University of Alberta

Mode of action, interaction and recovery of plant secondary metabolites for potential applications as food preservatives

by

Alma Fernanda Sánchez Maldonado

A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in

Food Science and Technology

Department of Agricultural, Food and Nutritional Science

ABSTRACT

Plants synthesize antimicrobial secondary metabolites that might have potential applications in food and pharmaceutical industries. Waste from food processing including peels, seeds, bark and cereal bran contain high amounts of these compounds. Their recovery from food by-products would be benefitial for the food industry, reducing costs and environmental damage. Phenolic compounds and glycoalkaloids are antimicrobial metabolites that coexist in plants of the Solanaceae family. However, in order to be efficiently utilized, their modes of action need to be completely elucidated. Furthermore, their recovery requires new sustainable and economically viable methods. This thesis partially elucidated the interactions between some of these compounds and their antimicrobial mechanisms of action. Moreover, an environmentally friendly method for their recovery from potato by-products was developed.

The investigation of the structure-function relationship of antibacterial phenolic acids showed that their activity is enhanced at lower pH values. The antibacterial activity of hydroxybenzoic acids is more dependent on their hydrophobicity compared to hydroxycinnamic acids. The double bond on the side chain plays an important role on the antibacterial activity of hydroxycinnamic acids. Lactic acid bacteria metabolize phenolic acids likely as a strain-dependent detoxification mechanism.

By studying the antifungal activity of potato secondary metabolites it was concluded that resistance to glycoalkaloids varies among fungal strains. Synergistic activity between phenolic acids and glycoalkaloids was found. The pattern of fungal sterols was related to their phylogenetic classification and to their resistance to potato glycoalkaloids.

A method for recovery and fractionation of phenolic acids and glycoalkaloids from potato peels using water/ethanol solvents was developed. The crude extract contained mainly phenolic

acids (chlorogenic, neochlorogenic and caffeic acids) and glycoalkaloids (α-chaconine and α-solanine). Solid phase fractionation allowed high recovery of glycoalkaloids and phenolic compounds. The phenolic acids fraction was free of toxic glycoalkaloids and, therefore, suitable for food applications. Alkaline hydrolysis of the crude extract followed by fractionation increased about five times the recovery of caffeic acid, enhancing the antimicrobial properties of the phenolic acids fraction.

PREFACE

A version of the Chapter 3 of this thesis has been published as Sánchez-Maldonado, A. F., Schieber, A., & Gänzle, M. G. (2011). Structure-function relationship of the antibacterial activity of phenolic acids and their metabolism by lactic acid bacteria. *Journal of Applied Microbiology, 111*(5), 1176-1184. I was responsible for the data collection and analysis as well as the manuscript composition. Dr. Andreas Schieber contributed to concept formation and manuscript edits. Dr. Michael Gänzle was the supervisory author and was involved with concept formation and manuscript composition.

A version of the Chapter 4of this thesis has been accepted for publication publication in *Food Research International*.The chapter includes experimental work of MSc Elizabeth Mudge, consisting of preliminary trials for the fractionation of phenolic acids and glycoalkaloids, performed under the supervision of Dr. Andreas Schieber.

Dedicada a mi mamá,
una mujer maravillosa

Dedicated to my mother,
a wonderful woman

ACKNOWLEDGEMENTS

During my degree program I went through many experiences that meaningfully changed my life. The most valuable thing is the amazing people I met and the time I shared with them.

First, I want to thank my supervisory committee members Dr. Michael Gänzle, Dr. Andreas Schieber, Dr. Marleny Saldaña, Dr. Stephen Strelkov and Dr. Luke Howard. Michael and Andreas, thank you for your excellent scientific guidance. It has been a privilege to work with you; I profoundly admire both of you. Michael, thank you for your time and willingness to assist me, your commitment to science inspires me. Andreas, thank you for making possible the completion of this work and giving me the opportunity to work in your lab in Bonn. I also thank Dr. Strelkov for allowing me to perform some experiments in his lab (4-60) and the people there for their technical assistance.

I want to acknowledge the University of Alberta (Canada), the Consejo Nacional de Ciencia y Tecnología and the Secretaria de Educación Pública (México), for the funding that allowed me to accomplish my degree.

I want to thank the people at the University of Bonn for their hospitality. Special thanks to Anaheim, Laura and Hannes for their support and gratifying friendship.

I am also very grateful to my colleagues in the lab 2-50, Petya, Chonggang, Yvonne, Yalu, Rigo, Christine,Yvonne, Janu, Peter and everyone else, thanks for the nice moments, help and advices. Thank you, Petya for all the great experiences we shared and for being there for me in good and bad days. Thank you, Chonggang for your friendship and support. Thanks to my students Aree, Kassandra and Ying, who taught me a lot and who made the lab work much more enjoyable.

I also thank my friends Diana and Ingrid for the irreplaceable times in Edmonton. Thank you, Nardhy for your support and advice. I also acknowledge my friends in Mexico, Arlette, Myrna, Deborah and Diana for their solid, fun and honest friendship.

I acknowledge Mikel for his love, support, generosity and for showing me new and amazing facets of life.

Finally, I want to acknowledge all my family. Special thanks to my sister Rosa, a beautiful person, who I deeply admire, and from whom I have been constantly learning since childhood; and to Javier for being always supportive and joyful. I am very grateful to my parents Miguel and Alma, for their love and care. Thank you to my dad, an indefatigable man, for his encouragement to follow my goals with constancy optimism and hard work. I thank my mom for all her time, strength and goodness, which have been essential for this and all my achievements.

TABLE OF CONTENTS

CHAPTER 1. INTRODUCTION. Plant defence mechanisms and enzymatic transformation products and their potential applications in food preservation: Advantages and limitations……. 1

1.1. General characteristics of phytoalexins and hytoanticipins…….. 2

1.2. Phenolic compounds……. 5

1.2.1 Phenolic acids…… 5

1.2.2. Phenolic lipids…….. 8

1.2.3. Prenylated flavonoids……... 9

1.2.4 Tannins.…… 11

1.2.5 Enzymatic conversion of phenolic compounds……. 13

1.3. Glucosinolates……... 16

1.4. Cyanogenic glycosides…….. 17

1.5. Oxylipins……... 18

1.6 Alkaloids……20

1.7 Food applications: Advantages and limitations…….. 22

1.8 Research hypothesis and objectives……... 26

CHAPTER 2. Antifungal activity of potato *(Solanum tuberosum* L.) glycoalkaloids alone and together with phenolic acids: Synergistic activity, membrane fluidity changes, and relationship between antifungal sterols and resistance to α-chaconine…….. 28

2.1 Introduction…… 29

2.2 Materials and methods…… 32

2.2.1 Chemicals…….. 32

2.2.2 Fungal strains and culture conditions…… 32

2.2.3. Determination of minimum inhibitory concentration (MIC)……34

2.2.4. Generalized polarization in fungal membranes…… 34

2.2.5. Saponification and extraction of non-saponifiable lipids……. 35

2.2.6. Thin layer chromatography (TLC)…….. 36

2.2.7. HPLC-MS analysis of sterols……... 36

2.2.8. Statistical analysis……37
2.3. Results……... 38
2.3.1. Minimum inhibitory concentration of potato secondary metabolites against eight fungal strains……..38
2.3.2. Measurement of membrane fluidity by generalized polarization (GP)…… 39
2.3.3. Characterisation and relative quantification of sterols…… 40
2.3.4. Principal component analysis of sterols and MIC relationship…… 43
2.4. Discussion…… 44

CHAPTER 3. Structure–function relationships of the antibacterial activity of phenolic acids and their metabolism by lactic acid bacteria…… 48
3.1. Introduction…….. 49
3.2 Materials and methods…… 51
3.2.1. Chemicals…… 51
3.2.2. Bacterial strains and culture conditions…… 51
3.2.3. Antibacterial activity…… 52
3.2.4. Metabolism of phenolic acids by LAB strains…… 53
3.3. Results……... 54
3.3.1. Effect of pH on the antibacterial activity of phenolic acids…… 54
3.3.2. Structure-function relationship of phenolic acids: Effect of number of hydroxyl (-OH) and methoxy (-OCH_3) groups in the aromatic ring…….. 55
3.3.3. Metabolism of phenolic acids by lactobacilli……... 56
3.3.4. Antimicrobial activity of phenolic acids compared to their metabolites produced by LAB…….. 58
3.4. Discussion…… 59
3.5. References …… 64

CHAPTER 4. Extraction and fractionation of phenolic acids and glycoalkaloids from potato peels using acidified water/ethanol-based solvents…….. 68
4.1. Introduction……... 69
4.2. Materials and methods…….. 72

4.2.1. External standards…… 72
4.2.2. Extraction of potato peels…… 72
4.2.3. Fractionation of phenolic acids and glycoalkaloids by solid-phase extraction……73
4.2.4. Alkaline hydrolysis of chlorogenic acid……... 73
4.2.5. Quantification of phenolic acids and glycoalkaloids…… 74
4.2.6. Quantification of quinic acid…… 77
4.2.7. Statistical Analysis……... 77
4.3. Results……... 77
4.3.1. Extraction of potato peels using three different solvents…… 77
4.3.2. MS identification of phenolic compounds and glycoalkaloids in the extracts……..... 78
4.3.3. Recovery of phenolic acids and glycoalkaloids from potato peels……... 79
4.3.4. Consecutive extractions of bioactive metabolites from potato peels…… 79
4.3.5. Fractionation of phenolic acids and glycoalkaloids by solid-phase extraction 80
4.3.6. Alkaline hydrolysis of the potato peel extract followed by fractionation of phenolic acids and glycoalkaloids…….. 82
4.3.7. Purity of extracts……... 83
4.4. Discussion…….. 84

CHAPTER 5. General discussion and conclusions……... 90
5.1 General Discussion…… 91
5.2 Conclusions……95

APPENDIX……... 97

References…… 107

LIST OF TABLES

Table 1-1. Examples of applications of phytoalexins, phytoanticipins and their enzymatic products as food preservatives……....23
Table 2-1. Taxonomical classification of the fungal strains analized …….. 32
Table 2-2. Strains, incidence or pathogenicity and culture conditions ……. 33
Table 2-3. Generalized polarization of fungal membranes after addition of glycoalkaloids. 40
Table 2-4. Retention times and fragments identified in the extracted sterols ……... 41
Table 3-1. MICs of hydroxybenzoic acids according to number of hydroxyl and methoxy groups in the aromatic ring……. 55
Table 3-2. MICs of hydroxycinnamic acids according to number of hydroxyl and methoxy groups in the aromatic ring……. 56
Table 3-3 Metabolism of phenolic acids by lactic acid bacteria. Shown are the percentage of phenolic acids remaining after incubation and major and metabolites produced during incubation……57
Table 3-4. Antibacterial activity of phenolic acids…… 60

LIST OF FIGURES

Figure 1-1. Basic chemical structures and examples of phenolic compounds……. 6

Figure 1-2. Phytoalexins and phytoanticipins and their enzymatic reactions to produce antimicrobial compounds. ……15

Figure 2-1. MIC of α-chaconine against 8 fungal strains……. 38

Figure 2-2. MIC of α-chaconine at different concentrations of caffeic acid against 4 fungal strains……..……39

Figure 2-3. Normalized amount of sterols from the fungal membrane of different strains, relative to *P. roqueforti* (analyte peak area/IS peak area)……. 42

Figure 2-4. Principal component analysis of the fungal strains related to their amount of normalized sterols from the membrane and their MIC……. 43

Figure 3-1. Hydroxybenzoic and hydroxycinnamic acids and their decarboxylated and reduced derivatives used in this study…… 50

Figure 3-2. MIC of protocatechuic acid, caffeic acid, ferulic acid and *p*-coumaric acid against *L. plantarum* and *L. hammesii* at different pH values……... 54

Figure 3- 3. Comparison of the antibacterial activity of phenolic acids and their metabolites. Effect of the reduction of the double bond hydroxycinnamic acids and effect of decarboxylation……..……... 59

Figure 4-1. Products of alkaline hydrolysis of chlorogenic acid…….. 70

Figure 4-2. Product of hydrolysis of α-chaconine and α-solanine……71

Figure 4-3. Phenolic acids and glycoalkaloids recovered from potato peels using 3 different solvents……... 78

Figure 4-4. Recovery of phenolic acids and glycoalkaloids from potato peels in 3 consecutive extractions with different solvents……80

Figure 4-5. Recovery of phenolic acids and glycoalkaloids before and after separation by solid phase extraction…… 81

Figure 4-6. Phenolic acids and glycoalkaloids recovered after extraction (E), extraction and alkaline hydrolysis (E+H) and extraction, alkaline hydrolysis and fractionation (E+H+F)…… 83

Figure A-1. Chromatogram of the sterols found in *P. roqueforti*, which contains all the sterols present in the strains investigated..…… 98
Figure A-2. Mass spectra of the sterols detected in the fungal membranes; compounds are: A, B, C, Ergosterol, E, F, G, H, I, J, K, L, M, N, O and P. ……98

LIST OF SYMBOLS AND ABBREVIATIONS

PPO	Polyphenol oxidase
LPX	Lipoxygenase
spp.	Species
Da	Daltons
PDA	Potato dextrose agar
PDB	Potato dextrose broth
GP	Generalized polarization
α-C	α-Chaconine
α-S	α-Solanine
S	Solanidine
CA	Caffeic acid
TLC	Thin layer chromatography
HPLC	High-performance liquid chromatography
MS	Mass spectrometry
Q TRAP	Quadrupole ion trap
APCI	Atmospheric pressure chemical ionization
DAD	Diode array detector
UV	Ultraviolet
MRM	Multiple reaction monitoring
GS1	Nebulizing gas
GS2	Heating gas
DP	Declustering potential

EP	Entrance potential
CE	Collision energy
CXP	Collision exit potential
IS	Internal standard
LAURDAN	2-(dimethylamino)-6-dodecanoylnaphthalene
m/z	Mass to charge ratio
PCA	Principal component analysis
LAB	Lactic acid bacteria
MRS	deMan-Rogosa-Sharpe
LB	Luria Berthani Broth
UFLC	Ultrafast liquid chromatography
LC	Liquid chromatography
EMS	Enhanced mass spectrometry
EPI	Enhanced product ion
IDA	Information dependent acquisition
CID	Collision induced dissociation
LOD	Limit of detection
LOQ	Limit of quantitation
FW	Fresh weight

CHAPTER 1

INTRODUCTION

Plant defence mechanisms and enzymatic transformation products and their potential applications in food preservation: Advantages and limitations

CHAPTER 1

1.1. General characteristics of phytoalexins and phytoanticipins

Plants have developed defence mechanisms against phytopathogens and herbivores during their evolution (Dixon 2001). These mechanisms can be inducible and/or constitutive, comprising synthesis of enzymes and biocidal compounds, the hypersensitive response and physical barriers such as lignin and hydroxyproline-rich cell wall proteins (Blée 2002; Dixon and Harrison 1994). Biocidal compounds produced by plants can be classified into phytoalexins and phytoanticipins (VanEtten et al. 1994). Phytoalexins are synthesized *de novo* in response to an elicitor from a microbial pathogen (VanEtten et al. 1994). Phytoanticipins are low molecular weight compounds that exist in the plant before interaction with pathogens or are generated from pre-existing chemicals after infection (VanEtten et al. 1994). Depending on the way of production, a compound may be both a phytoalexin and a phytoanticipin in different parts of the plants or in distinct plants, and they might be also called plant secondary metabolites (VanEtten et al. 1994; Dixon 2001), which are defined as those plant metabolites which are not part of the primary metabolism of the plant (Dixon 2001).

Chemicals synthesized for plant defence are vastly diverse (Dixon 2001). Their synthesis generally operates through the alkaloid, fatty acid, isopropanoid and phenylpropanoid pathways (Mandal et al. 2010). Defence response enzymatic reactions in plants are triggered by signals recognized by the plant such as insect oral secretions and injured plant cells (Howe and Jander 2008).

Antimicrobial phytoalexins and phytoanticipins include the following chemicals and/or their derivatives: phenolic compounds (acids, tannins, flavonoids), glucosinolates, cyanogenic glycosides, fatty acids and alkaloids (Ahuja et al. 2012).

The distribution of phytoalexins and phytoanticipins follow specific patterns at the cell, organ and plant levels. In the cell, compartments separate enzymes and substrates, protecting the plant from toxic reaction products. When compartments are destroyed by injury, enzymes reach the substrates and synthesize the corresponding biocidal compounds. Examples include the enzymatic reactions of glucosinolates, cyanogenic glycosides, phenolic compounds and fatty acids by myrosinase, β-glycosidases, polyphenol oxidase and lipoxygenase, respectively (Johnson 2002; Grubb and Abel 2006; Gruhnert et al. 1994; Thipyapong et al. 2004).

The distribution of defence compounds at the plant organ level is related to the likelihood of attack and the stage of life. In flowers the highest polyphenol oxidase activity occurs during development in anthers and ovaries (Thygesen et al. 1995). Most antimicrobial compounds are enriched in the outer parts of plant organs such as peels and bark (Zangerl and Bazzaz 1993), which is the first barrier for hervibores and microorganisms (Dai and Mumper 2010). Examples are glycoalkaloids, mostly concentrated in the peels of potatoes and tomatoes (Schieber and Aranda Saldaña 2009; Friedman et al. 1998), and phenolic compounds (ferulic acids and resorcinolic lipids), located predominantly in the pericarp of cereals (Dykes and Rooney 2007; Dai and Mumper 2010; Ross et al. 2004), the peels of potatoes (Schieber and Aranda Saldaña 2009; Friedman et al. 1998), and the peels and seeds of mangoes (flavonol and xanthone glycosides, gallotannins and benzophenone derivatives) (Ribeiro et al. 2008). The same pattern is observed for enzymatic activities. An example is polyphenol oxidase, the highest activity of which occurs in the exterior or the tuber (Thygesen et al. 1995).

Allocation of phytoalexins and phytoanticipins between organs and tissues relates to their importance for the plant survival/development, their susceptibility to attack (Zangerl and Bazzaz 1993) and their maturity. The probability of attack is determined by factors such as nutritional

value and availability (Zangerl and Bazzaz 1993). Reproductive parts are considered among the most valuable, due to short reproduction periods and difficulty of replacement. Seeds are reproductive organs and the most susceptible to attack due to their high amounts of energy and nutrients for the development of the embryo (Zangerl and Bazzaz 1993). Therefore, seeds are rich in phytoalexins and phytoanticipins. Examples include glucosinolates in mustard seeds (Brown et al. 2003), cyanogenic glycosides in seedling of sorghum (Bak et al. 2006), tannins in mango kernels (Engels et al. 2009; Ribeiro et al. 2008), proanthocyanidins in grapes seeds (Sivakumaran et al. 2004; Mayer et al. 2008), phenolic compounds in seeds of longan, avocado, tomato, and jack fruit (Balasundram et al. 2006), and long chain fatty acids in plant seeds (Jäger et al. 2002). Younger plants often have high amounts of plant defence compounds. Examples include high amounts of glucosinolates in young sprouts of cauliflower and broccoli (Fahey et al. 1997) and the tannins in younger fruits, which increases the astringency and deters herbivores (Santos-Buelga and Scalbert 2000). Plant defence enzymatic activity likely follows the same distribution arrangements, as observed in lipoxygenases in grain legume seeds (Baysala and Demirdoven 2007) and polyphenol oxidase, which is found in high levels in stolons, roots, flowers and developing tubers, but in low levels in stems and leaves (Thygesen et al. 1995). The presence of these deterrent compounds in certain plant parts reduces their attractiveness to animals and humans.

Processing of food for human consumption generally removes outer layers of the plant, which are rich in phytoalexins and phytoanticipins. By-products from food processing include peels, seeds, bark and cereals bran with high concentrations of natural antimicrobial compounds that can be used as food preservatives. Exceptions are cyanogenic glycosides and alkaloids due to their toxicity for humans. The recovery of antimicrobial compounds from industrial food

waste encompasses numerous profitable and sustainable advantages. Essential oils are also plant antimicrobial compounds. However, they have been recently reviewed with respect to their bioactivity and potential for food preservation (Bakkali et al. 2008; Burt et al. 2004; Holley and Patel 2005). Hence, they were not included in this treatise. The objective of this review is to outline the properties of phytoalexins and phytoanticipins, their occurrence in plants and their enzymatic products as alternatives for food preservation.

1.2. Phenolic compounds

Phenolic compounds are chemically diverse compounds ubiquitous in higher plants with antimicrobial, antioxidant and sensory properties (Dai and Mumper 2010). They can be classified into phenolic acids (C_6 -C_1, C_6 –C_3 backbones), flavonoids (C_6 –C_3 –C_6 backbones), xanthones (C_6 –C_1 –C_6), stilbenes (C_6 –C_2 –C_6), phenolic lipids and tannins (Schieber and Aranda Saldaña 2009; Engels 2012). They are generally present in edible fruits and vegetables; and consequently, in food processing byproducts (Balasundram et al. 2006).

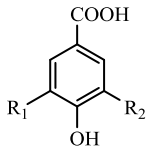
1.2.1. Phenolic acids

Phenolic acids are the simplest phenolic compounds synthesized through the phenylpropanoid pathway via shikimic acid (Mandal et al. 2010). They are classified according to their structure into hydroxybenzoic and hydroxycinnamic acids (Schieber and Aranda Saldaña 2009) (**Fig. 1-1a**).

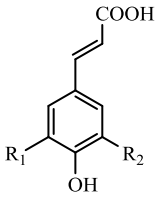
Most edible plants contain small amounts of free hydroxybenzoic and hydroxycinnamic acids; exceptions are radish, onions and green tea which are rich in free hydroxybenzoic acids (Manach et al. 2004). Hydroxybenzoic acids are commonly found in bigger molecules including hydrolysable tannins linked by ester bonds (Manach et al. 2004).

CHEMICAL STRUCTURES OF PHENOLIC COMPOUNDS | **EXAMPLES**

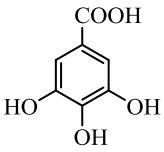
(a)



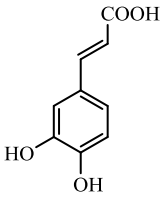
Hydroxybenzoic acids



Hydroxycinnamic acids

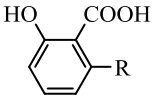


Gallic acid

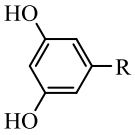


Caffeic acid

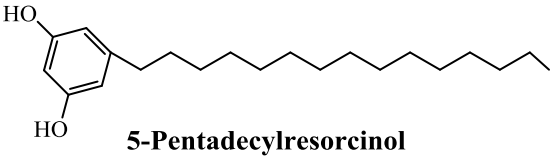
(b)



Anacardic acids

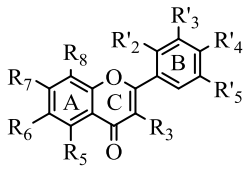


Alkylresorcinols

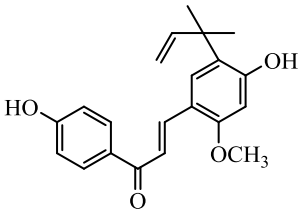


5-Pentadecylresorcinol

(c)

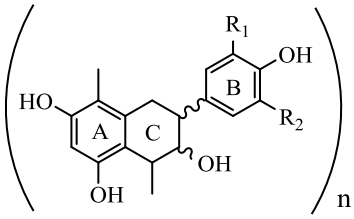


Prenylated flavonoids

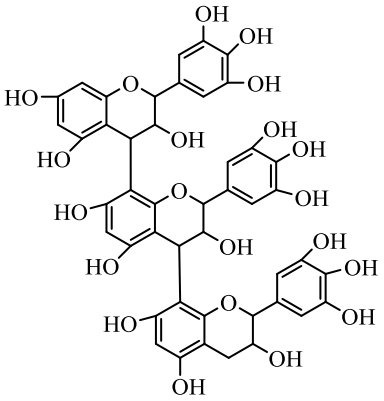


Licochalcone A

(d)

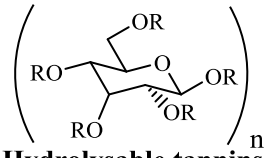


Proanthocyanidins/condensed tannins

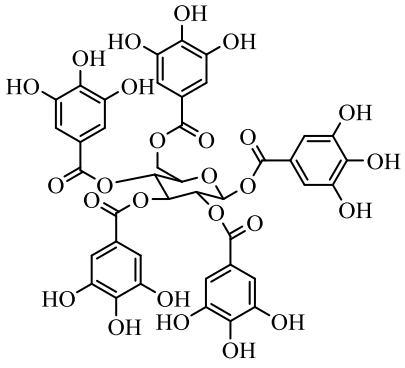


Prodelphinidin C2

(e)



Hydrolysable tannins



Penta-*O*-galloylglucose

Figure 1-1. Basic chemical structures and examples of phenolic compounds.

(a) Phenolic acids, Hydroxycinnamic and hydroxybenzoic acids, R=-H, -OH or $–OCH_3$. Examples are the hydroxybenzoic acid gallic acid found in green tea in the free and esterified form as gallotannins, and the hydroxycinnamic acid caffeic acid, found in potatoes esterified to quinic acid as chlorogenic acid.
(b) Phenolic lipids, R= Saturated/unsaturated alkyl-chain. The example is 5-pentadecyl resorcinol, found in rye.
(c) Prenylated flavonoids, R_6, R_8, R_3'and R_5'= one or more prenyl groups; R_2', R_4', R_3, R_5 and R=-H, -OH, other functional groups. The example is licochalcone A, found in *Glycyrrhiza inflata.*
(d) Proanthocyanidins, R_1, R_2=-H, propelargonidins; R_1=-H, R2=-OH, procyanidins; R_1, R_2=-OH, prodelphinidins. The example is prodelphinidin C2, found in barley.
(e) Hydrolysable tannins, R=Gallic acid, gallotannins; R=Ellagic acid, ellagitannins. The example is penta-*O*-galloyl glucose, found in mango kernels.

Hydroxycinnamic acids are found as esters of tartaric, shikimic and quinic acid or as glycosylated derivatives, formed mainly by units of *p*-coumaric, caffeic, ferulic and sinapic acids (Manach et al. 2004). They are found as monoesters, (caffeoylquinic acid or chlorogenic acid, *p*-coumaroyl quinic acid and feruloylquinic acid), di-esters, tri-esters and the unique tetra ester of caffeic acid or diesters that are mixtures of caffeic and ferulic acids (Clifford 1999). Caffeic acid is the most common phenolic acid in fruits and vegetables, which usually occurs esterified to quinic acid as chlorogenic acid, whereas ferulic acid is abundantly found in cereals esterified to hemicellulose in the cell wall (Dai and Mumper 2010).

Cueva et al. (2010) found strong antimicrobial activity of 13 phenolic acids against *Escherichia coli, Lactobacillus* spp., and *Staphylococcus aureus*. Antilisterial activity was demonstrated for hydroxycinnamic acids (Wen et al. 2003; Ramos-Nino et al. 1996) and hydroxybenzoic acids (Ramos-Nino et al. 1996). In addition, phenolic acids have shown lower antimicrobial effect against lactic acid bacteria, compared to other strains (Lee et al. 2006). The antibacterial activity of phenolic acids against lactobacilli is strain dependent (Cueva et al. 2010).

The antimicrobial mode of action of phenolic acids is not completely understood. It has been suggested that phenolic acids use their undissociated form to penetrate bacterial membranes

at pH below their pKa values (Ramos-Nino et al. 1996). Once inside the cell, acidification of the cytoplasm might interfere in the synthesis of ATP (Vattem et al. 2005). The number and position of the antimicrobial substitutes in the aromatic ring and the chain length play a role in their activity (Cueva et al. 2010).

1.2.2. Phenolic lipids

Phenolic lipids encompass a wide variety of compounds found in plants, algae, fungi, bacteria and animals (Kozubek and Tyman 1999). They are synthetized through the polyacetate pathway (Kozubek et al. 2001). In higher plants, phenolic lipids exist mainly in the Anacardiaceae (cashew, mango), Ginkgoaceae (*Ginkgo biloba*) and Gramineae (cereals) families (Kozubek et al. 2001). They are amphiphilic due to their hydrophilic phenolic moiety and their aliphatic chain (Stasiuk and Kozubek 2010) (**Fig. 1-1b**).

Alkylresorcinols, also known as resorcinolic lipids, are the most extensively studied phenolic lipids (Stasiuk and Kozubek 2010) due to their wide distribution among kingdoms (Kozubek et al. 2001). The bran of wheat and rye contain the highest concentrations of these compounds; less amounts are present in barley and triticale (Ross et al. 2004). Anacardic acids, existing in *Anacardium occidentale* (cashew nut) and *Ozoroa mucronata* have been also widely studied (Kubo et al. 2003). Alkylresorcinols are likely involved in the regulation of host pathogen relationships (Kozubek et al. 2001).They are synthesized during normal development of plants and as a response to stress factors such as UV light, wound and infection (Stasiuk and Kozubek 2010). They have antibacterial and antifungal properties. A fraction of alkylresorcinols from *Hordeum vulgare* showed antifungal activity against *Aspergillus niger* and *Penicillium crysogenum* (Garcia et al. 1997). Hexyl resorcinol inhibited growth of *Aspergillus parasiticus, Aspergillus versicolor, Penicillium chrysogenum,* and *Penicillium roqueforti* (Reiss 1989) and

reduced the number of live bacterial aggregates of *Streptococcus mutans* and *Actinomyces viscosus* after 24 hours of exposure (Kraal et al. 1979). An alkylresorcinol isolated from etiolated rice seeds completely inhibited the rice blast fungus *Pyricularia oryzae* (Suzuki et al. 1996). Anacardic acids and alkylresorcinols isolated from *Anacardium occidentale* nut shell oil showed strong antimicrobial activity only against Gram positive bacteria, when tested against *Bacillus subtilis, Brevibacterium ammoniagenes, Staphylococcus aureus* and *Streptococcus mutans* (Himejima and Kubo 1991). A mixture of anacardic acids was bactericidal against *Staphylococcus aureus* strains (Kubo et al. 2003).

The antimicrobial activity of phenolic lipids is mainly attributed to their amphiphilic properties. Higher degree of insaturations and a longer alkyl chain of phenolic lipids enhanced activity against Gram positive bacteria (Kubo et al. 2003; Himejima and Kubo 1991).

Removal of the carboxylic group, and subtraction or substitution of hydroxyl groups by methoxy groups in the benzene ring, decreased the antibacterial activity of phenolic lipids (Himejima and Kubo 1991).

1.2.3. Prenylated flavonoids

Flavonoids are the main phenolic compounds in animals' diet and are present in a wide range of vegetables such as berries, grapefruit, onion, apple, and broccoli (Dai and Mumper 2010) as well as tea and wine (Cushnie and Lamb 2005). In the plant, these compounds are synthesized via the shikimic acid/phenylpropanoid pathways (Cesco et al. 2012). The flavonoid structure consists of an elementary flavan nucleus formed by three arranged rings (C6-C3-C6) called A, B and C, comprising 15 carbon atoms (Dai and Mumper 2010). Their chemical arrangements differ in the hydroxylation, methoxylation, prenylation and glycosylation degrees and patterns (Dai and Mumper 2010). Cushnie and Lamb (2005) and Cushnie and Lamb (2011)

reviewed the most important aspects of antimicrobial flavonoids. Therefore, in this review we will describe only prenylated flavonoids, with a more specific approach.

Prenylated flavonoids are distinctive for having a prenyl chain, which refers to the 3,3-dimethylallyl substituent (3,3-DMA), a geranyl (*E*-3,7-dimethyl-2-isoprophenyl-hex-4-enyl), a 1,1-dimethylallyl and/or a lavandulyl (5-methyl-2-isoprophenyl-hex-4-enyl) and their oxidated and hydroxylated derivatives group, bound to the flavonoid backbone (Chen et al. 2014) (**Fig. 1-1c**). Most of them are C-prenylated but they exist in the O-prenylated form as well (Barron and Ibrahim 1996). The antibacterial and antifungal activities of prenylated flavonoids from several plant extracts such as *Eysenhardtia texana, Eriosema chinense* and *Glycyrrhiza inflata* have been demonstrated (Wätcher et al. 1999). Only Gram positive bacteria are susceptible to prenylated flavonoids. They were active against *Mycobacterium tuberculosis* (Sutthivaiyakit et al. 2009), *Bacillus cereus, Pseudomonas aeruginosa* (Pereira Ávila et al. 2008) and several strains of *Staphylococcus aureus* (Dzoyem et al. 2013). Licochalcone C and licochalcone A were active against food spoilage and pathogenic bacteria, including *Bacillus cereus, Bacillus subtilis,* and *Clostridium sporogenes* (Tsukiyama et al. 2002; Haraguchi et al. 1998).

The hydrophobic prenyl and/or geranyl moiety has been suggested as a key factor for penetration of prenylated flavonoids through the cell membrane (Tsukiyama et al. 2002; Haraguchi et al. 1998; Pereira Ávila et al. 2008). Mukne et al. (2001) reported a relationship between the prenyl and hydroxyl moieties in specific positions and the antibacterial activity of phenolic lipids. In contrast, Dzoyem et al. (2001) did not find any structure-activity relationship when comparing three prenylated flavonoids against one non prenylated flavonoid. Pereira Ávila et al. (2008) suggested that a hydroxyl group in position C-4 and an oxygenated substitution in position C-40 were important for the antimicrobial activity of prenylated flavonoids; while a

hydroxyl group in position C-20 seemed to confer stability to the molecule. Inhibition of biosynthetic pathways caused by loss of membrane potential has also been suggested as mode of antimicrobial action (Dzoyem et al. 2013).

1.2.4. Tannins

Tannins are phenolic compounds with a high molecular weight, usually from 500 to 3000 Da (Hassanpour et al. 2001). They are responsible for the astringency of fruits, legume seeds, cereal grains, wine, tea, cocoa and cider, due to their ability to precipitate proteins, including those present in the saliva (Santos-Buelga and Scalbert 2000). Tannins accumulate in almost any plant organ or tissue including bark, wood, fruits, root and leaves (Scalbert 1991). Algae also contain tannins called phlorotannins, which are highly hydrophilic polymers of phloroglucinol with biological activities (Li et al. 2011). Tannins are microorganism deterrents and confer protection to vital tissues against deterioration by external predators (Scalbert 1991). In fruits, the astringency and therefore the amount of tannins diminishes with maturity of the fruits which protects them from herbivores during early stages of their development (Santos-Buelga and Scalbert 2000). Tannins are structurally classified into proanthocyanidins and hydrolysable tannins (Scalbert 1991; Trentin et al. 2013).

Proanthocyanidins. These compounds are also called condensed tannins and are the most abundant phenolic compounds after lignin (Rasmussen et al. 2005). The most common proanthocyanidins in food are procyanidins and prodelphinidins (Santos-Buelga and Scalbert 2000) (**Fig. 1-1d**).

Proanthocyanidin fractions extracted from grape seeds consisting of catechin and epicatechin units had antibacterial activity against 10 pathogenic strains (Mayer et al. 2008). Proanthocyanidin fractions from *Dorycnium rectum* inhibited growth of Gram positive rumen

bacteria (Sivakumaran et al. 2004). Purified proanthocyanidins from *Quercus ilex* L. were antibacterial against human bacterial strains including *Salmonella enterica* and *Listeria monocytogenes* (Karioti et al. 2011). The antifungal activity of proanthocyanidins has been documented as well (Karioti et al. 2011; Anttila et al. 2013; Patel et al. 2011)

Studies regarding the mode of antibacterial action are scarce. Proanthocyanidins with low (≈ 3000 Da) and medium (≈ 12000 Da) molecular weights were more active than those with high (≈ 36000 Da) molecular weight against three Gram positive rumen bacteria strains ((Sivakumaran et al. 2004). The activity of double-linked proanthocyanidins against *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Neisseria gonorrhea* was enhanced with increasing units of flavanol (De Bruyne et al. 1999; Balde et al. 1991). The inhibition of tuberculosis bacilli by proanthocyanidins was related to monocyte iodination and interleukin-1 production (Sakagami et al. 1992).

Hydrolysable tannins. These compounds are phenolic polyesters that include gallotannins and ellagitannins (**Fig. 1-1e**) (Serrano et al. 2009). They showed antimicrobial activity against *Helicobacter pylori* (Funatogawa et al. 2004). Gallotannins from *Rhus trichocarpa* Miquel, *Galla chinensis* and *Mangifera indica* L. were active against several Gram positive and Gram negative pathogenic bacteria (Cho et al. 2010; Engels et al. 2011; Tian et al. 2009). Hydrolysable tannins failed to inhibit growth of lactic acid bacteria (Engels et al. 2011) and fungi (Tian et al. 2009).

