-3-G was not detected in any step of the process.

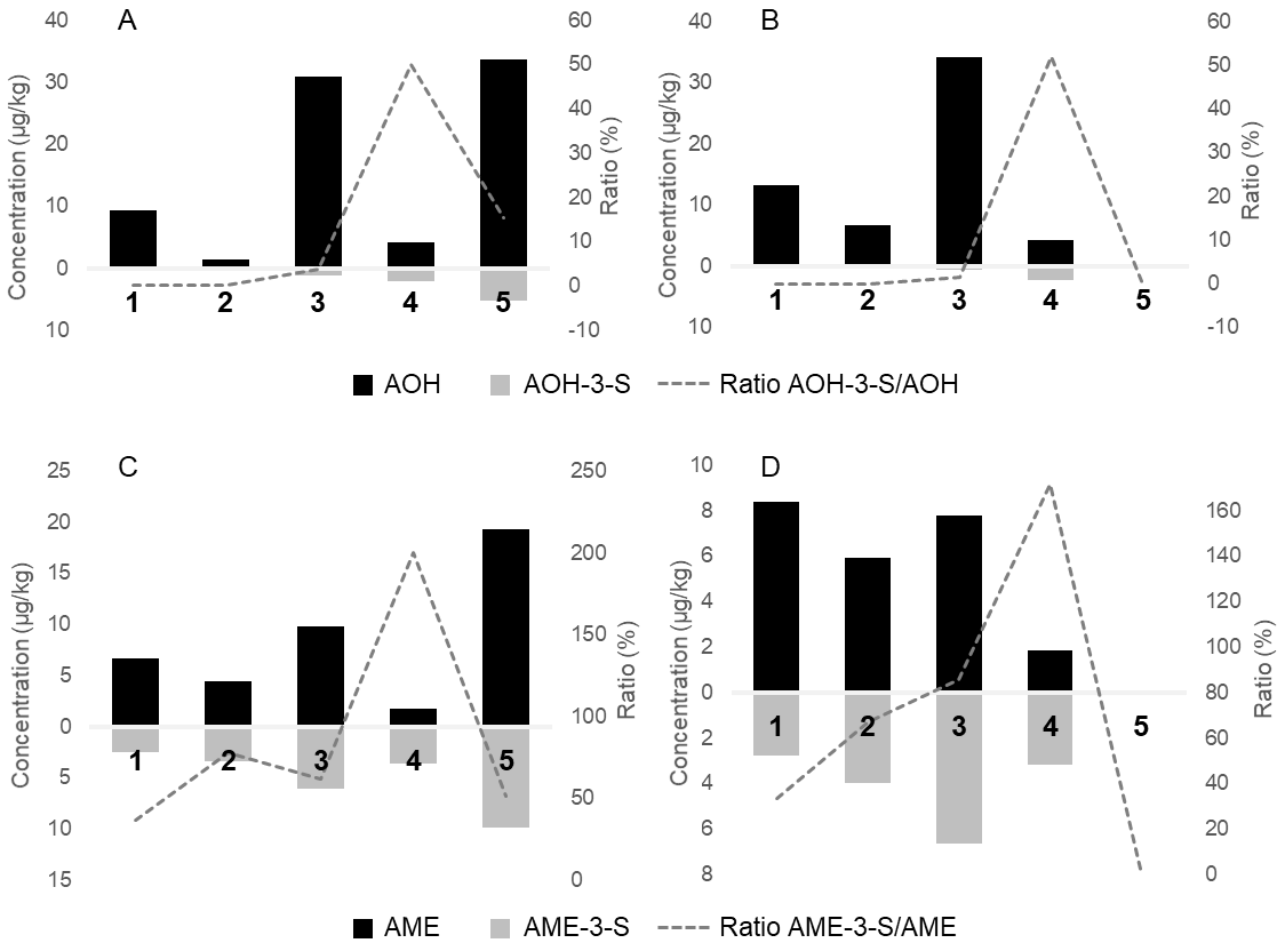

Figure V.6. Average concentration (bars) and ratio modified/native form mycotoxin (dash line) in each step of the five Red Delicious apple concentrate processes for alternariol (AOH) *vs*. alternariol 3-sulphate (AOH-3-S) in A) cloudy and B) clear processes, and alternariol monomethyl ether (AME) *vs*. alternariol monomethyl ether-3-sulpate (AME-3-S) in C) cloudy and D) clear processes.

This is the first report of these modified *Alternaria* toxins in apple concentrate. Both AOH-3-S and AME-3-S have been reported in tomato products before (Puntscher et al., 2019b; Walravens et al., 2016). No reports of quantifiable levels for AOH-3-G in commercially available foods are available up to now, although AOH-9-G has been detected in an organic tomato sauce from Italy (Puntscher et al., 2018). Considering that the presence of modified alternariol-derivatives has been confirmed in apple

concentrate, their concentration should be taken into account in the quantification of alternariol-derivatives in apple juices or other by-products made by the dilution of cloudy concentrates. Moreover, and due to the presence of multiple *Alternaria* metabolites in the apple concentrate, the combined toxic effects of them should be evaluated to perform an adequate risk assessment.

V.4. Conclusions

This is the first report stating the fate of free and modified forms of *Alternaria* toxins in the apple concentrate production and of the presence of AOH-3-S and AME-3-S in apple by-products. The results obtained indicate that the clarification stage in the apple concentrate process is of crucial importance to significantly reduce *Alternaria* toxins to safe levels in the final products. The major risk could be associated with cloudy apple by-products, especially if those are intended for infant foods; nevertheless, quantification of *Alternaria* mycotoxins in apple by commercial products is necessary. Although *Alternaria* mycotoxins are relatively stable, their contamination levels can be reduced to some extent during apple concentrate processing.

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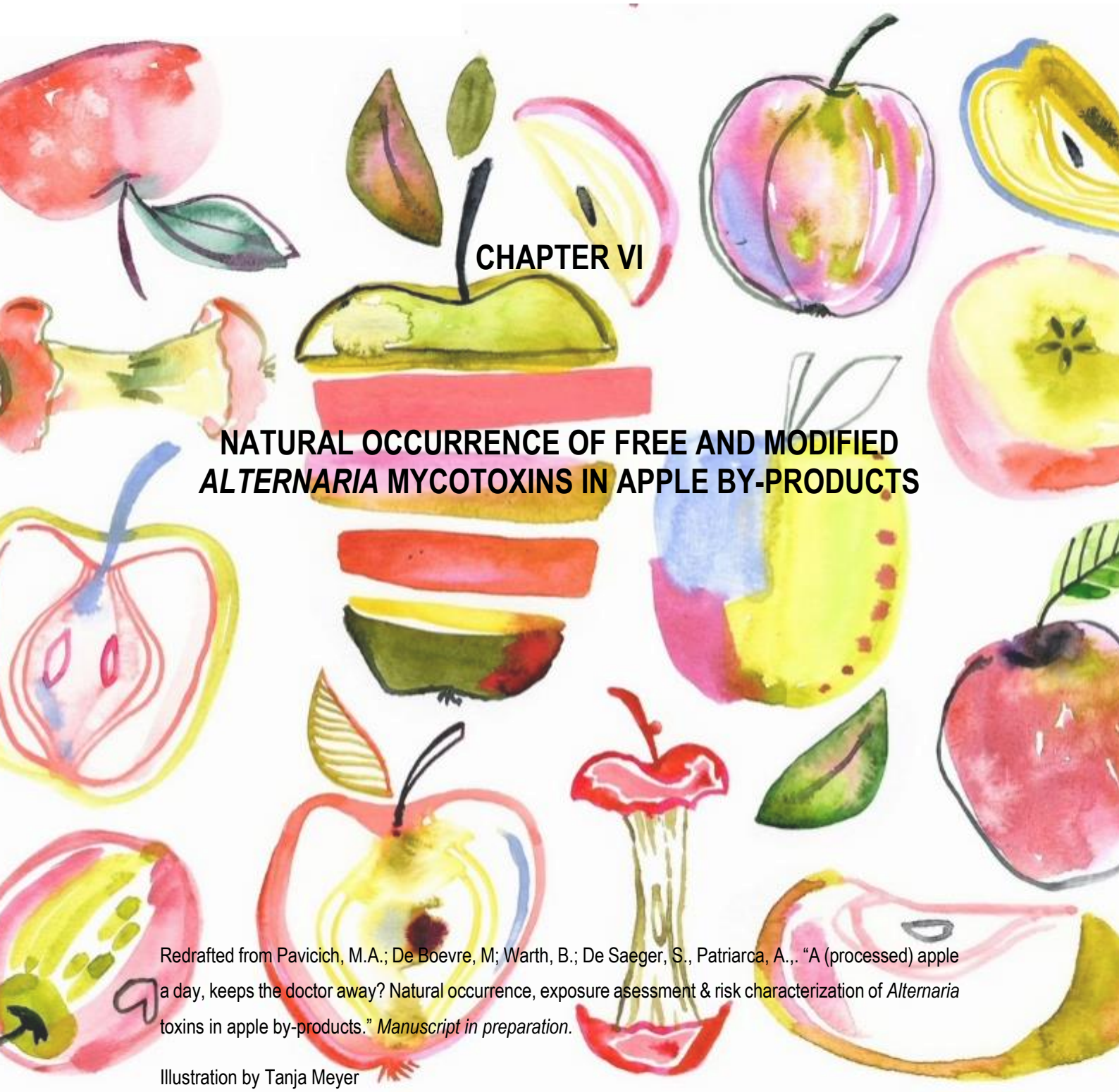

CHAPTER VI

NATURAL OCCURRENCE OF FREE AND MODIFIED *ALTERNARIA* MYCOTOXINS IN APPLE BY-PRODUCTS

Redrafted from Pavicich, M.A.; De Boevre, M; Warth, B.; De Saeger, S., Patriarca, A.,. "A (processed) apple a day, keeps the doctor away? Natural occurrence, exposure asessment & risk characterization of *Alternaria* toxins in apple by-products." *Manuscript in preparation.*

Illustration by Tanja Meyer

VI.1. Introduction

When analysing the concentrate production process in Chapter V, the raw material was contaminated with *Alternaria* toxins. Although the clarification step was key to reduce the concentration of most *Alternaria* mycotoxins, their original concentration increased in cloudy final products.

The apple concentrates can be destined to different apple by-products such as clear apple juice (AJ), cloudy AJ, apple marmalade, apple cider, and apple infant food, amongst others. Children are the main target consumers for certain apple by-products such as AJ and apple purees. The intake of cloudy apple products has been increasing lately since, as stated in Chapter V, a shift to less processed and more natural products is occurring and consumers perceive them as healthier. Regarding apple juice, Markowski et al. (2015) showed that the phenolic content of clear AJ was significantly lower than that of the cloudy one. A study by Ravn-Haren et al. (2013) showed the difference in LDL cholesterol when comparing a whole apple, apple pomace, cloudy and clear AJ, concluding that clear AJ may not have the health benefits of consuming a whole apple, and will have the highest glycaemic index of all the apple by-products. However, from the previous results, the mycotoxicological risk associated to non-clarified products seems to be higher, and none of these studies included the mycotoxin contamination aspect. Studies from other parts of the world showed the presence of *Alternaria* mycotoxins in apple and apple by-products (Delgado and Gómez-Cordovés, 1998; Gotthardt et al., 2019; Puntscher et al., 2020) but no information about these mycotoxins in the Argentinean market is available.

So far, there is a lack of data on the occurrence of *Alternaria* mycotoxins in Argentinean products, and, in consequence, no risk assessment has been performed on this population. Therefore, and considering the results obtained in this PhD thesis, it is crucial to evaluate the incidence of *Alternaria* toxins in clear and cloudy apple by-products, particularly when they are destined for children.

Therefore, the objective of this chapter was to analyse the natural occurrence of free and modified *Alternaria* mycotoxins in clear and cloudy apple by-products from the Argentinean market.

VI.2. Materials and methods

VI.2.1. Samples

Samples of apple by-products were analysed, namely clear and cloudy AJ, and non-clarified apple by-products categorized as apple infant food, and apple marmalades of several commercially available brands. The samples were collected in stores and supermarkets of the autonomous city of Buenos Aires. A total of 33 samples of AJ, 15 cloudy and 18 clear juices were purchased. For the analysis of non-clarified apple products, a total of 30 samples were collected from which 20 were infant food and 10 apple marmalades.

VI.2.2. Extraction and quantification

The extraction and LC-MS/MS quantification procedure for 10 *Alternaria* free and modified mycotoxins in apple products developed and validated by Walravens et al. (2016) and explained in detail in section 2 of chapter IV, was employed. For blanks, commercially available apple juice and puree free of *Alternaria* contamination were purchased in a supermarket in Ghent, Belgium.