The antimicrobial mode of action of hydrolysable tannins has been related to their high affinity to iron (Cho et al. 2010), likely due to their structural similarities to bacterial phenolic siderophores, forcing bacteria to compete for iron in order to survive (Engels et al. 2010; Engels et al. 2011; Cho et al. 2010). Hydrolysable tannins were able to damage the lipid bilayer of

Helicobacter pylori cells (Funatogawa et al. 2004). They also inactivated membrane proteins. Gallotannins interacted with respiratory enzymes of *B. subtilis* and protein transporters (Engels et al. 2011). Higher molecular weight and less polarity increased the antimicrobial activity of gallotannin extracts (Tian et al. 2009). The degree of galloylation does not affect the antimicrobial activity of hydrolysable tannins (Engels et al. 2011). According to (Cho et al. 2010), the gallate moiety in penta-*O*-galloylglucose accounts only for the antimicrobial activity against Gram positive bacteria. An attached aliphatic moiety such as in propyl gallate, confers antimicrobial activity against both Gram positive and Gram negative bacteria. Substitution of hydroxyl groups by methoxyl groups in the bezene ring inactivates the molecule.

1.2.5. Enzymatic conversion of phenolic compounds

Hydrolysis of phenolic acids by esterases. Since phenolic acids are mostly esterified to other molecules (Dai and Mumper 2010; Ishii 1997; Clifford 1999; Manach et al. 2004), hydrolysis is required for their recovery in the free form. Hydroxybenzoic acids from hydrolysable tannins can be recovered by chemical acidification (Serrano et al. 2009). However, the recovery of free hydroxybenzoic acids is not usually pursued, since hydrolysable tannins are also bioactive in their esterified form. In contrast, the antimicrobial activity of hydroxycinnamic acids has been studied mainly in their free form; therefore, different methods for hydrolysis of their esterified forms have been tested (Benoit et al. 2006). Acid and alkaline hydrolyses are usually used for extraction of bond hydroxycinnamic (Sun et al. 2001). In addition, enzymatic hydrolysis of hydroxycinnamic acids can be achieved using feruloyl esterases (cinnamoyl esterases or cinnamic acid hydrolases), from several microorganisms, malt and barley (Mathew and Abraham 2004; Crepin et al. 2004). They hydrolyse the ester bonds in the cell wall (Mathew and Abraham 2004; Crepin et al. 2004) and can work synergistically with other microbial

enzymes to recover free hydroxycinnamic acids (Faulds et al. 2006; Mathew and Abraham 2004).

Polyphenol oxidase activity. Phenolic compounds are susceptible to polyphenol oxidase activity, also called catechol oxidase or tyrosinase that causes enzymatic browning in fruits, vegetables and derivatives. This changes their nutritional and sensory characteristics and affects quality, shelf life and consumer acceptance (Yoruk and Marshall 2006). Polyphenol oxidases are type 3-copper containing oxidases, present in monomeric, dimeric and tetrameric forms (Dicko et al. 2006). Polyphenol oxidases exist in the thylakoids of chloroplasts, vesicles or other compartments in non-green plastids (Vaughn et al. 1988). They remain latent until released by physical damage (Thipyapong et al. 2004). Then, they catalyse enzymatic browning to produce quinones, followed by non-enzymatic polymerization to generate melanin pigments (**Fig. 1-2a**) (Yoruk and Marshall 2006).

Several studies propose a relationship between polyphenol oxidase and plant defence mechanisms. The amount and activity of polyphenol oxidase is higher in young fruits and leaves and decreases with maturity (Yoruk and Marshall 2006). Its enzymatic activity is induced by wounding and herbivore attack (Mayer 2006). Polyphenol oxidase likely triggers reactions for production of reactive oxygen species (ROS) (Thipyapong et al. 2004). In addition polyphenol oxidase produces reactive *o*-quinones in the mouth of herbivores, which bind to free amino and thiol groups, diminishing the nutritional value of dietary proteins (Constabel et al. 2000). The resistance of tomato plants to the pathogen *Pseudomonas syringae* decreased after inserting an antisense polyphenol oxidase cDNA (Thipyapong et al. 2004). In addition, the resistance of tomatoes to the same pathogen was augmented by polyphenol oxidase overexpression (Li et al.

2010). Raj et al. (2006) observed higher polyphenol oxidase expression in pearl millet resistant to *Sclerospora graminicola* (Sacc.) Schroet. specifically, after inoculation with the pathogen.

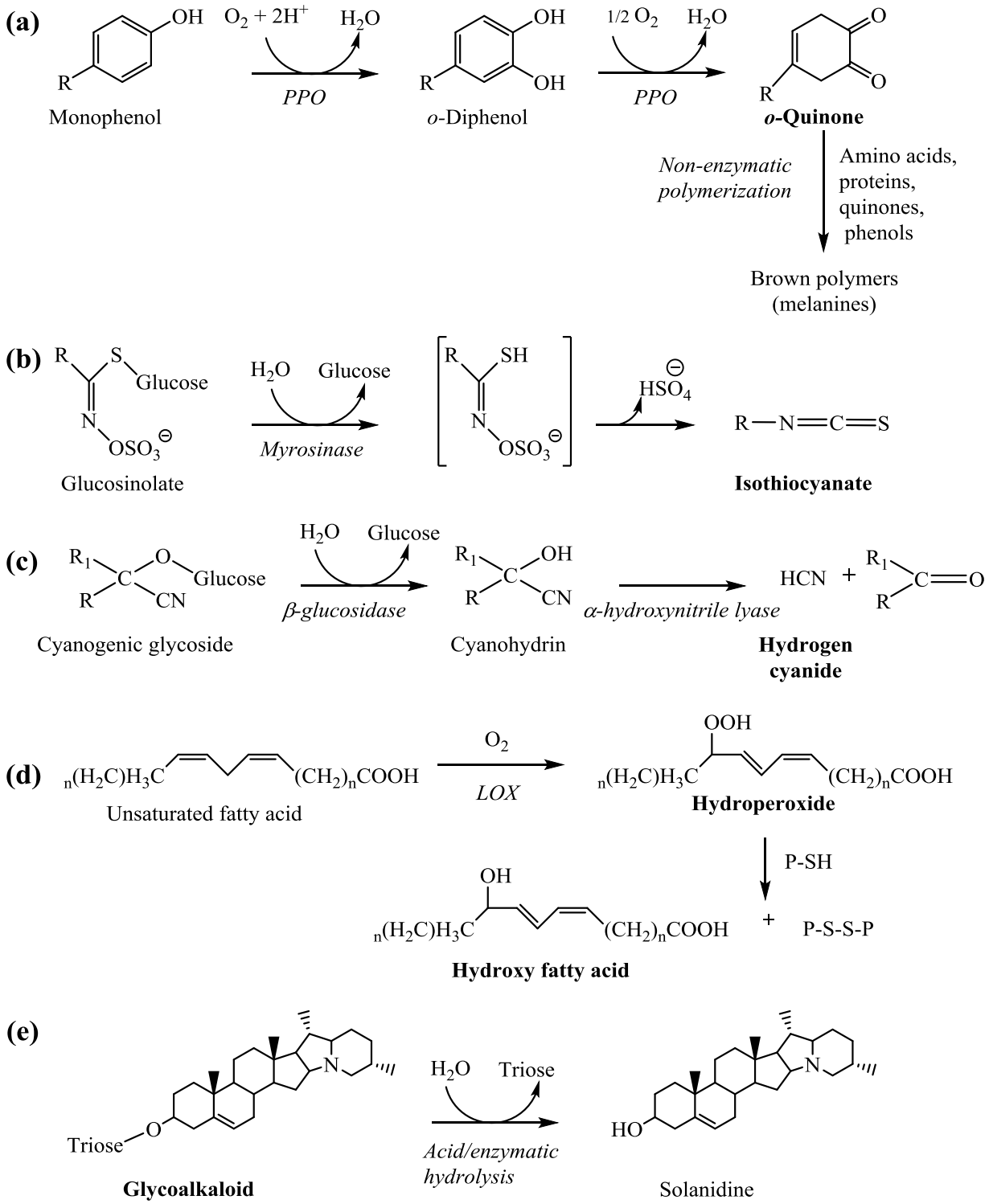


Figure 1-2. Phytoalexins and phytoanticipins and their enzymatic reactions to produce antimicrobial compounds. Compounds with antimicrobial activity are printed in bold.

- **(a)** Oxidation of phenolic compounds into **quinones** by the enzyme polyphenol oxidase (PPO) and subsequent non-enzymatic polymerization into brown polymers (melanines). Polyphenol oxidase is responsible for the enzymatic browning of plant tissue upon injury.
- **(b)** Hydrolysis of glucosinolates into **isothiocyanate** by the enzyme myrosinase. Hydrolysis is dependent on tissue injury destroying the separation between myrosinase and glucosinolates. Compounds with antimicrobial activity include allylisothiocyanate (R= CH_2=CH_2-CH_3) from mustard.
- **(c)** Hydrolysis of cyanogenic glycosides into cyanohydrin by the enzyme β-glucosidase and subsequent production of **hydrogen cyanide** and a ketone (R_1=alkyl chain) or an aldehyde (R_1=H) by the enzyme α- hydroxynitrile lyase. Conversion of cyanogenic glycosides is dependent on tissue injury destroying the separation between enzymes and glycosides. Examples include amygladin in bitter almonds and apple seeds which is converted to benzaldehyde, hydrogen cyanide, and two molecules of glucose.
- **(d)** Oxidation of unsaturated fatty acids into **hydroperoxides** by the enzyme lipoxygenase (LOX). Hydroxyperoxides of fatty acids are highly reactive molecules which are chemically converted to oxylipins, volatile aldehydes, or reduced by thiol groups from plants to produce **hydroxyfatty acids**.
- **(e)** Hydrolysis of **glycoalkaloids** to release the alkaloid solanidine. Different from other examples shown in this figure, the substrate of the conversion has antifungal activity; hydrolysis in acidic conditions or by microbial enzymes reduces the antifungal activity.

1.3. Glucosinolates

Glucosinolates are secondary metabolites synthesized mainly by plants within the Brassicaceae, Capparaceae and Caricaceae families (Fahey et al. 2001). They are synthesized from tryptophan, tyrosine, phenylalanine, isoleucine, leucine, valine, alanine and methionine (Grubb and Abel 2006).

Synthesis of glucosinolates is influenced by factors such as the presence of pathogens, wounding and soil fertility (Fahey et al. 2001). These compounds are mainly found in seeds (Brown et al. 2003). Their amount varies depending on the plant organs and stage of development (Bellostas et al. 2007; Brown et al. 2003; Fahey et al. 2001).Young sprouts of broccoli and cauliflower had different kinds of glucosinolates and their concentrations were 10 to 100 fold higher compared to mature plants (Fahey et al. 1997).

When the plant is physically injured, glucosinolates are released from vacuoles and hydrolysed by the enzyme myrosinase, located in the myrosin cells, to produce isothiocyanates as final products (Johnson 2002; Grubb and Abel 2006) (**Fig. 1-2b**). Most glucosinolate breakdown products have biocidal activities (Grubb and Abel 2006). Glucosinolates production increases after insects attack (Halkier and Gershenzon 2006). The insecticidal activity of glucosinolate enzymatic derivatives (allyl isothiocyanate, allyl isocyanate and allyl cyanide) against common pests makes them suitable as biodegradable natural pesticides (Wu et al. 2009).

The antibacterial activity of allyl isothiocyanate has been related to physical cell damage and inhibition of metabolic pathways (Luciano and Holley 2009). Allyl isothiocyanate decreased viable cells of S*almonella* spp., *Escherichia coli* O157:H7 and *Listeria monocytogenes* Scott A, causing metabolite leakage and increased β-galactosidase activity (Lin et al. 2000). Isothiocyanates are highly reactive species likely to perform nucleophilic reactions with oxygen, sulfur and nitrogen (Zhang and Talalay 1994). The mode of action of isothiocyanates was suggested to include the inactivation of thioredoxin reductase and acetate kinase in *E. coli.* (Luciano and Holley 2009; Luciano et al. 2008).

1.4. Cyanogenic glycosides

Cyanogenic glycosides are compounds biosynthetically related to glucosinolates. Plants contain either one or the other, with very rare exceptions (Olafsdottir et al. 2002). They are β-glycosides of α-hydroxynitriles derived from amino acids, synthesized by about 130 families of pteridophytes, gymnosperms and angiosperms (Bak et al. 2006). Ferns and gymnosperms have aromatic cyanogenic glycosides derived from tyrosine or phenylalanine. Angiosperms contain aliphatic and aromatic cyanogenic glycosides derived from valine/leucine or tyrosine/phenylalanine, respectively (Bak et al. 2006). These compounds are most likely present

in the vacuoles of the plant cell, separated from their hydrolytic enzymes (Gruhnert et al. 1994). After tissue injury, cyanogenic glycosides are released and hydrolysed by β-glucosidases and α-hydroxynitrile lyase, producing toxic hydrogen cyanide (**Fig. 1-2c**) (Bak et al. 2006), that binds to haemoglobin, leading to respiration inhibition (Gleadow and Woodrow 2002; Osbourn 1996; Scherer 2006). Due to their toxicity, cyanogenic glycosides are not suitable for food applications.

1.5. Oxylipins

Fatty acids are compounds ubiquitous in nature that function as structural units, energy storage and signals in metabolism pathways (Pohl et al. 2011). Long chain fatty acids such as linoleic, α-linolenic and γ-linolenic acids are frequently extracted from selected seed plants (Gill and Valivety 1997). The antifungal (Pohl et al. 2011) and antibacterial (Desbois and Smith 2010) activities of fatty acids, including their action mechanism has been recently reviewed. Therefore, this paper will focus on the antimicrobial activity of enzymatic oxidation products of fatty acids resulting from plant defence mechanisms.

Plant signalling upon environmental stimuli relies on fatty acids and their derivatives as chemical signals (Feussner and Wasternack 2002). Among the most important reactions for lipid modifications under abiotic and biotic stress is their oxidation into polyenoic fatty acids, also called oxylipins (Feussner and Wasternack 2002). Oxylipins include hydroperoxides, hydroxyl fatty acids, ketotrienes, ketodienes and epoxides usually synthesized by oxidation of polyunsaturated fatty acids (Prost et al. 2005). They significantly contribute to plant defence as signal molecules in wound-healing and protective compounds as cutin components (Blée 2002). Oxylipins have been related to stimulation of plant defence gene expression, regulation of plant cell death and antimicrobial activity (Prost et al. 2005).

In higher plants lipid oxidation of long chain fatty acids is carried out mainly by dioxygenases and lipoxygenases (Brash 1999). Lipoxygenases are non-heme iron containing molecules generally located in the cytosol and vacuoles of plant tissues and organs (Feussner and Wasternack 2002). They are abundant in grain legume seeds (Baysala and Demirdoven 2007). At least 3 reactions are catalysed by lipoxygenases, including oxygenation of substrates, secondary transformation of hydroperoxides and formation of epoxyleukotrienes (Andreau and Feussner 2009) (**Fig. 1-2d**). Lipoxygenases can be regiospecific enzymes, but the structural bases of this behaviour are unclear (Hornung et al. 2008). Plant lipoxygenases have mainly 9- or 13-regiospecificity.

Oxylipins showed cytotoxicity against bacteria, algae, fungi, echinoderms, molluscs and crustaceans, suggesting that their mode of action might be related to their chemical reactivity towards nucleophilic molecules, which is not specific (Adolph et al. 2004).

In some plant and food systems, lipid hydroperoxides produced by the oxidation of unsaturated fatty acids can be reduced to antifungal hydroxy fatty acids by reacting with thiol groups from glutathione (GSH), homocysteine and cysteine (**Fig. 1-2d)** (Terao et al. 1993). Trihydroxy fatty acids generated in tubers from linoleic acid have demonstrated antifungal activities against potato pathogens such as *Ceratocystis fimbriata* (Masui et al. 1989). Several natural plant oxylipins showed inhibitory effects against phytopathogens of the Brassicaceae family, including *Alternaria brassicae, Leptosphaeria maculans, Sclerotinia sclerotiorum* and *Verticillium longisporum* (Sjögren et al. 2003). Prost et al. (2005) reported that 26 out of 43 naturally occurring oxylipins were active against 3 or more out of 13 microbial pathogens, including bacteria, fungi and oomycetes. Only two oxylipins were completely inactive and eucaryotic microorganisms were particularly sensitive.

The mode of antimicrobial action of oxylipins and their function in the plant remain unclear. Their antimicrobial activity is strain dependent (Sjögren et al. 2003). In fungi, oxylipins do not affect the viability of the cells but delay mycelial growth (Prost et al. 2005). Their antimicrobial effect is likely related to their chemical or physical properties, rather than interaction with cell constituents (Prost et al. 2005). Compounds belonging to all stages of the oxylipin synthesis pathway have activities, suggesting that synthesis of antimicrobial compounds and plant signalling are combined (Prost et al. 2005). The effect of unsaturated aldehydes varies among organisms and interferes with key physiological cell processes, including cell proliferation and cell division, among others (Sjögren et al. 2003).

The mode of action of hydroxy fatty acids might be analogous to that of weak acids, which involves penetration through membrane bilayers, increases in membrane permeability and release of electrolytes and proteins (Sjögren et al. 2003; Pohl et al. 2011; Avis and Belanger 2001). The antifungal activity of the hydroxyl fatty acids likely relies on the hydroxyl group and at least one double bond. Their position and configuration also play a role (Black et al. 2013).

1.6 Alkaloids

Alkaloids are biocidal compounds synthesized by plants as part of their defence mechanism that contain nitrogen and are generally formed by ring structures, including pyridines, pyrroles, indoles, pyrrolidines, isoquinolines and piperidines (Bennett and Wallsgrove 1994). They have been used as phylogenetic classifiers due to their specificity in certain families and taxa (Bennett and Wallsgrove 1994). Steroidal alkaloids are typical in the Buxaceae, Liliaceae, Apocynaceae and Solanaceae families (Bennett and Wallsgrove 1994); (Wink 2003). They are (Bennett and Wallsgrove 1994).

The distribution of alkaloids in the plant organs differs between species and life stage. In tomatoes and potatoes, alkaloids are produced in any part of the plant (leaves, roots, tubers, and sprouts) (Friedman 2006; Friedman 2002). Their synthesis in potatoes begins in the germination period and reaches the maximum level during flowering. Unripe fruits and flowers have the highest amounts (Friedman 2006).

Alkaloids deter herbivores including mammals and insects (Phillips et al. 1996; Bennett and Wallsgrove 1994; Zúñiga and Corcuera 1986). They are toxic to some bacteria (Bennett and Wallsgrove 1994) and several fungi (Fewell et al. 1994; Fewell and Roddick 1993; Fewell and Roddick 1997).

Plants from the Solanaceae family synthesize membrane active antifungal alkaloids with a sugar moiety called glycoalkaloids (Keukens et al. 1992; Keukens et al. 1995; Roddick and Rijnenberg 1987; Blankemeyer et al. 1992). Membrane disruption was suggested to start with insertion of the alkaloid moiety in the bilayer, followed by interactions between the sugar moieties, formation of a sterol/alkaloid matrix, chemical rearrangement and lysis of the cell (Keukens et al. 1995).

Bacteria and fungi such as *Sphingomonas* sp., *Bacteroides* JY-6, *Pseudomonas paucimobilis*, *Clostridium stercorarium*, *Bacillus* sp., *Penicillium*, *Aspergillus*, *Mucor* and *Fusarium* detoxify glycoalkaloids by producing enzymes that hydrolyse their sugar moieties (Monti et al. 2004) (**Fig. 1-2e**).

Since some alkaloids are toxic to humans (Phillips et al. 1996), not all of them can be used for food applications. However, some toxic alkaloids have pharmaceutical applications, an example are glycoalkaloids,present in plants of the Solanaceae family can be utilized for

synthesis of steroidal hormones (Nikolic and Stankovic 2003). Their recovery can be done by acid hydrolysis (Friedman et al. 1998) or with microbial enzymes (Monti et al. 2004) (**Fig. 1-2e**).

1.7 Food applications: Advantages and limitations

Despite the antimicrobial activity of phytoalexins and phytoanticipins, there are relatively few studies evaluating the suitability of phytoalexins and phytoanticipins as preservatives in actual food systems (**Table 1**). Their stability, interactions and functional properties in food matrices and during food processing should be considered in order to improve food safety and quality.

The pH is an important factor that affects food properties and antimicrobial activity. Compounds that are more active in low pH matrices are phenolic acids (optimum pH = 4-5) (Ramos-Nino et al. 1996; Wen et al. 2003), prenylated flavonoids *(Tsukiyama* et al. *2002)* and allyl isothiocyanate (Zhang and Talalay 1994).

In addition to their antimicrobial activity, phenolic compounds have sensory and antioxidant properties, which has an influence on the characteristics of food products. However, some limitations should be taken into account. In the production of fermented products, metabolic transformations should be considered when selecting starter cultures (Teixeira et al. 2014), since lactic acid bacteria metabolize some antimicrobial phenolic acids during fementation (Svensson et al. 2010). Moreover, prenylated flavonoids are effective only against Gram positive bacteria (Himejima and Kubo 1991).

Table 1-1. Examples of applications of phytoalexins, phytoanticipins and their enzymatic products as food preservatives.

	FOOD APPLICATION	Ref.
PHENOLIC OMPOUNDS		
Pure compounds		
Caffeic acid	**Cellulose packaging (laccase-mediated grafting):** antimicrobial against *Staphylococcus aureus, Escherichia coli*	(1)
Caffeic acid	**Apple juice:** Inhibition of Shiga toxin-producing *E. coli* serotype O157:H7 (0.2 to 1g/L)	(2)
Oleuropein	**Reconstituted milk:** Inhibition of growth and enterotoxin production by *S. aureus* S-6	(3)
Carvacrol	**Biofilm for food packing:** Methyl cellulose/carvacrol films and methyl cellulose/carvacrol/montmorillonite nanocomposite films reduced *E. coli* and *S. aureus* counts by 0.9 and 0.7 log CFU/mL, respectively, in comparison to a control film.	(4)
Tannic acid	**Starch-based film for food packing:** Antimicrobial activity against *E. coli* and *Listeria monocytogenes.*	(5)
Plant extracts		
Grape seed extract (phenolic acids and flavonoids)	**Pea edible starch film for packing:** Antioxidant, antimicrobial, enhanced thickness and diminished puncture and tensile strength.	(6)
Pepperfruit (*Dennetia tripetala* G. Barker; Annonaceae) extract (phenolic acids)	**Antimicrobials in fresh, boiled or roasted beef:** Growth inhibition of *S. aureus, Salmonella* sp., *Pseudomonas aeruginosa, E. coli, Enterococcus faecalis, Serratia* spp., *Proteus* spp.and *Bacillus* spp.	(7)
Mango extract (gallotannins)	**Preservatives in spinach and lettuce:** Reduction of *L. monocytogenes* cell counts, antiadhesive effect of *E. coli* O157:H7 to the leaves.	(8)
Sanguisorba minor (high amounts of caffeic acid derivatives)	**Fruits:** Inhibition of more than 50% of *Monilinia laxa* growth in infected apricots and nectarines. Inhibition of 70% *of Penicillium digitatum* in oranges. Inhibition of more than 50% of *Botrytis cinerea* in berries.	(9)
Orobanche crenata (high amounts of phenolic compounds)	**Fruits:** Total inhibition of *Monilinia laxa* in infected apricots and nectarines. Inhibition of 90% *of P. digitatum* in oranges. Inhibition of more than 50% of *B. cinerea* in berries.	(10)
Olive oil extract (phenolic compounds)	**Reconstituted milk:** Inhibition of growth and enterotoxin production by *S. aureus* S-6.	(11)
Green tea extract	**Tapioca starch/decolorized hsian-tsao leaf gum coatings**: Antimicrobials in fruits-based saladas, aerobic count was reduced1 to 2 log.	(12)

Table 1-1. (Continued).

	FOOD APPLICATION	Ref.
Green tea extract	**Refrigerated romaine hearts and pork slices:** Antimicrobial, reduction of Gram positive bacterial counts by 4-6 log.	(13)
Glycyrrhiza inflata extract (prenylated flavonoid licochalcone A)	**Food preservative used in Japan**	(14)
GLUCOSINOLATES		
Pure compounds		
Ally isothiocyanate	**Refrigerated ground beef:** Elimination and inhibition of *E. coli* O157:H7, depending on inoculum	(15)
Allyl isothiocyanate	**Pears:** Inhibition from 20 to 80% of *Penicillium expansum*, including a thiabendazole resistant strain in pears (5 mg/L)	(16)
Allyl isothiocyanate	**Alfalfa seeds**: Reduction of 7 log in population of *E. coli* O157:H7	(17)
Allyl isothiocyanate (alone or combined with acetic acid)	**Cooked rice:** Total aerobic population was decreased or inhibited up to 48 hours (at 1 mg/L) extending shelf life	(18)
Allyl isothiocyanate	**Fresh beef, cure pork, sliced raw tuna, cheese, egg sandwich, noodles and pasta:** No spoilage after 7 days of incubation at 25°C packed into a sealed gas barrier plastic bag (concentration=20g/Kg)	(19)
Allyl isothiocyanate	**Corn and hulled rice:** After inoculation with *Aspergillus flavus, Penicillium citrinimum* and *Fusarium graminearum,* packed into a sealed gas barrier plastic bag no fungal growth was observed at day 60 of incubation at 25°C (concentration=20g/Kg).	(20)
Plant extracts		
Horseradish root extract (90% allyl isothiocyanate, 9% 2-phenethyl isothiocyanate)	**Roast beef:** bactericidal effect on *E. coli* O157:H7, *S. enterica, L. monocytogenes* and *S. aureus*	(21)
Mustard extract (allyl isothiocyanate)	**Bread:** antifungal effect on *A. flavus, Penicillium corylophilum, Penicillium discolor, Penicillium polonicum, Penicillium roqueforti* and *Endomyces fibulige*	(22)
Mustard extract (allyl isothiocyanate)	**Acidified chicken:** antimicrobial against *Lactobacillus alimentarius* and *E. coli*	(23)

Table 1-1. (Continued).

	FOOD APPLICATION	Ref.
LIPIDS		
Coriolic (13-hydroxy-9,11-octadecadienoic) acid	**Bread:** Inhibited growth of *P. roqueforti* and *Aspergillus niger* and it was comparable to propionic acid increasing shelf life.	(24)

References: (1)(Elegir et al. 2008); **(2)**(Reinders et al. 2001); **(3)**(Tassou and Nychas 1994); **(4)**(Tunç and Duman 2011); **(5)**(Pyla et al. 2010); **(6)**(Corrales et al. 2009); **(7)**(Ejechi and Akpomedaye 2005); **(8)**(Engels et al. 2012); **(9)**(Gatto *et al*. 2011); **(10)**(Gatto *et al*. 2011); **(11)**(Tassou and Nychas 1994); **(12)**(Chiu and Lai 2010); **(13)**(Chiu and Lai 2010); **(14)**(Tsukiyama et al. 2002);**(15)**(Muthukumarasamy e*t al*. 2003); **(16)**(Mari et al. 2002); **(17)**(Park *et al*. 2000); **(18)**(Kim et al. 2002); **(19)**(Isshiki et al. 1992); **(20)**(Isshiki et al. 1992); **(21)**(Ward et al. 1998); **(22)**(Nielsen and Rios 2000); **(23)**(Lemaya et al. 2002); **(24)**(Black et al. 2013).

Licochacone A is stable to salt and protease activity. Therefore, it is suitable for food with high salt concentrations, and fermented foods containing proteases, where other preservatives might be inactive (Tsukiyama et al. 2002). Amphiphilic phenolic lipids are appropriate surfactants and stabilizers. However, due to deactivation by divalent cations (Stasiuk et al. 2004) their use in foods containing divalent ions, such as calcium and magnesium, should be avoided. Furthermore, heat processes would inactivate anacardic acids by removing the carboxylic group (Himejima and Kubo 1991). Hydrolysable tannins are suitable for applications in fruit and vegetable products with no iron. This will avoid their inactivation by proteins and iron (Engels et al. 2011; Cho et al. 2010). However, their mechanism of action needs to be further studied.

Allyl isothiocyanate can be inhibited by binding to amino (Zhang and Talalay 1994) and thiol groups of proteins at high pH (Luciano et al. 2008). This limits their applications to low protein acidic products.

The stability of highly reactive oxylipins in foods requires further investigation. Hydroxy fatty acids are suitable for bread products due to their stability after baking (Black et al. 2013). Their production in dough involves linoleic acid, lipoxygenase and thiol groups (Black et al.

2013), which are related to the rheological properties of dough (Junqueira et al. 2008; Rombouts et al. 2012; Patient and Ainsworth 1994).

1.8 Research hypothesis and objectives

This review of the literature demonstrates that phytoanticipins and phytoalexins, and their enzymatic derivatives, have a remarkable potential for use as food preservatives; toxic alkaloids and cyanogenic glycosides are exceptions. The use of antimicrobial plant extracts in foods would help satisfy the increasing demand of natural ingredients, improve product properties and provide healthy choices to the consumers. In addition, their abundance in by-products of food processing enhances their environmental and economic benefits. However, this use in food applications is currently not exploited. Hurdles to their use are imposed by the lack of knowledge on the active components of plant extracts with antimicrobial activity, their association with compounds that are toxic to humans, and the lack of scalable and food-grade processes for their recovery from plants.

This project tested the following hypotheses:

- Differences in the structure of phenolic acids account for variances in their mode of action, and the structure of glycoalkaloids affects their antifungal activity.
- Differences in chemical structure of plant secondary metabolites allow their recovery and separation.

Objectives of this project were as follows:

1. To investigate the antifungal activity of glycoalkaloids from potatoes (*Solanum tuberosum* L.), alone and together with phenolic acids, and to assess the role of membrane sterols in the fungal resistance to α-chaconine.

2. To study the structure–function relationships of the antibacterial activity of phenolic acids and mechanisms of resistance by lactic acid bacteria.

3. To develop a method for extraction and separation of phenolic acids and glycoalkaloids from potato peels using water/ethanol-based solvent systems.

Taken together, this research will form an important basis for the development of applications of phytoalexins and phytoanticipins as food preservatives.

CHAPTER 2

Antifungal activity of potato *(Solanum tuberosum* L.) glycoalkaloids alone and together with phenolic acids:
Synergistic activity, membrane fluidity changes, and relationship between antifungal sterols and resistance to α-chaconine

CHAPTER 2

2.1. Introduction

Plants synthetize secondary metabolites as a natural defence mechanism, which provide protection against insects, nematodes, bacteria, viruses and fungi. Members of the Solanaceae family, such as potatoes, tomatoes, and eggplants produce glycoalkaloids and phenolic compounds (Friedman 2004; Friedman 2006; Schieber and Aranda Saldaña 2009). Glycoalkaloids are molecules derived from sterols that contain nitrogen and a glycosidic moiety attached to the 3-OH position (Friedman 2004). Some known glycoalkaloids are α-solanine, α-chaconine, α-tomatine, α-solasonine and α-solamargine (Friedman 2006). Potatoes (*Solanum tuberosum* L.) synthetize mainly the glycoalkaloids α-chaconine and α-solanine, which share the aglycone solanidine but differ in their glycan moieties, which are solatriose and chacotriose for α-solanine and α-chaconine, respectively. Furthermore, phenolic compounds such as chlorogenic, gallic, caffeic and protocatechuic acids have been found in potatoes (Im et al. 2008; Rodriguez de Soltillo et al. 2006). Chlorogenic acid generally constitutes 90% of the phenolic compounds in potato peels and the rest are mostly caffeic acid and other hydroxycinnamates (Schieber and Aranda Saldaña 2009; Im et al. 2008; Wu et al. 2012). Caffeic acid is also the product of hydrolysis of chlorogenic acid and as other phenolic compounds possesses antibacterial (Almajano et al. 2007) and antioxidant activities (Dai and Mumper 2010; Reddy et al. 2007). However, the antifungal activity compared of caffeic acid is weak when compared to other phenolic compounds such as alkyl esters (Merkl et al. 2010).

Glycoalkaloids from *Solanum* species possess anti-allergic, antipyretic, anti-inflammatory, hyperglycemic and antibiotic activities (Schieber and Aranda Saldaña 2009). They are toxic to animals and therefore have antihervibore function (Phillips et al. 1996). Furthermore, these compounds have shown antifungal activity. α-Solasonine and α-solamargine showed

synergistic and pH dependent activity against *Phoma medicaginis* and *Rhizoctonia solani* (Fewell et al. 1994). Moreover, α-solanine and α-solamargine demonstrated antifungal properties against 10 different strains (Cipollini and Levey 1997). α-Solanine and α-chaconine showed synergistic antifungal activity which was higher at pH 7 compared to pH 6; α-chaconine was more active than α-solanine (Fewell and Roddick 1993).

In order to estimate the effect of glycoalkaloids in cell membranes, their disruption effect has been studied in liposomes. α-Chaconine but not α-solanine disrupted phosphatidylcholine/cholesterol liposomes at pH 7.2. A synergistic effect was observed when both compounds were mixed (Roddick and Rijnenberg 1987). Sugar-sugar interactions between glycoalkaloids have been suggested as part of their membrane disruption mode of action. This statement is supported by the synergistic effect between α-solanine and α-chaconine, the leakage enhancing effect of glycolipids and the loss of activity after hydrolysing one or more of the monosaccharides from the glycosidic moiety (Keukens et al. 1995). α-Solanine, α-chaconine, and α-tomatine showed membrane disrupting activity and strong interaction with liposomes' model membranes containing sterols. The membrane sterols with planar ring structures and a 3 β-OH group interacted more with glycoalkaloids. The sugar moiety in the glycoalkaloid and side chain of the sterol played an important role in membrane disruption (Keukens et al. 1992). A mechanism for membrane disruption was suggested, starting with insertion of the aglycone in the bilayer, followed by sugar-sugar interactions between the glycoalkaloids forming a complex sterol-glycoalkaloid matrix, rearrangement of which causes lysis of the cell (Keukens et al. 1995).

Studies with animal, plant and fungal cells support the mode of action suggested by the liposome studies. Potato glycoalkaloids changed the membrane potential of frog embryo cells

(Blankemeyer et al. 1992). α-Chaconine and α-solanine have shown synergistic membrane disruptive properties in rabbit erythrocytes, beet cells, and protoplasm of *Penicillium notatum*, in all cases α-chaconine has been the most active (Roddick et al. 1988). These findings suggest that the sterol profile of fungal membranes might play a role in their interaction and resistance to glycoalkaloids. To our knowledge, no *in vivo* studies on fungi considering this interaction in the membrane have been conducted.

The sterol profile of fungi has been used as a chemotaxonomic tool. After analyzing the sterol profile of melanised fungi from hypersaline environments, Mejanelle et al. (2000) found four sterols that can be used to differentiate between the analysed species. The main sterols detected in 175 species of fungi were cholesterol, ergosterol, 24-methyl cholesterol, 24 ethyl cholesterol and brassicasterol, and their variability was related to their phylogenies (Weete et al. 2010).

In view of the biotic stress that potato tubers are constantly exposed to in their natural environment, it is surprising that the profile of secondary plant metabolites present in potato peels is relatively simple, including mostly chlorogenic acid, caffeic acid, α-chaconine and α-solanine (Friedman 2004; Schieber and Aranda Saldaña 2009; Wu et al. 2012). From this observation it can be hypothesized that phenolic acids and glycoalkaloids show synergistic antimicrobial effects.

Therefore, the objective of this study was to evaluate the antifungal effects of the secondary metabolites α-solanine, α-chaconine, solanidine and caffeic acid, alone or in combination, by determining minimum inhibitory concentrations (MICs), measuring membrane fluidity and studying the relationship between membrane sterol composition of several fungal species and their resistance to α-chaconine.

2.2. Materials and methods

2.2.1. Chemicals

Caffeic acid, cholesterol, cycloartenol, ergosterol and squalene were purchased from Sigma (St. Louis, MO, USA). α-Chaconine, α-solanine and solanidine were obtained from Extrasynthèse (Genay, France).

2.2.2. Fungal strains and culture conditions

Eight strains of fungi were used for the experiments (**Table 2-1)**. The strains *F. graminearum*, *M. pinodes*, *A. alternata*, *P. teres* and *P. tritici-repentis* were kindly provided by Dr. S. Strelkov (University of Alberta). The conditions for sporulation and the minimum inhibitory concentration (MIC) experiments were different according to the strain requirements (**Table 2-2**). Once the strains sporulated, the spores were scratched from the mycelial surface with sterile physiological solution containing 0.9% sodium chloride and 0.1% Tween 80 in water. The physiological solution was filtered and homogenized. The spores were counted using a hemocytometer and resuspended in media to a concentration of 10^4 spores/mL under sterile conditions.

Table 2-1. Taxonomic classification of the fungal strains analized.

KINGDOM	Subphylum	Class	Order	Family
A. niger	*Pezizomycotina*	*Eurotiomycetes*	*Eurotiales*	*Aspergillaceae*
P. roqueforti	*Pezizomycotina*	*Eurotiomycetes*	*Eurotiales*	*Aspergillaceae*
A. alternata	*Pezizomycotina*	*Dothideomycetes*	*Pleosporales*	*Pleosporaceae*
P. teres	*Pezizomycotina*	*Dothideomycetes*	*Pleosporales*	*Pleosporaceae*
P. tritrici-repentis	*Pezizomycotina*	*Dothideomycetes*	*Pleosporales*	*Pleosporaceae*
M. pinodes	*Pezizomycotina*	*Dothideomycetes*	*Pleosporales*	*Didymellaceae*
F. graminearum	*Pezizomycotina*	*Pyrenomycetes*	*Hypocreales*	*Nectriaceae*
M. plumbeus	*Mucoromycotina*	*Mucormycotina*	*Mucorales*	*Mucoraceae*

Table 2-2. Strains, incidence or pathogenicity and culture conditions

Strain	Incidence/ pathogenicity	Culture conditions
Aspergillus niger FUA5001* ***Penicillium roqueforti*** FUA5005, isolated from blue cheese (1)* ***Mucor plumbeus*** FUA5003*	Spoilage mold	**General:** 25°C, aerobic conditions, darkness. **Sporulation:** Potato dextrose agar, 7 d incubation **Extraction of membrane lipids:** Potato dextrose broth **MIC:** MRS broth
Fusarium graminearum FG001**	Fusarium head blight on wheat and barley	**General:** 25°C, sealed with parafilm, darkness. **Sporulation:** Potato dextrose agar, alternate 12 h light/ 12 h darkness, 5 d incubation **Extraction of membrane lipids:** As above **MIC:** Potato dextrose broth
Mycosphaerella pinodes Isolate 39, from infected peas (2)**	Ascochyta blight in peas	**General:** 25°C, sealed with parafilm, darkness, alternate 16 h light/ 8 h darkness **Sporulation:** Potato dextrose agar, 7 d incubation **Extraction of membrane lipids:** As above **MIC:** Potato dextrose broth
Alternaria alternata AA001**	Brown spot or brown spot in several plants including citrus	**General:** Sealed with parafilm, darkness. **Sporulation:** Half potato dextrose agar, 28 ºC, 3 d incubation. Plugs of 70 mm in diameter were subcultured in S-medium agar, and covered with distilled water, 18 ºC, 1 d incubation **Extraction of membrane lipids:** Potato dextrose agar**,** 18 ºC, 7 d incubation **MIC:** Potato dextrose broth, 18 ºC.
Pyrenophora teres* f. *teres Isolate SK51, from barley**	Net blotch of barley	**General:** 25 ºC, sealed with parafilm, alternate 12 h UV light/12 h darkness. **Sporulation:** V-8 juice-Potato dextrose agar, 7d incubation. **Extraction of membrane lipids:** As above **MIC:** Potato dextrose broth
Pyrenophora tririci-repentis 331-2, from infected wheat**	Tan spot in wheat	**General:** Darkness, aerobic conditions. **Sporulation:** V-8 juice agar, 20 ºC, darkness, 12 d incubation. Mycelia was covered with sterile MiliQ and softly streaked. Then 16 h incubation, light, 25 ºC. Followed by 24 h incubation, 16 ºC. **Extraction of membrane lipids:** V-8 juice agar, 20 ºC, darkness, 12 d incubation. **MIC:** Potato dextrose broth, 20 ºC, darkness, 12 d incubation.

References: (1) (Zhang et al. 2010); **(2)** (Liu et al. 2013)
*Dr. Michael G. Gänzle collection (University of Alberta)
**Dr. Strelkov collection (University of Alberta)

2.2.3. Determination of the minimum inhibitory concentration (MIC)

The MIC of the compounds was obtained based on the method used by (Parente et al. 1995; Gänzle et al. 1996), modified for fungi. Stock solutions of 20 g/L of caffeic acid and 2.4 g/L of α-solanine, α-chaconine and solanidine were prepared. α-Solanine was dissolved in methanol/acidified water (0.1 M HCl) (3:1, v/v). The other compounds were dissolved in pure methanol. The stock solutions and media were mixed (1:1, v/v), yielding pH values between 5.7 and 6 for all samples.

Two-fold serial dilutions of the compounds were prepared on microtiter plates. The solvents were evaporated over 2 h under a sterile flow of air. The microtiter plates were inoculated with liquid media containing 10^4 fungal spores/mL. The final concentration of the compounds ranged from 0.8 to 0.0015 g/L. Microtiter plates were incubated as indicated in **Table 2-2**. The mycelial growth was visually monitored every day and the inhibitory concentrations were determined 3 days after the positive controls showed mycelial growth. The MIC was defined as the minimum concentration of the compound that was able to inhibit mycelial growth. The antifungal effect of α-chaconine in combination with caffeic acid was also determined by obtaining the MIC by critical dilution assay (Gänzle et al. 1996; Parente et al. 1995). α-Chaconine was diluted to concentrations from 0.8 to 0.006 g/L. in media containing 0.05 to 6.7 g/L of caffeic acid, in a full factorial design. The media and conditions used were as indicated in **Table 2-2** for MIC. MICs are expressed as means ± standard deviations of three independent experiments.

2.2.4. Generalized polarization in fungal membranes

In order to determine the effect of α-chaconine, α-solanine and solanidine in the fungal membrane, as well as their synergism with caffeic acid, the generalized polarization of the fungal

membrane was measured, by using a modified method from Molina-Höppner et al. (2004). The strains were grown in potato dextrose agar (PDA) medium for two weeks with continuous shaking at room temperature and washed twice with physiological solution (0.85% NaCl). For each strain, 10 mg of mycelium was weighted in Eppendorf tubes, suspended in 300 µL of physiological solution and stained with 12 µL of saturated ethanolic solution of LAURDAN for 30 min in the dark. Mycelia were washed twice. After centrifugation, the pellets were resuspended in physiological solution to a final concentration of 1 g/L of α-chaconine, α-solanine or solanidine. The same compounds were also mixed with caffeic acid, 6.7 g/L and tested for synergistic effects on the membrane fluidity. Fluorescence spectra were measured at an excitation wavelength of 360 nm and emission wavelengths from 400 to 600 nm using a spectrofluorometer (Varioskan Flash, Thermo Electron Corporation, Nepean, Canada). Generalized polarization (GP) values of solutions were calculated using emission values at 440 nm and 490 nm as previously reported by Bagatolli et al. (2003):

$$GP = (I_{440} - I_{490})/(I_{440} + I_{490})$$

2.2.5. Saponification and extraction of non-saponifiable lipids

The fungal strains were grown as described in **Table 2-2.** Plugs of the mycelia (diameter 70 mm) were removed with a sterile scalpel and inoculated in flasks containing 100 mL of sterile potato dextrose broth (PDB). The flasks were incubated for 7 days at room temperature with shaking at 150 rpm. The content of the flasks was transferred to 50 mL sterile tubes and centrifuged at 4696 *g*. The media were discarded, the mycelia washed twice with distilled water, freeze-dried, and stored at -20 ºC.

Saponification and extraction were performed based on the method by (Headly et al. 2002) with some modifications. For this purpose, 200 mg of ground dried mycelia was mixed

with 6 mL of KOH/water/methanol (6:24:70, w/v/v), left under nitrogen for 1 min, closed tightly, shaken and held in a 90 ºC water bath for 2 h; the mix was shaken every 30 min. Unsaponifiable compounds were extracted 3 times with 600 mL of petroleum ether. The supernatants were removed and dried under nitrogen. The residues were dissolved in methanol to a final concentration of 1 mg/mL.

2.2.6. Thin layer chromatography (TLC)

Unsaponifiable lipids from the fungal membrane were separated by TLC, based on the method described by Marriot (1980) with some modifications. Standard solutions of 3 mg/mL of cholesterol, ergosterol and cycloartenol were prepared, as well as solutions of 3 mg/mL of each sample. Flexible plates coated with silica gel, 10 x 10 cm, were used. The solvent system used for separation consisted of heptane/diisopropyl ether/acetic acid (60:40:4, v/v/v). After air drying, the coated plates were developed in a solvent system of 20 % phosphomolybdic acid in ethanol at 70 ºC for 5 min.

2.2.7. HPLC-MS analysis of sterols

Analysis and relative quantification of sterols was performed by HPLC-MS using an Agilent 1200 series HPLC unit consisting of a degasser, binary pump, autosampler, thermostated column compartment, and diode array detector (Agilent Technologies, Palo Alto, CA, USA), coupled to an Applied Biosystems MDS SCIEX 4000 Q TRAP LC-MS/MS System (AB Sciex, Concord, ON, Canada) equipped with an atmospheric pressure chemical ionization (APCI) source operating in positive mode. Sterols were eluted using a Kinetex C18 100A (100 x 3.0 mm, 2.6 μm) column. Isocratic elution was used with a mobile phase consisting of methanol/water (95:5). The run time was 15 min. The flow rate was 0.4 mL/min and the injection volume was 5 μL. The wavelength for DAD detector was 208 nm. Multiple reactions

monitoring (MRM) mode was used for relative quantification. The following APCI conditions inlet were applied. Heated vaporization temperature, 400 °C; nebulizing gas (GS1), 50 psi; curtain gas, 10 psi; nebulizer current, 3 μA; declustering potential (DP), 20 V; entrance potential (EP), 10 V and collision exit potential (CXP), 10 V . The collision energy (CE) was optimized for each pair of ions for all compounds, using a sample of *P. roqueforti,* which contains all lipids identified in the samples. The values ranged from 30 to 50 eV. The two most abundant transitions (Q1→Q3) for each compound were selected for quantification and confirmation, being Q1 always the parent ion. In order to correct variations in the response and the amount of substance injected, cholesterol was added to each sample as an internal standard (IS) at a final concentration of 5 mg/L and therelative quantity of the compounds was calculated by dividing their analyte peak area by the IS peak area. The transitions (Q1→Q3) for all compounds are shown in **Table 2-4**. Due to possible variances in the response of the compounds the relative amouts of the sterols can be compared only between samples. Data were acquired and analyzed using Analyst 1.5 software. Results are expressed as means ± standard deviations of three independent experiments.

2.2.8. Statistical analysis

MICs are expressed as means ± standard deviations of three independent experiments.

SigmaPlot software (Systat Software, Inc., San Jose, CA, USA) was used to perform statistical analysis. The generalized polarization values were statistically analyzed by one-way ANOVA followed by Holm–Sidak method for multiple pairwise comparisons when required. For all analyses statistical significance was based on $P < 0.05$.

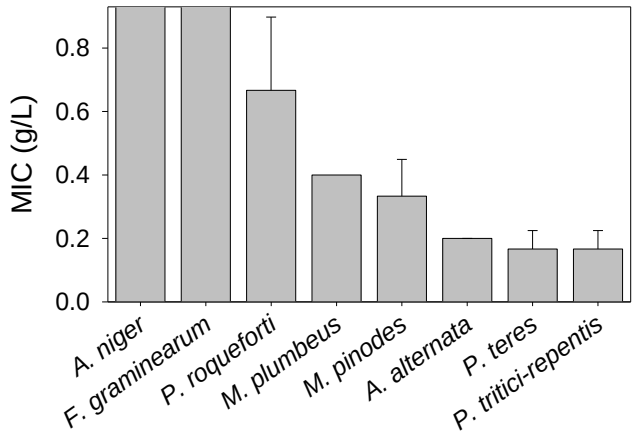
Principal component analysis, linear discriminant analysis and correlation analysis were performed using JMP software (version 8.0.1, SAS Institute, Inc.). The loading plot is a

graphical representation of the amount of variation within the data set and shows the correlation of the individual variables of the first two principal components (PC1 and PC2). The correlations between fungal sterols and MICs were assessed by Pearson correlation analysis.

2.3. Results

2.3.1. Minimum inhibitory concentration (MIC) of potato secondary metabolites against eight fungal strains

Caffeic acid at 6.7 g/L and and α-solanine and solanidine at 0.8 g/Lhad no antifungal activity against *A. niger, M. plumbeus* and *P. roqueforti*. α-Chaconine had antifungal activity at concentrations below 0.8 g/L. (**Fig. 2-1**) and *A. niger, F. graminearum* and *P. roqueforti* where the most resistant strains. *M. plumbeus* and *M. pinodes* were moderately resistant and *A. alternata, P. teres* and *P. tritici-pentis* were the most sensitive.



.

Figure 2-1. MIC of α-chaconine against 8 fungal strains.
Values are means ± standard deviation (n=3). Bars reaching the upper limit indicate MIC above the maximum concentration tested (0.8 g/L)

In order to evaluate potential synergism between the secondary metabolites present in potatoes, α-chaconine and caffeic acid, the MIC of α-chaconine was determined at different

concentrations of caffeic acid against five fungal strains (**Fig. 2-2**). Caffeic acid decreased the MIC of α-chaconine in a range of concentrations from 0.05 g/L to 6.7 g/L. For most strains, α-chaconine acted synergistically with caffeic acid as an antifungal agent. The MIC of *P. teres, F. graminearum,* and *A. niger* showed an about 1000-fold decrease at the highest concentration of caffeic acid. The MIC of *P. roqueforti* decreased about 10-fold and for *M. plumbeus* the decrease was two-fold.

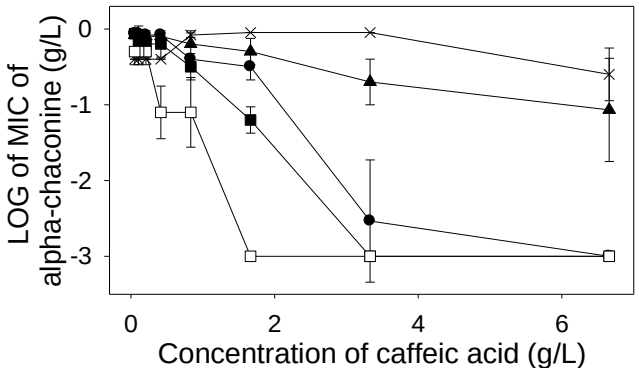


Figure 2-2. MIC of α-chaconine at different concentrations of caffeic acid against 4 fungal strains. *A. niger* (●), *F. graminearum* (■), *P. roqueforti* (▲), *M. plumbeus* (×), *P. teres* (□). Values are means ± standard deviation (n=3)

2.3.2. Measurement of membrane fluidity by generalized polarization (GP)

Generalized polarization was calculated for five fungal strains in order to determine the effect on the fluidity of the membrane caused by α-chaconine, α-solanine and solanidine, either alone or in combination with caffeic acid (**Table 2-3**). The changes in the GP were species dependent.

The addition of α-chaconine or α-solanine did not change the GP value of the membranes compared with the controls. The GP values decreased when solanidine was present and this change was significant for onestrain. In addition when caffeic acid and solanidine were present the GP decreased significantly for 3 out of five strains.

In all the cases but two, the addition of caffeic acid decreased the GP values. This variation was significant for α-chaconine against the two most resistant strains.

2.3.3. Characterisation and relative quantification of sterols

In order to determine whether the sterols pattern of different fungal strains relates to their resistance to α-chaconine, the unsaponifiable fraction extracted from the fungal membranes was separated by TLC. The major band, likely ergosterol or a derivative appeared at the same retention time for all strains. However, differences in the small bands were observed. Therefore, further analysis was carried out by HPLC-APCI-MS (**Fig. A-1, Fig. A-2**). A total of 16 different sterols were detected in all samples (**Table 2-4**). The mass spectra of these compounds were characteristic of sterols analysed by APCI, with $[M+H-H_2O]^+$ being the most abundant fragment in all cases (Sanchez-Machado et al. 2004; Romero-Gonzalez et al. 2010; Headly et al. 2002; Cabanete-Diaz et al. 2007).

Table 2-3. Generalized polarization of fungal membranes after addition of glycoalkaloids

Compound	**Generalized polarization values**									
	A. niger		***F. graminearum***		***M. plumbeus***		***A. alternata***		***P. teres***	
Blank *	0.60 ± 0.04	**a,b**	0.72 ± 0.09	**a**	0.60 ± 0.10	**a**	0.72 ± 0.11	**a**	0.76 ± 0.12	**a**
α-C	0.67 ± 0.09	**a**	0.69 ± 0.02	**a,b**	0.47 ± 0.05	**a,b**	0.69 ± 0.005	**a**	0.70 ± 0.05	**a**
α-C+CA	0.55 ± 0.01	**b**	0.49 ± 0.11	**c**	0.52 ± 0.19	**a**	0.50 ± 0.19	**a,b**	0.57 ± 0.18	**a**
α-S	0.53 ± 0.02	**b**	0.73 ± 0.04	**a**	0.71 ± 0.10	**a**	0.59 ± 0.04	**a**	0.71 ± 0.09	**a**
α-S+CA	0.51 ± 0.04	**b**	0.59 ± 0.06	**b,c**	0.50 ± 0.19	**a**	0.53 ± 0.20	**a,b**	0.62 ± 0.02	**a**
S	0.58 ± 0.05	**a,b**	0.49 ± 0.01	**c**	0.49 ± 0.10	**a**	0.61 ± 0.02	**a**	0.69 ± 0.11	**a**
S+CA	0.56 ± 0.03	**b**	0.53 ± 0.10	**c**	0.21 ± 0.13	**b**	0.39 ± 0.16	**b**	0.63 ± 0.23	**a**

α-chaconine (α-C), α-solanine (α-S), solanidine (S), caffeic acid (CA)
n=3; GP values in the same column with different letters differ significantly ($P < 0.05$)
***** $MeOH/H_2O$/formic acid (50:50:1)

Since ergosterol was the only standard available, the sterols detected in the samples were assigned with letters from A to P, according to their different *m/z.* The mass spectrum of compound F matched that of brassicasterol, since most ions, including that of 381 *m/z* and 297

m/z, matched with the mass spectrum reported by Mo et al. (2013) for brassicasterol. Compound A is probably dehydroergosterol or an isomer, which main *m/z* are 395 and 377, according to McIntosh et al. (2008).

Table 2-4. Retention times and fragments identified in the extracted sterols.

Compound	**Retention time** (min)	***m/z***		
		$[M+H]^+$	$[M+H-H_2O]^+$	Other
A	4.55	395	377	209
B	5.13	397	379	271
C	5.42	425	407	267
Ergosterol	5.96	397	379	295
E	5.96	445	427	409
F	5.95	399	381	297
G	6.48	411	393	295
H	6.54	459	441	423
I	6.86	399	381	213
J	7.05	413	395	325
K	7.06	383	365	145
L	7.57	423	405	149
M	7.58	409	391	149
N	8.39	427	409	241
O	8.41	441	423	149
P	8.44	437	419	149

The identity of the rest of the compounds was not elucidated. The compounds were relatively quantified by dividing the peak area in the MRM mode by the peak area of the internal standard, cholesterol in order to correct variations in the response and the amount of substance injected. Then, those amounts were normalized using *P. roqueforti,* which was the only strain containing all the sterols, as a reference strain, (**Fig. 2-3**). In all strains considered in this study, the most abundant sterols were ergosterol and compound E, the mass spectrum of which showed the *m/z* of 445, 427 and 409, which are observed in 24-brassinolide as well. Therefore compound E might be a brassinosteroid or a derivative. However, brassinosteroids are mainly found in plants and the identity of compound E thus remains to be confirmed.

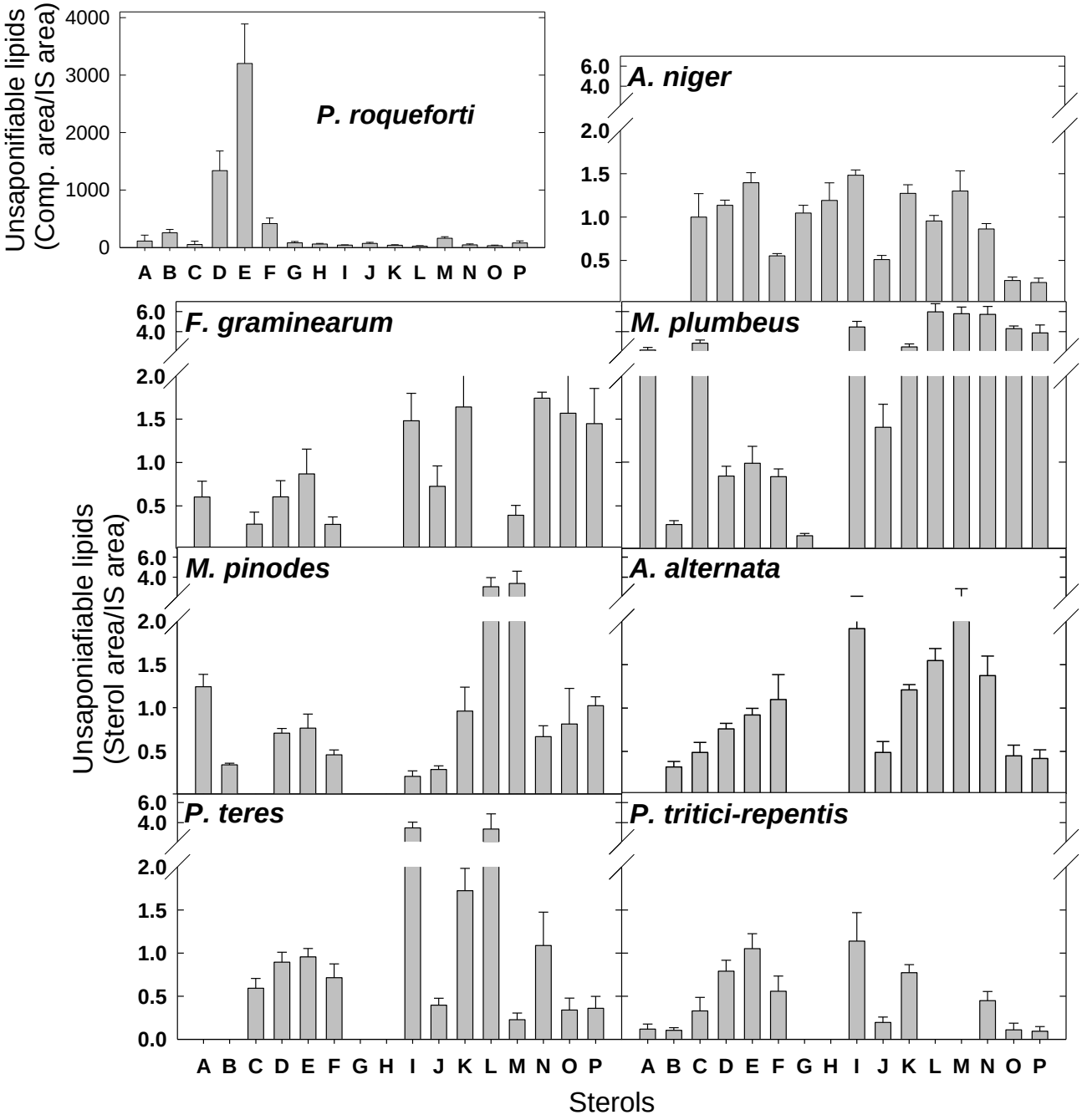


Figure 2-3. Normalized amount of sterols from the fungal membrane of different strains, relative to *P. roqueforti* (analyte peak area/IS peak area). Ergosterol (D). Values are means ± standard deviation (n=3). The main *m/z* of the compounds is as follow: A (395,377,209), B (397,379), C (425,407,267), D (ergostero) (397, 379, 295), E (445, 427, 409), F(399, 381, 297), G (411, 393, 295), H (459, 441, 423), I (399, 381, 213), J (413, 395, 325), K (383, 365, 145), L (423, 405, 149), M (409, 391, 149), N (427, 409, 241), O (441, 423, 149), P (437, 419, 149).

2.3.4. Principal component analysis of sterols and MIC relationship

PCA was used to determine correlations between the amounts and sterols found in the strains (**Fig. 2-4**).

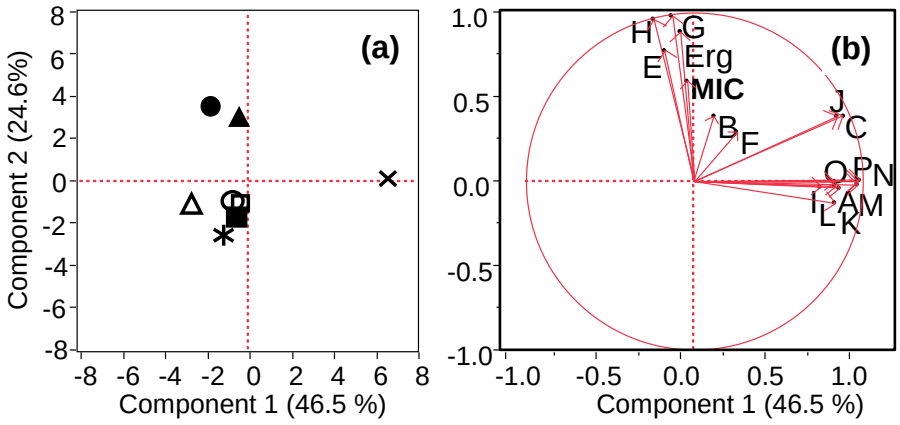


Figure 2-4. Principal component analysis of the fungal strains related to their amount of normalized sterols from the membrane and their MIC. (**a**) biplot; (**b**) loading plot. Strains: *A. niger* (●), *F. graminearum* (■), *P. roqueforti* (▲) *M. plumbeus* (✕), *M. pinodes* (□), *A. alternata* (○), *P. teres* (□), *P. tritici-repentis* (△). Ergosterol (Erg). The main *m/z* of the compounds is as follow: A (395,377,209), B (397,379), C (425,407,267), D (ergostero) (397, 379, 295), E (445, 427, 409), F(399, 381, 297), G (411, 393, 295), H (459, 441, 423), I (399, 381, 213), J (413, 395, 325), K (383, 365, 145), L (423, 405, 149), M (409, 391, 149), N (427, 409, 241), O (441, 423, 149), P (437, 419, 149).

The biplot (**Fig. 2-4a**) shows that strains clustered depending on their phylogenetic position (see **Table 2-1**). *A. alternata*, *P. teres*, and *P. tritici-repentis*, from the family Pleospora, are clustered together in the left low quadrant, as well as *M. pinodes*, which belongs to the same order, *Pleosporales*. *A. niger* and *P. roqueforti*, which belong to the same subphylum *Pezizomycotina* but to a different class, *Eurotiomycetes* clustered together, in the upper left side of the chart. *F. graminearum*, from the same subphylum, is also located in the left side. *M. plumbeus* is phylogenetically different from all other tested strains, it belongs to the phylum Zygomycota, and it is located in the right side of the biplot, far from all others. The loading plot (**Fig. 2-4b**), was used to analyze correlations between MIC and the presence and amounts of sterols in the fungal membranes. Variables in the same area were highly positively correlated. A

high MIC was correlated to the presence of sterols H, G, E and Ergosterol. Compounds G and H were present in the resistant strains, except for *F. graminearum*, but not in all sensitive strains. In order to confirm correlation between these compounds and high MIC values, Pearson correlation analysis was performed. Approaching significance P values of 0.09 were obtained for sterols G and H. Therefore, a high MIC value of α-chaconine against any of the tested fungal strains was related to a higher amount of the sterols H and G in the fungal membrane.

2.4. Discussion

The antifungal activity of solanidine, α-solanine and α-chaconine was determined for *A. niger, P. roqueforti* and *M. plumbeus.* α-Chaconine was the only active compound at concentrations below 0.8 g/L and its MICs values were determined also for *F. graminearum, M. pinodes, A. alternate, P. teres* and *P. tritici-repentis.* The differences in the MICs of α-chaconine against the strains were as high as nine fold, indicating species dependent antifungal activity. In agreement with our findings, the antifungal activity of α-chaconine and α-solanine was substantially different between fungal species, and α-chaconine demonstrated higher antifungal activity compared to α-solanine, the activity of which was negligible in some cases (Fewell and Roddick 1993; Fewell and Roddick 1997). In addition, differences in the antifungal activity of α-solanine against 10 fungal strains from several species were reported (Cipollini and Levey 1997). Furthermore, the lack of antifungal activity of the solanidine aglycone confirms the importance of the sugar moiety in the membrane disruption mechanism of glycoalkaloids proposed by Keukens et al. (1995). The crucial role of the sugar moiety can also be derived from the observation that filamentous fungi detoxify α-chaconine through the removal of sugars (Oda et al. 2002; Weltring et al. 1997).

CHAPTER 2

Synergism between numerous secondary metabolites in plants occurs as a defence mechanism to environmental stress (Ryabushkina 2005). The antifungal synergism between α-chaconine and caffeic acid observed in this study seems to be related to the defence mechanism against fungal phytopathogens. To our knowledge, synergistic antifungal activity between glycoalkaloids and phenolic compounds from potatoes has not been reported previously. Therefore, this new finding requires further investigation.

This is the first *in vitro* study of the disruption of fungal membranes by glycoalkaloids, and the results support previous findings from experiments carried out using liposome model systems (Roddick and Rijnenberg 1987; Keukens et al. 1992; Keukens et al. 1995)

LAURDAN (2-(dimethylamino)-6-dodecanoylnaphthalene) is a fluorescent dye that is used to study the variations of polarity in natural membranes, including hydration of the bilayer (Parasassi et al. 1998). The GP values in the liquid phase usually range between 0.3 and -0.3, whereas the values in the gel phase are between 0.5 and 0.6 (Sanchez et al. 2007; Hofstetter et al. 2012). GP values between 0.05 and 0.6 have been reported for bacteria (Ulmer et al. 2002). The GP values in the fungal cells were between 0.6 and 0.76, indicating a higher concentration of sterols in an organized gel phase, which is expected in eukaryotic cells.

The polarity in the membrane is determined by access of water to the polar-lipid interphase (Parasassi et al. 1998) and strongly dependent on the membrane fluidity, modulated by sterols. A decrease in the LAURDAN GP value occurs when there is a change from the gel phase to a liquid-crystalline phase (Parasassi et al. 1998), which implies an increase in the polarity. The polarity of the fungal membranes was not affected by the addition of α-chaconine or α-solanine. However, a decrease in the GP value indicating an increase in the polarity of the bilayer was observed when solanidine was present. These changes were significant for three out

of five strains. Since the mechanism of membrane disruption by glycoalkaloids proposed by (Keukens et al. 1995) implies first the insertion of the aglycone in the bilayer and then the complex formation between the sugar moieties, our results confirm that solanidine is able to insert in the phospholipidic bilayer by association with the sterols. In absence of the sugar moiety, hydration of the bilayer occurs. In contrast, if the hydrophilic sugar moieties are present as for α-chaconine and α-solanine, in spite of other changes occurring in the membrane, the water concentration of the membranes would remain unchanged, likely due to hydrophilic interaction between the sugar moieties with water molecules present in the surroundings.

The effect on the membrane caused by the three alkaloids was strongly enhanced by caffeic acid. This partially explains the synergistic antifungal effect of α-chaconine and caffeic acid observed (**Fig. 2-2**). Caffeic acid and solanidine in combination caused the highest polarity change in the membrane, indicating similar mode of action.

Saponification with KOH, followed by extraction using organic solvents such as petroleum ether, hexane, pentane or diethyl ether is the most common method for the recovery of sterols from different matrices such as oil, seaweeds, wetland and fungi (Cabanete-Diaz et al. 2007; Fujino and Ohnishi 1978; Headly et al. 2002; Sanchez-Machado et al. 2004). Usually no further sample treatment is needed. APCI-MS is a useful tool to identify sterols with standards; and HPLC is used for sterols quantification (Careri et al. 2001; Kemmo et al. 2007; Segura Carretero et al. 2008; Cabanete-Diaz et al. 2007; Headly et al. 2002; Sanchez-Machado et al. 2004; Mezine et al. 2003). Since identification of the sterols was not the aim of the study, APCI-MS was used for differentiation between the sterols present in the fungal membrane and MRM mode was used for relative quantification, since it provided higher sensitivity to detect minor components compared to HPLC.

Ergosterol was the second most abundant sterol in all fungal strains analysed, which belong to the phylum Ascomycota (**Fig. 2-3**). This observation is in agreement with previous findings that high concentrations of ergosterol are found in fungi from the phylum Ascomycota and Basidomycota (Pasanen et al. 1999; Newell et al. 1988). The other tentatively identified compound, brassicasterol, has also been found in fungi from the same phylum (Weete et al. 2010).

According to the PCA (**Fig. 2-4**), the sterol pattern of the analysed strains is correlated with their phylogenetic classification as previously observed by Weete et al. (2010). Nevertheless, there was an association between the MIC (resistance) against α-chaconine and the presence of compounds G and H. This agrees with previous findings that the type of sterols present in the membrane has an effect on the antifungal mode of action of α-chaconine (Keukens *et al*. 1992).

In conclusion, fungal species differ in resistance to α-chaconine and possibly other glycoalkaloids. This resistance is associated with their phylogenetic position and their sterols pattern in the fungal membrane.

CHAPTER 3

Structure–function relationships of the antibacterial activity of phenolic acids and their metabolism by lactic acid bacteria

CHAPTER 3

3.1. Introduction

Phenolic compounds are plant secondary metabolites, which possess aromatic rings with one or more hydroxyl or methoxy groups. Phenolic acids are a subclass of phenolic compounds, encompassing hydroxybenzoic acids (C_6-C_1 structures) and hydroxycinnamic acids (C_6-C_3 structures) (Schieber and Aranda Saldana 2008; Dai and Mumper 2010) Phenolic acids have antimicrobial activity and hold promise for application as preservatives in food and food packing materials. Phenolic acids or plant extracts containing these compounds gave satisfactory results when added to beef and food packing materials such as hand sheets and pea starch (Ejechi and Akpomedaye 2005; Elegir et al. 2008; Corrales et al. 2009).

The antimicrobial activity of phenolic acids is determined by their chemical structure, in particular the number and position of substitution in the benzene ring, and the saturated chain length (Cueva et al. 2010). Phenolic acids had lower antimicrobial activity compared with their butyl and methyl esters (Cueva et al. 2010). The antimicrobial effect increased with increasing length of the alkyl chain (Merkl et al. 2010). Oligomers show higher activity than the corresponding phenolic acid monomers (Elegir et al. 2008). Hydroxybenzoic and hydroxycinnamic acids occurring in plants exhibit diversity with respect the number of hydroxyl or methoxy groups **(Figure 3-1)**, however, current knowledge on structure-function relationships of the antimicrobial activity of phenolic acids does not account for this diversity of compounds. Moreover, additional derivatives of these compounds are produced from bacterial metabolism **(Figure 3-1)**.

Some lactic acid bacteria (LAB) such as *L. brevis, L. fermentum* and *L. plantarum* metabolize phenolic acids by decarboxylation and/or reduction. The products of phenolic acid

decarboxylases are vinylcatechol, vinylphenol, vinylguaiacol, pyrogallol and catechol, reduction of hydroxycinnamic acids yields dihydrocaffeic and dihydroferulic acids (Van Beek and Priest 2000; Curiel et al. 2010; Svensson et al. 2010) (**Figure 3-1)**. Lactobacilli that are capable of phenolic acid metabolism were isolated predominantly from fermented products with a high content of phenolic compounds such as olives, whisky, wine, and sorghum (Rozes and Peres, 1998; Van Beek and Priest 2000; Campos et al. 2009; Svensson et al. 2010).

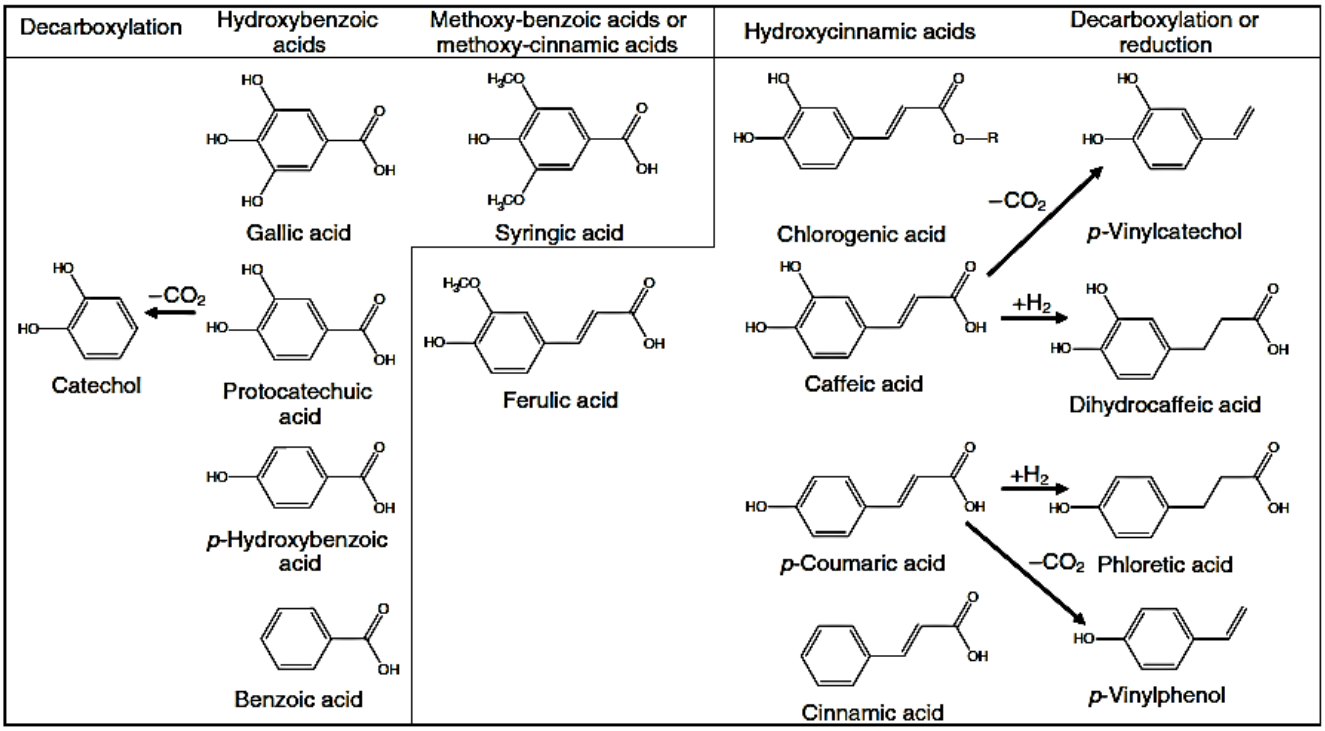


Figure 3-1. Hydroxybenzoic and hydroxycinnamic acids and their decarboxylated and reduced metabolites used in this study.