VI.3. Results

VI.3.1. Natural occurrence of Alternaria mycotoxins in apple juices

The number of positive samples, average concentration, standard deviation, and range of each mycotoxin in clear and cloudy apple juice is summarized in **Table VI.1**. Clear AJ were contaminated with AME, TeA, TEN, AME-3-S and AOH-3-G in levels above the LOD (LOD and LOQ presented in **Table IV.2**). From the 18 samples, 13 (72 %) were

contaminated with at least one mycotoxin, 8 (44 %) with only 1 (AME, AME-3-S, or TEN), 4 (22 %) presented co-occurrence of 2, and only 5 (28 %) were not contaminated with any of the investigated *Alternaria* mycotoxins. TEN was the most prevalent mycotoxin in clear AJ, being present in 8 (44 %) of the samples.

For cloudy AJ, the concentration and prevalence of *Alternaria* mycotoxins was higher than in the clear ones, except for AME-3-S and AOH-3-G. The mycotoxins found in these juices were the same as in the clear ones, with the addition of AOH. From the 15 samples, 14 (93 %) were contaminated with at least one *Alternaria* mycotoxin, 3 (20 %) presented concentrations above the LOD for 1 mycotoxin only, 5 (33 %) had co-occurrence of 2 mycotoxins, 1 (7 %) with 3, 4 (27 %) presented contamination with 4, and 1 (7 %) with 5 mycotoxins simultaneously. The predominant toxins in cloudy AJ were AME and TEN.

Table VI.1. Number of positive samples, average concentration, standard deviation, number of samples above LOQ, number of samples above LOD, and range of concentration for each mycotoxin in clear or cloudy apple juices. LOD and LOQ for each mycotoxin presented in **Table IV.2**.

	AOH	AME	ALT	TeA	TEN	ATX-I	AOH-3-S	AME-3-S	AOH-3-G	AME-3-G
Clear Apple Juice										
N° of positive samples	0/18	3/18	0/18	4/18	8/18	0/18	0/18	2/18	1/15	0/15
Average (µg/kg)	n.a.	0.9*	n.a.	13.9	2.7*	n.a.	n.a.	8.4	3.5	n.a.
Standard Deviation (µg/kg)	n.a.	0.0	n.a.	10.0	0.8	n.a.	n.a.	2.6	n.a.	n.a.
>LOQ (n)	0	0	0	4	2	0	0	2	1	0
>LOD (n)	0	3	0	4	8	0	0	2	1	0
Range (µg/kg)	<LOD	0.9*–0.95*	<LOD	6.2–28.0	1.9*–4.1	<LOD	<LOD	6.6–10.2	3.5	<LOD
Cloudy Apple Juice										
N° of positive samples	4/15	10/15	0/15	7/15	10/15	0/15	0/15	1/15	5/15	0/15
Average (µg/kg)	4.5	1.5	n.a.	28.2	2.6	n.a.	n.a.	1.5*	2.1*	n.a.
Standard Deviation (µg/kg)	1.6	0.5	n.a.	28.0	1.1	n.a.	n.a.	n.a	0.8	n.a.
>LOQ (n)	4	7	0	6	2	0	0	0	3	0
>LOD (n)	4	10	0	7	10	0	0	1	5	0
Range (µg/kg)	2.2–6.2	0.9*–2.2	<LOD	3.0*–79.8	1.2–4.6	<LOD	<LOD	1.5*	1.1*–2.9	<LOD

AOH: alternariol, AME: alternariol monomethyl ether, ALT: altenuene, TeA: tenuazonic acid, TEN: tentoxin, ATX-I: altertoxin-I, AOH-3-S: alternariol-3-sulphate, AME-3-S: alternariol monomethyl ether-3-sulphate, AOH-3-G: alternariol-3-glucoside, AME-3-G: alternariol monomethyl ether-3-glucoside. Average, standard deviation and range were calculated over positive samples. n.a.: not applicable

*values between LOD and LOQ.

VI.3.2. Natural occurrence of Alternaria mycotoxins in apple purees

Table VI.2 summarizes the contamination data on apple marmalades and infant food. Apple marmalades were contaminated with AME, TeA, TEN, and AOH-3-G in levels above the LOQ. From the 10 samples, 7 (70 %) were contaminated with at least one mycotoxin, 5 (50 %) with only 1, one (10 %) presented co-occurrence of 2, and one (10 %) of 3 mycotoxins. Only 3 (30 %) samples were not contaminated with any of the investigated *Alternaria* mycotoxins. AME and AOH-3-G were the most frequent mycotoxins in marmalades, being present in 4 (40 %) of the samples each.

For apple infant food, the concentration and prevalence of *Alternaria* mycotoxins was higher than in the marmalades, except for AOH-3-G, which was not detected in infant food. As well, the prevalence and range were higher than for clear and cloudy AJ. The mycotoxins found in apple infant food were AOH, AME, TeA and TEN. From the 20 samples, all were contaminated with at least 2 *Alternaria* mycotoxins, 8 (40 %) presented concentrations above the LOD for 3 mycotoxins, and 6 (30 %) had co-occurrence of 4 mycotoxins. The most frequent toxin in apple infant food was AME, with a 100 % prevalence.

Table VI.2. Number of positive samples, prevalence, average concentration, standard deviation, number of samples above LOQ, number of samples above LOD, and range of concentration for each mycotoxin in apple marmalades and apple infant food. LOD and LOQ for each mycotoxin informed in **Table IV.2**.

	AOH	AME	ALT	TeA	TEN	ATX-I	AOH-3-S	AME-3-S	AOH-3-G	AME-3-G
Apple Marmalades										
N° of positive samples	0/10	4/10	0/10	1/10	1/10	0/10	0/10	0/10	4/10	0/10
Average (µg/kg)	n.a.	4.3	n.a.	144.3	92.4	n.a.	n.a.	n.a.	13.3	n.a.
Standard Deviation (µg/kg)	n.a.	0.5	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	2.7	n.a.
>LOQ (n)	0	4	0	1	1	0	0	0	4	0
>LOD (n)	0	4	0	1	1	0	0	0	4	0
Range (µg/kg)	<LOD	3.9–5.0	<LOD	144.3	92.4	<LOD	<LOD	<LOD	10.3-16.8	<LOD
Apple Infant Food										
N° of positive samples	7/20	20/20	0/20	14/20	19/20	0/20	0/20	0/20	0/20	0/20
Average (µg/kg)	5.0	6.5	n.a.	48.9	30.7	n.a.	n.a.	n.a.	n.a.	n.a.
Standard Deviation (µg/kg)	4.4	2.2	n.a.	60.7	20.4	n.a.	n.a.	n.a.	n.a.	n.a.
>LOQ (n)	2	20	0	14	18	0	0	0	0	0
>LOD (n)	7	20	0	14	19	0	0	0	0	0
Range (µg/kg)	1.7*–13.7	4.4–14.7	<LOD	6.5–225.7	4.1–92.2	<LOD	<LOD	<LOD	<LOD	<LOD

AOH: alternariol, AME: alternariol monomethyl ether, ALT: altenuene, TeA: tenuazonic acid, TEN: tentoxin, ATX-I: altertoxin-I, AOH-3-S: alternariol-3-sulphate, AME-3-S: alternariol monomethyl ether-3-sulphate, AOH-3-G: alternariol-3-glucoside, AME-3-G: alternariol monomethyl ether-3-glucoside. Average, standard deviation and range were calculated over positive samples. n.a.: not applicable

*values between LOD and LOQ.

VI.4. Discussion

Processing industries perform quality controls to prevent that fruit with fungal spoilage are incorporated into the process line. Despite the widespread development of automated methods for measuring the quality of fruit (Brosnan and Sun, 2004; Opara et al., 2007), most fruit industries in Argentina still rely on visual inspection for the detection of fungal diseases in the fruit. In the previous chapter, it was demonstrated that the raw material for apple concentrate, was contaminated with these toxins, proving that the pre-processing selection performed by this industry is not sufficient to prevent the contamination with *Alternaria* mycotoxins. Both clear and cloudy concentrates contained detectable levels of these metabolites, but non-clarified products showed a higher risk. Apple concentrates are a supply to several industries; these are diluted or mixed with other ingredients to produce different apple by-products, but whether this step is sufficient to obtain safe final commercial products was still unknown.

VI.4.1. Natural occurrence of Alternaria metabolites in apple juices

A wide variety of *Alternaria* mycotoxins were present, not only in cloudy apple by-products, but also in clear ones. Five toxins (TEN, TeA, AME, AME-3-S and AOH-3-G) were present in clear AJ. TEN was the most frequent mycotoxin, but the levels were low. When the apples were inoculated with *Alternaria* species isolated from apple fruit (chapter IV), TEN was produced under all the conditions tested. However, it was not detected in the clear concentrate (chapter V), suggesting that the clarification step is key to reduce the concentration of this mycotoxin. Nevertheless, it was found in commercial clear juices, probably due the use of low quality raw material for their production. Other studies also reported TEN in low frequencies (Li et al., 2020) or traces (Zwickel et al., 2016) in apple juices.

TeA was the second most prevalent mycotoxin (22 %) and the one with the highest mean concentration in clear AJ. As well, in the inoculated apples, TeA was the toxin produced at the highest concentration under the different incubation conditions, and it was the only

mycotoxin present in the clear apple concentrates (chapter V). Studies from China and Europe also found TeA in apple juice, and usually in higher concentrations than the other *Alternaria* mycotoxins (Fan et al., 2016; Gross et al., 2011; Li et al., 2020; Prelle et al., 2013; Walravens et al., 2016; Zwickel et al., 2016). As proposed in chapter V, from the acidic and soluble aspect of TeA, its reduction compared to the other *Alternaria* toxins is less effective when solid parts are removed, resulting in higher concentrations in the final products.

AME was detected in 3 samples of clear AJ but in levels between LOD and LOQ, and from the available studies, it was only detected in AJ in China at low concentrations as well (Fan et al., 2016; Li et al., 2020). In the apples, AME was mostly synthetized at 25 °C, but also at 4 °C. Notably, AOH was synthetized in the apples, but was not detected in the clear AJ; as well, the clear concentrate did not had levels of AOH above LOD. Therefore, the clarification seems to reduce the concentration of AOH and AME but whether the levels in the final product are safe needs to be further evaluated.

The modified forms AME-3-S and AOH-3-G were found for the first time in commercially available AJ, and it is the first report of *Alternaria* modified mycotoxins in commercially available products in Argentina. The glucosyl forms are believed to be detoxification mechanisms of the plants and the sulphonated ones produced by the fungi (Puntscher et al., 2020). Nevertheless, sulphonated forms of these toxins were not found in the inoculated apples (chapter IV), but they were present during the production of apple concentrates (chapter V).

The cloudy AJ were contaminated with the same mycotoxins as the clear ones, with the addition of AOH. However, their concentration and frequency were higher in the cloudy ones. This is in accordance with the results from chapter V, in which the cloudy concentrates were significantly more contaminated than the clear ones. When cloudy apple concentrates were analysed, AOH, AME, TeA, and TEN were present. Consistently, the same mycotoxins were found in cloudy AJ, being TEN and AME the

most frequent ones and TeA the one present at the highest concentration. These results suggest that the dilution from concentrate to commercial product is not sufficient to reduce these mycotoxins to non-detectable levels. There are few studies in Europe and China analysing *Alternaria* mycotoxin contamination in apple juices but they do not specify if the samples were clear or cloudy AJ, and some of them did not find samples with levels above LODs (López et al., 2016). A possible explanation is that all the sampled AJ were clear, the variety of the raw material corresponded to non-susceptible ones (e.g. Granny Smith), or the fruit employed for juice making was of higher quality.

Patulin, another frequent mycotoxin in apple and apple by-products, is regulated in apple food by many food safety authorities. The European Commission admits 10 µg/kg for fruit baby food, 50 µg/kg for fruit juice, and 25 µg/kg for solid apple products in Europe. For this reason, *P. expansum* infection has been kept under surveillance in apple industries through quality control inspection, and fruit of higher quality is employed for production in Europe (Patriarca, 2019). Nevertheless, *Alternaria* toxins have been disregarded by food safety authorities. Prelle et al. (2013) analysed 70 food samples from different European countries, including apple juices. Even though they found more positive samples for *Alternaria* toxins among tomato products, the highest concentrations of these metabolites were found in commercial apple juices.