Lactobacillus spp. are more resistant to phenolic compounds when compared to other groups of bacteria such as *Clostridium* spp. and *Bacteroides* spp. (Lee et al. 2006). The tolerance of lactobacilli to phenolic acids and their ability to metabolize phenolic acids are strain or species specific (Van Beek and Priest 2000; Curiel et al. 2010; Svensson et al. 2010; Cueva et al. 2010). However, the strain-specific tolerance of LAB to phenolic acids has not been related to the

metabolism of phenolic acids. Moreover, the antibacterial activity of the products of phenolic acid metabolism by LAB remains unknown. Knowledge on structure-function relationships of phenolic acids is important for application of these compounds as food preservatives as well as the selection of starter cultures for food fermentations. Therefore, this study aimed to elucidate the structure-function relationships of phenolic acids with model organism isolated from food. Moreover, it was determined whether phenolic acid metabolism by LAB is a mechanism of detoxification of noxious compounds that LAB encounter in their natural habitat.

3.2. Materials and methods

3.2.1. Chemicals

Caffeic, *p*-coumaric, ferulic, gallic, protocatechuic and *p*-hydroxybenzoic acids were obtained from Extrasynthèse (Genay, France). Chlorogenic, cinnamic, syringic, benzoic, dihydrocaffeic and phloretic acids, and catechol were purchased from Sigma (St. Louis, MO, U.S.A.). MRS (Difco) and LB media were obtained from BD (Mississauga, ON, Canada).

3.2.2. Bacterial strains and culture conditions

The following strains and incubation conditions were used to evaluate the antibacterial activity of phenolic acids: *Lactobacillus plantarum* TMW 1.460 (MRS medium, 30°C, microaerophilic conditions), isolated from spoiled beer (Ulmer et al. 2000); *Lactobacillus hammesii* DSM 16381 (MRS medium, 30°C, microaerophilic conditions), isolated from sourdough (Valcheva et al. 2006); *Lactobacillus fermentum* FUA 3165 and *Lactobacillus reuteri* FUA 3168 (both MRS medium, 34°C, microaerophilic conditions), both isolated from fermented sorghum (Svensson et al. 2010); *Bacillus subtilis* FAD 110 (LB medium, 37°C, aerobic

conditions), isolated from ropy bread (Röcken et al. 1993); and *Escherichia coli* AW1.7 (LB medium, 37°C, aerobic conditions), isolated from beef (Dlusskaya et al. 2011).

3.2.3. Antibacterial activity

The minimum inhibitory concentration (MIC) of phenolic acids was determined by a critical dilution assay as described by Parente et al., (1995) and Gänzle et al., (1996). Stock solutions of each phenolic acid (20 g/L) were prepared in 50% methanol (v/v) and 50 % of phosphate buffer (50 mmol/L, pH 6.5). Caffeic acid was dissolved in 100% methanol. Media and stock solutions were adjusted to pH 4.0, 5.5, and 7.0 with 2N HCl or NaOH in order to evaluate the effect of the pH on the activity of phenolic acids. The stock solutions (100 µL) and media (100 µL of LB or MRS) were mixed and two-fold serial dilutions of the phenolic acids were prepared on microtiter plates (Parente et al., 1995; Gänzle et al., 1996). Solvents were evaporated under a sterile flow of air.

Indicator strains were subcultured twice in liquid media under the conditions listed above. Media were inoculated with 10% of stationary cultures obtained after overnight incubation and 50 µL inoculated media were added to microtiter plates. This procedure resulted in phenolic acids concentrations in growth media ranging from 6.7 g/L to 6.5 mg/L and an initial cell count of indicator strains of about 10^7 cfu/mL. Microtiter plates were incubated for 24 h. After incubation, bacterial growth was determined by optical density at 630 nm. The MIC was defined as the lowest concentration of phenolic acids inhibiting bacterial growth (Gänzle et al., 1996). MICs are expressed as means ± standard deviations of at least two independent experiments. For evaluation of structure-function relationships of antibacterial phenolic acids, the MICs are reported as means ± standard deviations of four independent experiments and

significance was determined by Student's t test using SigmaPlot software (SystatSoftware, Inc., San Jose, CA), where P was < 0.05.

3.2.4. Metabolism of phenolic acids by LAB strains

Modified MRS media (Svensson et al., 2010) were supplemented with caffeic, ferulic, *p*-coumaric, protocatechuic or *p*-hydroxybenzoic acids at a concentration of 1 mmol/L to match conditions employed in previous investigations related to phenolic acid metabolism by LAB (Svensson et al., 2010). Chlorogenic acid was added at a concentration of 1.5 mmol/L. Media were inoculated with 5% overnight cultures of *L. plantarum*, *L. hammesii*, *L. fermentum* or *L. reuteri* and incubated for 24 h at 30 or 34°C. Sterile media containing the corresponding phenolic acids served as a control. After incubation, cells were removed by centrifugation and the supernatant was acidified with hydrochloric acid to pH 1.5. Liquid-liquid extraction with ethyl acetate was carried out three times. The extracts were analysed by LC-DAD-MS using a Shimadzu ultrafast liquid chromatography (UFLC) system with a Kinetex PFP column (100 x 3.0 mm, 2.6 µm) and a SPD-M20A Prominence diode array detector. The mobile phase solvents consisted of (A) 0.1% (v/v) formic acid in water, and (B) 0.1% formic acid in water/acetonitrile (10:90, v/v). The gradient was as follows: 0-20% B (0-1.5 min), 20% B (1.5-4.5 min), 20-90% B (4.5-7.5 min), 90% B (7.5-8 min), 90-0%B (8-14 min). The injection volume was 5 µL and the flow rate was 0.9 mL/min. The analytes were quantified at 280 nm. Bacterial conversion of phenolic acids during incubation was calculated as percentage of the initial substrate concentration. Metabolism is expressed as means ± standard deviation of duplicate independent experiments analysed in duplicate.

3.3. Results

3.3.1. Effect of pH on the antibacterial activity of phenolic acids

In order to evaluate the effect of pH on the antimicrobial activity of phenolic acids, the MICs of five phenolic acids (chlorogenic, protocatechuic, caffeic, ferulic and *p*-coumaric acids) were determined at pH 4.0, 5.5 and 7.0. Two strains of lactic acid bacteria, *L. plantarum* and *L. hammesii*, were used as indicator strains. *B. subtilis* and *E. coli* did not grow at a pH of 4.0, independent of the presence of phenolic acids (data not shown). The MIC of chlorogenic acid was higher than the highest concentration used, 6.7 g/L. The antimicrobial activity of phenolic acids increased at lower pH (**Figure 3-2)**. This effect was more pronounced for *L. plantarum* than for *L. hammesii*.

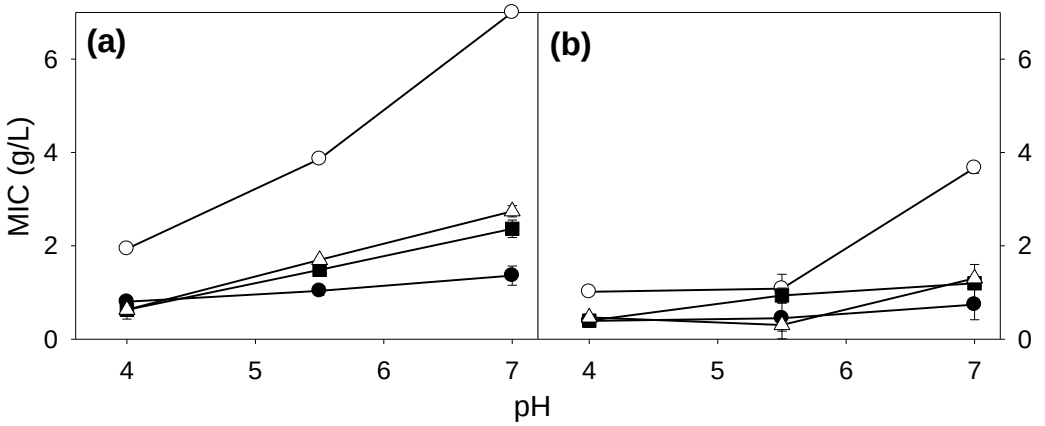


Figure 3-2. MIC of protocatechuic acid (○), caffeic acid (●), ferulic acid (△) and *p*-coumaric acid (■) against *L. plantarum* **(a)** and *L. hammesii* **(b)** at different pH values. Symbols on the upper axis indicate a MIC that is higher than the highest concentration used (6.7 g/L).

3.3.2. Structure-function relationship of phenolic acids: Effect of number of hydroxyl (-OH) and methoxy (-OCH_3) groups in the aromatic ring

The effect of hydroxyl (-OH) and methoxy (-OCH_3) groups on the antimicrobial activity of phenolic acids was studied by comparison of the MICs of benzoic acid, cinnamic acid, hydroxybenzoic acids (*p*-hydroxybenzoic, protocatechuic, gallic and syringic acids) and hydroxycinnamic acids (*p*-coumaric, caffeic and ferulic acids) against *L. plantarum* and *L. hammesii* as well as *E. coli* and *B. subtilis*. The antimicrobial activity of hydroxycinnamic acids was comparable or higher than of hydroxybenzoic acids with same number of hydroxyl groups (**Tables 3-1 and 3-2)**. The antimicrobial activity of hydroxybenzoic acids decreased significantly with increasing number of hydroxyl groups **(Table 3-1)**. The activity of gallic acid was approximately two to tenfold lower compared to other hydroxybenzoic acids. In comparison, the effect of the number of hydroxyl groups on the antimicrobial effect of hydroxycinnamic acids was relatively minor (less than four-fold change in activity). Caffeic acid was only about half as active as cinnamic and *p*-coumaric acids (**Table 3-2).**

Table 3-1. MICs of hydroxybenzoic acids according to number of hydroxyl and methoxy groups in the aromatic ring.

# of -OH groups	# of -OCH_3 groups	MIC (g/L) *L. pl*	*L. hm.*	*E. coli*	*B.sub.*
		Hydroxybenzoic acids			
0	**0**	1.45 ± 0.04^{A}	0.97 ± 0.65^{A}	0.07 ± 0.01^{A}	0.04 ± 0.02^{A}
1	**0**	1.57 ± 0.15^{A}	1.12 ± 0.64^{A}	0.12 ± 0.08^{A}	0.13 ± 0.02^{B}
2	**0**	3.87 ± 0.02^{C}	1.19 ± 0.41^{A}	0.31 ± 0.06^{B}	$0.35\pm0.01^{C,D}$
3	**0**	3.74 ± 0.10^{B}	4.56 ± 0.86^{B}	0.49 ± 0.30^{B}	0.64 ± 0.20^{D}
		Methoxy-hydroxybenzoic acids			
1	**2**	1.75 ± 0.42^{A}	1.15 ± 0.65^{A}	0.39 ± 0.08^{B}	$0.26\pm0.12^{B,C}$

Values are mean ± standard deviation (n=4).
MICs values in the same column with different superscripts differ significantly ($p<0.05$).

The antimicrobial activity of syringic acid with one hydroxyl and two methoxy groups was higher than the activity of gallic acid but comparable to other hydroxybenzoic acids. Thus, the antimicrobial activity of hydroxybenzoic acids increased through substitution of a hydroxyl group with a methoxy group (**Table 3-1)**. In contrast, the presence of methoxy groups did not significantly affect the antimicrobial activity of cinnamic acids (**Table 3-2)**. The MICs of ferulic acid were comparable to MICs of other hydroxycinnamic acids, except for *L. plantarum*.

Table 3-2. MICs of hydroxycinnamic acids according to number of hydroxyl and methoxy groups in the aromatic ring.

# of -OH groups	# of $-OCH_3$ groups	MIC (g/L)			
		L. plantarum	***L. hammesii***	***E. coli***	***B. subtilis***
		Hydroxycinnamic acids			
0	**0**	0.79 ± 0.11^{A}	0.86 ± 0.11^{A}	0.11 ± 0.01^{A}	0.07 ± 0.04^{A}
1	**0**	1.21 ± 0.20^{B}	1.04 ± 0.63^{A}	0.12 ± 0.02^{A}	0.25 ± 0.13^{B}
2	**0**	$1.52\pm0.14^{B,D}$	0.63 ± 0.38^{A}	0.23 ± 0.12^{A}	0.30 ± 0.11^{B}
		Methoxy-hydroxycinnamic acids			
1	**1**	1.68 ± 0.14^{D}	0.89 ± 0.59^{A}	0.16 ± 0.08^{A}	$0.38\pm0.25^{A,B}$

Values are mean ± standard deviation (n=4).
MICs values in the same column with different superscripts differ significantly ($p<0.05$).

3.3.3. Metabolism of phenolic acids by lactobacilli

In comparison to *E. coli* and *B. subtilis*, lactobacilli were substantially more tolerant to phenolic acids. The metabolism of phenolic acids by lactobacilli was investigated to determine whether metabolic conversion detoxifies phenolic acids and thus contributes to tolerance. The concentration of 1 mmol/L matched conditions employed in previous investigations (Svensson et al., 2010) and corresponds to 0.13 to 0.2 g/L, well below the MIC of these phenolic acids against lactobacilli (**Tables 3-1 and 3-2**). Metabolites of six phenolic acids (chlorogenic, caffeic, *p*-coumaric, ferulic, protocatechuic and *p*-hydroxybenzoic acids) by four strains (*L. plantarum, L.*

hammesii, *L. fermentum* and *L. reuteri*) were quantified using UFLC-DAD-MS (Svensson et al., 2010). The metabolites were identified by comparing their mass spectra to literature data **(Table 3-3)**. None of the strains metabolized *p*-hydroxybenzoic acid (data not shown). *L. plantarum* consumed all phenolic acids except chlorogenic acid. *L. hammesii* consumed more than 80% of caffeic acid, *p*-coumaric acid and protocatechuic acid. *L. fermentum* metabolized *p*-coumaric and ferulic acids, *L. reuteri* metabolized only chlorogenic acid.

Table 3-3 Metabolism of phenolic acids by lactic acid bacteria. Shown are the percentage of phenolic acids remaining after incubation and major and metabolites produced during incubation.

Phenolic acids	*L. plantarum*		*L. hammesii*		*L. fermentum*		*L. reuteri*	
	Rem. %	Metabolites	Rem.%	Metabolites	Rem. %	Metabolites	Rem. %	Metabolites
Chlorogenic acid	96 ± 7.4	Caffeic acid	98 ± 2.3	Caffeic acid	75 ± 1.0	Caffeic acid	64 ± 4.5	Caffeic acid
Caffeic acid	0.2 ± 0.07	Vinyl catechol	9 ± 1.5	Vinyl catechol	63 ± 26	Dihydrocaffeic acid Vinyl catechol	94 ± 16	NI*
***p*-Coumaric acid**	0.2 ± 0.01	p-Vinyl phenol	16 ± 2.2	*p*-Vinyl phenol	1 ± 0.1	Phloretic acid *p*-Vinyl phenol	85 ± 10	NI*
Ferulic acid	1 ± 0.1	Dihydro ferulic acid	94 ± 7.2	ND*	1 ± 0.3	Dihydroferulic acid	86 ± 6.0	NI*
Protocatechuic acid	0.2 ± 0.06	Catechol	0.5 ± 0.05	Catechol	82 ± 4.2	NI*	91 ± 3.4	ND*

Values are mean ± standard deviation (n=2) of the remaining amount of phenolic acids after 24 hours of incubation with each LAB strain.
*NI, Not identified; ND, Not detected

Caffeic acid results from chlorogenic acid hydrolysis. *L. plantarum*, *L. hammesii* and *L. fermentum* decarboxylated caffeic acid to vinylcatechol. *L. fermentum* alternatively reduced caffeic acid to dihydrocaffeic acid. These compounds were identified based on mass spectra reported by Svensson et al. (2010). Comparable to the metabolism of *p*-coumaric acid by other lactobacilli (Van Beek and Priest 2000; Rodriguez et al. 2008), the decarboxylation of *p*-coumaric acid by *L. plantarum*, *L. hammesii* and *L. fermentum* gave *p*-vinylphenol, which was

identified by the $[M-H]^-$ ion at *m/z* 119. *L. fermentum* also reduced *p*-coumaric acid to phloretic acid, which was previously identified as a metabolite of *p*-coumaric acid (Barthelmebs et al. 2000). *L. plantarum* and *L. fermentum* reduced ferulic acid to dihydroferulic acid as previously described by Svensson et al. (2010). Decarboxylation of protocatechuic acid by *L. plantarum* and *L. hammesii* generated catechol, which was identified by the $[M-H]^-$ ion at *m/z* 109 (Albarran et al. 2010).

3.3.4. Antimicrobial activity of phenolic acids compared to their metabolites produced by LAB.

The MIC of phenolic acids was compared to the MIC of their metabolites to determine whether metabolism reduces their antimicrobial activity. Caffeic and *p*-coumaric acids were compared with their reduced products, dihydrocaffeic and phloretic acids, respectively. Protocatechuic acid was compared with its decarboxylated product, catechol. Six bacterial strains (*L. plantarum, L.hammesii, L. fermentum, L. reuteri, E. coli* and *B. subtilis*) were used as indicator strains. The antimicrobial activity of reduced metabolites was two to five -fold lower than that of the substrates, except for *B. subtilis* (**Figure 3-3)**. Decarboxylation of protocatechuic acid decreased the antimicrobial activity against *L. fermentum, E. coli* and *B. subtilis*, but did not alter the MIC against *L. plantarum, L. hammesii* and *L. reuteri.*

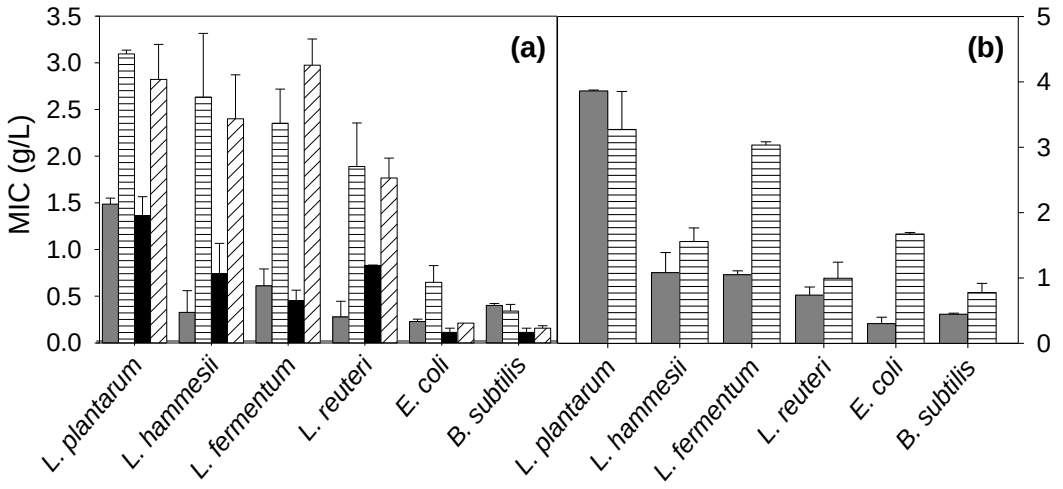


Figure 3- 3. Comparison of the antibacterial activity of phenolic acids and their metabolites. (**a**) Effect of the reduction of the double bond hydroxycinnamic acids. Shown is the MIC of caffeic acid (■) and *p*-coumaric acid (■) and the corresponding reduced metabolites dihydrocaffeic acid (▤) and phloretic acid (▨), respectively. (**b**) Effect of decarboxylation. Shown is the MIC of protocatechuic acid (■) and the decarboxylated metabolite, catechol (▤)**.**

3.4. Discussion

Despite the substantial body of literature related to the antibacterial activity of phenolic acids **(Table 3-4)**, a systematic evaluation of the effect of hydroxyl and methoxy groups on the antibacterial activity of phenolic acids has not been reported. Moreover, different studies used different methodologies for determination of antibacterial activity, and organisms of the same species or genus exhibit substantial strain-to-strain variation in the sensitivity to phenolic acids. This study determined structure-function relationships of hydroxycinnamic and hydroxybenzoic acids by taking into account the effects of hydroxyl- and methoxy groups as well as the contribution of carboxyl groups and the double bond in hydroxycinnamic acids. Our data generally agree with MICs of phenolic acids that were previously published (**Table 3-4**). Our results particularly confirm, and extend for a large selection of structurally diverse phenolic acids

that lactobacilli are more resistant against their antibacterial activity compared to *E. coli, B. subtilis,* and other bacteria relevant in food (**Table 3-4**, Lee et al. 2006).

In analogy to other weak organic acids, benzoic acid and hydroxybenzoic acids exert antimicrobial activity by diffusion of the undissociated acid across the membrane, resulting in acidification of the cytoplasm and, eventually, cell death (Herald and Davidson 1983; Ramos-Nino et al. 1996; Phan et al., 2002; Campos et al., 2009). Consequently, the pK_a and the lipophilicity was proposed to determine the solubility of phenolic acids in bacterial membranes, and thus their antimicrobial activity (Herald and Davidson 1983; Ramos-Nino et al. 1996; Campos et al., 2009). Hydroxybenzoic acids and hydroxycinnamic acids are weak organic acids but differ in their lipophilicity. Factors that affect the lipophilicity of phenolic acids include the pH, which determines the charge of the carboxyl group, ring substitutions (hydroxyl and methoxy groups), and the saturation of the side chain of cinnamic acids.

Table 3-4. Antibacterial activity of phenolic acids

Phenolic acid	MIC (g/L)			References
	Lactobacillus spp.	*E. coli*	*Bacillus* spp.	
Hydroxybenzoic acids				
Benzoic acid *	1-1.8	0.50	0.05-0.12	(1), (2)
***p*-Hydroxybenzoic acid**	0.125-13.8	0.34-0.55	0.40-0.69	(3), (4), (5), (2), (6)
Protocatechuic acid	7.7-14.1	0.55-2.67	0.4-2.67	(4), (7), (5), (6)
Gallic acid	< 0.5[a)]	0.60	0.02-1.6	(3), (7), (8)
Syringic acid	4.95-4.99	0.55	0.40	(4), (5)
Hydroxycinnamic acids				
Chlorogenic acid		0.10		(9)
Caffeic acid	0.5[a)]-1	0.32-2.67	0.22-1.60	(10), (11), (12), (7), (13), (6)
***p*-Coumaric acid**	0.5 [a)]-1	0.45	0.40	(10), (4)
***t*-Cinnamic acid ***	7.4	1.33		(5), (14)
Ferulic acid	0.5 [a)]-1	0.45-1.94	0.40-1.94	(10), (4), (6)

*Benzoic and t-cinnamic acids were included in this study to investigate the effects of aromatic substitution on the antibacterial activity.[a)] MIC corresponding to growth inhibition by 50% or more. **References: (1)**(Chipley 2005; **(2)**(Cueva et al. 2010); **(3)**(Campos et al. 2003); **(4)**(Tuncel and Nergiz 1993); **(5)**(Landete et al. 2008); **(6)**(Merkl et al. 2010); **(7)**(Taguri et al. 2006); **(8)**(Wansi, et al. 2010); **(9)**(Xia et al. 2010); **(10)**(Stead 1993); **(11)**(Wen et al.2003); **(12)**(Lee et al. 2006); **(13)**(Almajano et al. 2007)

A decrease in the pH increased the antibacterial activity of phenolic acids. The same trend has been reported for hydroxycinnamic acids and benzoic acid (Otto and Conn 1944; Herald and Davidson 1983; Wen et al. 2003; Almajano et al. 2007). The concentration of undissociated, more lipophilic phenolic acids increases with decreasing pH. The activity of undissociated phenolic acids is higher compared to dissociated phenolic acids, because they are more soluble in the cytoplasmic membrane (Ramos-Nino et al. 1996). However, our results demonstrate that dissociation of phenolic acids does not fully account for the effect of pH on their activity. The pH also had a strong effect on the MIC of phenolic acids when these were calculated on the basis of the undissociated portion (data not shown). Thus, dissociation is not the only factor responsible for their antibacterial activity.

The number of hydroxyl groups altered the antibacterial activity of hydroxybenzoic acids but did not affect the activity of hydroxycinnamic acids. Likewise, an increase of the lipophilicity by substitution of hydroxyl groups with methoxy groups increased the activity of hydroxybenzoic acids but not of hydroxycinnamic acids. This result contrasts previous estimations that 80% of the antibacterial activity of phenolic acids is determined by their pK_a and lipophilicity (Herald and Davidson 1983; Ramos-Nino et al. 1996). Hydroxycinnamic acids are more lipophilic than hydroxybenzoic acids because of their unsaturated chain (Campos et al. 2003). It is thus possible that their lipophilicity is less affected by substitutions of the aromatic ring. However, the double bond of the side chain, which is the main difference in the structure of hydroxybenzoic and hydroxycinnamic acids, likely contributes to the antibacterial activity of hydroxycinnamic acids.

The reduction of the double bond of hydroxycinnamic acids substantially decreased the antibacterial activity against LAB. This unexpected result further confirms that the double bond

of hydroxycinnamic acids plays an important role in their mode of action. The reduction of the double bond, which strongly affects their antibacterial activity, has only a minor effect on the lipophilicity of the overall molecule. In contrast, the number of hydroxyl groups did not affect the antibacterial activity of hydroxycinnamic acids but has a more pronounced effect on the lipophilicity. Decarboxylation of protocatechuic acid decreased the antibacterial activity against some indicator strains (*L. fermentum* FUA3168, *E. coli* AW1.7, and *B. subtilis* FAD110). However, the MIC against other lactobacilli (*L. plantarum* TMW 1.460, *L. hammesii* DSM13681, and *L. reuteri* FUA3168) remained unchanged. The role of the carboxylic group on the activity of protochatechuic acid was thus not more pronounced than the role of hydroxyl groups.

LAB exhibit a strong strain to strain variation with respect to their tolerance to phenolic acids (Campos et al. 2003). Because the antibacterial activity of phenolic acid metabolites was generally lower when compared to the original substrates (**Figure 3-3**), this variability likely relates to the strain specific metabolism. In keeping with prior observations, lactobacilli metabolized phenolic acids by strain-specific decarboxylation and / or reduction (Van Beek and Priest 2000; De las Rivas et al. 2009; Svensson et al. 2010). *L. plantarum* TMW 1.460 and *L. fermentum* FUA3165 produced decarboxylases and reductases. However, metabolism of *L. fermentum* FUA3165 differed from *L. plantarum* TMW 1.460 as the former strain also reduced caffeic and *p*-coumaric acids. *L. hammesii* DSM13681 only produced decarboxylases. Chlorogenic acid was hydrolyzed by *L. reuteri* FUA3165 and *L. fermentum* FUA3165 to produce caffeic acid, indicating esterase activity of these two strains. Caffeic acid was also identified in supernatants of other strains, but the low conversion of chlorogenic acid by these strains may be attributable to factors other than enzyme activity. Among LAB, chlorogenic acid esterase activity

was previously shown only for *Lactobacillus gasseri* (Coteau et al. 2001). *L. reuteri* FUA3168 had the highest esterase activity among the strains tested in this work, and converted more than 50% of the caffeic acid. This strain or its esterase could serve as suitable catalyst for enzymatic conversion of chlorogenic acid and other phenolic acid esters for food and pharmaceutical purposes.

Although the assay systems for determination of tolerance to phenolic acids and phenolic acid metabolism differed, *L. plantarum* TMW1.460, the strain with the highest metabolic activity towards phenolic acids, was also the most tolerant strain. *L. reuteri* FUA3168 was the most sensitive among the four LAB strains tested in this work (**Figure 3-3** and data not shown), and also exhibited the lowest metabolic activity towards phenolic acids. *L. fermentum* FUA3165 and *L. hammesii* DSM13681 exhibited intermediate sensitivity, and intermediate potential for metabolism of phenolic acids. This relationship between metabolic capacity and sensitivity to phenolic acids further indicates that metabolism of phenolic acids by LAB contributes to their detoxification.

In conclusion, the antibacterial mode of action of hydroxybenzoic and hydroxycinnamic acids differs. The antibacterial activity of hydroxybenzoic acids decreases with an increasing number of hydroxyl groups and is thus primarily correlated to their lipophilicity. The antibacterial activity of hydroxycinnamic acids, particularly their activity against lactobacilli, depends to a much lesser extent on the substitutions of the aromatic ring with hydroxy or methoxygroups but is strongly dependent on the double bond of the side chain. LAB metabolism of phenolic acids by decarboxylation and / or reduction thus likely is primarily a mechanism for detoxification of noxious compounds encountered by LAB in plant substrates. This knowledge on structure-function relationships of antibacterial phenolic acids facilitates the selection of

phenolic acids, or plant extracts containing phenolic acids for use as food preservatives as well as the selection of starter cultures for fermentation of substrates that are rich in phenolic acids, such as sorghum or olives.

3.5. References

Albarran, G., Boggess, W., Rassoloy, V. & Schuler, R. H. (2010). Absorption spectrum, mass spectrometric properties, and electronic structure of 1,2-benzoquinone. *Journal of Physical Chemistry 114*, 7470–7478.

Almajano, M. P., Carbo, R., Delgado, M. E. & Gordon, M. H. (2007). Effect of pH on the antimicrobial activity and oxidative stabiliy of oil-in-water emulsions containing caffeic acid. *Journal of Food Science 72*, 258-263.

Barthelmebs, L., Divies, C. & Cavin, J.F. (2000). Knockout of the *p*-coumarate decarboxylase gene from *Lactobacillus plantarum* reveals the existence of two other inducible enzymatic activities involved in phenolic acid metabolism. *Applied and Environmental Microbiology 66*, 3368-3375.

Campos, F. M., Couto, J. A. & Hogg, T. A. (2003). Influence of phenolic acids and inactivation of *Oenococcus oeni* and *Lactobacillus hilgardii. Journal of Applied Microbiology 94*, 167-174.

Campos, F. M., Couto, J. A., Figuereido, A. R., Toth, I. V., Rangel, A.O.S.S. & Hogg, T.A. (2009). Cell membrane damage induced by phenolic acids on wine lactic acids bacteria. *International Journal of Food Microbiology 135*, 144-151.

Chipley, J. R. (2005) Sodium benzoate and benzoic acid 12-48 *In* Davidson, P. M., Sofos, J. N., Branen, A. L. (ed), Antimicrobials in food. CRC Taylor and Francis, USA.

Corrales, M., Han, J.H. & Tauscher, B. (2009). Antimicrobial properties of grape seed extracts and their effectiveness after incorporation into pea starch films. *International Journal of Food Science and Technology 44*, 425-433.

Coteau, D., Mc Cartney, A. L., Gibson, G. R., Williamson, G. & Faulds, C. B. (2001). Isolation and characterization of human colon bacteria able to hydrolyse chlorogenic acid. *Journal of Applied Microbiology 90*, 873-881.

Cueva, C., Moreno-Arribas, M. V., Martinez-Alvarez, P. J., Bills, G., Vicente, M. F., Basilio, A., Lopez Rivas, C., Requena, T., Rodriguez, J. M. & Bartolome, B. (2010). Antimicrobial activity of phenolic acids against commensal, probiotic and pathogenic bacteria. *Research in Microbiology 161*, 372-382.

Curiel, J.A., Rodriguez, H., Landete J. M., De las Rivas, B. & Munoz, R. (2010). Ability of *Lactobacillus brevis* to degrade food phenolic acids. *Food Chemistry,* 120, 225-229.

Dai, J. & Mumper, R. S. (2010). Plant phenolics: Extraction, analysis and their antioxidant and anticancer properties. *Molecules 15*, 7313-7352.

De las Rivas, B., Rodriguez, H., Curiel, J. A., Landete, J. M. & Muñoz, R. (2009). Molecular screening of wine lactic acid bacteria degrading hyroxycinnamic acids. *Journal of Agriculture and Food Chemistry 57,* 490-494.

Dlusskaya, E.A., McMullen, L.M. & Gänzle, M.G. (2011). Characterization of an extremely heat-resistant *Escherichia coli* obtained from a beef processing facility. *Journal of Applied Microbiology 110,* 840–849.

Ejechi, B. O. & Akpomedaye, D.E. (2005). Activity of essential oil and phenolic extracts of pepperfruit (*Dennetia tripetala* G. Baker; Anonaceae) against some food-borne microorganisms. *African Journal of Biotechnology 4*, 258-261.

Elegir, G., Kindl, A. & Sadocco, P., Orlandi, M. (2008). Development of antimicrobial cellulose packing through laccase-mediated grafting of phenolic compounds. *Enzyme Microbiology and Technology 43*, 84-92.

Gänzle, M. G., Hertel, C. & Hammes, W. P. (1996). Antimicrobial activity of bacteriocin-producing cultures in meat products: modelling of the effect of pH, NaCl, and nitrite concentrations on the antimicrobial activity of sakacin P against *Listeria ivanovii* DSM20750. *Fleischwirtschaft 76*, 409-412.

Herald, P.J. & Davidson, P. M. (1983). Antibacterial activity of selected hydroxycinnamic acids. *Journal of Food Science 48*, 1378-1379.

Landete, J. M., Curiel, J. A., Rodriguez, H., De las Rivas, B. & Muñoz, R. (2008). Study of the inhibitory activity of phenolic compounds found in olive products and their degradation by *Lactobacillus plantarum* strains. *Food Chemistry 107*, 320-326.

Lee, H. C., Jenner, A. M., Low, C.S. & Lee, Y. K. (2006). Effect of tea phenolics and their aromatic fecal bacteria metabolites on intestinal microbiota. *Research in Microbiology 157*, 876-884.

Merkl, R., Hrádková, I., Filip, V. & Šmidrkal, J. (2010). Antimicrobial and antioxidant properties of phenolic acids alkyl esters. *Czech Journal of Food Science 28*, 275–279.

Otto, R. & Conn, J. E. (1944). Effect of increase acidity on antiseptic efficiency. *Industrial and Engineering Chemistry 36*, 2185-187.

Parente, E., Brienza, C., Moles, M. & Ricciardi, A. (1995). A comparison of methods for the measurement of bacteriocin activity. *Journal of Microbiology Methods 22*, 95-108.

Phan, T.N., Nguyen, P.T., Abranches, J. & Marquis, R.E. (2002). Fluoride and organic weak acids as respiration inhibitors for oral streptococci in acidified environments. *Oral Microbioly and Immunology 17*, 19-124.

Ramos-Nino, M. E., Clifford, M. N. & Adams, M. R. (1996). Quantitative structure activity relationship for the effect of benzoic acids, cinnamic acids and benzaldehydes on *Listeria monocytogenes. Journal of Applied Bacteriology 80*, 303-310.

Röcken, W. & Spicher, G. (1993). Fadenziehende Bakterien;Vorkommen, Bedeutung und Gegenmassnahmen. *Getreide Mehl Brot 47*, 30–35.

Rodriguez, H., Landete, J. M., De las Rivas, B. & Munoz, R. (2008). Metabolism of food phenolic acids by *Lactobacillus plantarum* CECT 748^{T}. *Food Chemistry 107*, 1393-1398.

Rozes, N. & Peres, C. (1998). Effects of phenolic compounds on the growth and the fatty acid composition of *Lactobacillus plantarum*. *Applied Microbiology and Biotechnology 49*, 108-111

Schieber, A. & Aranda Saldana, M. D. (2009). Potato peels: A source of nutritionally and pharmacologically interesting compounds - A review. *Food* 3, *23-29.*

Stead, D. (1993) The effect of hydroxycinnamic acids on the growth of wine-spoilage lactic acid bacteria. *Journal of Applied Bacteriology 75*, 135-141.

Svensson, L., Sekwati-Monang, B., Lopes Lutz, D., Schieber, A. & Gänzle, M. G. (2010). Phenolic acids and flavonoids in nonfermented and fermented red sorghum (*Sorgum bicolor* (L.) Moench). *Journal of Agriculture and Food Chemistry 58*, 9214-9220.

Taguri, T., Tanaka, T. & Kouno, I. (2006). Antibacterial spectrum of plant polyphenols and extracts depending upon hydroxyphenyl structure. *Biological and Pharmaceutical Bulletin 29*, 2226-2235.

Tuncel, G. & Nergiz, C. (1993). Antimicrobial effect of some olive phenols in a laboratory medium. *Letters in Applied Microbiology 17*, 300-302.

Ulmer, H. M., Gänzle, M. G. & Voguel, R. F. (2000). Effects of high pressure on survival and metabolic activity of *Lactobacillus plantarum* TMW1.460. *Applied and Environmental Microbiology 66*, 3966–3973.

Van Beek, S. & Priest, F. G. (2000). Decarboxylation of substituted cinnamic acids by lactic acid bacteria isolated during malt whisky fermentation. *Applied and Environmental Microbiology 66*, 5322-5328.

Valcheva, R., Korakli, M., Onno, B., Prevost, H., Ivanova, I., Ehrmann, M. A., Dousset, X., Gänzle, M. G. & Vogel, R. F. (2006). *Lactobacillus hammesii* sp. nov., isolated from

French sourdough. *International Journal of Systematic and Evolutionary Microbiology 55*, 763–767.

Wansi, J. D., Chiozen, D. D., Tcho, A. T., Toze, F. A. A., Devkota, K. P., Ndjakou, L. B. & Sewald, N. (2010). Antimicrobial and antioxidant effects of phenolic constitutens from *Klainedoxa gabonensis*. *Pharmaceutical Biology 48)*, 1124-1129.

Wen, A., Delaquis, P., Stanich, K. & Toivonen, P. (2003). Antilisterial activity of selected phenolic acids. *Food Microbiology 20*, 305-311.

Xia, D., Wu, Z., Shi, J., Yang, Q. & Zhang, Y. (2010) Phenolic compounds from the edible seeds extract of Chinese Mei (*Prunus mume* Sieb, et Zucc) & their antimicrobial activity. *LWT-Food Science and Technology 44*(1), 347-349.

CHAPTER 4

Extraction and fractionation
of phenolic acids and glycoalkaloids from potato peels
using acidified water/ethanol-based solvents

CHAPTER 4

4.1. Introduction

Potatoes (*Solanum tuberosum* L.) are among the most important staple crops consumed by humans (Mattila and Hellstrom 2007). In the last years, the production of value-added potato products has increased to satisfy the demand of consumers for convenience foods, whereas fresh potato consumption is continuously decreasing. Processing leads to the production of significant amounts of waste (Schieber and Aranda Saldaña 2009; Anonymous 2008). Processed potato products account only for 50 to 60% of the raw material. The byproducts include cull potatoes and processing waste (Charmley et al. 2006). Peels constitute the main fraction of the processing waste. While considered waste, potato peels also contain valuable components (Wu et al. 2012). Phenolic compounds and glycoalkaloids are particularly interesting because they are suitable for application in the food and pharmaceutical industries after extraction and purification (Schieber and Aranda Saldaña 2009; Wu et al. 2012).

Phenolic acids are the main phenolic compounds in potatoes (Wu et al. 2012; Schieber and Aranda Saldaña 2009). They have shown antioxidant and antibacterial activities (Sánchez-Maldonado et al. 2011; Rodriguez de Soltillo et al. 1998). Therefore, these compounds hold promise for application as preservatives in foods, feeds, and packing materials. Plant extracts containing phenolic acids were suitable as food preservatives (Ejechi and Akpomedaye 2005; Elegir et al. 2008; Corrales et al. 2009). However, chlorogenic acid constitutes 90% of the phenolic compounds in potato peels (Schieber and Aranda Saldaña 2009; Im et al. 2008). Chlorogenic acid exists in the form of three main isomers, which include chlorogenic acid (5-*O*-caffeoylquinic acid), neochlorogenic acid (3-*O*-caffeoylquinic acid) and cryptochlorogenic acid (4-*O*-caffeoylquinic acid) (Lee and Finn 2007; Shui et al. 2005; Nandutu et al. 2007). Chlorogenic acid isomers do not have strong antibacterial activity but can be hydrolysed to

quinic and caffeic acids (**Fig. 4-1**). Caffeic acid shows antimicrobial activity against Gram positive and Gram negative bacteria at concentrations ranging from 0.1 – 1 g / L (Sánchez-Maldonado et al. 2011; Rodriguez de Soltillo et al. 1998). Quinic acid, the second product of chlorogenic acid hydrolysis, is a starting material for the synthesis of drugs such as Oseltamivir for influenza treatment (Yeung et al. 2006).

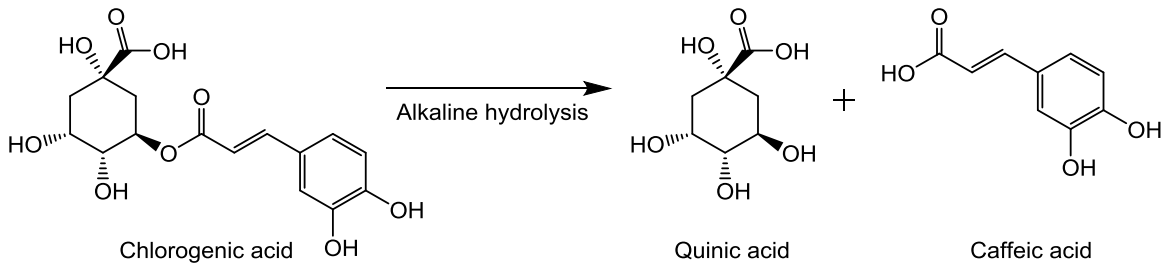


Figure 4-1. Products of alkaline hydrolysis of chlorogenic acid

Glycoalkaloids are plant steroidal compounds that contain nitrogen and a sugar moiety attached to the 3-OH position (**Fig. 4-2**). α-Chaconine and α-solanine are the main glycoalkaloids found in potatoes (Friedman, 2004). The aglycone solanidine is an intermediate for synthesis of hormones such as progesterone and cortisone derivatives (Nikolic and Stankovic 2003). Additionally, glycoalkaloids and their aglycones have been shown to possess anti-allergic, antipyretic, anti-inflammatory, hyperglycemic, and antibiotic properties (Friedman, 2006). Furthermore, potato glycoalkaloids have antifungal activities (Fewell and Roddick 1993; Fewell and Roddick 1997). However, they are toxic for humans and should be absent in potato products or potato extracts used for food applications (Rodriguez-Saona, Wrolstad & Pereira, 1999). For fresh potatoes, a maximum of 200 mg of glycoalkaloids per kilogram is acceptable for human consumption (Friedman 2006; Fewell and Roddick 1993).

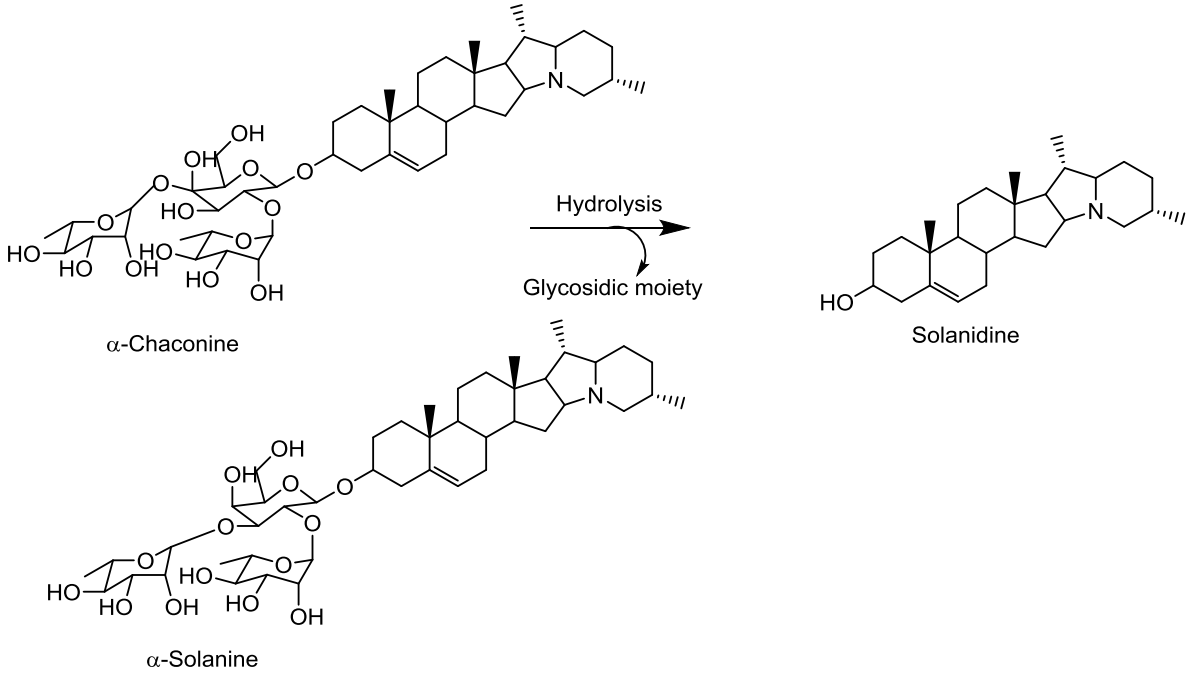


Figure 4-2. Product of hydrolysis of α-chaconine and α-solanine.

Conventional methods for the extraction of phenolic compounds from plant material use organic solvents such as methanol, acetone, ethanol, and ethyl acetate (Dai and Mumper 2010). Glycoalkaloids from potatoes are traditionally extracted with chloroform/methanol mixtures (Bushway & Ponnampalam, 1981; Friedman, Roitman & Kozukue, 2003). These methods are detrimental for the environment. More recently water, ethanol or their mixtures have been used for the recovery of phenolic compounds from potato peels, in some cases for their applications in foods (Kannat et al. 2005; Onyeneho and Hettiarachchy 1993; Singh and Rajini 2004). Water/acetic acid mixtures also have been used to extract glycoalkaloids (Friedman, Roitman & Kozukue, 2003; Machado, Toledo & Garcia, 2007; Sotelo & Serrano, 2000). However, to our knowledge no studies aimed to extract and utilize both phenolic acids and glycoalkaloids from the same source, most likely because the extract containing both requires fractionation in order to obtain phenolic extracts free of toxic glycoalkaloids for food applications and the glycoalkaloids fraction for pharmaceutical purposes. The use of these food-grade solvents facilitates the

incorporation of phenolics extracts in food products and minimizes the environmental damage caused by toxic solvents. In addition, recovery of these compounds from potato peels is an advantage for the food industry, since organic waste causes disposal problems and is of ecological concern (Kim and Kim 2010). Therefore, this is the first study aiming to develop a sustainable method for the simultaneous extraction of these compounds from potato peels using food grade acidified water/ethanol based solvents. Furthermore, experiments aimed to achieve separation of polyphenols and glycoalkaloids from potato peels to allow applications of both fractions in the food and pharmaceutical industries, respectively.

4.2. Materials and methods

4.2.1. External standards

Chlorogenic acid *(*5-*O*-caffeoylquinic acid*)* and caffeic acid were purchased from Sigma (St. Louis, MO, USA). α-Chaconine, α-solanine and solanidine were obtained from Extrasynthese (Genay, France).

4.2.2. Extraction of potato peels

Potatoes from the cultivar ´Russet´ purchased in a local grocery store in Edmonton, Alberta, Canada, were used for this study. After manual peeling, 30 g of fresh peels were simultaneously crushed and mixed with 75 mL of extraction solvent in a domestic blender. Peels and solvent were left in the dark for 30 minutes, stirred for an additional 30 min, sonicated for 20 minutes, and centrifuged at 4696 *g*. The supernatant was recovered and filtered. Extraction was performed three times per batch and samples from each extraction were collected. Three different solvents were used for extraction, acetic acid was used to equal the pH to that of the control solvent (3.2). Solvent A contained 25% water, 70% methanol, and 5% acetic acid; solvent B contained 24% water, 67% ethanol, and 9% acetic acid; solvent C contained 46%

water, 51% ethanol and 3% acetic acid. The organic solvent was evaporated under vacuum at 40 °C using a Rotavapor RE21 (Büchi, Flawil, Switzerland). The dry potato peel extract was resuspended in 15 mL of water. A 0.04 mg/L standard solution of chlorogenic acid was extracted under the same conditions as the potato peels, in order to evaluate the stability of chlorogenic acid during the process.

4.2.3. Fractionation of phenolic acids and glycoalkaloids by solid-phase extraction

Phenolic acids were fractionated from the glycoalkaloids using a Sep Pak Vac 6 cc C18 cartridge. The pH of solvents and water was adjusted to 7. Prior to use, the column was conditioned by elution with 5 mL of ethanol followed by 5 mL of water. Two mL of the extract previously re-suspended in water was passed through the column and washed with 5 mL of water (pH 7). Subsequently, 20 mL of the corresponding solvent was added, phenolic acids were eluted with water/ethanol (80:20, v/v) and glycoalkaloids were eluted with water/ethanol (20:80, v/v). In order to determine the volume of solvent required for complete elution, the fractions were collected successively in 2 mL tubes, and the concentration of phenolic acids and glycoalkaloids was determined subsequently.

4.2.4. Alkaline hydrolysis of chlorogenic acid

Three mL of the extract obtained from solvent C, previously dissolved in 15 mL of water, was centrifuged and the supernatant was mixed with 750 μL of NaOH solution (10 M) and flushed under nitrogen for 2 min. The vial was hermetically closed and the solution stirred for 4 hours at room temperature. Subsequently, the solution was adjusted to pH 4 with HCl and used for fractionation as described in section 4.2.3. To evaluate whether alkaline hydrolysis results in the loss of caffeic acid, a 13 mg/mL standard solution of chlorogenic acid was subjected to alkaline hydrolysis under the same conditions as previously mentioned.

4.2.5. Quantification of phenolic acids and glycoalkaloids

The separation and quantification of phenolic compounds from potato peels was performed using an ultrafast liquid chromatography (UFLC) system consisting of a LC 20 AD XR pump, SIL-20 AC XR Prominence autosampler, a Prominence column oven and a Prominence SPD-M20 diode array detector (Shimadzu, Kyoto, Japan). Separations were performed on a Kinetex PFP column (100 x 3.0 mm, 2.6 μm). The injection volume was 5 μL and the flow rate was 0.9 mL/min. The temperature of the oven was 25 °C. The mobile phase consisted of (A) 0.1% (v/v) formic acid in water and (B) 0.1% (v/v) formic acid in water/acetonitrile (10:90), according to the method of Cruz et al. (2008). The gradient program was as follows: 0–20% B (0-1.5 min), 20% B (1.5-4.5 min), 20–90% B (4.5-7.5 min), 90% B (7.5-8 min) and 90–0% B (8-14 min). Phenolic acids were detected at 280 and 320 nm. Quantification of chlorogenic and caffeic acids was performed using external standards dissolved in a mixture of methanol/water/formic acid (80:20:0.1). Chlorogenic acid isomers, neochlorogenic acid and chlorogenic acid, were quantified based on the standard curve of chlorogenic acid. Calibration curves, with a correlation coefficient ≥ 0.99, were established using concentration ranges from 0.005 to 0.15 and from 0.01 to 0.32 g/L for caffeic acid and chlorogenic acid, respectively. Fresh standard solutions were prepared on the same day of the analysis for each run.

Phenolic compounds in the extracts were characterized by ultrafast liquid chromatography -mass spectrometry (UFLC-MS), under the same LC conditions mentioned above. The UFLC system was coupled to an Applied Biosystems MDS SCIEX 4000 Q TRAP LC/MS/MS System (AB Sciex, Concord, Ontario, Canada) equipped with an ESI Turbo V™ source operating in negative mode with the pneumatically assisted electrospray probe using high-

purity nitrogen gas (99.995%) as the nebulizing (GS1) and heating gas (GS2). The values for optimum spray voltage, source temperature, GS1, GS2, and curtain gases were -4 kV, 600 ºC, and 50, 30, and 25 psi, respectively. Identification of phenolic compounds was performed using an information-dependent acquisition (IDA) method, enhanced mass spectrometry-enhanced product ion (EMS-4EPI). Q1 and Q3 were operated at low and unit mass resolution. The spectra were obtained over a range from *m/z* 50 to 1300 in 2 s. Linear ion trap (LIT) fill time was 20 ms. The IDA threshold was 100 cps. The enhanced product ion (EPI) spectra were collected from the eight most intense peaks above this parameter. The EPI scan rate was 1000 amu/s. Collision-induced dissociation (CID) spectra were acquired using nitrogen as the collision gas under two different collision energies. The collision energy (CE) was -20 eV and collision energy spread (CES) 0 eV. Declustering potential (DP), entrance potential (EP), and collision exit potential (CXP) were -70 V, -10 V and -7 V, respectively.

The analysis of glycoalkaloids was performed using the same UFLC-MS system described above, performed in positive MS mode. Quantification was done by MS using Multiple Reaction Monitoring mode, UFLC gradient was used only to achieve separation. A Kinetex C18 100A (100 x 3.0 mm, 2.6 µm) column was used as the stationary phase. The injection volume was 5 µL and the flow rate 0.6 mL/min. The temperature of the oven was 25 °C. The mobile phase consisted of (A) 0.5% (v/v) formic acid in water/acetonitrile (95:5) and (B) 0.5% (v/v) formic acid in water/acetonitrile (5:95). The gradient was as follows: 20% B (0-12.5 min), 20-90% B (12.5-13.5 min), 90% B (13.5-14.5 min), 90-20% B (14.5-16 min) and 20% B (16-20 min). An information-dependent acquisition (IDA) method, MRM - EPI, was used to profile and quantify the glycoalkaloids. Q1 and Q3 were operated at low and unit mass resolution. The spectra were obtained over a scan range from *m/z* 50 to 1000 in 2 s. LIT fill time

was set at 20 ms. The IDA threshold was set at 100 cps, above which EPI were collected from the eight most intense peaks. For the MRM the values for optimum spray voltage, source temperature, GS1, GS2, and curtain gases were +4.5 kV, 600 ºC, 60, 45, and 15 psi, respectively. The MRM scan rate was 1000 amu/s. Optimization of DP, EP, CE and CXP was done specifically for each transition and the values used were in the range of 55-70 V, 8-14 V, 60-100 eV and 10-40 V, respectively. The two most abundant transitions for each compound were selected (Q1→Q3), for quantification and confirmation. For α-chaconine, α-solanine and solanidine the transitions for quantification were (Q1 852→Q3 706), (Q1 868→Q3 398) and (Q1 398→Q3 98), respectively. For the EPI, the scan rate was 4000 amu/s and the values for optimum spray voltage, source temperature, GS1, GS2, and curtain gases were +5 kV, 600 ºC, 50, 30, and 10 psi, respectively. Standard solutions dissolved in methanol/water/formic acid (80:20:0.1) were used for the calibration curves that gave a correlation coefficient ≥ 0.99. The concentration ranges were 100 to 10000 ppb for α-chaconine, and 50 to 5000 ppb for both α-solanine and solanidine.

The limits of detection (LOD) and quantitation (LOQ) of phenolic acids and glycoalkaloids were determined according to the International Conference on Harmonization (ICH) (Chandran and Singh 2007; Nandutu et al. 2007) as LOD=3σ/S and LOQ=10σ/S, where σ is the standard deviation of response and S is the slope of the calibration curve. The LOD and LOQ of chlorogenic acid were determined as 0.36 and 1.20 ng/L, respectively. The LOD and LOQ for caffeic acid were 0.16 ng/L and 0.55 ng/L, respectively. The LOD for α-chaconine, α-solanine and solanidine were 3.22, 5.42 and 0.01 µg/L, respectively. The respective LOQ were 10.7, 18.07 and 0.033 µg/L, in the same order. Data are reported as means ± standard deviations of triplicate independent experiments.

4.2.6. Quantification of quinic acid

After alkaline hydrolysis, quinic acid was quantified according to the method for organic acids published by Teixeira et al. (2012). An Agilent 1200 series HPLC unit comprised of a degasser, binary pump, autosampler, thermostated column compartment, and diode array detector (Agilent Technologies, Palo Alto, CA, USA) was used. Separation was performed using an Aminex HPX-87 column (Bio-Rad, Mississauga, ON, Canada) at 70 ºC. Quinic acid was detected at 210 nm. An isocratic gradient with a flow rate of 0.4 mL/min, during 60 min was used. The solvent consisted of 5 mM H_2SO_4. No peaks were detected, indicating that the amount of quinic acid in the samples was below the detection and quantification limits of the method. For the standards, only concentrations above 1 mmol/L were detected.

4.2.7. Statistical Analysis

Data are reported as means ± standard deviations of triplicate independent experiments. SigmaPlot software (Systat Software, Inc., San Jose, CA, USA) was used to perform all statistical analysis. To determine statistically significant differences between the three extraction methods, data were subjected to two-way analysis of variance (ANOVA). For the rest of the experiments, the recovery of each compound was statistically analyzed by one-way ANOVA followed by Holm–Sidak method for multiple pairwise comparisons when required. For all analyses statistical significance was based on $P < 0.05$.

4.3. Results

4.3.1. Extraction of potato peels using three different solvents

Three solvents were tested for the extraction of phenolic acids and glycoalkaloids from fresh potato peels to compare their recovery using acidified aqueous methanol and ethanol based

mixtures. There was no significant difference between the phenolic compounds and glycoalkaloids extracted from potato peels with any of the solvents (**Fig. 4-3**).

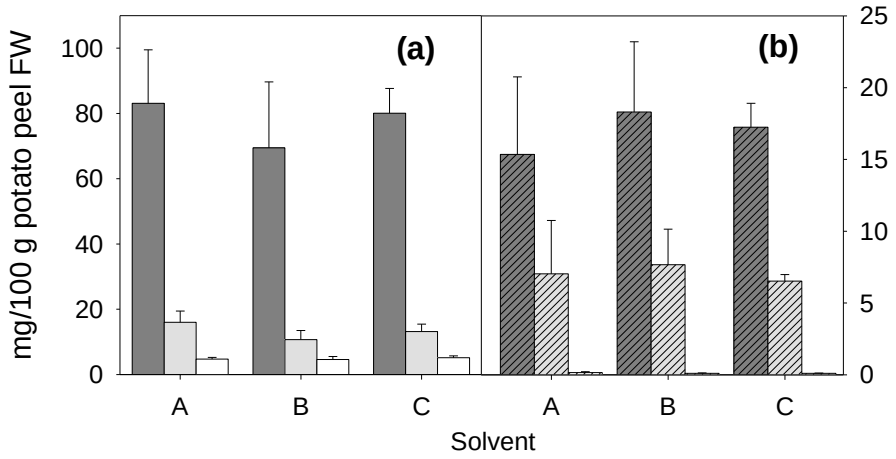


Figure 4-3. Phenolic acids and glycoalkaloids recovered from potato peels using 3 different solvents (A, B or C). **(a)** Phenolic acids: chlorogenic acid (▪), neochlorogenic acid (▫), caffeic acid (□). **(b)** Glycoalkaloids: α-chaconine (▨), α-solanine (▨), solanidine (▨). Data are means ± standard deviations (n=3). Significant differences were determined by two-way ANOVA ($P < 0.05$). The yields of the compounds were compared as a function of the solvent used. There were no significant differences.

4.3.2. MS identification of phenolic compounds and glycoalkaloids in the extracts

UFLC-MS analysis of phenolic constituents showed four main compounds in the potato peel extract. Mass spectra of the most abundant peaks matched those of caffeic acid, chlorogenic acid and neochlorogenic acid. Their maximum absorption wavelength was 324, 326 and 322 nm, respectively, which is typical for chlorogenic acid isomers (Nandutu et al. 2007). As previously reported by others (Nandutu et al. 2007; Clifford et al. 2003; Matsui et al. 2007), neochlorogenic acid eluted before chlorogenic acid under reversed-phase HPLC conditions and the MS spectra of both isomers provided *m/z* 191 as the base peak, as well as peaks at *m/z* 135 and 179. However, for chlorogenic acid the peak at *m/z* 179 was very weak. In addition, *m/z* 173 was seen only in the mass spectrum of chlorogenic acid. The mass spectrum of the fourth peak showed *m/z* 529 as the parent ion and base peak. This compound may correspond to a

caffeoylferuloylquinic acid, as previously reported by Nandutu et al. (2007). However, no fragmentation was observed to support the identity of this compound. α-Chaconine, α-solanine and solanidine were identified in the extracts by UFLC-MS. Their mass spectra were compared to those of the corresponding standards. α-Chaconine was identified by the fragments at *m/z* 852, 706 and 398, α-solanine by the fragments at *m/z* 868, 722, 398, and solanidine showed fragments at *m/z* 398, 382 and 98.

4.3.3. Recovery of phenolic acids and glycoalkaloids from potato peels

The amount of neochlorogenic, chlorogenic and caffeic acids recovered from potato peels was 13.3, 77.6, and 4.8 mg/100 g of potato peel fresh weight, respectively. The recovery of α-chaconine, α-solanine and solanidine was 17.0, 7.1 and 0.1 mg/100 g of potato peel fresh weight (FW), respectively. The recovery was calculated as average of the yield obtained with three different solvents.

4.3.4. Consecutive extractions of bioactive metabolites from potato peels

To determine how many extraction steps are needed for the quantitative recovery of secondary metabolites from potato peels, three consecutive extractions were performed with the same batch of fresh potato peels. Samples from each extraction were collected and quantified by UFLC-MS (**Fig. 4-4**). No significant differences between the three solvents was found for the consecutive extractions. After the second extraction, 97% of chlorogenic acid, 94% of neochlorogenic acid and 89% of caffeic acid were recovered. To evaluate whether the increase in the proportion of caffeic acid in the second and third extraction was due to chlorogenic or neochlorogenic acid hydrolysis, chlorogenic acid was extracted from a standard solution using the same conditions as for potato peels (data not shown). No hydrolysis into caffeic acid was observed and the extraction efficiency was higher than 99%. The recovery of α-chaconine and α-

solanine after the second extraction was higher than 99%. In addition, 95% of solanidine had been recovered after the second extraction.

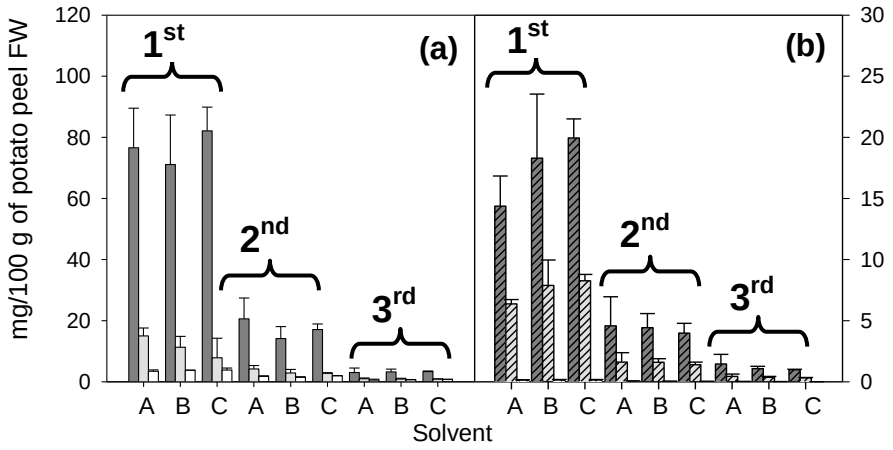


Figure 4-4. Recovery of phenolic acids and glycoalkaloids from potato peels in 3 consecutive extractions with different solvents. (**a**) Phenolic acids: chlorogenic acid (▓), neochlorogenic acid (▒), caffeic acid (□). (**b**) Glycoalkaloids: α-chaconine (▨), α-solanine (▨), solanidine (▨). Data are means ± standard deviations (n=3). Significant differences were determined by two-way ANOVA ($P < 0.05$). The yields of the compounds were compared as a function of the solvent used for each extraction (first, second and third). There were no significant differences.

4.3.5. Fractionation of phenolic acids and glycoalkaloids by solid-phase extraction

To accomplish fractionation of phenolic acids and glycoalkaloids from the potato peels extract, solid-phase extraction with a Sep Pak Vac 6cc C18 cartridge was used. The extract obtained using solvent C was employed for this purpose (**Fig. 4-5**). Statistical analysis showed that quantitative recovery of chlorogenic acid was achieved. However, there was a significant difference between the amounts of neochlorogenic acid and caffeic acid before and after fractionation. The amount of neoclorogenic acid decreased, while caffeic acid increased.

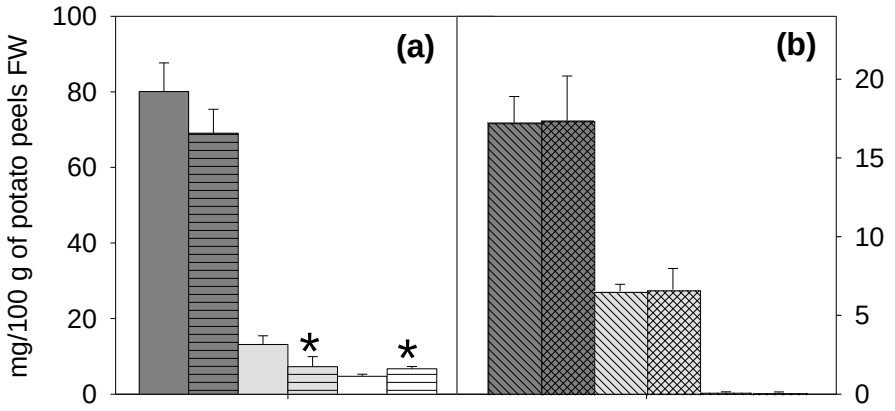


Figure 4-5. Recovery of phenolic acids and glycoalkaloids before and after fractionation by solid phase extraction. (a) Phenolic acids; in crude extract: chlorogenic acid (▩), neochlorogenic acid (▩), caffeic acid (▩); recovered in water/ethanol (80:20) fraction: chlorogenic acid (▩), neochlorogenic acid (▩), caffeic acid (▩). (b) Glycoalkaloids; in crude extract: α-chaconine (▩), α-solanine (▩),solanidine (▩); recovered in water/ethanol (20:80) fraction: (▩) α-chaconine, α-(▩) solanine, (▩) solanidine. Data are means ± standard deviations (n=3). Significant differences were determined by one-way ANOVA followed by Holm–Sidak method for multiple pairwise comparisons ($P < 0.05$). Comparisons between extraction and separation were performed for each compound. * Indicates significant difference in the recovery after fractionation compared to the crude extract.

For complete elution, phenolic compounds and glycoalkaloids required 10 and 8 mL of solvent, respectively (data not shown). The fractionation allowed complete recovery of glycoalkaloids and no hydrolysis to solanidine was observed. There was no significant difference between the amount of glycoalkaloids before and after fractionation.The concentration of glycoalkaloids in the fraction containing phenolic acids was below the detection limit of 3.2, 5.4 and 0.01 µg/L of α-chaconine, solanine and solanidine respectively. *Vice versa*, the concentrations of phenolic acids in the glycoalkaloids fraction were below their respective detection limits.

4.3.6. Alkaline hydrolysis of the potato peel extract followed by fractionation of phenolic acids and glycoalkaloids

The extract obtained from solvent C was subsequently subjected to alkaline hydrolysis and fractionated by solid-phase extraction. The crude extract, the hydrolysed extract and the recovered fractions were analyzed by UFLC-MS. The initial extract contained 226 μmol chlorogenic acid/100 g potato peel FW and 37 μmol neochlorogenic acid/100 g of potato peel FW. After alkaline hydrolysis, no chlorogenic acid isomers were detected and the extract contained 179 μmol caffeic acid/100 g potato peel FW. After fractionation, 139 μmol caffeic acid/100 g potato peel FW were recovered. In order to evaluate the efficiency of the hydrolysis and the recovery after fractionation, the amounts in μmol/100 g potato peel FW of chlorogenic acid, neochlorogenic acid and caffeic acid present in the initial extract were summarized and compared to the yield of caffeic acid after hydrolysis and after hydrolysis and fractionation. If the reaction efficiency and the recovery after fractionation were both 100 %, these three values should be equal. There was a significant difference between the sum of the three initial compounds and the yield of caffeic acids after hydrolysis. However, no significant difference was observed between caffeic acid after hydrolysis and after hydrolysis and fractionation. To evaluate whether alkaline hydrolysis results in the loss of caffeic acid, a chlorogenic acid standard was subjected to alkaline hydrolysis**.** After hydrolysis, 44% of the molar concentration of chlorogenic acid was recovered as caffeic acid, indicating high losses of caffeic acid during hydrolysis.

Following alkaline hydrolysis, the amount of α-chaconine and α-solanine significantly decreased to about 50% of their initial quantity (**Fig. 4-6**). Moreover, no solanidine was detected in the hydrolysed extract, indicating not only hydrolysis but also degradation of these alkaloids.

However, the recovery of glycoalkaloids did not change significantly after hydrolysis compared to after hydrolysis and fractionation.

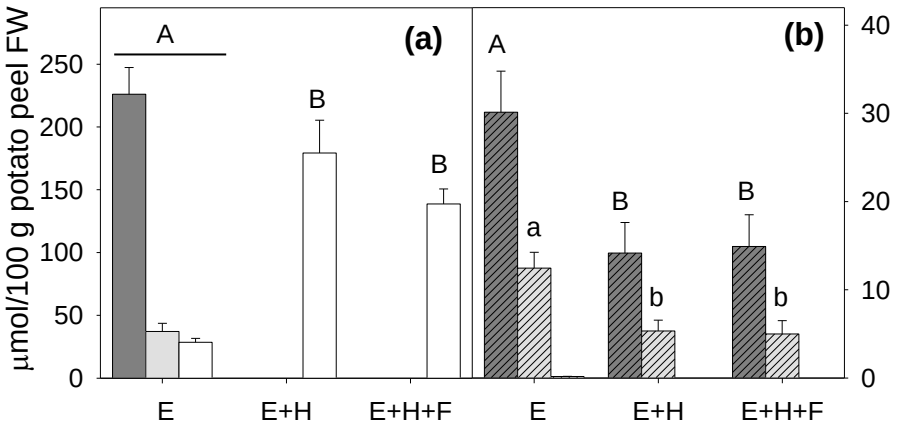


Figure 4-6. Phenolic acids and glycoalkaloids recovered after extraction (E), extraction and alkaline hydrolysis (E+H) and extraction, alkaline hydrolysis and fractionation (E+H+F). (**a**) Phenolic acids: Chlorogenic acid (■), neochlorogenic acid (□), caffeic acid (□). (**b**) Glycoalkaloids: α-chaconine (▨), α-solanine (▨). Data are means ± standard deviations (n=3).Significant differences were determined by one-way ANOVA followed by Holm–Sidak method for multiple pairwise comparisons ($P < 0.05$). For panel (**a**) since hydrolysis of chlorogenic and neochlorogenic acids releases caffeic acid, the summarized amounts of chlorogenic, neochlorogenic and caffeic acids were compared to the amount of caffeic acid recovered after extraction and alkaline hydrolysis, and after extraction, alkaline hydrolysis and fractionation. For panel (**b**) the yield of each compound was compared between extraction, extraction and alkaline hydrolysis and extraction, alkaline hydrolysis and fractionation (capital letters were used to compare amounts of α-chaconine and non capital letters to compare α-solanine). Different letters in the same panel indicate significant difference. Solanidine was not quantified after hydrolysis, since its concentration was below the lowest concentration of the calibration curve.

4.3.7. Purity of extracts

To obtain an approximation of the purity of the extracts regarding the amount of the phenolic compounds in it before and after hydrolysis, the percentages of the compounds were calculated related to the total peak area of the chromatograms at 280 nm and 210 nm. These wavelengths were used since not only hydroxycinnamates, which are selectively detected at 320 nm, but a wide range of compounds have absorbance at this wavelength. Before alkaline hydrolysis, the summarized amounts of neochlorogenic acid, chlorogenic acid and caffeic acid

accounted for 75% and 69% of the material absorbing at 280 nm in the crude extract and phenolic acids fraction, respectively. In the hydrolyzed phenolic acids fraction, caffeic acid accounted for 80% of the UV absorbance at 280. At 210 nm and before alkaline hydrolysis, neochlorogenic acid, chlorogenic acid and caffeic acid together accounted for 74% and 59% in the crude extract and the phenolics fraction. After alkaline hydrolysis, caffeic acid in the phenolic acids fraction was equivalent to 72% of the total material absorbing at 210 nm.