VI.4.2. Natural occurrence of Alternaria metabolites in apple purees

Even though non-clarified apple by-products such as infant food or marmalades can be made of or contain apple concentrate, they are produced by a different process than the juices. Apple infant food or apple sauce is usually made by smashing the whole apple, eliminating skin, stem and seeds, incorporating additives to prevent browning, pasteurizing the mix, and packing it; whereas marmalades or apple jams are made by crushing the fruits without skin, seeds and stem, adding a significant amount of sugar and additives and cooking the mix for a prolonged period (Emelike and Akusu, 2019).

For non-clarified apple products, the concentration and prevalence of mycotoxins was higher than the juices. Since there is no clarification step in these products, a higher concentration of mycotoxins was expected. Apple marmalades were contaminated with AME, TEN, TeA, and AOH-3-G, being AME the most frequent one and TeA the one found at the highest concentrations. Apple infant food was contaminated with AOH, AME, TEN and TeA, and again AME was the most prevalent being present in all the tested samples. The concentration and prevalence were higher in the apple infant food than in any other apple by-product, raising particular concern since infants are a vulnerable group. Few studies investigated the presence of these mycotoxins in non-clarified apple products. In China, AME and TEN were present in apple jam (Li et al., 2020) and a study by Ackermann et al. (2011) found AOH in all the apple sauce samples studied (n=10). More recently, Gotthardt et al. (2019) found AOH, AME, TEN and TeA in infant food. However, no occurrence data from Argentina was available previous to this study. This information is crucial to assess the risk consumers are exposed to.

Notably, no modified forms of *Alternaria* mycotoxins were detected in infant food. On the other hand, AOH-3-G was found in the marmalades, but its parent form was not found. It was shown that high temperature and the presence of sugar led to formation of fumonisin B1-sugar when fumonisin B1 (FB1), a toxin produced by *Fusarium* spp. was present in maize (Schaarschmidt and Fauhl-Hassek, 2021). Therefore, the presence of the modified forms in this product might be attributed to higher temperatures in the treatments and the addition of sugar. However, more information about the modification of *Alternaria* mycotoxins is needed and the toxicity of these compounds should also be tested, in order to perform more accurate risk assessments.

Although TEN and AME were the most frequent *Alternaria* toxins in apple products, TeA was present in higher concentrations. The highest levels reported before this study correspond to an apple juice sample from the German market (58 µg/kg) (Gross et al., 2011), another from China (49.61 µg/kg) (Fan et al., 2016), and one from an Italian

supermarket (45.3 µg/kg) (Prelle et al., 2013). Nevertheless, the results of this study are above these levels: the highest concentration in AJ was 79.8 µg/kg, and in an apple infant food 225.7 µg/kg. Even though these values do not exceed the regulation of 500 µg/kg established for infant food in Bavaria (Rychlik et al., 2016), the risk could not be overlooked, since infants are a particularly susceptible group.

Considering that a single apple fruit can contain high levels of one or several *Alternaria* toxins, the wide variation observed in toxins concentration in the different by-products analysed is expected. Many factors can influence their levels in processed food, mainly the raw material contamination, but also the effect of processing steps, such as thermal treatment, clarification, enzymatic treatment, etc. Even in clarified products, *Alternaria* toxins were detected and quantified for the first time in Argentina, implying that if the raw material is highly contaminated, toxins might remain in the final product. A risk assessment to know if the levels found in this first study are safe or not is therefore imperative.

VI.5. Conclusions

To our knowledge, this is the first report of *Alternaria* mycotoxins and their modified forms in commercial apple by-products from the Argentinean market. All the different food categories analysed were contaminated with *Alternaria* toxins, but higher levels were found in non-clarified products destined to infants, implying a potential risk associated to these products. These results justify performing a risk assessment for the infant population in Argentina based on the current results.

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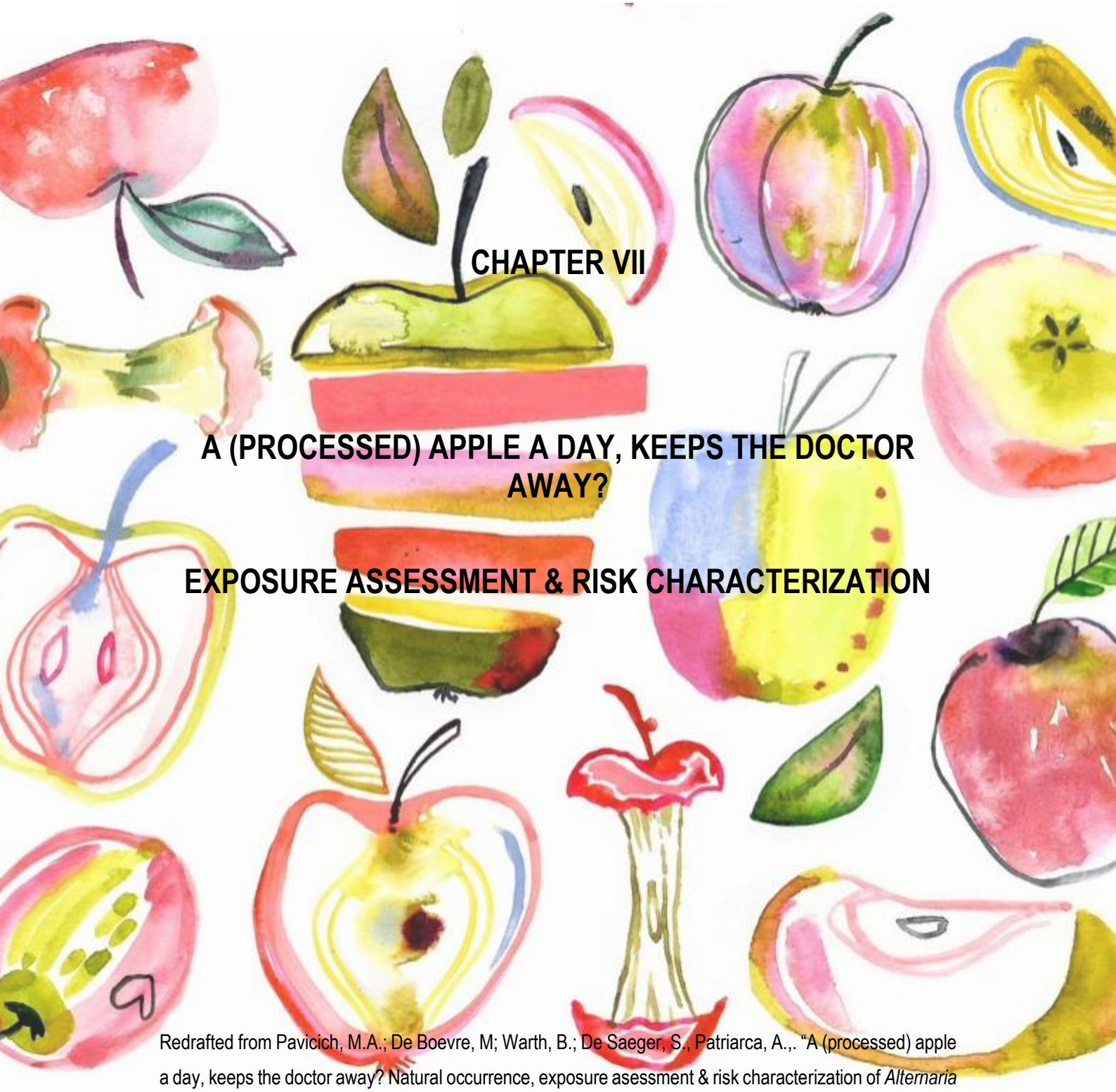

CHAPTER VII

A (PROCESSED) APPLE A DAY, KEEPS THE DOCTOR AWAY?

EXPOSURE ASSESSMENT & RISK CHARACTERIZATION

Redrafted from Pavicich, M.A.; De Boevre, M; Warth, B.; De Saeger, S., Patriarca, A.,. "A (processed) apple a day, keeps the doctor away? Natural occurrence, exposure asessment & risk characterization of *Alternaria* toxins in apple by-products." *Manuscript in preparation.*

Illustration by Tanja Meyer

VII.1. Introduction

In Chapter VI, it was demonstrated that Argentinean apple by-products were contaminated with *Alternaria* mycotoxins, but whether those levels were of risk to consumers still needs to be evaluated. Several studies have been done on toxicity of the main *Alternaria* mycotoxins (AOH, AME, TeA) establishing a Threshold of Toxicological Concern (TTC). A TTC of 2.5 ng/kg of body weight per day (BW/d) was set for AOH and AME and 1500 ng/kg of BW/d for TeA (Arcella et al., 2016). This value is suitable for performing risk assessments when other toxicity data is lacking (Patlewicz et al., 2018). Currently, there is no legislation on these mycotoxins in Argentina, and the only *Alternaria* toxin legislated worldwide in a food commodity is TeA, with a limit of 500 µg/kg in sorghum/millet infant food in Bavaria, Germany, after showing the TTC was exceeded by the consumption of this kind of food (Rychlik et al., 2016; Solfrizzo, 2017). Further information is imperative to implement safe limits for consumers around the globe. The TTC of 2.5 ng/kg of BW/d estimated for both AOH and AME does not consider the possible additive effect of conjugated forms, such as their sulphates or glycosides, nor the effect that other co-occurring mycotoxins might have.

Risk assessments are a valuable tool to evaluate the risk to which consumers are exposed. The risk assessment process consists of 4 stages: 1: hazard identification; 2: hazard characterization; 3: exposure assessment; 4: risk characterization (Meerpoel et al., 2021). Exposure assessment can be performed using different approaches. The deterministic approach considers occurrence, consumption, and body weight data as point estimates, using fixed values, while in probabilistic exposure assessments, the variables are described as distributions. In this way, all possible values assumed for each variable are considered and each possible outcome in each scenario is weighted by its occurrence probability (Abdallah et al., 2020). As well, exposure to mycotoxins can be estimated based on occurrence data in food combined with food consumption data, or via human biomarkers of exposure in biological samples such as blood or urine

(Meerpoel et al., 2021). When dietary exposure assessments of chemical substances are made on occurrence data, the lower bound (LB) and upper bound (UB) approach is recommended to manage left-censored data (EFSA, 2010).

In a risk assessment performed on the European population, data of natural contamination of foods with *Alternaria* toxins showed that the TTC levels for AOH and AME were exceeded in some European countries (Arcella et al., 2016) and that the most exposed groups were vegetarians and toddlers. Children are of particular concern, being a vulnerable group; they have a higher exposure per kg of BW to contaminants, their enzymatic activity is not fully developed and they have lower ability to break down chemical compounds (Boon et al., 2009; Oueslati et al., 2018; Pustjens et al., 2021). Moreover, children may be more sensitive to neurotoxic, endocrine disturbance, and immunological toxic effects up to 4 years old (Huybrechts et al., 2011). This should be of relevance, since alternariols have estrogenic capacity (Vejdovszky et al., 2017), and particularly, AOH has androgenic effects (Stypuła-Trębas et al., 2017) that might produce a bigger impact during childhood. Therefore, children should be addressed as a separate group in risk assessments.

Given the lack of data on the occurrence of *Alternaria* mycotoxins in Argentina and on *Alternaria* human biomarkers, no risk assessment has been performed on this population so far. Therefore, and according to the results obtained in this PhD thesis, it is crucial to assess the risk for consumers associated with the intake of apple products. Since apple infant food showed the highest contamination, and considering that AJ are also consumed by children, the analysis was focused on population group.

Therefore, the objective of this chapter was to perform an exposure assessment and risk characterisation from the consumption of apple by-products, based on the most relevant *Alternaria* mycotoxins, for the Argentinean population from 6 months to 5 years old.

VII.2. Materials and methods

VII.2.1. Data treatment

The risk characterization was done separately for AOH, AME and TeA for 3 different apple by-products: clear apple juice, cloudy apple juice, and apple-based infant food. The risk was evaluated for the Argentinean population under 5 years old, divided in two groups: kids from 6 to 23 months old (6-23 MO) and kids from 2 to 5 years old (2-5 YO), because consumption data informed by the National Survey of Nutrition and Health of Argentina (MSAL, 2012) is divided in these groups. The occurrence data for AOH, AME and TeA for apple by-products in Chapter VI of this PhD thesis were used for the exposure assessment and lower and upper bound concentration scenarios were constructed with toxins concentrations obtained for the different food matrices. In the lower bound (LB) scenario, the non-detectable values were replaced by 0 and the values below LOQ, by half the LOD value for each compound. For the upper bound (UB) scenario, the non-detectable values were replaced by the LOD and the values <LOQ, by the LOQ as performed by Walravens (2017). These scenarios were then used for the deterministic and probabilistic assessments.

The consumption values for the group ages 6-23 months old and 2-5 years old were taken from the National Survey of Nutrition and Health (MSAL, 2012). For apple juice (clear and cloudy) the values for fruit juices were used, and for apple infant food, the data for fruit marmalade was used since no specific consumption data for these products was available. The average body weight values for the different age ranges were taken from the Argentinean Society of Nutrition.

VII.2.2. Deterministic exposure assessment and risk characterisation

For the deterministic assessment of apple juice, two consumption scenarios were tested: average and high consumption. In the first one, mean consumption data were taken from the National Survey of Nutrition and Health (MSAL, 2012) for the afore mentioned products for the age groups of 6-23 MO and 2-5 YO. In the high consumption scenario,

a LogNorm distribution was simulated based on the consumption data of fruit juices or marmalade obtained from MSAL (2012) using the @Risk® 5.5 (Palisade Corporations, California, USA) add-on to Microsoft Excel 2016. The percentiles 95 (P95) of consumption for the high consumption scenarios were obtained from the distributions of each age and food group.

For apple infant food, in addition to these two scenarios where fruit marmalade consumption from MSAL (2012) was used, a third one was proposed since this product was first accessible in the Argentinean market long after the survey took place, so no consumption data were available. In this scenario, the recommended consumption by the manufacturer was considered, in which the whole pack (90 g) is equal to one fruit.

The daily intake of the different mycotoxins by body weight was obtained multiplying the mean (LB or UB) concentration of each mycotoxin by the consumption of each food group (mean, P95, and whole pack as appropriate) by the mean body weight for each age group, taken from the Argentinean Society of Nutrition as detailed in **Equation VII.1**. To perform the risk characterisation, the obtained exposure values were compared with the TTC for each mycotoxin, being 2.5 ng/kg of BW/d for AOH and AME and 1500 ng/kg of BW/d for TeA.

Equation VII.1. Daily mycotoxin intake used to calculate the exposure to mycotoxins using a deterministic approach.

$$\text{Daily intake}_{(\text{mycotoxin})} = [\ X\ ]_{(\text{mycotoxin})} \times C \times BW$$

Where:

$[\ X\ ]_{(\text{mycotoxin})}$ = mean concentration of each mycotoxin in ng/g
C = consumption of each food group in g
BW = mean body weight for each age group in kg

VII.2.1.3. Probabilistic exposure assessment and risk characterisation

The probabilistic exposure assessment was performed using the @Risk® 5.5 add-on for Microsoft Excel. The food intake of the different age groups was modelled using a LogNorm distribution from MSAL (2012) data and the resulting distribution was divided by a Uniform one that considered the weights of each age group. The @Risk function best fit distribution was applied to the LB scenario for each mycotoxin concentration and the resulting distribution was also applied to model the UB scenario. Then, the estimated ng mycotoxin intake by kg of body weight/day was calculated by first-order Monte Carlo simulation considering 10,000 iterations, multiplying the modelled mycotoxin concentration in each food category by the modelled food intake/kg of BW/d. Using the distribution for each food category and age group, the percentage of kids exceeding the TTC value was calculated in the risk characterization.

From the resulting distributions, the number of kids exceeding the TTC values were calculated multiplying the number of kids reported to consume the food commodity (MSAL, 2012) by the percentage exceeding the TTC.

VII.3. Results

VII.3.1. Deterministic exposure assessment and risk characterisation for apple juices

Table VII.1 shows the estimated intake of AOH, AME and TeA, using a deterministic approach, per juice category and age group in the LB and UB scenarios, through mean and high consumption of clear or cloudy AJ. In the LB scenarios, neither of the TTC values for AOH or AME (2.5 ng/kg of BW/d) were exceeded through the mean or high consumption of clear AJ alone. In the UB scenarios the TTC value for AME was exceeded from the mean and high consumption of clear AJ. On the contrary, through the intake of cloudy AJ, the TTC values for the alternariols were surpassed regardless the scenario or consumption. On the other hand, the TTC value of 1500 ng/kg of BW/d for

TeA was not exceeded by the consumption of clear or cloudy AJ in neither of these scenarios.

Table VII.1. Deterministic exposure assessment to alternariol, alternariol monomethyl ether and tenuazonic acid through the intake of clear and cloudy apple juice for Argentinean kids from 6 months to 5 years old. Mean concentrations from chapter VI of each mycotoxin in the lower bound (LB) and upper bound (UB) scenarios were considered for mean (average) and high consumers (P95).

Mycotoxin	Food category	Age group	Mean consumption		High consumption (P95)	
			LB (ng/kg of BW/d)	UB (ng/kg of BW/d)	LB (ng/kg of BW/d)	UB (ng/kg of BW/d)
AOH	Clear AJ	6-23 MO	n.a.	n.a.	n.a.	n.a.
		2-5 YO	n.a.	n.a.	n.a.	n.a.
	Cloudy AJ	6-23 MO	19.5	40.7	48.0	100.2
		2-5 YO	17.9	37.3	39.4	82.3
AME	Clear AJ	6-23 MO	0.4	7.7	1.1	18.9
		2-5 YO	0.4	7.1	0.9	15.5
	Cloudy AJ	6-23 MO	14.9	19.1	36.7	47.0
		2-5 YO	13.7	17.5	30.2	38.6
TeA	Clear AJ	6-23 MO	54.8	72.7	135.0	179.1
		2-5 YO	50.3	66.7	110.9	147.2
	Cloudy AJ	6-23 MO	231.4	251.0	570.3	618.7
		2-5 YO	212.4	230.4	468.5	508.2

AOH: alternariol, AME: alternariol monomethyl ether, TeA: tenuazonic acid. AJ: apple juice. 6-23 MO: Argentinean kids from 6 to 23 months old; 2-5 YO: Argentinean kids from 2 to 5 years old. n.a.: not applicable

VII.3.2. Probabilistic exposure assessment and risk characterisation for apple juices

The best fitted distribution for the concentration of AOH in cloudy AJ, and AME and TeA in both clear and cloudy AJ, was exponential. The probabilistic exposure could not be

calculated for AOH in clear AJ since all samples had concentrations below the LOD; thus, values were 0 in the LB scenario and LOD in the UB scenario.

Table VII.2 shows the estimated intake of mycotoxins by the different age groups through the consumption of the different food categories in the LB and UB scenarios. The mean and P95 estimations were taken from the corresponding distributions. **Table VII.3** shows the percentage of 6 to 23 month old, and 2 to 5 year old Argentinean kids exceeding the TTC for AOH, AME and TeA calculated from the distributions.

Table VII.2. Probabilistic exposure assessment of alternariol, alternariol monomethyl ether and tenuazonic acid trough the consumption of clear and cloudy apple juice for Argentinean kids from 6 months to 5 years old for the lower bound (LB) and upper bound (UB) scenarios. Mean and P95 were obtained from the corresponding distribution.

Mycotoxin	Food category	Age group	Mean		P95	
			LB (ng/kg of BW/d)	UB (ng/kg of BW/d)	LB (ng/kg of BW/d)	UB (ng/kg of BW/d)
AOH	Clear AJ	6-23 MO	n.a.	n.a.	n.a.	n.a.
		2-5 YO	n.a.	n.a.	n.a.	n.a.
	Cloudy AJ	6-23 MO	6.0	38.3	33.4	142.7
		2-5 YO	4.8	30.9	27.4	111.6
AME	Clear AJ	6-23 MO	0.1	7.5	0.5	28.3
		2-5 YO	0.1	6.0	0.4	21.6
	Cloudy AJ	6-23 MO	9.9	18.2	42.9	66.9
		2-5 YO	7.6	14.5	32.9	51.9
TeA	Clear AJ	6-23 MO	13.7	70.5	78.4	261.8
		2-5 YO	10.9	55.9	65.6	196.0
	Cloudy AJ	6-23 MO	151.3	255.3	689.9	925.6
		2-5 YO	120.0	203.5	544.2	718.4

AOH: alternariol, AME: alternariol monomethyl ether, TeA: tenuazonic acid. 6-23 MO: Argentinean kids from 6 to 23 months old; 2-5 YO: Argentinean kids from 2 to 5 years old.

n.a.: not applicable.

Table VII.3. Percentage of Argentinean kids from 6 to 23 months old or 2 to 5 years old exceeding the Toxicological Threshold of Concern (TTC) of alternariol, alternariol monomethyl ether and tenuazonic acid from clear and cloudy apple juice in the lower bound (LB) and upper bound (UB) scenarios.

Mycotoxin	Food category	Age group	% Exceeding TTC	
			LB	UB
AOH	Clear AJ	6-23 MO	n.a.	n.a.
		2-5 YO	n.a.	n.a.
	Cloudy AJ	6-23 MO	25.4	87.3
		2-5 YO	24.7	86.3
AME	Clear AJ	6-23 MO	0.4	59.3
		2-5 YO	0.2	56.6
	Cloudy AJ	6-23 MO	50.2	76.9
		2-5 YO	48.8	75.4
TeA	Clear AJ	6-23 MO	0	0
		2-5 YO	0	0
	Cloudy AJ	6-23 MO	0.7	1.5
		2-5 YO	0.3	0.8

AOH: alternariol, AME: alternariol monomethyl ether, TeA: tenuazonic acid. 6-23 MO: Argentinean kids from 6 to 23 months old; 2-5 YO: Argentinean kids from 2 to 5 years old. n.a.: not applicable

VII.3.3. Deterministic exposure assessment and risk characterisation for apple infant food

Table VII.4 shows the estimated intake of AOH, AME and TeA through the consumption of apple infant food discriminated by age group in the LB and UB scenarios, with mean and high consumption (P95) from MSAL (2012) and complete pack consumption of 90 g, using a deterministic approach. For AOH, in the LB scenario and mean consumption, neither of the age groups exceeded the TTC value of 2.5 ng/kg of BW/d; nevertheless, it

was exceeded in all the others. The TTC value for AME was surpassed in all the proposed scenarios, while for TeA, the intake remained below the TTC value of 1500 ng/kg of BW/d in all the proposed scenarios.

Table VII.4. Deterministic exposure assessment for Argentinean kids from 6 months to 5 years old to alternariol, alternariol monomethyl ether and tenuazonic acid through the consumption of apple infant food. Mean concentrations of each mycotoxin in the lower bound (LB) and upper bound (UB) scenarios were considered for mean (average) and high consumers (P95) according to MSAL (2012) and complete pack consumption of 90 g.

Mycotoxin	Food category	Age group	Mean consumption MSAL		High consumption (P95) MSAL		Complete pack consumption	
			LB (ng/kg of BW/d)	UB (ng/kg of BW/d)	LB (ng/kg of BW/d)	UB (ng/kg of BW/d)	LB (ng/kg of BW/d)	UB (ng/kg of BW/d)
AOH	Apple infant food	6-23 MO	1.8	4.4	4.1	9.8	10.8	25.8
		2-5 YO	1.7	4.2	4.1	9.8	7.4	17.8
AME	Apple infant food	6-23 MO	9.4	9.4	21.1	21.1	55.5	55.5
		2-5 YO	8.9	8.9	21.0	21.0	38.3	38.3
TeA	Apple infant food	6-23 MO	42.9	43.5	95.8	97.1	252.4	255.8
		2-5 YO	40.7	41.2	95.3	96.6	174.1	176.4

AOH: alternariol, AME: alternariol monomethyl ether, TeA: tenuazonic acid. 6-23 MO: Argentinean kids from 6 to 23 months old; 2-5 YO: Argentinean kids from 2 to 5 years old.

VII.3.4. Probabilistic exposure assessment and risk characterisation for apple infant food

The best fitted distribution for mycotoxin concentration in infant food was exponential for AOH and TeA, and LogLogistic for AME. **Table VII.5** summarizes the exposure to

mycotoxins through apple infant food in the different age groups. The results regarding the exposure values are similar to those obtained from the deterministic approach. **Table VII.6** shows the percentage of kids exceeding the corresponding TTC values for each mycotoxin.

Table VII.5. Probabilistic exposure assessment for Argentinean kids from 6 months to 5 years old to alternariol, alternariol monomethyl ether and tenuazonic acid through the intake of apple infant food for the lower bound (LB) and upper bound (UB) scenarios with consumption values from MSAL (2012) and the consumption of the complete pack of 90 g. Mean and P95 were obtained from the corresponding distribution with 10,000 iterations.