4.4. Discussion

This study compared the recovery of phenolic acids and glycoalkaloids from fresh potato peels comparing a water/methanol-based solvent and two water/ethanol-based solvents. Additionally, the suitability of solid phase extraction with ethanolic solvents for the fractionation of phenolic acids and glycoalkaloids was evaluated. The recovery of phenolic acids without modification or after alkaline hydrolysis of chlorogenic acid isomers into caffeic acid was analysed. UFLC analysis was used for quantification of phenolic acids and UFLC-MS analyses were used for identification of phenolic acids and identification and quantification of glycoalkaloids.

The three solvent systems resulted in comparable recoveries of bioactive compounds. Among the solvents that were evaluated in this study, solvent C with the highest proportion of water (46% water) is the most environmentally benign and least costly alternative. The recovery of chlorogenic, neochlorogenic and caffeic acids (**Fig. 4-3**) reported in this study is two- to threefold higher compared to data in the literature. Rodriguez de Soltillo et al. (2006) found 24-30 mg/100 g of chlorogenic acid and 1.4-2.7 mg/100 g of caffeic acid from fresh potato peels and also reported the presence of protocatechuic and gallic acids. Mattila and Hellstrom (2007) detected mainly chlorogenic acid (15 to 26 mg/100 g) and caffeic acid (4.1 mg/100 g to 4.4

mg/100 g) from fresh potato peels. Because the profile and quantity of phenolic compounds varies with the plant source, variety, season, climate, and several other factors, these differences likely represent different levels of phenolic compounds in the raw material used. The recovery of glycoalkaloids obtained in this study (**Fig. 4-3**) is well in agreement with previous studies, which achieved between 0.9 to 37 mg/100 g of α-chaconine and from 0.4 to 17 mg/100 g of α-solanine in fresh potato peels (Friedman et al. 2003).

Consecutive extractions of potato peels revealed that 97%, 94% and 89% of chlorogenic acid, neochlorogenic acid, and caffeic acid, respectively, were recovered after the second extraction, indicating that the extraction is more efficient for chlorogenic acid than for caffeic acid. Higher amounts of caffeic acid in the third extraction are not likely to be resultant of the hydrolysis of bound phenolic components in potato peels to hydroxycinnamatesas, as reported by Nara et al. (2006), since no hydrolysis was observed when a chlorogenic acid standard was extracted under the same conditions as the samples (data not shown). Therefore, the higher efficiency for extraction of chlorogenic acid is attributable to the higher affinity of the solvents to dissolve this compound that is more polar than caffeic acid. Between 95% to 99% of all glycoalkaloids were extracted after the second extraction. This indicates that in general, two consecutive extractions with solvent C are sufficient for the recovery of more than 90 % of the total bioactive secondary metabolites from potato peels.

Alternative procedures for the extraction of bioactive compounds from plants include subcritical water extraction, which eliminates the need for organic solvents. Subcritical water was used to extract phenolic compounds from bitter melon (Budrat & Shotipruk, 2009), rosemary plants (Ibañez et al., 2003) and oregano (Rodriguez-Meizoso et al. 2006). This process employs high pressure and high temperature and thus accelerates chemical reactions including

the release of bound phenolic compounds and the degradation of caffeic and chlorogenic acid. Singh and Saldaña (2011) compared the recovery and profile of phenolic acids extracted from potato peels using subcritical water extraction to the recovery achieved with methanol extraction. The total amount of phenolic acids obtained from subcritical water extraction was significantly higher (81 mg/100 g FW) compared to the methanol extracts (46 mg/100 g FW) and ethanol extracts (30 mg/100 g FW). Nevertheless, the recovery of chlorogenic and caffeic acids with subcritical water was only 50% and 75%, respectively, compared to methanol extraction. In contrast, subcritical water extracted hydroxybenzoic acids, which were not recovered with methanol. Similarly, catechin was extracted from bitter melons with subcritical water but not with solvent extraction (Budrat & Shotipruk 2009). The amount of phenolic acids reported in this study cannot directly be compared to literature data because of the variability in the content of phenolic compounds in the feedstock. However, the use of acidified solvents significantly increased the recovery of phenolic compounds when onion waste was extracted (Khiari, Makris & Kefalas, 2009). Moreover, the use of acidified ethanolic solvents avoids side reactions and the resulting extract is relatively pure and stable. As shown by purity analysis, the methods utilized in this study generate relative pure mixtures, consisting mainly of chlorogenic and caffeic acids.

Solid phase extraction of the crude extract containing phenolic compounds and glycoalkaloids allowed complete recovery of all target compounds. Slight increases in caffeic acid after fractionation may indicate hydrolysis of a small fraction of chlorogenic acid and other phenolic compounds present in low quantities. The reason might be that fractionation was carried out at pH 7 and ester hydrolysis occurs faster in alkaline conditions (Kim et al. 2006) In contrast, glycoalkaloids were stable during fractionation, and hydrolysis to the aglycone solanidine was not observed. Previous attempts to separate glycoalkaloids and phenolic acids from potato peel

extract by alkaline precipitation of glycoalkaloids resulted in degradation of 30 % of phenolic compounds and 90% of glycoalkaloids (Rodriguez-Saona, Wrolstad & Pereira, 1999). In addition, the amount of solvent required for elution of both fractions is relatively small and comparable with other protocols carried out with acetonitrile (Machado et al. 2007; Abreu et al. 2007). Therefore, solid phase extraction performed in this study results in a significant improvement in the recovery of phenolic compounds and glycoalkaloids as separate fractions.

Alkaline hydrolysis of the crude extract achieved virtually quantitative hydrolysis of chlorogenic and neochlorogenic acids. However, the recovery of caffeic acid represented only 57% of the theoretical yield, indicating degradation of caffeic acid during hydrolysis. Degradation of caffeic acid was also observed during alkaline hydrolysis of a standard in the same conditions as performed for the extract. Although alkaline hydrolysis is a common method for the determination of bound phenolic acids (Kim, Tsao, Yang & Cui, 2006; Mattila & Kumpulainen, 2002), degradation of more than 50% of caffeic acid during hydrolysis of chlorogenic acid has been reported (Krygier et al. 1982; Maillard & Berset, 1995; Nardini et al. 2002). Under alkaline conditions, *o*-dihydroxy benzenes are oxidized to their corresponding quinones when oxygen is present. The degradation of caffeic acid during alkaline hydrolysis can be mitigated by the addition of antioxidants such as ascorbic acid, or by chelating metal ions with EDTA (Nardini et al., 2002). Enzymatic hydrolysis with bacterial esterases is also an alternative to increase caffeic acid recovery. Lactobacilli have the strain-specific capacity to hydrolyze chlorogenic acid (Rodriguez de Soltillo et al., 1998; Sánchez-Maldonado et al., 2011) and hydroxycinnamoyl esterases of lactic acid bacteria were recently characterized (EstebanTorres et al. 2013). It is recommended to find a suitable method for quantification of low concentrations of quinic acid in order to evaluate the yield and stability of this compound

after hydrolysis and fractionation, since the detection limit of the method used in this study was above the concentrations in the extract.

Glycoalkaloids and solanidine were also degraded during alkaline hydrolysis. However, all glycoalkaloids present in the hydrolyzed extract were recovered using solid phase extraction, indicating no degradation at pH 7. Rodriguez-Saona et al. (1999) reported minimum precipitation of glycoalkaloids in a potato peel extract at pH 7 but increased precipitation at pH above 8. Quantitative recovery of glycoalkaloids thus requires solid-phase extraction prior to alkaline hydrolysis of chlorogenic acids.

Caffeic acid, a product of chlorogenic acid hydrolysis, has demonstrated substantial antimicrobial activity (Sánchez-Maldonado et al. 2011), and both chlorogenic and caffeic acids have been highly correlated to the antioxidant activity of potato peel extracts (Nara et al. 2006). Therefore, due to their purity and stability, the phenolic acid fractions obtained in this study before or after hydrolysis can likely be applied as food preservatives. In addition, solid phase fractionation provided a high recovery of glycoalkaloids from potato peels, allowing their utilization as raw materials in the pharmaceutical industry.

This study demonstrates that acidified ethanol-based solvents efficiently recover phenolic acids and glycoalkaloids from potato peels and are thus suitable alternatives to the use of environmentally harmful solvents, allowing the recovery of valuable plant secondary metabolites from potato peels. Simultaneous fractionation and hydrolysis of esterified phenolic acids was also achieved. However, use of antioxidants during alkaline hydrolysis or enzymatic hydrolysis should be considered to allow quantitative recovery of caffeic and quinic acids. Hydrolysis should be performed preferably after fractionation of phenolic acids and glycoalkaloids to avoid degradation of the latter compounds. Solid-phase extraction of phenolic acids and glycoalkaloids

is a suitable method that will allow the use of phenolic acids extracts as food preservatives without any toxicological concerns, while recovered glycoalkaloids can be utilized for pharmaceutical purposes. Thus, this study provides a valuable contribution to sustainable production by assiting with the utilization of by-products as a source of biologically active compounds.

CHAPTER 5
General discussion and conclusions

CHAPTER 5

5.1 General Discussion

The food industry is challenged by the consumer's demand of products with fewer additives and the highest quality and safety (Sloan 2014; Tiwari et al. 2009; Cleveland et al. 2001). Recent outbreaks caused by foodborne pathogens as well as the high consumption of chemical food preservatives, have augmented the safety concerns associated with current food preservation approaches (Cleveland et al. 2001). Substitution of chemical additives by natural components is a challenge for food technologists, since chemical additives simplify processing, enhance organoleptic properties, extend shelf life and ensure safety of the products. In addition, the disposal procedures for food processing waste, which usually include landfilling or incineration, can be significantly costly and damaging for the environment (Kim and Kim 2010). Consequently, the development of natural ingredients and the implementation of alternatives for waste utilization are currently two important issues for the food industry.

The recovery of phytoalexins and phytoanticipins and their enzymatic derivatives from food by-products and their application as preservatives would offer significant rewards for the food industry. It might allow the creation of foods with long shelf-life and enhanced sensory features that contain mostly natural constituents while mitigating environmental problems caused by discarded plant parts, such as seeds, peels and bran of cereals.

In order to use new antimicrobials in foods, the industry requires information to address specific points, including: (i) Mode of action of the antimicrobial compound (inhibitory spectrum, structure-function relationships, effects on microorganisms, interactions with other chemicals in foods). (ii) Sustainable and economical methods to recover active compounds or food extracts. (iii) Acceptance by consumers.

CHAPTER 5

The utilization of phytoalexins, phytoanticipins and their enzymatic derivatives is hampered by the lack of knowledge related to these points. Therefore, this thesis aimed to improve the understanding of the antimicrobial mode of action of phenolic acids and glycoalkaloids occurring together in some plants. Moreover, it aimed to develop an environmentally friendly method using water/ethanol-based solvents for their recovery and fractionation from industrial waste; with the purpose of obtaining phenolic extracts free of toxic glycoalkaloids and glycoalkaloid extracts for pharmaceutical purposes.

The results of this project with respect to the mode of action of phenolic acids highlight their outstanding potential as food preservatives. Although glycoalkaloids are not suitable for food applications because of their toxicity, the procedures used in this work for the investigation on their antifungal mode of action can be transferred to other natural extracts as part of their validation process.

Validation is the first step before the commercialization of antimicrobials. Challenge tests are generally used during antimicrobials validation, since experimentation in the laboratory cannot mimic the conditions in the final products (Baranyi and Roberts 2000). Factors considered include selection of culture media and microorganisms, preparation of the inoculum, maintenance of organisms, changing conditions during growth and aeration of bacteria and sampling (Baranyi and Roberts 2000). In most cases, determination of the inhibitory spectrum would be the first step in looking for the mode of antimicrobial action, by distinguishing sensitive microorganisms from insensitive (Engels et al. 2011; Schwaber et al. 2005). For phenolic acids, this was done previously (Cueva et al. 2010). However, in this thesis the variability of resistance of lactic acid bacteria and other species to phenolic acids was screened. Furthermore, to our knowledge this is the first work that studies several fungal phytopathogenic

and spoilage strains for sensitivity to glycoalkaloids. The differences found in the resistance of the microorganisms to α-chaconine provide essential information for designing future research trials for their potential utilization as antifungal agents in non-food systems.

Studying single pathogens allows the assessment of specific effects of an antimicrobial (Baranyi and Roberts 2000). This was accomplished by studying the susceptibility of few bacterial strains to several phenolic acids at different pH values and the metabolism of these compounds by lactic acid bacteria. In addition, specific information was obtained by investigating the interaction between glycoalkaloids and phenolic acids as antifungals and their effects on the membrane polarity of five selected strains with different resistance.

The knowledge of the structure-function relationship is crucial for determination of applications of food antimicrobials. Mango gallotannins are an example. The antimicrobial activity of gallotannins is compromised by iron and proteins (Engels et al. 2011; Engels et al. 2012), which limits their application in protein-rich or iron-rich foods such as dairy products or meat. However, knowledge of the mode of action allowed the successful application as a biopreservative on ready-to-eat produce (Engels et al., 2012). This thesis investigated the structure-function relationship of antimicrobial glycoalkaloids and phenolic compounds. For potato glycoalkaloids, the results supported the action mechanisms proposed by Keukems et al. (1995), regarding the importance of the interaction between the aglycone and membrane sterols and the complex formation by the sugar moieties. In addition, a relationship between structure of fungal sterols and resistance to glycoalkaloids was found.

The structure-function relationship of phenolic acids was related to the pH of the matrix, which is a key factor for their application as food preservatives. Hydroxycinnamic acids are less dependent on the pH for their activity. Therefore, they are suitable for less acidic foods. In

contrast, hydroxybenzoic acids require low pH values to be active. The results indicated that depending on the pH, the antimicrobial activity of these compounds can be comparable to that of benzoic acid. The MICs of phenolic acids at pH 5.5 against *E. coli* and *B. subtilis* ranged from 0.12 to 0.49 and from 0.13 to 0.64, respectively, whereas the MICs of benzoic acid against *E. coli* and *B. subtilis* were 0.12 at pH 5.6 and 0.5 at pH 6.3, respectively (Chipley 2005). This shows the suitability of phenolic acids as substitutes for chemical antimicrobials currently used in food, including benzoic and sorbic acid. Likewise, their interaction with lactic acid bacteria was explored. The results indicated that lactic acid bacteria are more resistant than *E. coli* and *B. subtilis*, likely due to their ability to metabolize phenolic acids as a strain-dependent detoxification mechanism. This information is important with respect to control of lactic acid bacteria spoilage in non-fermented (Samelis et al. 2000) and fermented (Suzuki et al. 2006) foods and for selection of starter cultures in fermented products (Rodríguez et al. 2009; Svensson et al. 2010), where the metabolic pathways influence the quality of the products (Teixeira et al. 2014).

Environmental approaches and reduction of costs are necessary when developing new processing technologies. A methanolic extraction of potato phenolic acids and glycoalkaloids for industrial applications is ecologically detrimental and the treatment of the toxic residues is expensive. Moreover, extraction with methanol recovers phenolic compounds mixed with toxic glycoalkaloids, which prevents their use as food additives. As a solution, the research presented in this thesis accomplished the development of a sustainable method using innocuous solvents for recovery and separation of phenolic acids and glycoalkaloids from plant matrices. In addition, although glycoalkaloids are not suitable for food applications, their aglycones can be

utilized for the synthesis of steroidal hormones (Nikolic and Stankovic 2003), which makes worth their recovery.

When offering new food products and technologies, the main factors for acceptance by the consumers are the perception of benefit, risks and naturalness (Siegrist 2008). The labeling of a food product, including the ingredient list, is an important determinant for its success in the market (McCluskey and Loureiro 2003). A label that includes natural plant extracts as ingredients certainly creates a more natural and beneficial perception of the products. The use of food grade solvents for obtaining phenolic acid fractions allows their direct application in foods, and facilitates their labeling as natural ingredients. Bacteriocins are examples of natural preservatives which are already used. They are labeled as "natural preservatives" in countries of the European Union (Cleveland et al. 2001). Another example is fermented celery extract used in natural and organic cured meat products. It substitutes the addition of nitrites by fermenting nitrate-rich celery extract with a nitrate-reducer culture, making a safe product by inhibiting *Clostridium botulinum* growth (Sebranek et al. 2012; Sullivan et al. 2012).

5.2 Conclusions

Overall, the results of this project are consistent with the hypotheses that differences in the structure of phenolic acids account for variances in their mode of action, the structure of glycoalkaloids affects their antifungal activity, and differences in the chemical structure of plant secondary metabolites allow their recovery and separation. Moreover, the findings of this work provide valuable knowledge for future research trials leading to the successful application of natural plant extracts from food by-products as preservatives, which is highly promising. This will allow the development of natural products of high quality and

acceptance by the consumers while diminishing waste disposal costs and generation of organic solvents waste, which may contribute to the preservation of the environment.

APPENDIX

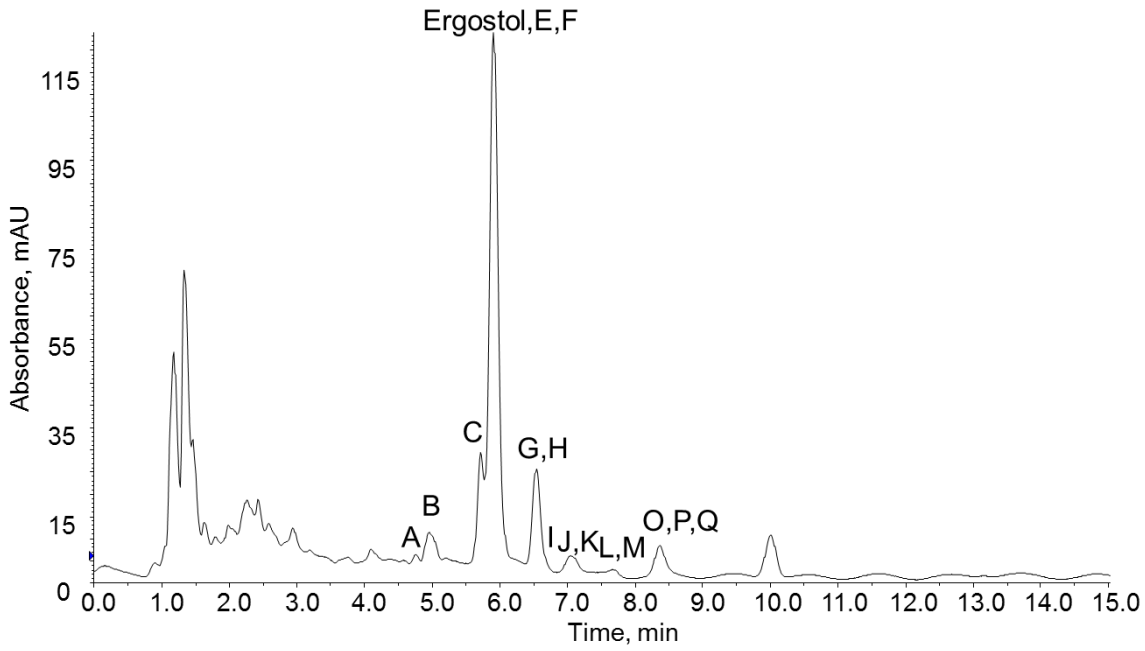


Figure A-1. Chromatogram of the sterols found in *P. roqueforti*, which contains all the sterols present in the strains investigated. The wavelength was 208 nm.

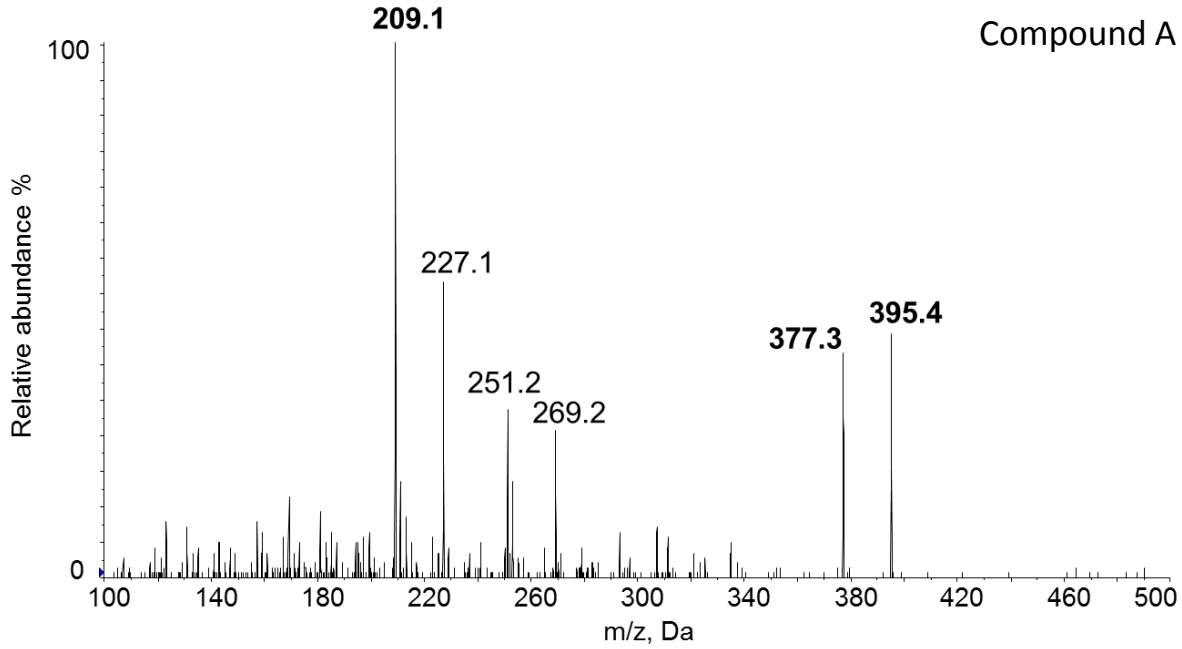


Figure A-2. Mass spectra of the sterols detected in the fungal membranes; compounds are: A, B, C, Ergosterol, E, F, G, H, I, J, K, L, M, N, O and P. The *m/z* in bold were used for the relative quantification in the MRM for the transitions (Q1→Q3), Q1 always being the parent ion.

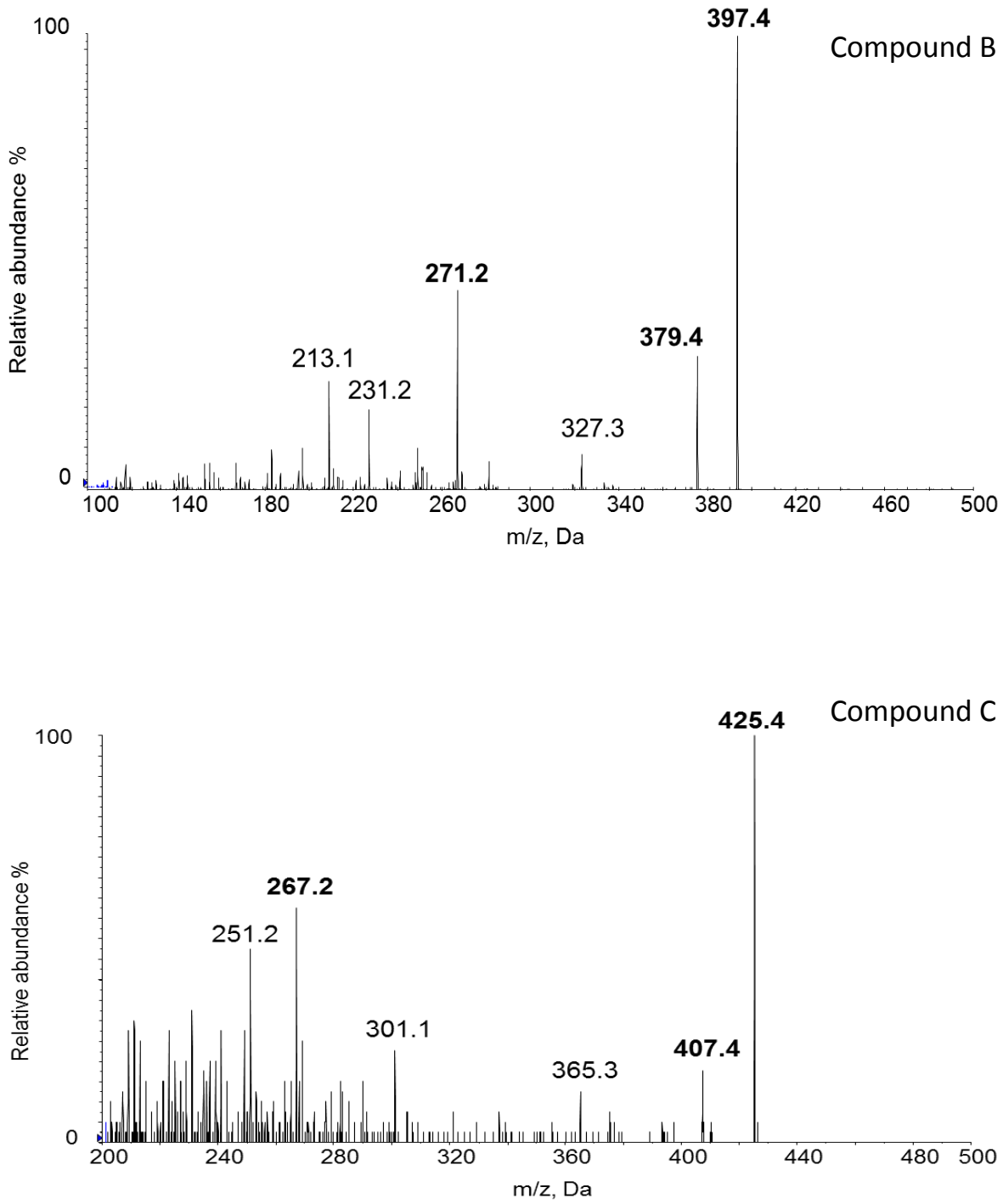


Figure A-2. (Continued).

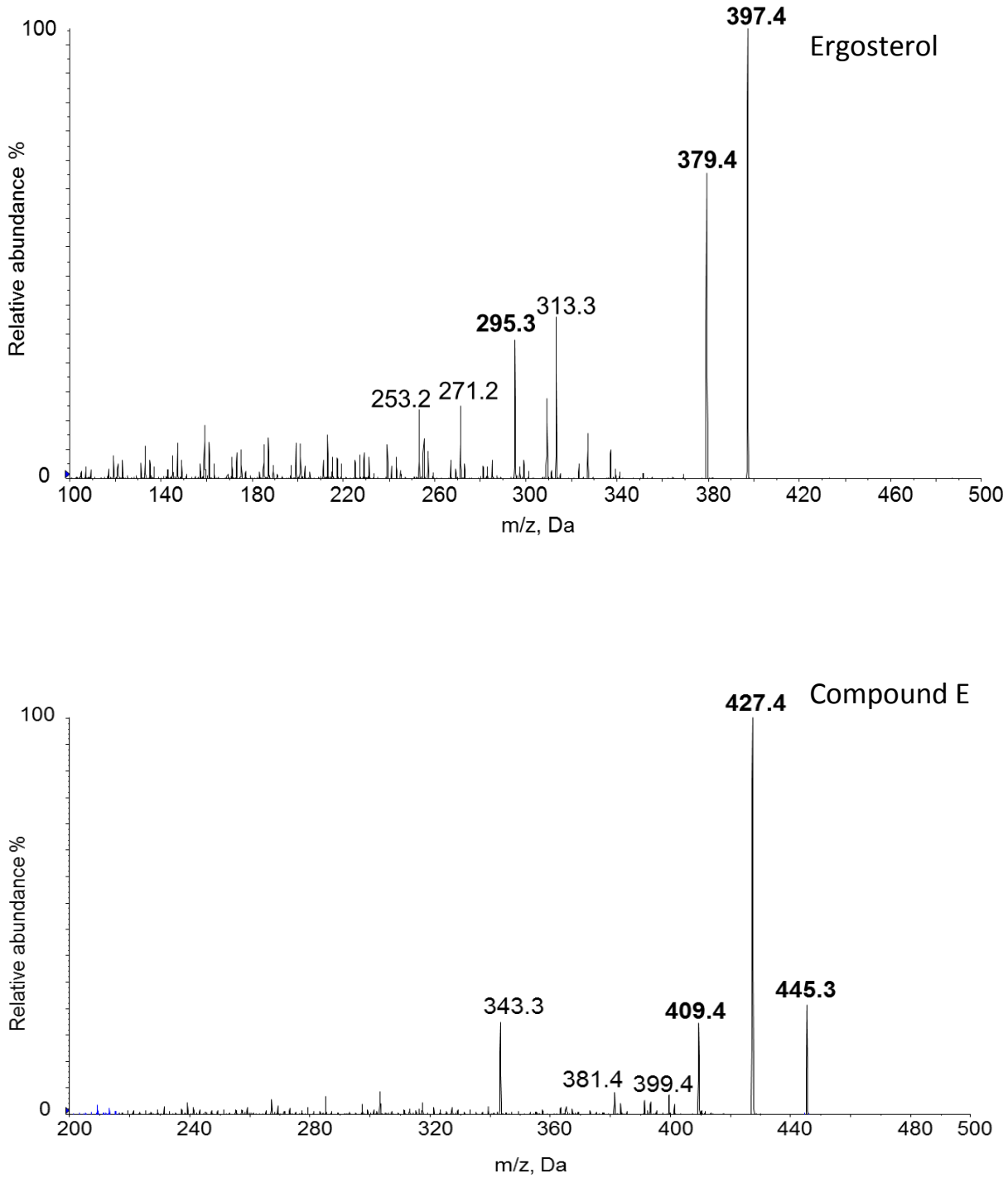


Figure A-2. (Continued).

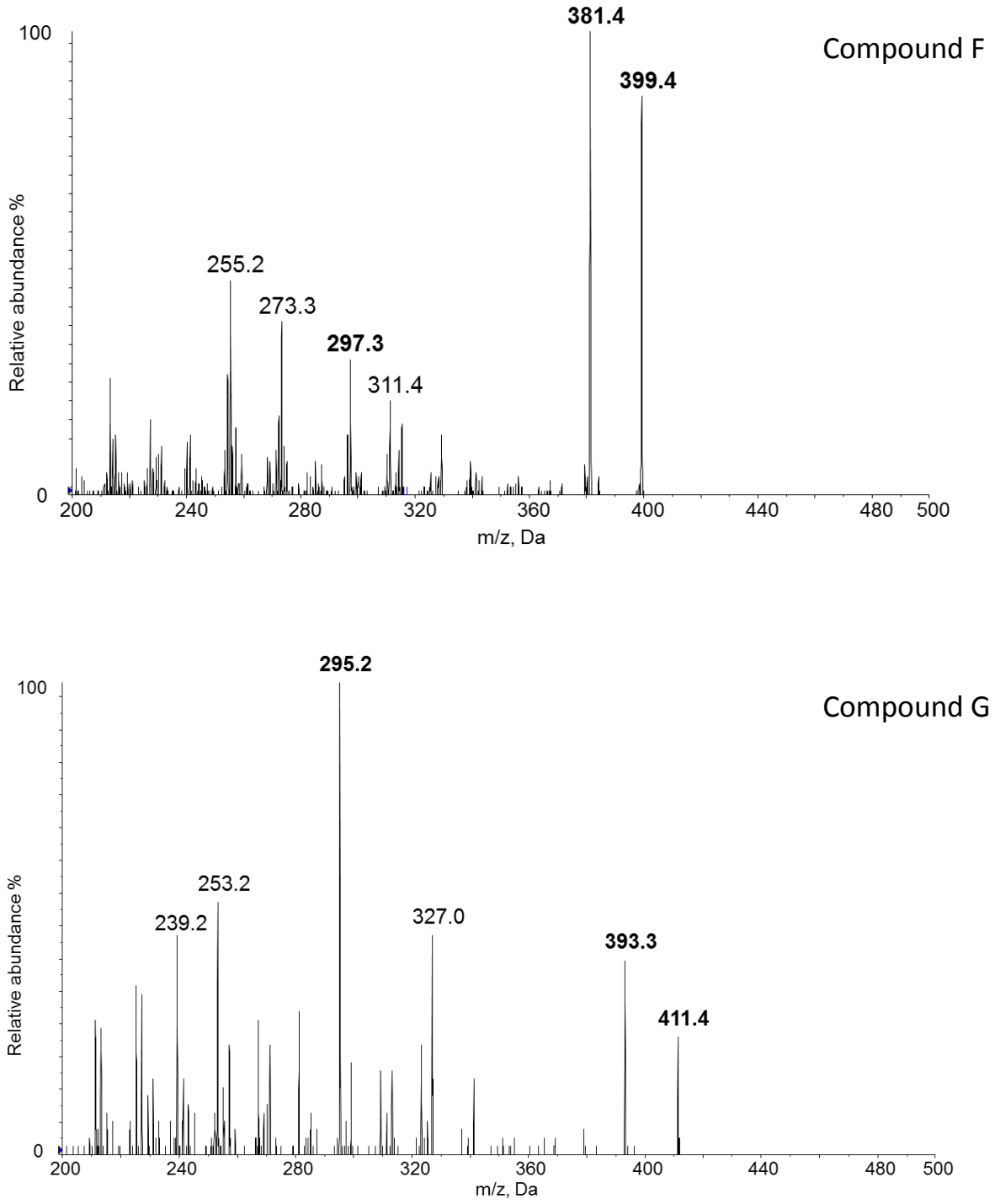


Figure A-2. (Continued).

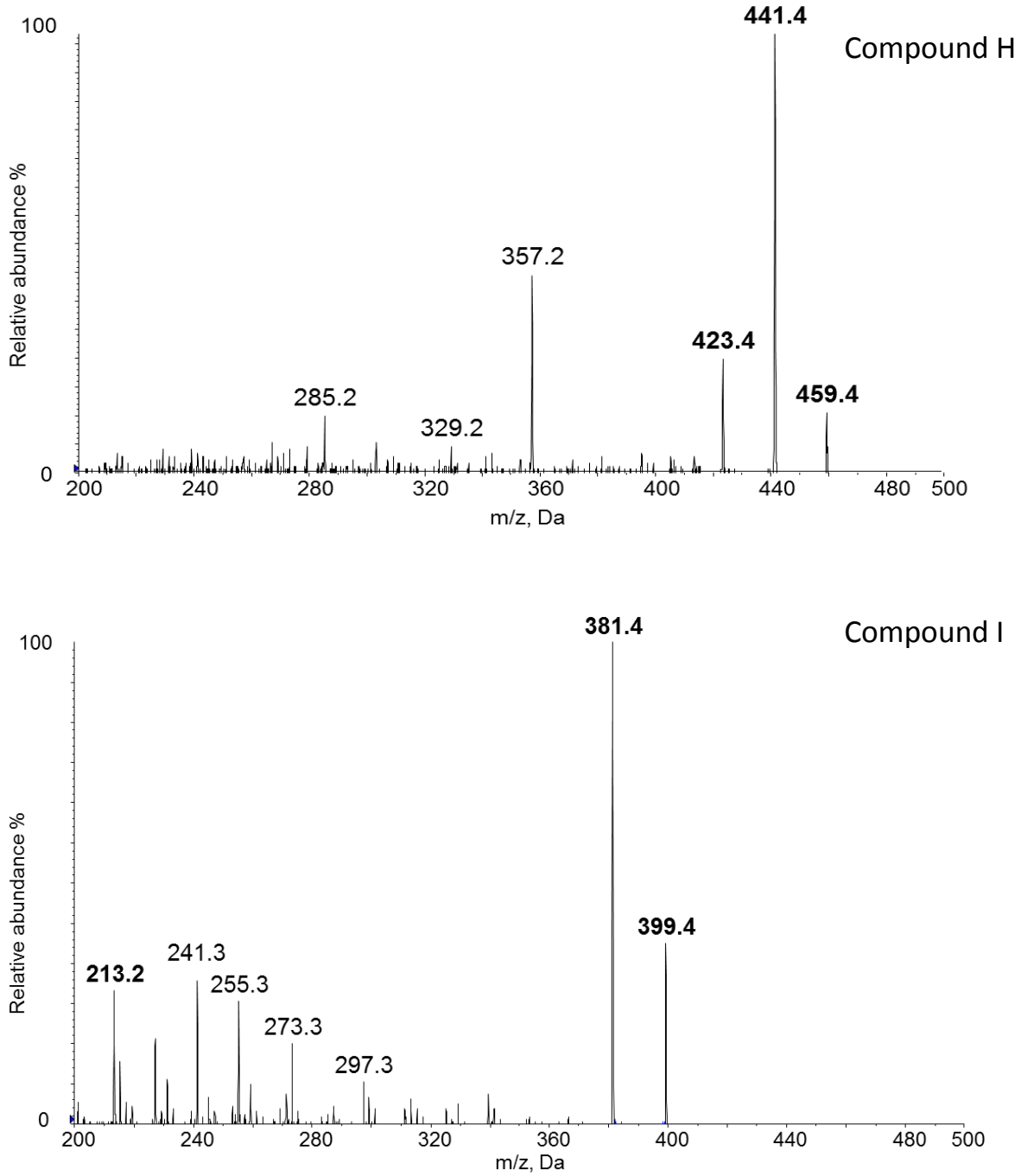


Figure A-2. (Continued).