			Mean (MSAL consumption)		**P95 (MSAL consumption)**		**Mean (Complete pack consumption)**		**P95 (Complete pack consumption)**	
Mycotoxin	**Food category**	**Age group**	**LB (ng/kg of BW/ d)**	**UB (ng/kg of BW/ d)**	**LB (ng/kg of BW/ d)**	**UB (ng/kg of BW/ d)**	**LB (ng/kg of BW/ d)**	**UB (ng/kg of BW/ d)**	**LB (ng/kg of BW/ d)**	**UB (ng/kg of BW/ d)**
AOH	Apple infant food	6-23 MO	0.7	4.3	3.4	14.9	3.9	25.1	21.2	78.6
		2-5 YO	0.5	3.5	2.9	13.1	2.3	15.1	12.8	47.1
AME	Apple infant food	6-23 MO	8.9	8.9	21.9	22.2	52.1	52.3	80.6	81.0
		2-5 YO	7.3	7.3	19.0	19.2	31.2	31.3	51.5	51.6
TeA	Apple infant food	6-23 MO	20.6	42.1	98.4	152.2	122.3	249.0	582.3	773.6
		2-5 YO	16.8	35.1	83.3	125.0	72.4	149.0	346.7	472.9

AOH: alternariol, AME: alternariol monomethyl ether, TeA: tenuazonic acid. 6-23 MO: Argentinean kids from 6 to 23 months old; 2-5 YO: Argentinean kids from 2 to 5 years old.

Table VII.6. Percentage of Argentinean kids from 6 to 23 months old or 2 to 5 years old exceeding the toxicological threshold of concern (TTC) of alternariol, alternariol monomethyl ether and tenuazonic acid through apple infant food in the lower bound (LB) and upper bound (UB) scenarios considering consumption data from MSAL (2012) and the consumption of the whole pack of 90 g.

			% Exceeding TTC (MSAL consumption)		% Exceeding TTC (complete pack consumption)	
Mycotoxin	**Food category**	**Age group**	**LB**	**UB**	**LB**	**UB**
AOH	Apple infant food	6-23 MO	8.0	47.2	28.6	86.6
		2-5 YO	6.3	39.7	24.2	81.1
AME	Apple infant food	6-23 MO	91.2	91.3	95.5	95.8
		2-5 YO	84.9	85.4	95.5	95.8
TeA	Apple infant food	6-23 MO	0	0	0.2	0.4
		2-5 YO	0	0	0	0

AOH: alternariol, AME: alternariol monomethyl ether, TeA: tenuazonic acid. 6-23 MO: Argentinean kids from 6 to 23 months old; 2-5 YO: Argentinean kids from 2 to 5 years old.

The number of kids at risk is summarized in **Table VII.7**. For juices, 19,474 kids from 6-23 MO and 73,189 kids from 2-5 YO are reported to consume these products. Because the population has been growing, and the consumption is not updated, the values for the UB scenario for each product and mycotoxin are used based on the probabilistic approach. For clear AJ, only AME represented a risk and 11,548 kids from 6-23 MO and 41,425 kids from 2-5 YO would be exposed to this mycotoxin in levels above the TTC by consuming clear AJ. From consuming cloudy AJ, a total of 17,001 kids between 6-23 MO and 63,162 children from 2-5 YO would be exceeding the TTC of AOH. The number of children exceeding the TTC of AME from cloudy AJ consumption arises to 14,976 for

the 6-23 MO group, and 55,185 from the 2-5 YO. Much lower number of children would be exceeding the TTC of 1500 ng/kg BW d for TeA by consuming cloudy AJ, but they represent 292 and 586 kids from 6-23 MO and 2-5 YO, respectively.

For apple infant food, the consumption data for fruit marmalades was used because it was the most similar product, but this is a rough underestimation. Since infant food was first available in the country long after the survey took place, the percentage of kids exceeding the TTC was calculated considering the complete pack consumption. The total of children from 6-23 MO consuming these products are 26,228, and the children from 2-5 YO are 215,898. Therefore, the number of children from 6-23 MO and 2-5 YO surpassing the TTC of AOH from the sole consumption of apple infant food are 22,716 and 175,093 kids from 6-23 MO and 2-5 YO, respectively. For AME, over 91 % and 85 % are exceeding the TTC, representing 25,126 and 206,830 kids. For TeA, only 0.4 % of kids in the UB and consuming the whole pack of infant food and from the 6-23 MO age group are exceeding the TTC, representing 105 kids.

Table VII.7. Number of Argentinean kids from 6 to 23 months old or 2 to 5 years old exceeding the toxicological threshold of concern (TTC) of alternariol, alternariol monomethyl ether and tenuazonic acid from consuming clear and cloudy apple juice (AJ) and apple infant food in the upper bound scenario considering consumption data from MSAL, (2012) for juices and the consumption of the whole apple infant food pack of 90 g.

		Number of children exceeding the TTC		
Food category	**Age group**	**AOH**	**AME**	**TeA**
Clear AJ	6-23 MO	0	11,548	0
	2-5 YO	0	41,425	0
Cloudy AJ	6-23 MO	17,001	14,976	292
	2-5 YO	63,162	55,185	586
Apple infant food	6-23 MO	22,716	25,126	105
	2-5 YO	175,093	206,830	0

AOH: alternariol, AME: alternariol monomethyl ether, TeA: tenuazonic acid. 6-23 MO: Argentinean kids from 6 to 23 months old; 2-5 YO: Argentinean kids from 2 to 5 years old.

VII.4. Discussion

The natural occurrence of *Alternaria* mycotoxins in apple by-products in Argentina was detected for the first time and described in chapter VI. Those results suggested a potential risk associated with the consumption of these products, especially for children since they are the targeted consumers of AJ and apple infant food and a vulnerable group. Based on the data generated in chapter VI, it was possible to characterize the risk associated to the presence of AOH, AME and TeA in apple products for children in Argentina for the first time. Deterministic and probabilistic approaches were made to assess the exposure to *Alternaria* mycotoxins for infants from 6 to 23 months old and 2

to 5 years old by the consumption of apple by-products. Even though the results from the deterministic and probabilistic approaches were similar, the deterministic one seemed to overestimate the exposure in most of the cases, except in the UB 95% scenarios. In deterministic approaches, however, the uncertainty and variability of food consumption and contamination data are not considered. A deterministic exposure assessment can be firstly used for screening, but probabilistic assessments consider all the possible scenarios and avoid overestimations due to variation and uncertainties (Abdallah et al., 2020). As well, the probabilistic assessment gives more insights to exposure as they consider every possible value each variable can take and weigh every scenario by the probability of its occurrence. The results from the probabilistic assessment are in line with the deterministic one, therefore will be discussed together.

It is worth mentioning the limitations to the model. First, the lack of updated consumption data could lead to biased results; changes in the consumption pattern might have a strong impact on the exposure calculations and should be further evaluated. Clear and cloudy AJ were assumed to have the same consumption in this assessment since no distinction was made in the MSAL (2012), and apple infant food was only recently available on the local market; therefore, approximations had to be made to estimate the risk, sacrificing precision. As well, a bigger sample size is advisable, with periodic follow up of mycotoxin concentrations. No other occurrence data of these products were available and information of *Alternaria* mycotoxin contamination in Argentina is scarce for most food products. Another factor of uncertainty in the model was derived from samples with non-detectable levels of mycotoxins. Therefore, the LB and UB scenario provides a best- and worst-case scenario estimation (EFSA, 2010).

For all the food commodities analysed, a higher risk was associated with the alternariols, which are estrogenic disruptors. In apple juice AME posed a risk in clear and cloudy AJ, and AOH only in cloudy AJ. Since AOH was not present in clear AJ, no risk is assumed, but frequent monitoring of this mycotoxin is suggested to check stability of this result in

time. A different result is the one obtained from cloudy AJ, in which in all the scenarios, AOH intake by cloudy AJ by far exceeded the TTC of 2.5 ng/kg of BW/d for the different age groups. The daily intake represented from 2 to 57 times the TTC value and 87.3 % (17001) and 86.3% (63162) of consumers were exposed to higher levels of AOH than the TTC. For AME, the TTC value was exceeded in all the proposed scenarios from consuming cloudy AJ, surpassing the TTC value from 3 to 27 times exposing 79.9 % (14,976) and 75.4 % (55,185) of children. For TeA, even though the TTC was not exceeded in any of the deterministic scenarios, the consumption of cloudy AJ represented from 8 to 62 % of the total TTC value for both age groups.

Overall, a higher risk was found for non-clarified products. Particularly, regardless the concentration scenario or consumption of apple infant food, over 90 % of children between 6-23 MO and 84 % of children between 2-5 YO are exposed to levels above the TTC of AME, raising special concern. The deterministic approach showed that the consumption of this product alone was enough to exceed the TTC value of AOH in the UB of mean consumption, and in the LB and UB of high consumption. When a more realistic consumption (whole pack) was tested, the TTC of AOH was exceeded 3 to 10 times for both age groups. In the worst-case probabilistic scenario 86.6 % (22716) and 86.1 % (175093) of the consumers from 6-23 MO and 2-5 YO, respectively, exceeded the TTC, thus elevating concern.

Even though TeA was found at the highest concentrations among all the products, it did not pose a high risk alone for children consuming apple products. This result does not consider the intake of TeA from other food commodities and the contribution that other foods might have on the intake of AOH and AME should not be overlooked as well as. Other food commodities in Argentina have been reported as contaminated with AOH, AME and TeA, like vegetables or cereals, usually mixed with fruits to obtain other infant foods (Azcarate et al., 2016; Castañares et al., 2019; da Cruz Cabral et al., 2016; Romero Bernal et al., 2019; Terminiello et al., 2006). The data generated here should be

evaluated performing a risk characterisation including other foods consumed in Argentina. As well, modified mycotoxins should also be considered in risk assessments, since modified forms of alternariols have been found in apple by-products (chapter VI) and they may hydrolyse in the digestive tract to form unconjugated mycotoxins (Chen et al., 2021); thus, their potential toxicity could be equivalent to that of alternariols.

Even though some studies evaluated the risk from exposure to *Alternaria* mycotoxins in the European population (Arcella et al., 2016; Sprong et al., 2016; Vin et al., 2020), and China (Zhao et al., 2015), the occurrence data and consumption patterns differ from the Argentinean one. Nevertheless, data of natural contamination of foods with *Alternaria* toxins showed that the TTC levels for AOH and AME were exceeded in some European countries (Arcella et al., 2016) and also in China (Zhao et al., 2015). In a recent study in the Netherlands, it was shown that the TTC value for the alternariols was exceeded when analysing the occurrence data and consumption from some foods for kids from 1-2 YO, and that apple infant food and fruit juices were responsible for the high intake of AOH (Pustjens et al., 2021).

Considering the estrogenic potential of alternariols (Vejdovszky et al., 2017), the combinatory toxic effects they may exert (Fernández-Blanco et al., 2016), and the overall vulnerability of children, the numbers here presented should raise attention. The impact that the early life exposure to these mycotoxins could imply on the Argentinean population should be considered, checking the estrogenic post-puberty effects. Taking into account that other food commodities alone may pose a risk for consumers from *Alternaria* contamination, like tomato products (Walravens et al., 2016), a holistic assessment including other foods in the Argentinean market should be made. *Alternaria* mycotoxins are no longer emerging (Aichinger and Marko, 2021), and their presence in food commodities, especially when they are intended for infants should be addressed by implementing not only better controls by producers, but regulation by national and international authorities.

VII.5. Conclusions

The risk of exposure to *Alternaria* toxins from consuming apple by-products was characterized for children, showing a high exposure to alternariols and a higher risk associated to non-clarified products, particularly infant food. Even though the risk posed for TeA was low in the present analysis, it should not be underestimated, since this mycotoxin was found in higher concentrations and is broadly distributed in other food commodities.

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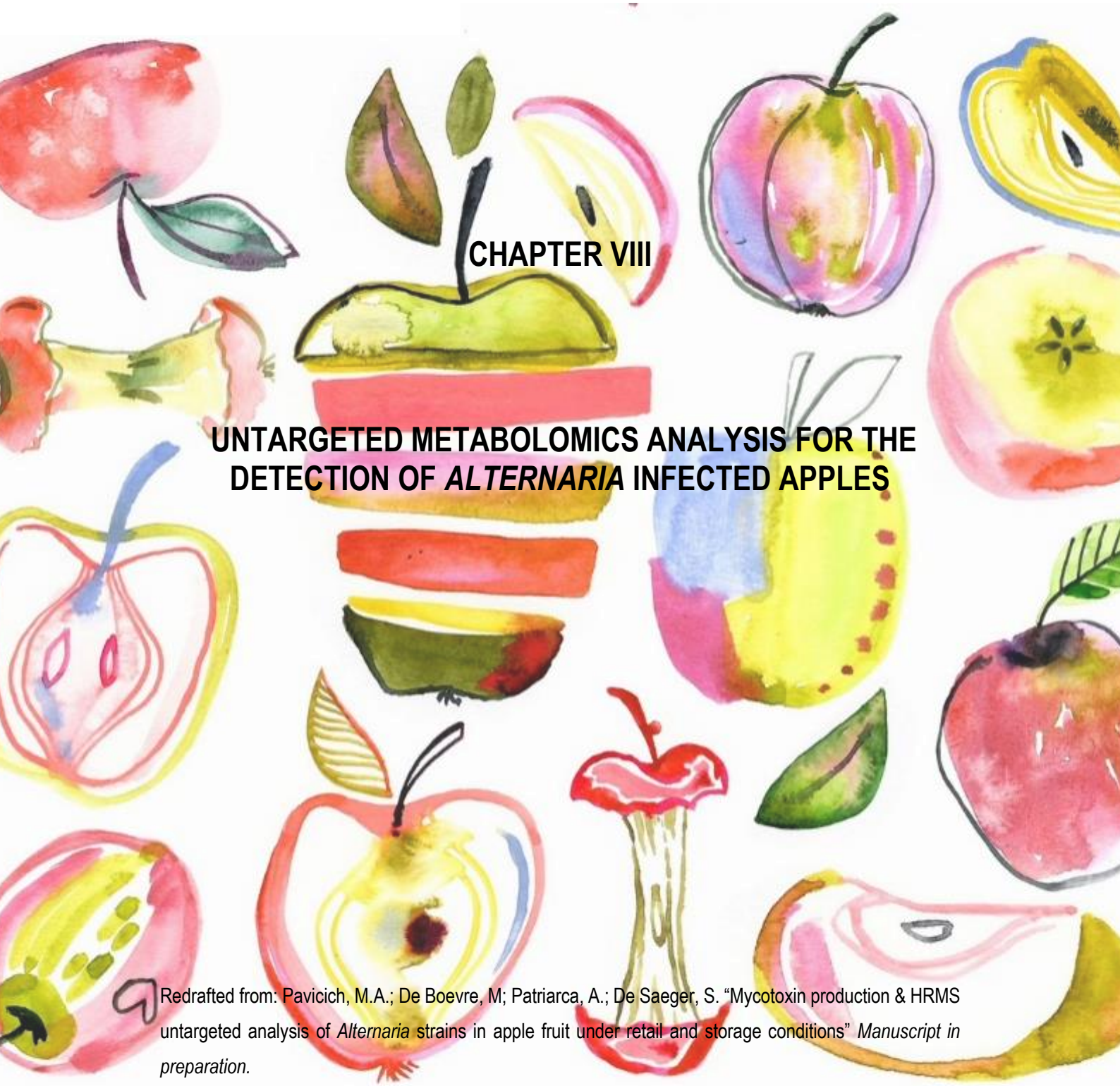

CHAPTER VIII

UNTARGETED METABOLOMICS ANALYSIS FOR THE DETECTION OF *ALTERNARIA* INFECTED APPLES

Redrafted from: Pavicich, M.A.; De Boevre, M; Patriarca, A.; De Saeger, S. "Mycotoxin production & HRMS untargeted analysis of *Alternaria* strains in apple fruit under retail and storage conditions" *Manuscript in preparation.*

Illustration by Tanja Meyer

VIII.1. Introduction

The results of this PhD thesis showed that *Alternaria* species and mycotoxins in apple and by-products should raise concern. In chapter VII, it was suggested to establish legislative maximum levels for *Alternaria* mycotoxins in apple by-products since a risk for consumers was demonstrated, particularly for the vulnerable group of children. Nonetheless, legislation alone does not prevent the accumulation of toxins; controls in the process of apple by-products are necessary for producers to be able to comply with the future legislation.

The incidence and severity of *Alternaria* in the field is relatively low, increasing with time according to the storage temperature (chapters II and IV). The prevention of MC by spraying fungicide once fungal spores enter the seed chamber via the calyx sinus after flowering is not effective (Shtienberg, 2012; Gao et al., 2013). Not only *Alternaria* spp. are less sensitive against commonly applied fungicides in comparison to other fungi affecting apple fruit (Grantina-Ievina et al.,2016), but once the fungus is inside the fruit, it is protected against contact fungicides, thus improving the conditions for its development (Reuveni et al., 2002). Moreover, fungicide applications to reduce other fungi have seemed to raise the incidence of the *Alternaria* disease, as they eliminate competitors for MC causal agents (Snowdon, 1991).

The incidence of *Alternaria* during the postharvest stage increased. Thus, storing fruits for shorter periods and processing freshly harvested apples would be a promising strategy for reducing mycotoxin concentration in the final products, but not always possible or profitable.

The application of untargeted metabolomics towards food safety strategies is still in early stages, but the work done to date shows promising results. These studies employ various analytical hardware and software, many using high-resolution mass spectrometry (HRMS) (Peter et al., 2021), and enable to identify unique metabolic markers that can be applied in the early detection of a phytopathogen or its metabolites as well as a

fingerprint of infection (Adeniji et al., 2020). Therefore, untargeted metabolomics can be used to develop strategies to tackle the issue of the contamination of food and feed with mycotoxins through an understanding of the plant-pathogen interaction (Castro-Moretti et al., 2020; Richard-Forget et al., 2021).

The aim of this chapter was to set the bases of a method for a control strategy based on liquid chromatography tandem HRMS to detect apples infected with *Alternaria* spp. and their mycotoxins and thus prevent their incorporation into the process line.

VIII.2. Materials and methods

VIII.2.1. Samples & Extraction

The artificially contaminated apples from chapter IV were employed for this analysis. To assess metabolite differences, blank samples free of fungal infection and mycotoxin and incubated for 1 month at 25 °C and 9 months at 4 °C were used. A portion of 2.0000 ± 0.002 g of each fruit was placed in an extraction tube and extracted with 10 ml of a mixture of methanol, ultra-pure water, and acetic acid (MeOH/H_2O/AA, 79:20:1, v/v/v) for 30 minutes in a shaker. The samples where then centrifuged at 4,000 g for 15 min and a 1 ml aliquot was taken from the supernatant and evaporated till dryness under a N_2 steam. Samples were resuspended in the injection solvent H_2O/ACN (70:30, v/v) and filtered through a 0.22 µm centrifugal PTFE filter (Merck Millipore, Darmstadt, Germany).

VIII.2.2. UPLC/HRMS analysis

UPLC/HRMS conditions were adapted from chapter III in combination with previous experiments at the CEMPH (Abdallah et al., 2020). An aliquot of 5 µL was injected into an ACQUITY UPLC system coupled to a Synapt G2−Si High-Definition instrument, a hybrid quadrupole orthogonal acceleration time of flight equipped with traveling wave ion mobility separation mass spectrometer (Waters Corporation, Milford, MA, USA). The column was an HSS T3 (1.8 µm, 2.1 × 100 mm) held at 40 °C and samples were

maintained at 10 °C (Waters Corporation). A linear gradient elution program with solvent A (ultra−pure water:20 mmol L^{-1} formic acid) and B (ACN: 20 mmol L^{-1} formic acid) was applied with a flow rate of 0.35 mL/min as follows: 90% A and 10% B for 0.5 min, and an increase to 100% B from 0.5 to 10.0 min, and 100% B maintained from 10.0 to 13.0 min, with a direct back to 90% A from 13.0 to 13.1 min, and maintaining starting conditions from 13.1 to 15 min. Ultra-pure water, acetonitrile and formic acid were LC-MS grade and obtained as indicated in chapter IV. The instrument was operated in resolution mode and calibration was done with sodium formate clusters. Leucine enkephalin was used as lock mass for mass correction with a scan time of 0.1 s, and a frequency of 20 s. Data type was continuum and acquired in MS^E mode on ESI^+ and ESI^- in separate runs in the scan range m/z 50 to 1,200 Da. Mass spectrometry parameters were: capillary voltage 2.8 kV; sample cone voltage 40 V; source offset 80 °C; source temperature 130 °C; desolvation gas flow 800 L/h at a temperature of 550 °C, and cone gas flow 50 L/h. Nitrogen was employed as desolvation and cone gas at a pressure of 6.5 bar. Argon was used as the collision gas at a 9.28×10^{-3} mbar. Collision energy ramp was used for fragmentation of ions for the low and high mass from 11/13 V (start/end) to 50/120 V (start/end), respectively.

VIII.2.3. Data processing

Progenesis QI (Waters Corporation) was used to analyse the large amount of data generated. The adduct ions $[M+H]^+$, $[M+Na]^+$, $[M+NH_4]^+$, $[M+H-H_2O]^+$ were selected in the positive mode and $[M-H]^-$, and $[M+HCOO]^-$ in the negative mode. Then, pre-processing by retention time alignment and peak picking which involved the information of retention time, m/z, and peak area, was done. Composite ion maps were obtained, and with the statistical tools included in Progenesis QI, metabolites were filtered according to ANOVA p-value <0.05 to build principal component analysis.

VII.3. Results

Composite ion maps which contained 21,804 and 21,506 compounds in ESI^+ and ESI^- mode, respectively, were obtained. Metabolite filtering according to the ANOVA p-value <0.05 decreased the number to 9,308 and 10,505 metabolites in positive and negative mode, respectively. This data reduction allowed to focus on the metabolites that clearly discriminate infected from non-infected apples by principal component analysis (PCA).

Figure VIII.1 shows the PCA of metabolites of the infected and non-infected (control) apples detected in ESI^+, and **Figure VIII.2** in ESI^-. In the ESI^+ analysis, the two components explained 43.3 % of the differences. For ESI^-, both components explained 44.6 % of the differences. In both analyses, the control samples at 25 ºC and 4 ºC showed significant differences with the infected apples, independently from the strain, site of inoculation or incubation temperature. The control samples were situated in the right extreme of the PCA, where principal component 1 (PC1) took its higher positive values in both analyses. Controls were segregated from the contaminated apples.

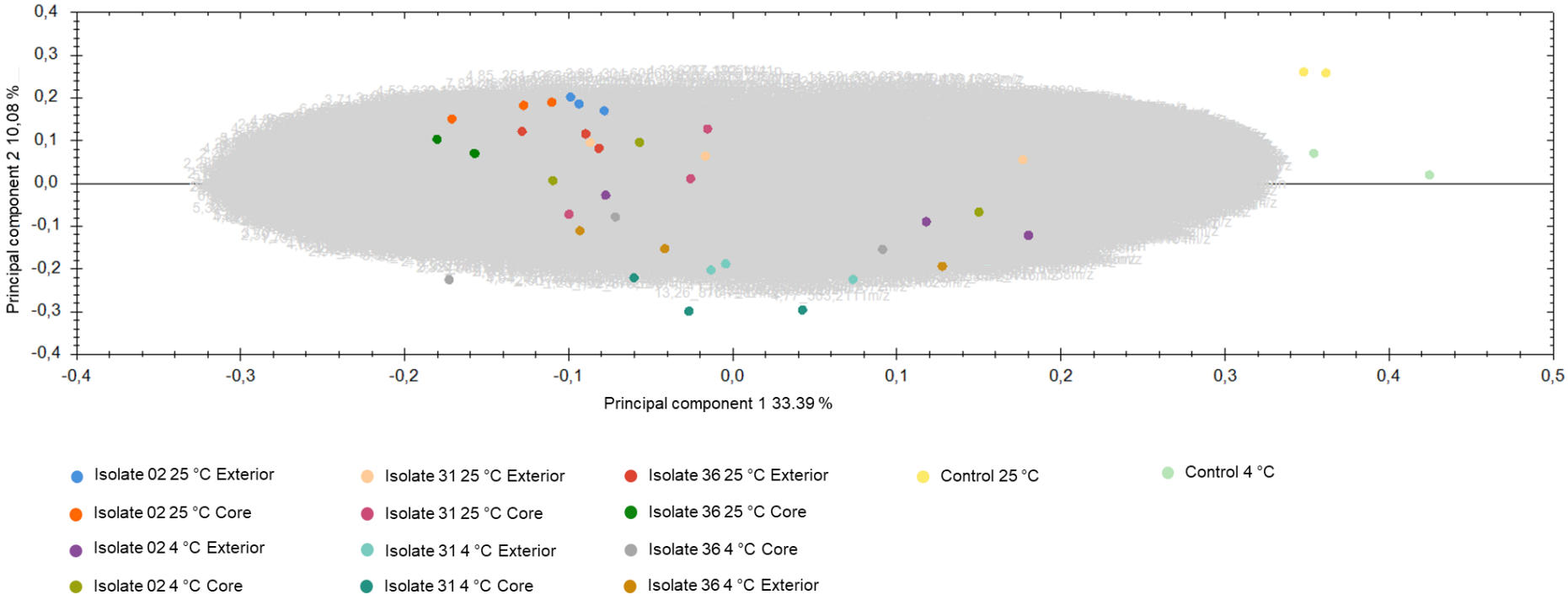

Figure VIII.1. Principal component analysis of metabolites of contaminated and uncontaminated apples in ESI^+ under the different incubation conditions. Incubation at 25 °C was done for 1 month, and at 4 °C for 9 months.