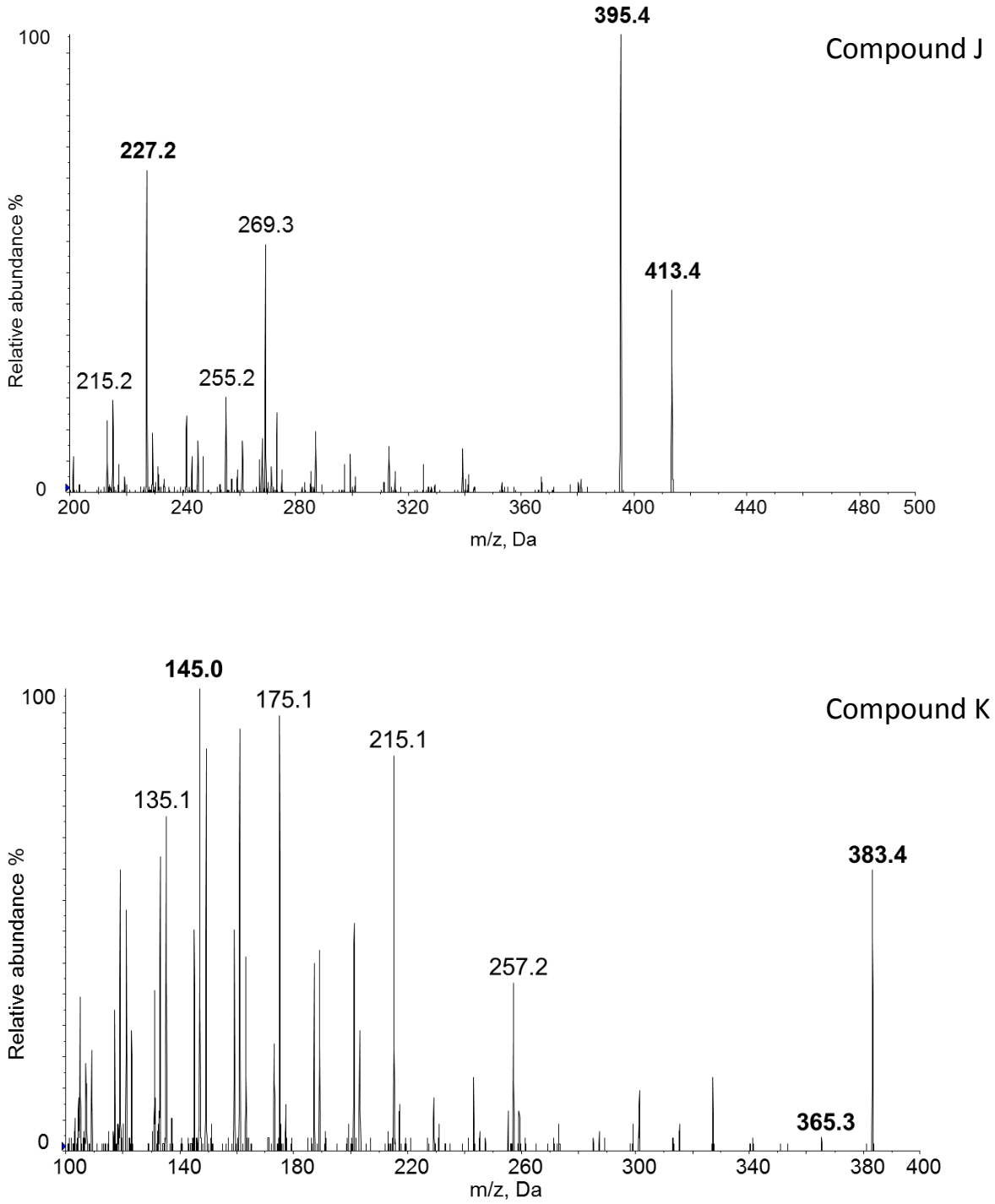


Figure A-2. (Continued).

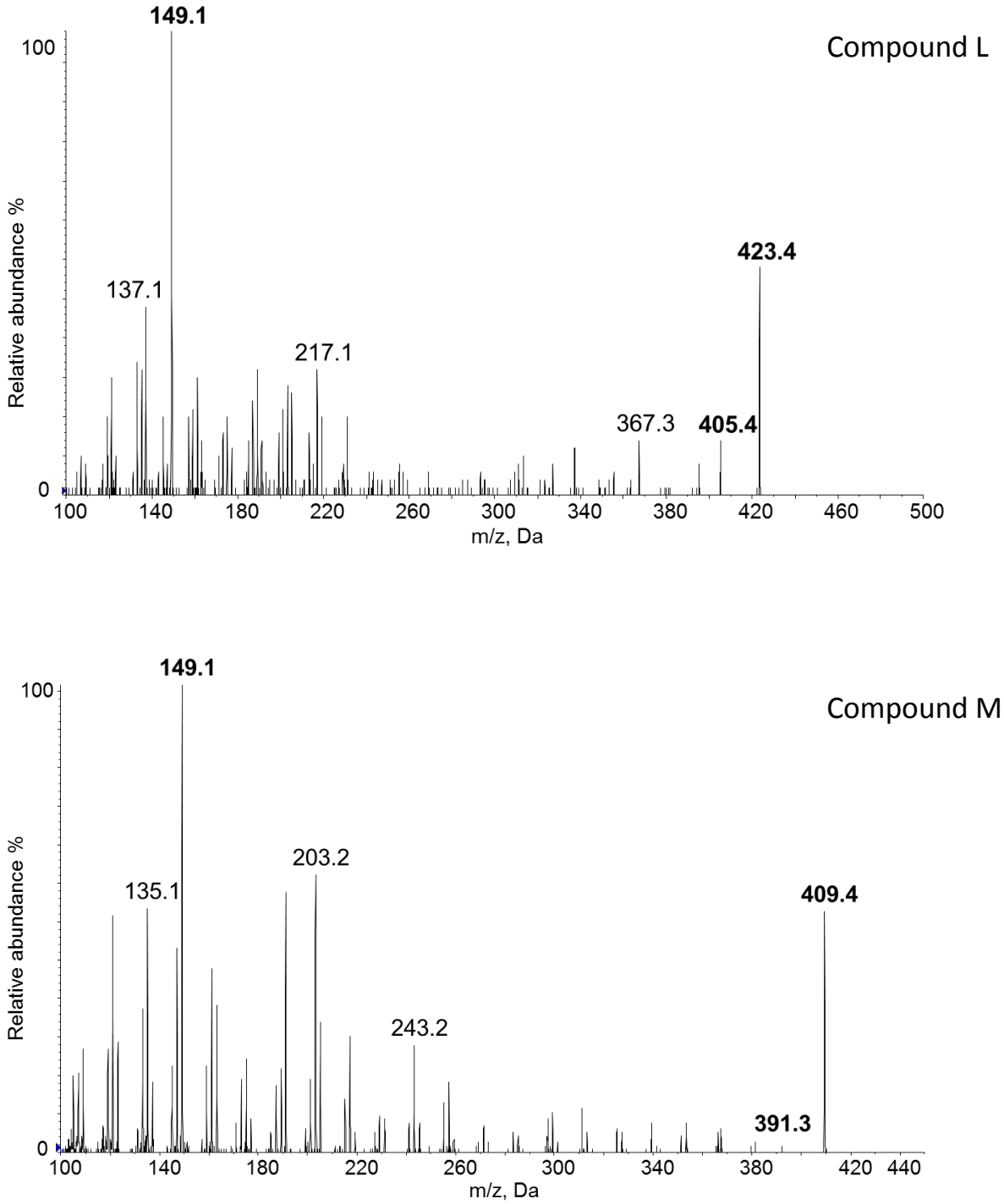


Figure A-2. (Continued).

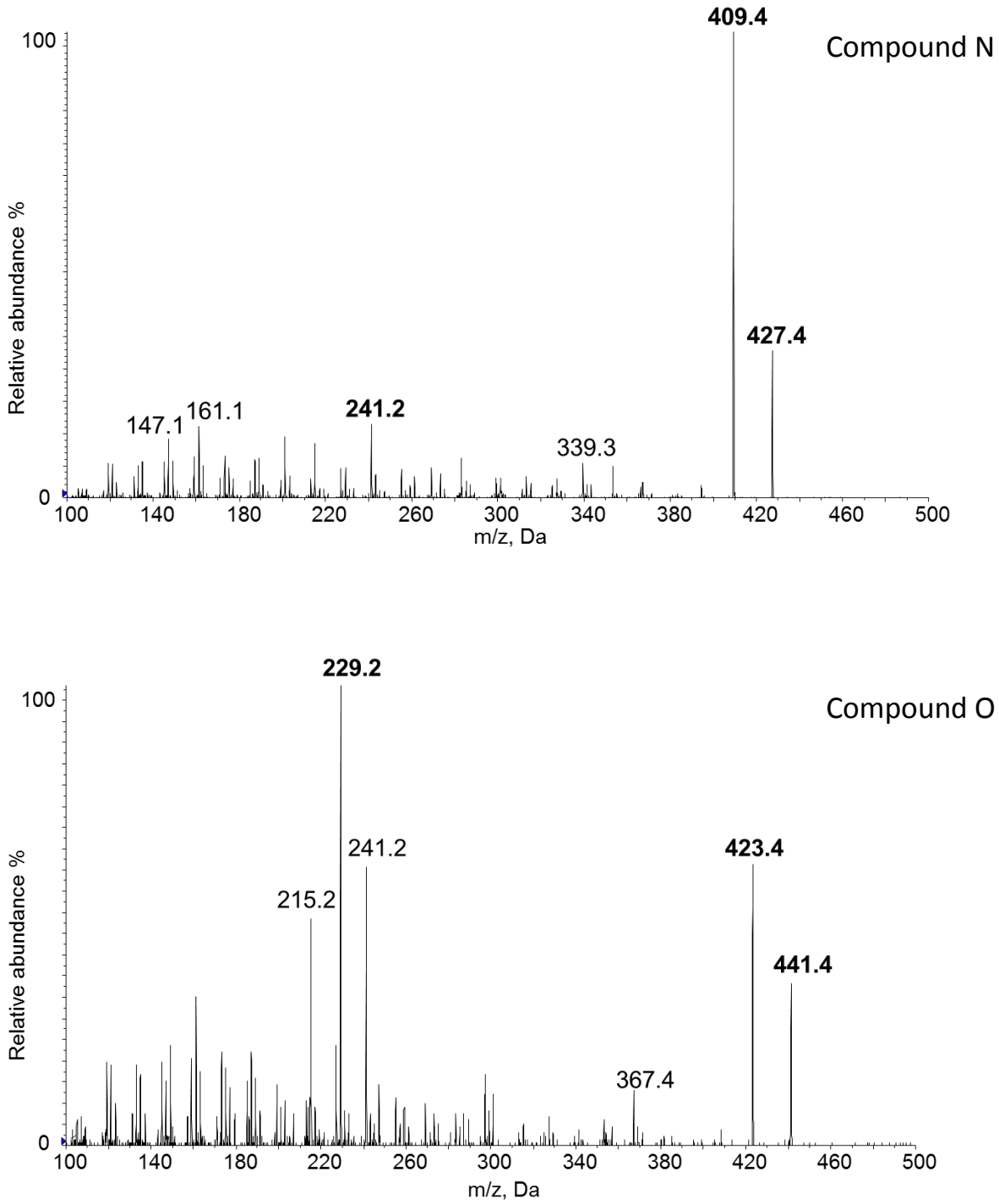


Figure A-2. (Continued).

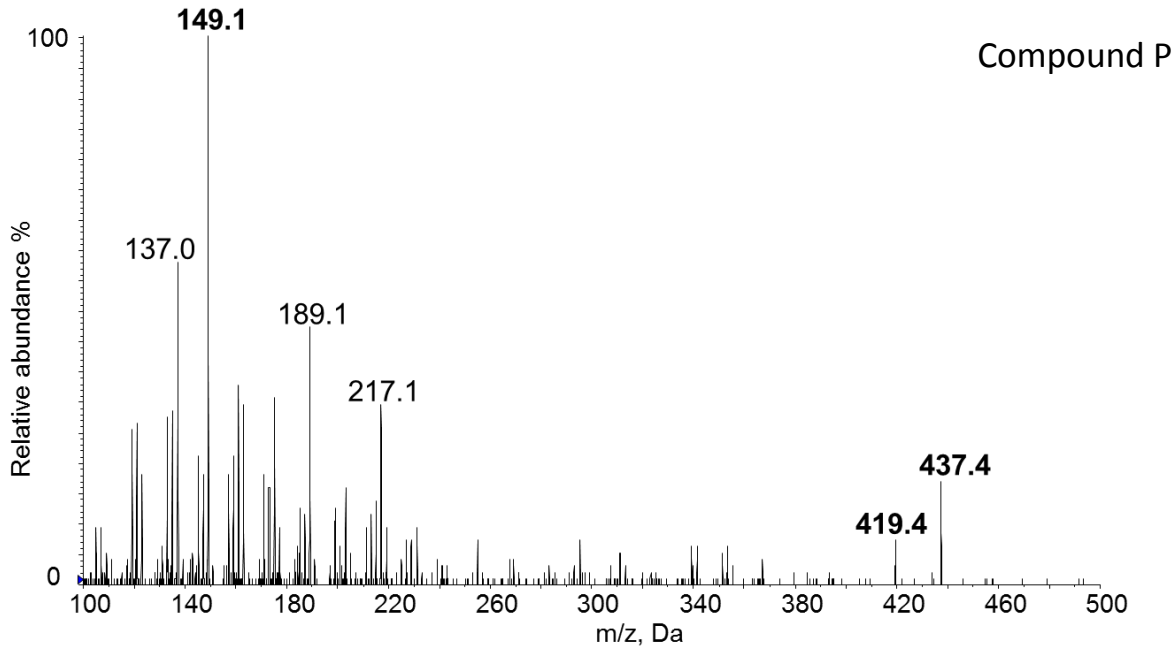


Figure A-2. (Continued).

REFERENCES

Abreu, P., Relva, A., Matthew, S., Gomes, Z., & Morais, Z. (2007). High-performance liquid chromatographic determination of glycoalkaloids in potatoes from conventional, integrated, and organic crop systems. *Food Control, 18*, 40-44.

Adolph, S., Bach, S., Blonde, M., Cueff, A., Moreau, M., Pohnert, G. & Zuccaro, A. (2004). Cytotoxicity of diatom-derived oxylipins in organisms belonging to different phyla. *The Journal of Experimental Biology, 207*, 2935-2946.

Ahuja, I., Kissen, R., & Bones, A. M. (2012). Phytoalexins in defense against pathogens. *Trends in Plant Science, 17*, 73-90.

Albarran, G., Boggess, W., Rassoloy, V. & Schuler, R. H. (2010). Absorption spectrum, mass spectrometric properties, and electronic structure of 1,2-benzoquinone. *Journal of Physical Chemistry 114* , 7470–7478.

Almajano, M. P., Carbo, R., Delgado, M. E., & Gordon, M. H. (2007). Effect of pH on the antimicrobial activity and oxidative stabiliy of oil-in-water emulsions containing caffeic acid. *Journal of Food Science, 72*, 258-263.

Andreau, A., & Feussner, I. (2009). Lipoxygenases-structure and reaction mechanism. *Phytochemistry, 70*, 1504-1510.

Anttila, A., Pirttilä, A. M., Häggman, H., Harju, A., Venäläinen, M., Haapala, A. & Julkunen-Tiitto, R. (2013). Condensed conifer tannins as antifungal agents in liquid culture. *Holzforschung, 67*, 825-832.

Avis, T. J., & Belanger, R. B. (2001). Specificity and mode of action of the antifungal fatty acid cis-9-heptadecenoic acid produced by *Pseudozyma flocculosa*. *Applied and Environmental Microbiology, 67*, 956-960.

Azima, A., Shaikh, H. A., & Ahmad, R. (1982). Effect of feeding greened potatoes on different visceral organs and blood plasma of rabbits. *Journal of the Science of Food and Agriculture, 33*, 1275-1279.

Bagatolli, L. A., Sánchez, S. A., Hazlett, T., & Gratton, E. (2003). Giant vesicles, laurdan, and two photon fluorescence microscopy: Evidence of lipid lateral separation in bilayers. *Methods in Enzymology, 360*, 481-500.

Bak, S., Paquette, S. M., Morant , M., Morant, A. V., Saito, S., Bjarnholt, N. & Møller, B. L. (2006). Cyanogenic glycosides: A case study for evolution and application of cytochromes P450. *Phytochemistry Reviews, 5*, 309-329.

Bakkali, F., Averbeck, S., Averbeck, D., & Idaomar, M. (2008). Biological effects of essential oils – A review. *Food and Chemical Toxicology, 46*, 446-475.

REFERENCES

Balasundram, N., Sundram, K., & Samman, S. (2006). Phenolic compounds in plants and agri-industrial by-products: Antioxidant activity, occurrence, and potential uses. *Food Chemistry, 99*, 191-203.

Balde, A. M., Calomme, M., Pieters, L., Claeys, M., Vanden Berghe, D., & Vlietinck, A. (1991). Structure and antimicrobial activity relationship of doubly-linked procyanidins. *Plant Medica, 57*, A42-A43.

Baranyi, J. & Roberts, T.A. 2000, "Principles and application of predictive modelling of the effects of preservative factors on microorganisms In: T. Ch. 18: 342-358. Ed. Publishers Inc. 2000 " in *The microbiology safety and quality of foods*, eds. B.M. Lund, A.C. Baird-Parker & G.W. Gould, Publishers Inc, USA, pp. 342-358.

Barron, D., & Ibrahim, R. K. (1996). Isoprenylated flavonoids- A survey. *Phytochemistry, 43*, 921-982.

Barthelmebs, L., Divies, C. & Cavin, J.F. (2000). Knockout of the *p*-coumarate decarboxylase gene from *Lactobacillus plantarum* reveals the existence of two other inducible enzymatic activities involved in phenolic acid metabolism. *Applied and Environmental Microbiology 66*, 3368-3375.

Baysala, T., & Demirdoven, A. (2007). Lipoxygenase in fruits and vegetables: A review. *Enzyme and Microbial Technology, 40*, 491-496.

Bellostas, N., Kachlicki, P., Sørensen, J. C., & Sørensen, H. (2007). Glucosinolate profiling of seeds and sprouts of *B. oleracea* varieties used for food. *Scientia Horticulturae*, 1-9.

Bennett, R. N., & Wallsgrove, R. M. (1994). Secondary metabolites in plant defence mechanisms. *New Phytologist, 127*, 617-633.

Benoit, I., Navarro, D., Marnet, N., Rakotomanomana, N., Lesage-Meessen, L., Sigoillot, J. & Asth, M. (2006). Feruloyl esterases as a tool for the release of phenolic compounds from agro-industrial by-products. *Carbohydrate Research, 341*, 1820-1827.

Black, B. A., Zannini, E., Curtis, J. M., & Gänzle, M. G. (2013). Antifungal hydroxy fatty acids produced during sourdough fermentation: Microbial and enzymatic pathways, and antifungal activity in bread. *Applied and Environmental Microbiology, 79*, 1866-1873.

Blankemeyer, J. T., Stringer, B. K., Rayburn, J. R., Bantle, J. A., & Friedman, J. (1992). Effect of potato glycoalkaloids, α-chaconine and α-solanine, on membrane potential of frog embryos. *Journal of Agricultural and Food Chemistry, 40*, 2022-2025.

Blée, E. (2002). Impact of phyto-oxylipins in plant defense. *Trends in Plant Science, 7*, 315-321.

Bourgaud, F., Gravot, A., Milesi, S., & Gontier, E. (2001). Production of plant secondary metabolites: A historical perspective. *Plant Science, 161*, 839-861.

REFERENCES

Brash, A. R. (1999). Lipoxygenases: Occurrence, functions, catalysis, and acquisition of substrate . *Journal of Biological Chemistry, 274*, 23679-23682.

Bravo, H. R., Copaja, S. V., & Lamborot, M. (2013). Chapter 3: Phytotoxicity of phenolic acids from cereals. In A. J. Price, & J. A. Kelton (Eds.), *Herbicides-advances in research* (pp. 37-49). Available online, DOI: http://dx.doi.org/10.5772/55942: Intech Open Science Open Minds.

Bridle, P., & Timberlake, C. F. (1997). Anthocyanins as natural food colours-selected aspects. *Food Chemistry, 58*, 103-109.

Brown, P. D., Tokuhisa, J. G., Reichet, M., & Gershenzon, J. (2003). Variation of glucosinolate accumulation among different organs and developmental stages of *Arabidopsis thaliana*. *Phytochemistry, 62*, 471-481.

Budrat, P., & Shotipruk, A. (2009). Enhanced recovery of phenolic compunds from bitter melon *(Momordica charantia*) by subcritical water extraction. *Separation and Purification Technology, 66*, 125-129.

Burt, S. (2004). Essential oils: Their antibacterial properties and potential applications in foods—a review. *International Journal of Food Microbiology, 94*, 223-253.

Bushway, R. J., & Ponnampalam, R. (1981). α-Chaconine and α-solanine content of potato products and their stability during several modes of cooking. *Journal of Agricultural and Food Chemistry, 29*, 814-817.

Cabañete-Diaz, B., Segura Carretero, A., Fernandez-Gutierrez, A., Belmonte Vega, A., Garrido Frenich, A., Martinez Vidal, J. L., & Duran Martos, J. (2007). Separation and determination of sterols in olive oil by HPLC-MS. *Food Chemistry, 102*, 593-598.

Campos, F. M., Couto, J. A. & Hogg, T. A. (2003). Influence of phenolic acids and inactivation of *Oenococcus oeni* and *Lactobacillus hilgardii. Journal of Applied Microbiology 94*, 167-174.

Campos, F. M., Couto, J. A., Figuereido, A. R., Toth, I. V., Rangel, A.O.S.S. & Hogg, T.A. (2009). Cell membrane damage induced by phenolic acids on wine lactic acids bacteria. *International Journal of Food Microbiology, 135*, 144-151.

Careri, M., Elviri, L., & Mangia, A. (2001). Liquid chromatography-UV determination and liquid chromatography-atmospheric pressure chemical ionization mass spectrometric characterization of sitosterol and stigmasterol in soybean oil. *Journal of Chromatography A, 935*, 249-257.

Carvalho, M. B., Martins, I., Leitão, M. C., Garcia, H., Rodrigues, C., San Romão, V., Silva Pereira, C. (2009). Screening pentachlorophenol degradation ability by environmental

fungal strains belonging to the phyla ascomycota and zygomycota. *Journal of Industrial Microbiology and Biotechnology, 36*, 1249-1256.

Cesco, S., Mimmo, T., Tonon, G., Tomasi, N., Pinton, R., Terzano, R. & Nannipieri, P. (2012). Plant-borne flavonoids released into the rhizosphere: Impact on soil bio-activities related to plant nutrition. A review. *Biology and Fertility of Soils, 48*, 123-149.

Chandran, S., & Singh, R. S. P. (2007). Comparison of various international guidelines for analytical method validation. *Pharmazie, 62*, 4-14.

Charmley, E., Nelson, D., & Zvomuya, F. (2006). Nutrient cycling in the vegetable processing industry utilization of potato by-products. *Canadian Journal of Soil Science, 86*, 621-629.

Chen, X., Mukwaya, E., Wong, M., & Zhang, Y. (2014). A systematic review on biological activities of prenylated flavonoids. *Pharmaceutical Biology, 52*, 655-660.

Chipley, J. R. (2005). Sodium benzoate and benzoic acid. In P. M. Davidson, J. N. Sofos & A. L. Branen (Eds.), *Antimicrobials in food* (Third Edition ed., pp. 11-48). Boca Raton, Florida: CRC Press Taylor & Francis Group.

Chiu, P. E., & Lai, L. S. (2010). Antimicrobial activities of tapioca starch/decolorized hsian-tsao leaf gum coatings containing green tea extracts in fruit-based salads, romaine hearts and pork slices. *International Journal of Food Microbiology 1, 139*, 23-30.

Cho, J., Sohn, M., Lee, J., & Kim, W. G. (2010). Isolation and identification of pentagalloylglucose with broad-spectrum antibacterial activity from *Rhus trichocarpa* Miquel. *Food Chemistry 123 (2010) 501–506, 123*, 501-506.

Cho, Y., & Anh, H. K. (1999). Purification and characterization of polyphenol oxidase from potato: I. purification and properties. *Journal of Food Biochemistry, 23*, 577-592.

Cipollini, M. L., & Levey, D. J. (1997). Antifungal activity of *Solanum* fruit glycoalkaloids: Implications for frugivory and seed dispersal. *Ecology, 78*, 799-809.

Cleveland, J., Montville, T. J., Nes, I. F., & Chikindas, M. L. (2001). Bacteriocins: Safe, natural antimicrobials for food preservation. *International Journal of Food Microbiology, 71*, 1-20.

Clifford, M. N. (1999). Chlorogenic acids and other cinnamates nature, occurrence and dietary burden. *Journal of the Science of Food and Agriculture, 79*, 362-372.

Clifford, M. N., Johnson, K. L., Knight, S., & Kuhnert, N. (2003). Hierarchical scheme for LC-MS^n identification of chlorogenic acids. *Journal of Agricultural and Food Chemistry, 51*, 2900-2911.

REFERENCES

Constabel, C. P., Yip, L., Patton, J. J., & Christopher, M. E. (2000). Polyphenol oxidase from hybrid poplar, cloning and expression in response to wounding and herbivory. *Plant Physiology, 124*, 285-296.

Corrales, M., Han, J.H. & Tauscher, B. (2009). Antimicrobial properties of grape seed extracts and their effectiveness after incorporation into pea starch films. *International Journal of Food Science and Technology 44*, 425-433.

Coteau, D., Mc Cartney, A. L., Gibson, G. R., Williamson, G. & Faulds, C. B. (2001). Isolation and characterization of human colon bacteria able to hydrolyse chlorogenic acid. *Journal of Applied Microbiology 90*, 873-881.

Crepin, V. F., Faulds, C. B., & Connerton, I. F. (2004). Functional classification of the microbial feruloyl esterases. *Applied Microbiology and Biotechnology, 63*, 647-652.

Cruz, J., Novak, O., & Strnad, M. (2008). Rapid analysis of phenolic acids in beverages by UPLC-MS/MS. *Food Chemistry, 111*, 789-794.

Cueva, C., Moreno-Arribas, M. V., Martinez-Alvarez, P. J., Bills, G., Vicente, M. F., Basilio, A., Lopez Rivas, C., Requena, T., Rodriguez, J. M. & Bartolome, B. (2010). Antimicrobial activity of phenolic acids against commensal, probiotic and pathogenic bacteria. *Research in Microbiology, 161*, 372-382.

Curiel, J.A., Rodriguez, H., Landete J. M., De las Rivas, B. & Munoz, R. (2010). Ability of *Lactobacillus brevis* to degrade food phenolic acids. *Food Chemistry*, 120, 225-229.

Cushnie, T. P. T., & Lamb, A. J. (2005). Antimicrobial activity of flavonoids. *International Journal of Antimicrobial Agents, 26*, 343-356.

Cushnie, T. P. T., & Lamb, A. J. (2011). Recent advances in understanding the antibacterial properties of flavonoids. *International Journal of Antimicrobial Agents, 38*, 99-107.

Dai, J. & Mumper, R. S. (2010). Plant phenolics: Extraction, analysis and their antioxidant and anticancer properties. *Molecules, 15*, 7313-7352.

De Bruyne, T., Pieters, L., Deelstra, H., & Vlietinck, A. (1999). Condensed vegetable tannins: Biodiversity in structure and biological activities. *Biochemical Systematics and Ecology, 27*, 445-459.

De las Rivas, B., Rodriguez, H., Curiel, J. A., Landete, J. M. & Muñoz, R. (2009). Molecular screening of wine lactic acid bacteria degrading hyroxycinnamic acids. *Journal of Agriculture and Food Chemistry 57*, 490-494.

Desbois, A. P., & Smith, V. J. (2010). Antibacterial free fatty acids: Activities, mechanisms of action and biotechnological potential. *Applied Microbiology and Biotechnology, 85*, 1629-1642.

REFERENCES

Dicko, M. H., Gruppen, H., Traoré, A. S., Voragen, A. G. J., & van Berkel, W. J. H. (2006). Phenolic compounds and related enzymes as determinants of sorghum for food use. *Biotechnology and Molecular Biology Review, 1*, 21-38.

Dixon, R. A. (2001). Natural products and plant disease resistance. *Nature, 411*, 843-847.

Dixon, R. A., & Harrison, M. J. (1994). Early events in the activation of plant defense responses. *Annual Review of Phytopathology, 32*, 479-501.

Dlusskaya, E.A., McMullen, L.M. & Gänzle, M.G. (2011). Characterization of an extremely heat-resistant *Escherichia coli* obtained from a beef processing facility. *Journal of Applied Microbiology 110*, 840–849.

Doohan, F. M., Brennan, J., & Cooke, J. M. (2003). Infuences of climatic factors on fusarium species pathogenic to cereals. *European Journal of Plant Pathology,109*, 755-768.

Dykes, L., & Rooney, L. W. (2007). Phenolic compounds in cereal grains and their health benefits. *Cereal Foods World, 52*, 105-111.

Dzoyem, J. P., Hamamoto, H., Ngameni, B., Tchaleu Ngadjui, B., & Sekimizu, K. (2013). Antimicrobial action mechanism of flavonoids from dorstenia species. *Drug Discoveries & Therapeutics, 7*, 66-72. doi:10.5582/ddt.2013.v7.2.66

Ejechi, B. O., & Akpomedaye, D. E. (2005). Activity of essential oil and phenolic extracts of pepperfruit (*Dennettia tripetala* G. baker; Annonaceae) against some food-borne microorganisms. *African Journal of Biotechnology, 4*, 258-261.

Elegir, G., Kindl, A. & Sadocco, P., Orlandi, M. (2008). Development of antimicrobial cellulose packing through laccase-mediated grafting of phenolic compounds. *Enzyme Microbiology and Technology 43*, 84-92.

Engels, C. (2012). *Structural and functional characterization of plant polyphenols as natural food preservatives.* Universität Hohenheim and University of Alberta, 1-3.

Engels, C., Schieber, A., & Gänzle, M. G. (2010). Fractionation of gallotannins from mango (*Mangifera indica* L.) kernels by high-speed counter-current chromatography and determination of their antibacterial activity. *Journal of Agricultural and Food Chemistry, 58*, 775-780.

Engels, C., Schieber, A., & Gänzle, M. G. (2011). Inhibitory spectra and modes of antimicrobial action of gallotannins from mango kernels (*Mangifera indica* L.). *Applied and Environmental Microbiology, 77*, 2215-2223.

Engels, C., Weiss, A., Carle, R., Schmidt, H., Schieber, A., & Gänzle, M. G. (2012). Effects of gallotannin treatment on attachment, growth, and survival of *Escherichia coli* O157:H7

and *Listeria monocytogenes* on spinach and lettuce. *European Food Research and Technology, 234*, 1081-1090.

Esteban-Torres, M., Reveron, I., Mancheno, J. M., De las Rivas, B., & Muñoz, R. (2013). Characterization of a feruloyl esterase from *Lactobacillus plantarum*. *Applied and Environmental Microbiology, 79*, 5130-5136.

Fahey, J. W., Zalcmann, A. T., & Talalay, P. (2001). The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. *Phytochemistry, 56*, 5-51.

Fahey, J. W., Zhang, Y., & Talalay, P. (1997). Broccoli sprouts: An exceptionally rich source of inducers of enzymes that protect against chemical carcinogens . *Proceedings of the National Academy of Sciences USA, 94*, 10367-10372.

Fao. (2008). Retrieved November/11th, 2013, from ftp://ftp.fao.org/docrep/fao/011/i0500e/i0500e02.pdf

Faulds, C. B., Mandalari, G., Lo Curto, R. B., Bisignano, G., Christakopoulos, P., & Waldron, K. W. (2006). Synergy between xylanases from glycoside hydrolase family 10 and family 11 and a feruloyl esterase in the release of phenolic acids from cereal arabinoxylan. *Applied Microbiology and Biotechnology,71*, 622-629.

Feussner, I., & Wasternack, C. (2002). The lipoxygenase pathway. *Annual Review of Plant Biology, 53*, 275-297.

Fewell, A. M., & Roddick, J. G. (1993). Interactive antifungal activity of the glycoalkaloids α-solanine and α-chaconine. *Phytochemistry, 33*, 323-328.

Fewell, A. M., & Roddick, J. G. (1997). Potato glycoalkaloid impairment of fungal development. *Mycological Research, 101*, 597-603.

Fewell, A. M., Roddick, J. G., & Weissenberg, M. (1994). Interactions between the glycoalkaloids solasonine and solamarginein relation to inhibition of fungal growth. *Phytochemistry, 37*, 1007-1011.

Fraizer, P. J. (1983). Lipid-protein interaction during dough development. In P. J. Barbes (Ed.), *Lipids in cereal technology* (pp. 189-212). London: Academic Press Inc.

Friedman, M. (2002). Tomato glycoalkaloids: Role in the plant and in the diet. *Journal of Agricultural and Food Chemistry, 50*, 5751-5780.

Friedman, M. (2004). Analysis of biologically active compounds in potatoes (S*olanum tuberosum*), tomatoes (*lycopersicon esculentum*), and jimson weed (*Datura stramonium*) seeds. *Journal of Chromatography A, 1054*143-155.

REFERENCES

Friedman, M. (2006). Potato glycoalkaloids and metabolites: Role in the plant and in the diet. *Journal of Agricultural and Food Chemistry, 54*, 8655-8681.

Friedman, M., Kozukue, N., & Harden, L. A. (1998). Preparation and characterization of acid hydrolysis products of the tomato glycoalkaloid α-tomatine.*Journal of Agricultural and Food Chemistry, 46*, 2096-2101.

Friedman, M., Roitman, J. N., & Kozukue, N. (2003). Glycoalkaloid and calystegine contents in eight potato cultivars. *Journal of Agricultural and Food Chemistry,51*, 2964-2973.

Fujino, Y., & Ohnishi, M. (1978). Characterization and composition of sterols in the free and esterified sterol fractions of *Aspergillus oryzae*. *Lipids, 14*, 663-668.

Funatogawa, K., Hayashi, S., Shimomura, H., Yoshida, T., Hatano, T., Ito, H., & Hirai, Y. (2004). Antibacterial activity of hydrolyzable tannins derived from medicinal plants against *Helicobacter pylori*. *Microbiology and Immunology, 48*, 251-261.

Gänzle, M. G., Hertel, C. & Hammes, W. P. (1996). Antimicrobial activity of bacteriocin-producing cultures in meat products: modelling of the effect of pH, NaCl, and nitrite concentrations on the antimicrobial activity of sakacin P against *Listeria ivanovii* DSM20750. *Fleischwirtschaft, 76*, 409-412.

García, S., García, C., Heinzen, H., & Moyna, P. (1997). Chemical basis of the resistance of barley seeds to pathogenic fungi. *Phytochemistry, 44*, 415-418.

Gatto, M. A., Ippolito, A., Linsalata, V., Cascarano, N. A., Nigro, F., Vanadia, S., & Di Venere, D. (2011). Activity of extracts from wild edible herbs against postharvest fungal diseases of fruit and vegetables. *Postharvest Biology and Technology, 61*, 72-82.

Gill, I., & Valivety, R. (1997). Polyunsaturated fatty acids, part 1: Occurrence, biological activities and applications. *Trends in Biotechnology, 15*, 401-409.

Giusti, M. M., & Wrolstad, R. E. (2003). Acylated anthocyanins from edible sources and their applications in food systems. *Biochemical Engineering Journal, 14*, 217-225.

Gleadow, R. M., & Woodrow, I. E. (2002). Constraints on effectiveness of cyanogenic glycosides in herbivore defense. *Journal of Chemical Ecology, 28*, 1301-1313.

Grandmougin-Ferjania, A., Dalpe, Y., Hartmannc, M. A., Laruellea, F., & Sanchollea, M. (1999). Sterol distribution in arbuscular mycorrhizal fungi. *Phytochemistry, 50*, 1027-1031.

Graveland, A. (1968). Combination of thin layer chromatography and gas chromatography in the analysis on a microgram scale of lipids from wheat flour and wheat flour doughs. *Journal of the American Oil Chemists' Society, 45*, 834-840.

REFERENCES

Grubb, C. D., & Abel, S. (2006). Glucosinolate metabolism and its control. *Trends in Plant Science, 11*, 89-100.