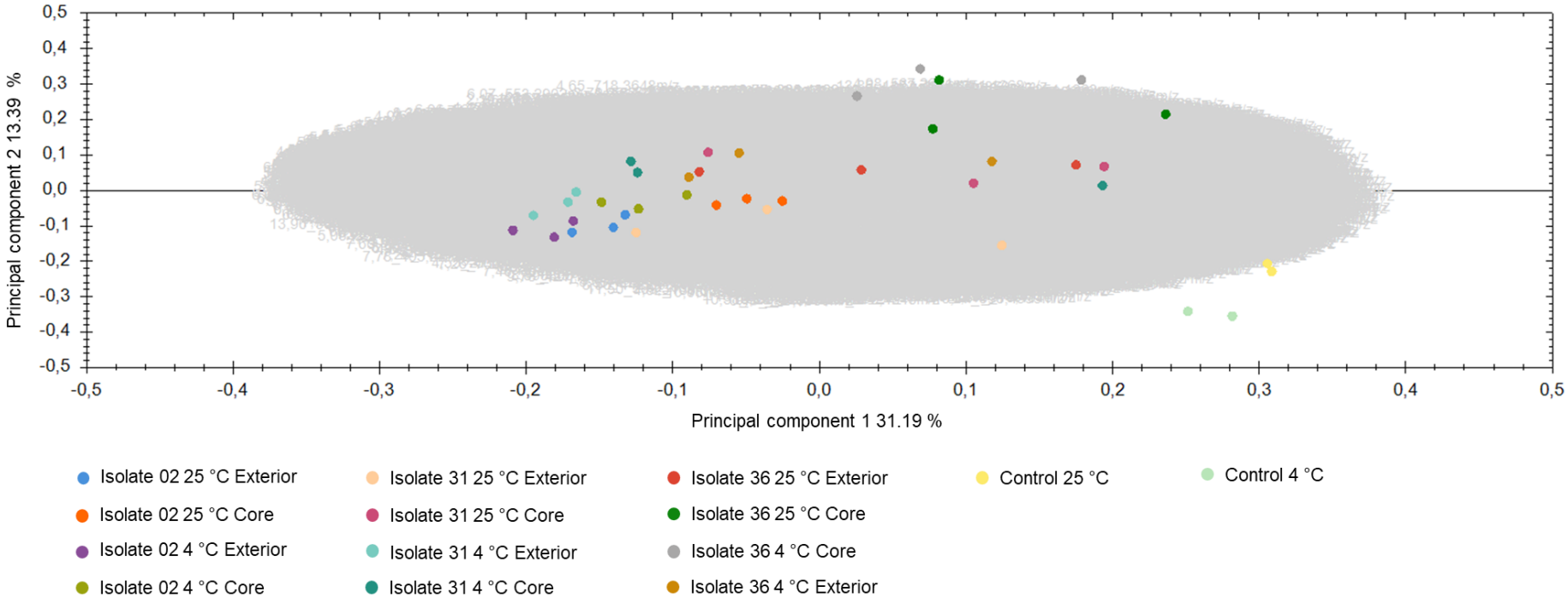

Figure VIII.2. Principal component analysis of metabolites of contaminated and uncontaminated apples in ESI^- under the different incubation conditions. Incubation at 25 °C was done for 1 month, and at 4 °C for 9 months.

VIII.4. Discussion

The results of this PhD thesis suggested the need for control strategies to prevent the presence of *Alternaria* mycotoxins in apple by-products. As mentioned, the application of fungicides both in pre- and post-harvest stages does not seem to solve the problem, and moreover, fungicide resistance is gaining more relevance (Chavan et al., 2017; Yang et al., 2019). There is a growing understanding that knowledge about the diversity in natural ecosystems can contribute to more sustainable crop production (Kristoffersen et al., 2020). Since long-term storage increases the incidence of MC, an effective selection of raw material could prevent the incorporation of infected fruit into the process line. Several automated methods have been proposed for non-destructive detection of this disease. Hu et al. (2019) recently developed a frequency domain diffuse optical tomography method for detecting underlying lesions of apple. The mouldy lesions not deeper than 20 mm from the peel were resolvable on the absorption images, but the model still presented several limitations. Yang and Yang (2010) and Kadowaki et al. (2012) developed non-destructive methods based on X-Ray for detecting early stages of core rot in Japanese pear and suggested it could also be applied for apples. Methods based on transmittance spectroscopy were developed for the detection of single fruit with MC (Tian et al., 2020; Zhou et al., 2016), as well as an online detection method based on visible and near infrared spectroscopy full-transmittance spectra of MC apples (Tian et al., 2020). All these methods are promising for fresh retail apples or to be applied by packhouses to prevent storage of fruit with early stages of MC. Nonetheless, none of them detect mycotoxin accumulation and therefore would not provide a solution for processing industries.

The untargeted approach presented here, involving HRMS analysis, allowed to detect metabolites from the non-infected apples, the fungi, and metabolites from the apple and fungi interaction (infected apples). It clearly separated *Alternaria* infected from non-infected fruits, regardless incubation temperature or place of inoculation, even in the

conditions in which mycotoxin accumulation was lower (*e.g*., exterior infection, 9 months storage) in both ESI positive and negative. This separation can be related to a group of metabolites produced by *Alternaria´*s interaction with the apple that are not present in the healthy apples incubated under any conditions. This was more evident when using ESI^+ mode, but both modes were able to distinguish infection. In the PCA, PC1 discriminated infected from non-infected apples, and the conditions that favoured mycotoxin production were displaced to the left, taking negative values in PC1. Under these conditions, a more diverse metabolite production was detected, originating from the apple-fungal interaction.

This HRMS method could be used by apple concentrate industries to prevent the presence of *Alternaria* mycotoxins in the final product. As explained in chapter V, after raw material is incorporated in the process line, the first step consists in grinding the fruit. A sample of this ground product can be analysed, and a in a 15-minute chromatographic run, a contaminated batch could be detected. Consequently, mycotoxin quantification should be performed on those batches to decide its fate. When low levels of *Alternaria* toxins are detected, the batch could be destined to clear products, since clarification reduces their concentration, with the rigorous control of TeA, which cannot be eliminated in this step. Even though the consumption of apple by-products alone did not pose a risk of surpassing TeA´s TTC, it contributes to the total intake and should not be overlooked. If high levels of mycotoxin are found in contaminated batches, then an alternative use as compost should be evaluated (Maldonado et al., 2021). Additionally, detoxification methods such as UV radiation, which proved to degrade patulin in apple juice (Chandra et al., 2017), or treatments with fungal enzymes that were effective in the degradation of other mycotoxins (Loi et al., 2018) could be investigated.

In the untargeted analysis, metabolites were not identified further than their retention time and m/z in the given chromatographic and mass spectrometry conditions. Progenesis QI allows to search for metabolites in different data bases, but information of

fungal metabolites is scarce, and a build in-house data base is recommended; this is a future objective for the CEMPH. These promising preliminary results encourage further studies, such as scouting for biomarkers of early *Alternaria* infection. Similar methods based on gas chromatography tandem HRMS using an untargeted approach allowed the chemical separation of grapes infected with different pathogens from non-infected ones and the identification of marker compounds (Schueuermann et al., 2019). Additionally, models for apple processing industries using different apple varieties can be constructed considering the metabolomic differences between them.

VIII.5. Conclusions

The bases for a HRMS model were proposed for the detection of contaminated apple batches to prevent their processing and consequent mycotoxin accumulation in apple by-products. The model allowed resolution between contaminated and uncontaminated fruits, and it is a promising perspective for future developments to reduce the risk associated with apple processed food.

VIII.6. References

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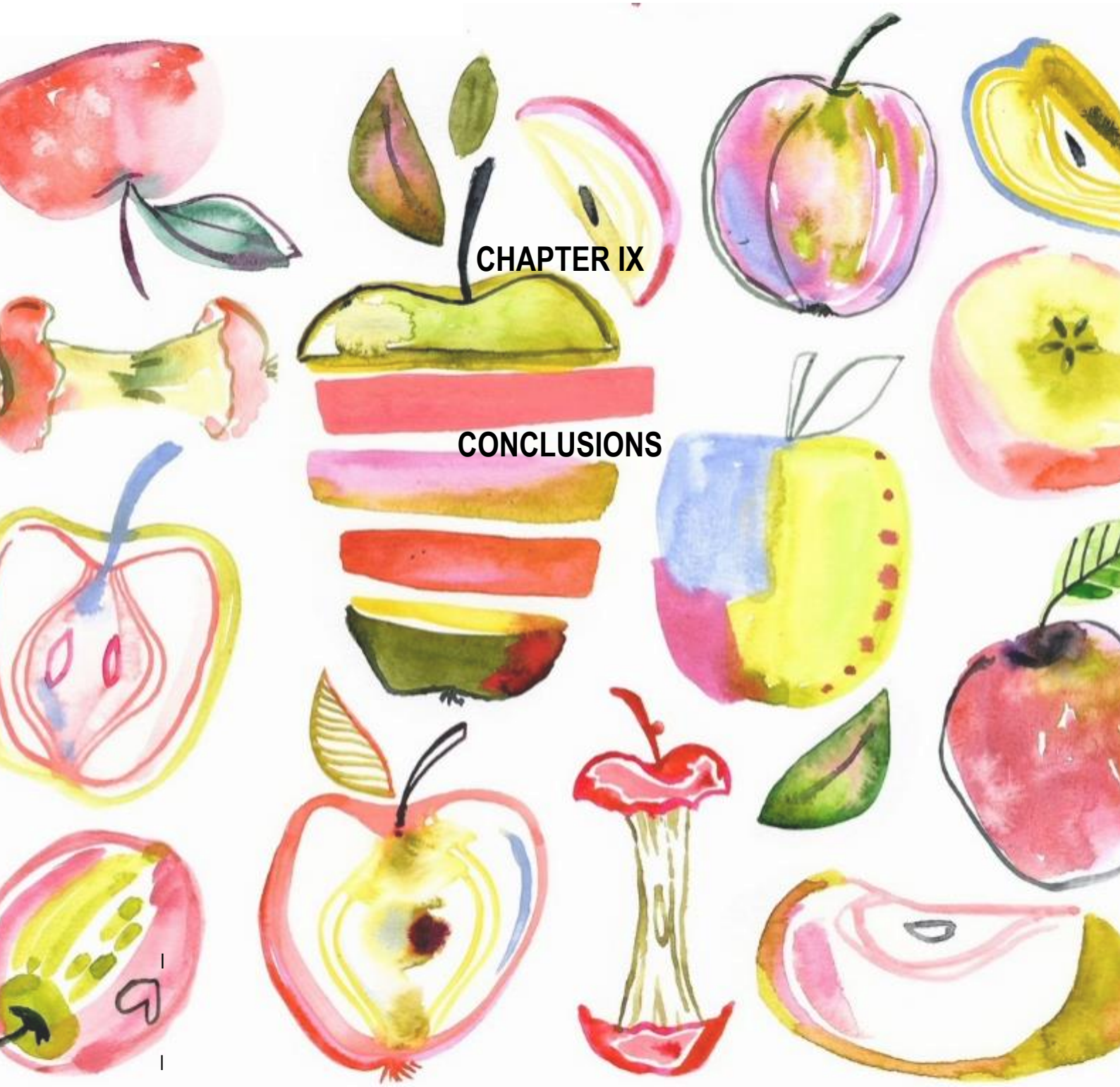

CHAPTER IX

CONCLUSIONS

Illustration by Tanja Meyer

IX.1. Conclusions

IX.1.1. General conclusions

The results from this joint PhD thesis between Universidad de Buenos Aires and Ghent University allowed to characterize the problem of *Alternaria* in apple from field to fork, as well as to set the bases for new technologies to tackle the mycotoxin issue. *Alternaria* mycotoxins are still considered emerging since scarce information about their natural occurrence and toxicity is available. Therefore, the results here presented contribute to widening the knowledge on this topic, with perspectives for their control and legislation.

IX.1.2. Specific conclusions

I. The main fungal infectants of apple fruit from the Alto Valle of Rio Negro region intended for fresh consumption (C) as well as for industrial food processing (I) were identified. Both C and I apples were highly contaminated in the exterior and interior of the fruit. As well, the high incidence of MC in apple fruit in Argentina was demonstrated. Long-term cold storage selected the toxicogenic fungal genera in apples over the whole mycobiota and *Alternaria* was found as the main causal agent of MC in the field and during the postharvest stage. The incidence and severity of MC increased during storage. (Chapter II)

II. The *Alternaria* isolates infecting apple fruit corresponded to *Alternaria* section *Alternaria* and low morphological variability was observed. Nevertheless, a high metabolic and toxigenic capacity was observed in the *Alternaria* apple population. Most of the secondary metabolites produced *in vitro* were either mycotoxins, or modified forms. Isolates from MC showed more diverse chemical profiles and the ability to synthesise compounds from different chemical families. (Chapter III)

III. The *Alternaria* strains isolated from apple fruit were able to produce mycotoxins under retail and long-term cold storage conditions in the interior

and the exterior of the fruit. The risk of mycotoxin accumulation was higher at 25 ºC, but the long-term cold storage usually performed by processing industries did not prevent the accumulation of secondary *Alternaria* toxic metabolites in apples. (Chapter IV)

IV. The first report stating the fate of free and modified forms of *Alternaria* toxins in the apple concentrate production and the first report of the presence of AOH-3-S and AME-3-S in apple concentrate was made. The results obtained indicate that the clarification stage in the apple concentrate process is of crucial importance to significantly reduce *Alternaria* toxins to safe levels in the final products. The major risk could be associated with cloudy apple by-products, especially if those are intended for infant foods. Although *Alternaria* mycotoxins are relatively stable, their contamination levels can be reduced to some extent during apple concentrate processing. (Chapter V)

V. The first report of *Alternaria* mycotoxin and their modified forms occurrence in apple by-products from the Argentinean market was made. All the different food categories analysed were contaminated with *Alternaria* toxins, but higher levels were found in non-clarified products destined to infants. (Chapter VI)

VI. The risk of exposure to *Alternaria* toxins from consuming apple by-products was characterized for children, showing a high exposure to alternariols and a higher risk associated to non-clarified products, particularly infant food. Even though the risk posed for TeA was low in the present analysis, it should not be underestimated, since this mycotoxin was found in higher concentrations and is broadly distributed in other food commodities. (Chapter VII)

VII. The bases for a HRMS model were proposed for the detection of contaminated apple batches to prevent their processing and consequent mycotoxin accumulation in apple by-products. The model allowed resolution between contaminated and uncontaminated fruits, and it is a promising perspective for future developments to reduce the risk associated with apple

processed food. (Chapter VIII)

APPENDICES

Annex Table III.1. Retention time (RT), monoisotopic mass, m/z of ions and adducts in positive (ESI+) and negative (ESI-) mode, and UV data used to identification of metabolites in chapter III.

Metabolite	RT (min)	Calculed monoisotopic mass	[M+H]+	[M+Na]+	[M+NH4]+	[M-H]-	additional ions positive mode	additional ions negative mode	UV data
3-Hydroxyalternariol 5-O-methyl	8.1	288,0634	289,0707	311,0526	306,0972	287,0561	271, 243	272,0000	203, 236, 260, 340
4-Hydroxi-alternariol monomethylether	8.1	287,9927	289,0000	310,9819	306,0265	286,9854		287, 272, 257, 229, 188	187, 185
4Z-Infectopyrone	7.08	264,0998	265,1071	287,0890	282,1336	263,0925	288,0000		219, 268, 350
Altechromone A	5.6	190,0630	191,0703	213,0522	208,0968	189,0557	175, 162,150, 122		210, 242, 251, 293
Altechromone B	4.58	232,0727	233,0809	255,0619	250,1065	231,0654	218, 193, 176		249, 255, 261, 291
Altenuene	6.3	292,0947	293,1020	315,0839	310,1285	291,0874	275, 257, 229, 201	337, 327	240, 280, 320
Altenuic acid II	4.95	322,0683	323,0761	345,0581	340,1027	321,0605			
Altenuic acid III	5.3	322,0688	323,0761	345,0580	340,1026	321,0615	233, 277, 321		
Altenuisol	7.78	274,0477	275,0550	297,0369	292,0815	273,0401	274, 91	258, 547	217, 255, 276 (sh.)
Altenusin	6.82	290,0790	291,0863	313,0683	308,1129	289,0718	296,273, 245, 239, 227, 198	271, 245, 230	217sh, 258, 290
Alterlactone	7.00	288,0627	289,0700	311,0519	306,0965	287,0554	599, 271	287, 243, 228	206, 221, 254
Alternarienonic acid	5.4	278,0790	279,0863	301,0682	296,1128	277,0717	279, 261	233,0000	218, 260 (sh), 302
Alternariol	7.35	258,0528	259,0601	281,0420	276,0866	257,0455	244, 213	213,0000	204, 256, 288, 300, 340
Alternariol 5-O-methyl ether-4′-O-sulphate	7.1	352,0253	353,0326	375,0145	370,0591	351,0180	273,0000	351, 271	203, 254, 285, 337
Alternariol 5-O-sulphate	4.00	413,9927	415,0000	436,9819	432,0265	412,9854	437, 416, 438		
Alternariol monomethylether	9.08	272,0685	273,0758	295,0577	290,1023	271,0612	258, 230	256,0000	204, 256, 288, 300, 340
Alterperylenol	7.54	350,0790	351,0863	373,0682	368,1128	349,0717	333, 315, 305	331, 313, 303, 261	215, 255, 285, 364
Altersetin	12.3	399,2409	400,2482	422,2301	417,2747	398,2336	201, 145	354,0000	233, 287
Altertoxin analog	8.4	347,9927	349,0000	370,9819	366,0265	346,9854			
Altertoxin-I	7.42	352,0947	353,1020	375,0839	370,1285	351,0874	317, 271	398, 397, 333, 315, 297, 263	216, 260, 284, 356

Annex Table III.1. Retention time (RT), monoisotopic mass, m/z of ions and adducts in positive (ESI+) and negative (ESI-) mode, and UV data used to identification of metabolites in chapter III. (Continuation)

Metabolite	RT (min)	Calculed monoisotopic mass	[M+H]+	[M+Na]+	[M+NH4]+	[M-H]-	additional ions positive mode	additional ions negative mode	UV data
Altertoxin-II	8.8	350,0790	351,0863	373,0682	368,1128	349,0717	333, 315	331, 313	218, 260 (sh), 360
Altertoxin-III	9.9	348,0634	349,0707	371,0526	366,0972	347,0561	poor ionization	319, 277	240, 268, 354
cis-dehydrocurvularin	7.33	290,1154	291,1227	313,1046	308,1492	289,1081	273, 245, 123	201, 175	205, 227, 293
Dehydroaltenusin	6.86	288,0634	289,0707	311,0526	306,0972	287,0561	271, 245, 243, 227	243, 228	217, 249, 300
Desmethylaltenusin	4.67	276,0561	277,0710	299,0526	294,0899	275,0566	233,0000	231, 174.955	203, 224, 255, 293
Dihydrotentoxin	7.68	416,2424	417,2496	439,2316	434,2762	415,2351	440,0000	462, 461, 417, 440	202, 284
Infectopyrone I	6.8	264,0998	265,1071	287,0890	282,1336	263,0925	288,0000		220, 265, 348
Isopropyl tetramic acid	5.44	183,0895	184,0968	206,0787	201,1233	182,0822	207,0000	183, 207	225, 280
Novae-zelandin A	4.1	224,0685	225,0758	247,0577	242,1023	223,0612			210, 280
Novae-zelandin B	7.9	194,0943	195,1016	217,0835	212,1281	193,0870			207, 288
Phomapyrone A	9.83	234,1256	235,1329	257,1148	252,1594	233,1183	257, 258		215, 257, 350
Phomapyrone B	5.9	224,1048	225,1121	247,0940	242,1386	223,0975	248,0000		220, 255, 345
Phomapyrone D	6.2	222,0892	223,0965	245,0784	240,1230	221,0819	246, 257		220, 256, 348
Phomapyrone E or G	8.4	250,1205	251,1278	273,1097	268,1543	249,1132	274,0000		220, 257, 350
Phomapyrone F	10.25	232,1099	233,1172	255,0991	250,1437	231,1026	256,0000		220, 256, 349
Pyrenochaetic acid A	4.9	234,0892	235,0965	257,0784	252,1230	233,0819	191,0000		
Stemphyltoxin-III	8.9	348,0634	349,0707	371,0526	366,0972	347,0561			
Tentoxin	7.61	414,2267	415,2340	437,2159	432,2605	413,2194	358, 312	459, 438, 215	202, 284
Tenuazonic acid	6.56	197,1052	198,1125	220,0944	215,1390	196,0979	181, 153, 142	139, 112	225, 280

Annex Table IV.1. Multifactorial analysis of variance of mycotoxins concentrations under the different conditions evaluated and the influence of strain, temperature, inoculation site and their interactions on mycotoxin production.

	Df	Sum Sq	Mean Sq	F value	p value
Inoculation	1	439688	439688	6.3578	0.0125000
Temperature	1	892003	892003	12.8981	0.0004178
Mycotoxins	7	1373547	196221	2.8373	0.0077518
Isolate	2	518140	259070	3.7461	0.0253536
Mycotoxin:inoculation	7	517223	73889	1.0684	0.3853486
Mycotoxin:temperature	7	949428	135633	1.9612	0.0622810
Inoculation:temperature	1	412005	412005	5.9575	0.0155620
Mycotoxin:isolate	14	1784324	127452	1.8429	0.0350452
Inoculation:isolate	2	217451	108725	1.5721	0.2102617
Temperature:isolate	2	464731	232365	3.3599	0.0367919
Mycotoxin:inoculation:temperature	7	523716	74817	1.0818	0.3764978
Mycotoxin:inoculation:isolate	14	1004782	71770	1.0378	0.4176537
Mycotoxin:temperature:isolate	14	1597714	114122	1.6502	0.0691726
Inoculation:temperature:isolate	2	291781	145891	2.1095	0.1240968
Mycotoxin:inoculation:temperature:isolate	14	957295	68378	0.9887	0.4663447
Residuals	192	13278270	69158		

Annex Table IV.2. Multifactorial analysis of variance of mycotoxins concentrations under the different conditions evaluated and the influence of strain, temperature, inoculation site and their interactions on mycotoxin production for isolate 02.

	Df	Sum Sq	Mean Sq	F value	p value
Inoculation	1	161671	161671	4.3775	0.0403885
Temperature	1	786264	786264	21.2891	1.949e-05
Mycotoxins	7	1767176	252454	6.8355	4.601e-06
Inoculation:temperature	1	211538	211538	5.7277	0.0196432
Inoculation:mycotoxins	7	400271	57182	1.5483	0.1673322
Temperature:mycotoxins	7	1172228	167461	4.5342	0.0003715
Inoculation:temperature:mycotoxins	7	376248	53750	1.4553	0.1993549
Residuals	64	2363687	36933		

Annex Table IV.3. Multifactorial analysis of variance of mycotoxins concentrations under the different conditions evaluated and the influence of strain, temperature, inoculation site and their interactions on mycotoxin production for isolate 31.

	Df	Sum Sq	Mean Sq	F value	p value
Inoculation	1	1927	1926.94	2.1424	0.1482
Temperature	1	38	37.63	0.0418	0.8386
Mycotoxins	7	8909	1272.67	1.4150	0.2149
Inoculation:temperature	1	2316	2315.75	2.5747	0.1135
Inoculation:mycotoxins	7	8687	1241.00	1.3798	0.2292
Temperature:mycotoxins	7	6494	927.76	1.0315	0.4182
Inoculation:temperature:mycotoxins	7	4512	644.59	0.7167	0.6581
Residuals	64	57562	899.41		

Annex Table IV.4 Multifactorial analysis of variance of mycotoxins concentrations under the different conditions evaluated and the influence of strain, temperature, inoculation site and their interactions on mycotoxin production for isolate 36.

	Df	Sum Sq	Mean Sq	F value	p value
Inoculation	1	493540	493540	2.9093	0.09292
Temperature	1	570432	570432	3.3626	0.07134
Mycotoxins	7	1381787	197398	1.1636	0.33611
Inoculation:temperature	1	489933	489933	2.8881	0.09409
Inoculation:mycotoxins	7	1113047	159007	0.9373	0.48434
Temperature:mycotoxins	7	1368420	195489	1.1524	0.34260
Inoculation:temperature:mycotoxins	7	1100251	157179	0.9265	0.49228
Residuals	64	10857020	169641		