Gruhnert, C., Biehl, B., & Selmar, D. (1994). Compartmentation of cyanogenic glucosides and their degrading enzymes. *Planta, 195*, 36-42.

Halkier, B. A., & Gershenzon, J. (2006). Biology and biochemistry of glucosinolates. *Annual Review of Plant Biology, 57*, 303-333.

Hamberg, M. (1999). An epoxy alcohol synthase pathway in higher plants: Biosynthesis of antifungal trihydroxy oxylipins in leaves of potato. *Lipids, 34*, 1131-1142.

Haraguchi, H., Tanimoto, K., Tamura, Y., Mizutani, K., & Kinoshita, T. (1998). Mode of antibacterial action of retrochalcones from *Glycyrrhiza inflata*. *Phytochemistry, 48*, 125-129.

Hassanpour, S., Maherisis, N., Eshratkhah, B., & Mehmandar, F. B. (2001). Plants and secondary metabolites (tannins): A review. *International Journal of Forest, Soil and Erosion, 1*, 47-53.

Headly, J. V., Peru, K. M., Verma, B., & Robarts, R. D. (2002). 1. Mass spectrometric determination of ergosterol in a prairie natural wetland. *Journal of Chromatography A*, (958), 149-156.

Helmja, K., Vaher, M., Gorbatšova, J., & Kaljurand, M. (2007). Characterization of bioactive compounds contained in vegetables of the solanaceae family by capillary electrophoresis. *Proceedings of the Estonian Academy of Sciences, 56*, 172-186.

Herald, P.J. & Davidson, P. M. (1983). Antibacterial activity of selected hydroxycinnamic acids. *Journal of Food Science 48*,1378-1379.

Himejima, M., & Kubo, I. (1991). Antibacterial agents from the cashew *Anacardium occidentale* (Anacardiaceae) nut shell oil. *Journal of Agricultural and Food Chemistry, 39*, 418-421.

Hofstetter, S., Denter, C., Winter, R., McMullen, L. M., & Gänzle, M. G. (2012). Use of the fluorescent probe LAURDAN to label and measure inner membrane fluidity of endospores of *Clostridium* spp. *Journal of Microbiological Methods, 91*, 93-100.

Holley, R. A., & Patel, D. (2005). Improvement in shelf-life and safety of perishable foods by plant essential oils and smoke antimicrobials. *Food Microbiology, 22*, 273-292.

Hornung, E., Kunze, S., Liavonchanka, A., Zimmermann, G., Kuhn, D., Fritsche, K., Feussner, I. (2008). Identification of an amino acid determinant of pH regiospecificity in a seed lipoxygenase from *Momordica charantia*. *Phytochemistry, 69*, 2774-2780.

REFERENCES

Howe, G. A., & Jander, G. (2008). Plant immunity to insect herbivores. *Annual Review of Plant Biology, 59,* 41-66.

Huo, F., & Liu, H. (2010). Fragmentation investigation of brassinosteroid compounds by ion trap and quadrupole time-of-flight mass spectrometry. *Rapid Communications in Mass Spectrometry, 24,* 3325-3334.

Ibañez, E., Kubátová, A., Señoráns, F. J., Cavero, S., Reglero, G., & Hawthorne, S. B. (2003). Subcritical water extraction of antioxidant compounds from rosemary plants. *Journal of Agricultural and Food Chemistry, 51,* 375-382.

Im, H. W., Suh, B. S., Lee, S. U., Kozukue, N., Ohnisi-Kameyama, M., Levin, C. E., & Friedman, M. (2008). Analysis of phenolic compounds by high-performance liquid chromatography and liquid chromatography/mass spectrometry in potato plant flowers, leaves, stems, and tubers and in home-processed potatoes. *Journal of Agricultural and Food Chemistry, 56,* 3341-3349.

Ishii, T. (1997). Structure and functions of feruloylated polysaccharides. *Plant Science, 127,* 111-127.

Isshiki, K., Tokuoka, K., Mori, R., & Chiba, S. (1992). Preliminary examination of allyl isothiocyanate vapor for food preservation. *Bioscience, Biotechnology, and Biochemistry, 56,* 1476-1477.

Jäger, A. K., Van Staden, J., & McGaw, L. J. (2002). Antibacterial effects of fatty acids and related compounds from plants. *South African Journal of Botany,68,* 417-423.

Johnson, I. T. (2002). Glucosinolates in the human diet, bioavailability and implications for health. *Phytochemistry Reviews, 1,* 183-188.

Junqueira, R. M., Cocato, M. L., Colli, C., & Castro, I. A. (2008). Synergism between lipoxygenase-active soybean flour and ascorbic acid on rheological and sensory properties of wheat bread. *Journal of the Science of Food and Agriculture, 88,* 194-198.

Kannat, S., Chander, R., Radhakrishna, P., & Sharma, A. (2005). Potato peel extracts, a natural antioxidant for retarding lipid peroxidation in radiation processed lamb meat. *Journal of Agricultural and Food Chemistry, 53,* 1499-1504.

Kemmo, S., Ollilainen, V., Lampi, A. M., & Piironen, V. (2007). Determination of stigmasterol and cholesterol oxides using atmospheric pressure chemical ionization liquid chromatography/mass spectrometry. *Food Chemistry, 101,* 1438-1445.

Keukens, E. A. J., De Vrije, T., Fabrie, C. H. J. P., Demel, R. A., Jongen, W. M. F., & De Kruijff, B. (1992). Dual specificity of sterol-mediated glycoalkaloid induced membrane disruption. *Biochimica et Biophysica, 1110,* 127-136.

REFERENCES

Keukens, E. A. J., De Vrije, T., Van den Boom, C., De Waard, P., Plasman, H. H., Thiel, F. De Kruijff, B. (1995). Molecular basis of glycoalkaloids induced membrane disruption. *Biochimica et Biophysica Acta, 1240,* 216-228.

Khiari, Z., Makris, D. P., & Kefalas, P. (2009). An investigation on the recovery of antioxidant phenolics from onion solid wastes employing water/ethanol-based solvent systems. *Food and Bioprocess Technology, 2,* 337-343.

Kim, K. H., Tsao, R., Yang, R., & Cui, S. W. (2006). Phenolic acid profiles and antioxidant activities of wheat bran extracts and the effect of hydrolysis conditions. *Food Chemistry, 95,* 466-473.

Kim, M.-H., & Kim, J.-W. (2010). Comparison through a LCA evaluation analysis of food waste disposal options from the perspective of global warming and resource recovery. *Science of the Total Environment, 408,* 3998-4006.

Kim, Y. S., Ahn, E. S., & Shin, D. H. (2002). Extension of shelf life by treatment with allyl isothiocyanate in combination with acetic acid on cooked rice. *Journal of Food Science, 67,* 274-279.

Kozubek, A., & Tyman, J. H. P. (1999). Resorcinolic lipids, the natural non-isoprenoid phenolic amphiphiles and their biological activity. *Chemical Reviews,99,* 1-24.

Kozubek, A., Zarnowski, R., Stasiuk, M., & Gubernator, J. (2001). Natural amphiphilic phenols as bioactive compounds. *Cellular and Molecular Biology Letters,6*(2A), 351-355.

Kraal, J. H., Hussain, A. A., Gregorio, S. B., & Akaho, E. (1979). Exposure time and the effect of hexylresorcinol on bacterial aggregates . *Journal of Dental Research, 58*), 2125-2131.

Krygier, K., Sosulsky, F., & Hogge, L. (1982). Free, esterified and insoluble-bound phenolic acids. 1. extraction and purification procedure. *Journal of Agricultural and Food Chemistry, 30,* 330-334.

Kubo, I., Nihei, K., & Tsujimoto, K. (2003). Antibacterial action of anacardic acids against methicillin resistant *Staphylococcus aureus* (MRSA). *Journal of Agricultural and Food Chemistry, 51,* 7624-7628.

Lamari, L., & Bernier, C. C. (1989). Evaluation of wheat lines and cultivars to tan spot *(Pyrenophora tritici-repentis)* based on lesion type. *Canadian Journal of Plant Pathology, 11,* 49-56.

Landete, J. M., Curiel, J. A., Rodriguez, H., De las Rivas, B. & Muñoz, R. (2008). Study of the inhibitory activity of phenolic compounds found in olive products and their degradation by *Lactobacillus plantarum* strains. *Food Chemistry 107,* 320-326.

REFERENCES

Lee, H. C., Jenner, A. M., Low, C.S. & Lee, Y. K. (2006). Effect of tea phenolics and their aromatic fecal bacteria metabolites on intestinal microbiota. *Research in Microbiology 157*, 876-884.

Lee, J., & Finn, C. E. (2007). Anthocyanins and other polyphenolics in American elderberry (*Sambucus canadensis*) and European elderberry (*S. nigra*) cultivars. *Journal of the Science of Food and Agriculture, 87*, 2665-2675.

Lemaya, M. J., Choquettea, J., Delaquis, P. J., Gariepy, C., Rodrigue, N., & Saucier, L. (2002). Antimicrobial effect of natural preservatives in a cooked and acidified chicken meat model. *International Journal of Food Microbiology, 78*, 217-226.

Li, L., & Steffens, J. C. (2002). Overexpression of polyphenol oxidase in transgenic tomato plants results in enhanced bacterial disease resistance. *Planta,215*, 239-247.

Li, L., Lee, W., Lee, W. J., Auh, J. H., Kim, S. S., & Yoon, J. (2010). Extraction of allyl isothiocyanate from wasabi (*Wasabia japonica* Matsum) using supercritical carbon dioxide. *Food Science and Biotechnolgy, 19*, 405-410.

Li, Y., Wijesekara, I., Li, Y., & Kim, S. (2011). Phlorotannins as bioactive agents from brown algae. *Process Biochemistry, 46*, 2219-2224.

Lin, C., Preston, J. F., & Wei, C. (2000). Antibacterial mechanism of allyl isothiocyanate. *Journal of Food Protection, 63*, 727-734.

Liu, J., Cao, T., Feng, J., Chang, K., Hwang, S., & Strelkov, S. E. (2013). Characterization of the fungi associated with ascochyta blight of field pea in Alberta, Canada. *Crop Protection, 54*, 55-64.

Liu, S., Ruan, W., Li, J., Xu, H., Wang, J., Gao, Y., & Wang, J. (2008). Biological control of phytopathogenic fungi by fatty acids. *Mycopathologia, 166*, 93-102.

Luciano, F. B., & Holley, R. A. (2009). Enzymatic inhibition by allyl isothiocyanate and factors affecting its antimicrobial action against *Escherichia coli* O157:H7. *International Journal of Food Microbiology, 131*, 240-245.

Luciano, F. B., Hosseinian, F. S., Beta, T., & Holley, R. A. (2008). Effect of free-SH containing compounds on allyl isothiocyanate antimicrobial activity against *Escherichia coli* O157:H7. *Journal of Food Science, 73*, M214-M220.

Maillard, M. N., & Berset, C. (1995). Evolution of antioxidant activity during kilning: Role of insoluble bound phenolic acids of barley and malt. *Journal of Agricultural and Food Chemistry, 43*, 1789-1793.

Manach, C., Scalbert, A., Morand, C., Remesy, C., & Jimenez, L. (2004). Polyphenols: Food sources and bioavailability. *American Journal of Clinical Nutrition, 79*, 727-747.

REFERENCES

Mandal, S. M., Chakraborty, D., & Dey, S. (2010). Phenolic acids act as signaling molecules in plant-microbe symbioses. *Plant Signaling and Behavior, 5*, 359-368.

Manohan , D., & Wai, W. C. (2012). Characterization of polyphenol oxidase in sweet potato (I*pomoea batatas* (L.). *Journal for the Advancement of Science & Arts, 3*, 14-31.

Mari, M., Leoni, O., Lori, R., & Cembali, T. (2002). Antifungal vapour-phase activity of allyl-isothiocyanate against *Penicillium expansum* on pears. *Plant Pathology, 51*, 231-236.

Marriot, M. S. (1980). Inhibition of sterol byosynthesis in *Candida albicans* by imidazole-containing antifungals. *Journal of General Microbiology, 117*, 253-255.

Masuda, H., & Naohideb, K. (2004). Bactericidal activity of wasabi (*Wasabia japonica*) against *Helicobacter pylori* il shik shina. *International Journal of Food Microbiology, 94*, 255-261.

Masui, H., Kondo, T., & Kojima, M. (1989). An antifungal compound 9,12,13-trihydroxy-(E)-10-octadecenoic acid, from *Colocasia antiquorum* inoculated with C*eratocistys fimbriata*. *Phytochemistry, 28*, 2613-2615.

Mathew, S., & Abraham, E. (2004). Ferulic acid: An antioxidant found naturally in plant cell walls and feruloyl esterases involved in its release and their applications. *Critical Reviews in Biotechnology, 24*, 59-83.

Matsui, Y., Nakamura, S., Kondou, N., Takasu, Y., Ochiai, R., & Masukawa, Y. (2007). Liquid chromatography-electrospray ionization-tandem mass spectrometry for simultaneous analysis of chlorogenic acids and their metabolites in human plasma. *Journal of Chromatography B, 858*, 96-105.

Mattila, P., & Hellstrom, J. (2007). Phenolic acids in potatoes, vegetables and some of their products. *Journal of Food Composition Analysis, 20*, 152-160.

Mattila, P., & Kumpulainen, J. (2002). Determination of free and total phenolic acids in plant derived foods by HPLC with diode-array detection. *Journal of Agricultural and Food Chemistry, 50*, 3660-3667.

Mayer, A. M. (2006). Polyphenol oxidases in plants and fungi: Going places? A review. *Phytochemistry, 67*, 2318-2331.

Mayer, R., Stecher, G., Wuerzner, R., Colonia Silva, R., Sultana, T., Trojer, L. & Bonn, G. K. (2008). Proanthocyanidins: Target compounds as antibacterial agents. *Journal of Agricultural and Food Chemistry, 56*, 6959-6966.

McCluskey, J. J., & Loureiro, M. L. (2003). Consumer preferences and willingness to pay for food labeling: A discussion of empirical studies. *Journal of Food Distribution Research, 34*, 95-102.

REFERENCES

McIntosh, A. L., Atshaves, B. P., Huang, H., Gallegos, A. M., Kier, A. B., & Schroeder, F. (2008). Fluorescence techniques using dehydroergosterol to study cholesterol trafficking. *Lipids, 43*, 1185-1208.

Mejanelle, L., Lopez, J. F., Gunde-Cimerman, N., & Grimalt, J. O. (2000). Sterols of melanized fungi from hypersaline environments. *Organic Geochemistry, 31*, 1031-1040.

Merkl, R., Hrádková, I., Filip, V. & Šmidrkal, J. (2010). Antimicrobial and antioxidant properties of phenolic acids alkyl esters. *Czech Journal of Food Science 28*, 275–279.

Mezine, I., Zhang, H., Macku, C., & Lijana, R. (2003). Analysis of plant sterol and stanol esters in cholesterol-lowering spreads and beverages using high-performance liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry. *Journal of Agricultural and Food Chemistry, 51*, 5639-5646.

Milner, S. E., Brunton, N. P., Jones, P. W., O' Brien, N. M., Collins, S. G., & Maguire, A. R. (2011). Bioactivities of glycoalkaloids and their aglycones from *Solanum* species. *Journal of Agricultural and Food Chemistry, 59*, 3454-3484.

Mo, S., Dong, L., Hurst, W. J., & Van Breemen, R. B. (2013). Quantitative analysis of phytosterols in edible oils using APCI liquid chromatography–tandem mass spectrometry. *Lipids, 48*, 949-956.

Mohn, T., Cutting, B., Ernst, B., & Hamburger, M. (2007). Extraction and analysis of intact glucosinolates—A validated pressurized liquid extraction/liquid chromatography–mass spectrometry protocol for *Isatis tinctoria*, and qualitative analysis of other cruciferous plants. *Journal of Chromatography A, 1166*, 142-151.

Molina-Höppner, A., Doster, W., Vogel, R. F., & Gänzle, M. G. (2004). Protective effect of sucrose and sodium chloride for *Lactococcus lactis* during sublethal and lethal highpressure treatments. *Applied and Environmental Microbiology, 70*, 2013-2020.

Monti, D., Pisvejcova, A., Kren, V., Lama, M., & Riva, S. (2004). Generation of an α-L-rhamnosidase library and its application for the selective derhamnosylation of natural products. *Biotechnology and Bioengineering, 87*, 763-771.

Mukne, A. P., Viswanathan, V., & Phadatar, A. G. (2001). Structure pre-requisites for isoflavones as effective antibacterial agents. *Pharmacognosy Reviews,5*, 13-18.

Müller, M. M., Kantola, R., & Kitunen, V. (1994). Combining sterol and fatty acid profiles for the characterization of fungi. *Mycological Research, 98*, 593-603.

Muthukumarasamy, P., Han, J. H., & Holley, R. A. (2003). Bactericidal effects of *Lactobacillus reuteri* and allyl isothiocyanate on *Escherichia coli* O157:H7 in refrigerated ground beef. *Journal of Food Protection, 66*, 2038-2044.

REFERENCES

Nandutu, A. M., Clifford, M., & Howell, N. K. (2007). Analysis of phenolic compounds in Ugandan sweet potato varieties (NSP, SPK AND TZ). *African Journal of Biochemistry Research, 1*, 29-36.

Nara, K., Miyoshi, T., Honma, T., & Koga, H. (2006). Antioxidative activity of bound-form phenolics in potato peel. *Bioscience, Biotechnology and Biochemistry,70*, 1489-1491.

Nardini, M., Cirillo, E., Natella, F., Mencarelli, D., Comisso, A., & Scaccini, C. (2002). Detection of bound phenolic acids: Prevention by ascorbic acid ethylenediaminetetraacetic acid of degradation of phenolic acids during alkaline hydrolysis. *Food Chemistry, 79*, 119-124.

Nielsen, P. V., & Rios, R. (2000). Inhibition of fungal growth on bread by volatile components from spices and herbs, and the possible application in active packaging, with special emphasis on mustard essential oil. *International Journal of Food Microbiology, 60*, 219-229.

Nikolic, N. C., & Stankovic, M. Z. (2003). Kinetics of solanidine hydrolytic extraction from potato (*Solanum tuberosum* L.) haulm in solid-liquid systems. *Journal of the Serbian Chemical Society, 68*, 9-16.

Oda, Y., Saito, K., Ohara-Takada, A., & Mori, M. (2002). Hydrolysis of the potato glycoalkaloid a-chaconine by filamentous fungi. *Journal of Bioscience and Bioengeneering, 94*, 321-325.

Olafsdottir, E. S., Jørgensen, L. B., & Jaroszewskic, J. W. (2002). Cyanogenesis in glucosinolate-producing plants: *Carica papaya* and *Carica quercifolia*. *Phytochemistry, 60*, 269-273.

Onyeneho, S. N., & Hettiarachchy, N. S. (1993). Antioxidant activity, fatty acids and phenolic acids compositions of potato peels. *Journal of the Science of Food and Agriculture, 62*, 345-350.

Osbourn, A. E. (1996). Preformed antimicrobial compounds and plant defense against fungal attack. *The Plant Cell, 8*, 1821-1831.

Otto, R. & Conn, J. E. (1944). Effect of increase acidity on antiseptic efficiency. *Industrial and Engineering Chemistry 36*, 2185-187.

Ou, S., Wang, Y., Tang, S., Huang, C., & Jackson, M. G. (2005). Role of ferulic acid in preparing edible films from soy protein isolate. *Journal of Food Engineering,70*, 205-210.

Parasassi, T., Giusti, E., Gratton, E., Monaco, E., Raimond, M., Ravagnan, G., & Sapora, O. (1994). Evidence for an increase in water concentration in bilayers after oxidative damage of phospholipids induced by ionizing radiation *International Journal of Radiation Biology, 65*, 329-334.

REFERENCES

Parasassi, T., Krasnowska, E. K., Bagatolli, L., & Gratton, E. (1998). Laurdan and prodan as polarity-sensitive fluorescent membrane probes. *Journal of Fluorescence, 8*, 365-373.

Parente, E., Brienza, C., Moles, M. & Ricciardi, A. (1995). A comparison of methods for the measurement of bacteriocin activity. *Journal of Microbiology Methods 22*, 95-108.

Park, C. M., Taormina, P. J., & Beuchat, L. R. (2000). Efficacy of allyl isothiocyanate in killing enterohemorrhagic *escherichia coli* O157:H7 on alfalfa seeds. *International Journal of Food Microbiology, 56*, 13-20.

Pasanen, A. L., Yli-Pietila, K., Pasanen, P., Kalliokoski, P., & Tarhanen, J. (1999). Ergosterol content in various fungal species and biocontaminated building materials. *Applied and Environmental Microbiology, 65*, 138-142.

Patel, K. D., Scarano, F. J., Kondo, M., Hurta, R., & Neto, C. C. (2011). Proanthocyanidin-rich extracts from canberry fruit (*Vaccinium macrocarpon* Ait.) selectively inhibit the growth of human pathogenic fungi *Candida* spp. and *Cryptococcus neoformans*. *Journal of Agricultural and Food Chemistry, 59*, 12864-12873.

Patient, D., & Ainsworth, P. (1994). The chemistry of flour and bread. *Nutrition & Food Science, 94*, 22-24.

Peever, T. L., Ibañez, A., Akimitsu, K., & Timmer, L. W. (2002). Worldwide phylogeography of the citrus brown spot pathogen, *Alternaria alternata*. *Phytopathology, 92*, 794-802.

Penney, V., Henderson, G., Blum, C., & Johnson-Green, P. (2004). The potential of phytopreservatives and nisin to control microbial spoilage of minimally processed fruit yogurts. *Innovative Food Science and Emerging Technologies, 5*, 369-375.

Pereira Ávila, H., Albino Smânia, E. F., Delle Monache, F., & Smânia Júnior, A. (2008). Structure–activity relationship of antibacterial chalcones. *Bioorganic & Medicinal Chemistry, 16*, 9790-9794.

Phan, T.N., Nguyen, P.T., Abranches, J. & Marquis, R.E. (2002). Fluoride and organic weak acids as respiration inhibitors for oral streptococci in acidified environments. *Oral Microbioly and Immunology 17*, 19-124.

Phillips, B. J., Hughes, J. A., Phillips, J. C., Walters, D. G., Anderson, D., & Tahourdin, C. S. (1996). A study of the toxic hazard that might be associated with the consumption of green potato tops. *Food and Chemical Toxicology, 34*, 439-449.

Pohl, C., H., Kock, J., & Thibane, V. S. (2011). Antifungal free fatty acids: A review. *Science Against Microbial Pathogens: Communicating Current Research and Technological Advances*,61-71.

REFERENCES

Pohl, E. E., Voltchenko, A. M., & Rupprecht, A. (2008). Flip-flop of hydroxy fatty acids across the membrane as monitored by proton-sensitive microelectrodes. *Biochimica et Biophysica Acta, 1778*, 1292-1297.

Prost, I., Dhondt, S., Rothe, G., Vicente, J., Rodriguez, M. J., Kift, N. & Fournier, J. (2005). Evaluation of the antimicrobial activities of plant oxylipins supports their involvement in defense against pathogens. *Plant Physiology, 139*, 1902-1913.

Pyla, R., Kim, T., Silva, J. L., & Jung, Y. (2010). Enhanced antimicrobial activity of starch-based film impregnated with thermally processed tannic acid, a strong antioxidant. *International Journal of Food Microbiology, 137*(2-3), 154-160.

Raj, S. N., Sarosh, B. R., & Shetty, H. S. (2006). Induction and accumulation of polyphenol oxidase activities as implicated in development of resistance against pearl millet downy mildew disease. *Functional Plant Biology, 33*, 563-571.

Ramanathan, L., & Das, N. P. (1992). Studies on the control of lipid oxidation in ground fish by some polyphenolic natural products. *Journal of Agricultural and Food Chemistry, 40*, 17-21.

Ramos-Nino, M. E., Clifford, M. N. & Adams, M. R. (1996). Quantitative structure activity relationship for the effect of benzoic acids, cinnamic acids and benzaldehydes on *Listeria monocytogenes. Journal of Appiedl Bacteriology 80*, 303-310.

Rasmussen, S. E., Frederiksen, H., Krogholm, K. S., & Poulsen, L. (2005). Dietary proanthocyanidins: Occurrence, dietary intake, bioavailability, and protection against cardiovascular disease. *Molecular Nutrition & Food Research, 49*, 159-174.

Reddy, M. K., Gupta, S. K., Jacob, M. R., Khan, S. I., & Ferreira, D. (2007). Antioxidant, antimalarial and antimicrobial activities of tannin-rich fractions, ellagitannins and phenolic acids from *Punica granatum* L. *Planta Medica, 73*, 461-467.

Reinders, R. D., Biesterveld, S., & Bijker, P. G. H. (2001). Survival of *Escherichia coli* O157:H7 ATCC 43895 in a model apple juice medium with different concentrations of proline and caffeic acid. *Applied and Environmental Microbiology, 67*, 2863-2866.

Reiss, J. (1989). Influence of alkylresorcinol from rye and related compounds in the growth of fungal molds. *Cereal Chemistry, 66*, 491-493.

Renuka Devi, J., & Berla Thangam, E. (2010). Extraction and separation of glucosinolates from *Brassica oleraceae* var *rubra*. *Advances in Biological Research,4*, 309-313.

Ribeiro, S. M. R., Barbose, L. C. A., Queiroz, J. H., Knödler, M., & Schieber, A. (2008). Phenolic compounds and antioxidant capacity of brazilian mango (*Mangifera indica* L.) varieties. *Food Chemistry, 110*, 620-626.

REFERENCES

Röcken, W. & Spicher, G. (1993). Fadenziehende Bakterien;Vorkommen, Bedeutung und Gegenmassnahmen. *Getreide Mehl Brot 47*, 30–35.

Roddick, J. G., & Rijnenberg, A. L. (1987). Synergistic interaction between the potato glycoalkaloids α-solanine and α-chaconine in relation to lysis of phospholipid/sterol liposomes. *Phytochemistry, 26*, 1325-1328.

Roddick, J. G., Rijnenberg, A. L., & Osman, S. F. (1988). Synergistic interaction between potato glycoalkaloids α-solanine and α-chaconine in relation to desestabilization of cell membranes: Ecological implications. *Journal of Chemical Ecology, 14*, 889-902.

Rodríguez de Soltillo, D., Hadley, M., & Holm, E. T. (2006). Phenolics in aqueous potato peel extract: Extraction, identification and degradation. *Journal of Food Science, 59*, 649-651.

Rodríguez de Soltillo, D., Hadley, M., & Wolf-Hall, C. (1998). Potato peel extract, a nonmutagenic antioxidant with potential antimicrobial activity. *Journal of Food Science, 63*, 1-4.

Rodríguez, H., Curiel, J. A., Landete, J. M., De las Rivas, B., & López de Felipe, F. (2009). Food phenolics and lactic acid bacteria. *International Journal of Food Microbiology, 132*, 79-90.

Rodriguez, H., Landete, J. M., De las Rivas, B. & Munoz, R. (2008). Metabolism of food phenolic acids by *Lactobacillus plantarum* CECT 748^{T}. *Food Chemistry 107*, 1393-1398.

Rodríguez-Meizoso, I., Marin, F. R., Herrero, M., Señorans, F. J., Reglero, G., Cifuentes, A., & Ibañez, E. (2006). Subcritical water extraction of nutraceuticals with antioxidant activity from oregano. chemical and functional characterization. *Journal of Pharmaceutical and Biomedical Analysis, 41*, 1560-1565.

Rodríguez-Saona, L. E., Wrolstad, R. E., & Pereira, C. (1999). Glycoalkaloid content and anthocyanin stability to alkaline treatment of red fleshed potato extracts. *Journal of Food Science, 64*, 445-450.

Rombouts, I., Lagrain, B., Brijs, K., & Delcour, J. A. (2012). Cross-linking of wheat gluten proteins during production of hard pretzels. *Amino Acids, 42*, 2429-2438.

Romero-Gonzalez, R., Garrido Frenich, A., & Martinez Vidal, J. L. (2010). Chapter 64: Liquid chromatography-mass spectrometry determination of sterols in olive oil. In P. Victor, & W. Ronald (Eds.), *Olives and olive oil and health and disease prevention* (1st ed., pp. 591-601). San Diego CA, USA: Elsevier.

Ross, A. B., Kamal-Eldin, A., & Åman, P. (2004). Dietary alkylresorcinols: Absorption, bioactivities, and possible use as biomarkers of whole-grain wheat– and rye–rich foods. *Nutrition Reviews, 62*, 81-95.

REFERENCES

Rozes, N. & Peres, C. (1998). Effects of phenolic compounds on the growth and the fatty acid composition of *Lactobacillus plantarum*. *Applied Microbiology and Biotechnology 49*, 108-111

Ryabushkina, N. A. (2005). Synergism of metabolite action in plant responses to stresses. *Russian Journal of Plant Physiology, 52*, 547-552.

Sakagami, H., Asano, K., Tanuma, S., Hatano, T., Yoshida, T., & Okuda, T. (1992). Stimulation of monocyte iodination and IL-1 production by tannins and related compounds. *Anticancer Research, 12*, 377-388.

Samelis, J., Kakouri, A., & Rementzis, J. (2000). Selective effect of the product type and the packaging conditions on the species of lactic acid bacteria dominating the spoilage microbial association of cooked meats at 48°C. *Food Microbiology, 17*, 329-340.

Sánchez, S. A., Tricerri, M. A., Gunther, G., & Gratton, E. (2007). Laurdan generalized polarization: From cuvette to microscope. In A. Méndez-Vilas, & J. Díaz (Eds.), *Modern Research and Educational Topics in Microscopy.* (pp. 1007-1014) FORMATEX.

Sánchez-Machado, D. I., Lóópez-Hernandez, J., Paseiro-Losada, P., & López-Cervantes, J. (2004). An HPLC method for the quantification of sterols in edible seaweeds [1.] *Biomedical Chromatography, 18*, 183-190.

Sánchez-Maldonado, A. F., Schieber, A., & Gänzle, M. G. (2011). Structure-function relationship of the antibacterial activity of phenolic acids and their metabolism by lactic acid bacteria. *Journal of Applied Microbiology, 111*, 1176-1184.

Santos-Buelga, C., & Scalbert, A. (2000). Proanthocyanidins and tannin-like compounds – nature, occurrence, dietary intake and effects on nutrition and health. *Journal of the Science of Food and Agriculture, 80*, 1094-1117.

Sarpeleh, A., Wallwork, H., Tate, M. E., Catcheside, D. E., & Able, A. M. (2008). Initial characterisation of phytotoxic proteins isolated from *Pyrenophora teres*. *Physiological and Molecular Plant Pathology, 72*, 73-79.

Satoh, N., Akiba, T., Yokoshima, S., & Fukuyama, T. (2007). A practical synthesis of (-)-Oseltamivir. *Angewandte Chemie, 119*, 5836-5838.

Scalbert, A. (1991). Antimicrobial properties of tannins. *Phytochemistry, 30*, 3875-3883.

Scherer, G. (2006). Carboxyhemoglobin and thiocyanate as biomarkers of exposure to carbon monoxide and hydrogen cyanide in tobacco smoke. *Experimental and Toxicologic Pathology, 58*, 101-124.

Schieber, A., & Aranda Saldaña, M. D. (2009). Potato peels: A source of nutritionally and pharmacologically interesting compounds-A review. *Food, 3*(Special issue), 23-29.

REFERENCES

Schwaber, M. J., Navon-Venezia, S., Schwartz, D., & Carmeli, Y. (2005). High levels of antimicrobial coresistance among extended-spectrum--lactamase-producing Enterobacteriaceae. *Antimicrobial Agents and Chemotherapy, 49*, 2137-2139.

Sebranek, J. G., Jackson-Davis, A. L., Myers, K. L., & Lavieri, N. A. (2012). Beyond celery and starter culture: Advances in natural/organic curing processes in the United States. *Meat Science, 92*, 267-273.

Segura Carretero, A., Carrasco-Pancorbo, A., Cortacero, S., Gori, A., Cerretani, L., & Fernandez-Gutierrez, A. (2008). 1. Simplified method for HPLC-MS analysis of sterols in vegetable oil. *European Journal of Lipid Science and Technology, 110*, 1142-1149.

Serrano, J., Puupponen-Pim, R., Dauer, A., Aura, A., & Saura-Calixto, F. (2009). Tannins: Current knowledge of food sources, intake, bioavailability and biological effects. *Molecular Nutrition & Food Research, 53*, S310-S329.

Shui, G., Leong, L. P., & Wong, S. P. (2005). Rapid screening and characterisation of antioxidants of *Cosmos caudatus* using liquid chromatography coupled with mass spectrometry. *Journal of Chromatography B, 827*, 127-138.

Siegrist, M. (2008). Factors influencing public acceptance of innovative food technologies and products. *Trends in Food Science & Technology, 19*, 603-608.

Singh, N., & Rajini, P. S. (2004). Free radical scavenging activity of an aqueous extract of potato peel. *Food Chemistry, 85*, 611-616.

Sivakumaran, S., Molan, A. L., Meagher, L. P., Kolb , B., Foo, L. Y., Lane, G. A. & Tavendale, M. (2004). Variation in antimicrobial action of proanthocyanidins from *Dorycnium rectum* against rumen bacteria. *Phytochemistry, 65*, 2485-2497.

Sjögren, J., Broberg, A., Schnürer, J., & Kenne, L. (2003). Antifungal 3-hydroxy fatty acids from *Lactobacillus plantarum* MiLAB 14. *Applied and Environmental Microbiology, 69*, 7554-7557.

Sloan, A. E. (2014). The top ten functional food trends. *Food Technology*, 22-45.

Sohn, H., Son, K. H., Kwon, C. S., Kwon, G. S., & Kang, S. S. (2004). Antimicrobial and cytotoxic activity of 18 prenylated flavonoids isolated from medicinal plants: *Morus alba* L., *Morus mongolica* Schneider, *Broussnetia papyrifera* (L.) Vent, *Sophora flavescens* ait and *Echinosophora koreensis* Nakai. *Phytomedicine, 11*, 666-672.

Sotelo, A., & Serrano, B. (2000). High-performance liquid chromatography determination of the glycoalkaloids α-solanine and α-chaconine in 12 commercial varieties of mexican potato. *Journal of Agricultural and Food Chemistry, 48*, 2472-2475.

REFERENCES

Stasiuk, M., & Kozubek, A. (2010). Biological activity of phenolic lipids. *Cellular and Molecular Life Sciences, 67*, 841-860.

Stasiuk, M., Jaromin, A., & Kozubek, A. (2004). The effect of merulinic acid on biomembrane. *Biochimica Et Biophysica Acta, 1667*, 215-221.

Stead, D. (1993) The effect of hydroxycinnamic acids on the growth of wine-spoilage lactic acid bacteria. *Journal of Applied Bacteriology, 75***,** 135-141.

Sullivan, G. A., Jackson-Davis, A. L., Schrader, K. D., Xi, Y., Kulchaiyawat, C., Sebranek, J. G., & Dickson, J. S. (2012). Survey of naturally and conventionally cured commercial frankfurters, ham, and bacon for physio-chemical characteristics that affect bacterial growth. *Meat Science 92*, 808-815.

Sun, R., Sun, X., & Zhang, S. (2001). Quantitative determination of hydroxycinnamic acids in wheat, rice, rye, and barley straws, maize stems, oil palm frond fiber, and fast-growing poplar wood. *Journal of Agricultural and Food Chemistry, 49*, 5122-5129.

Sutthivaiyakit, S., Thongnak, O., Lhinhatrakool, T., Yodchun, O., Srimark, R., Dowtaisong, P., & Chuankamnerdkarn, M. (2009). Cytotoxic and antimycobacterial prenylated flavonoids from the roots of *Eriosema chinense*. *Journal of Natural Products, 72*, 1092-1096.

Suzuki, K., Iijima, K., Sami, M., & Yamashita, H. (2006). A review of hop resistance in beer spoilage lactic acid bacteria. *Journal of the Institute of Brewing, 112*, 173-191.

Suzuki, Y., Esumi, Y., Hyakutake, H., Kono, Y., & Sakurai, A. (1996). Isolation of 5-(8'Z-heptadecenyl)-resorcinol from etiolated rice seedlings as an antifungal agent. *Phytochemistry, 41*, 1485-1489.

Svensson, L., Sekwati-Monang, B., Lopes Lutz, D., Schieber, A., & Gänzle, M. G. (2010). Phenolic acids and flavonoids in nonfermented and fermented red sorghum (*Sorghum bicolor* (L.) moench). *Journal of Agricultural and Food Chemistry, 58*(16), 9214-9220.

Taguri, T., Tanaka, T. & Kouno, I. (2006). Antibacterial spectrum of plant polyphenols and extracts depending upon hydroxyphenyl structure. *Biological and Pharmaceutical Bulletin 29*, 2226-2235.

Tassou, C. C., & Nychas, G. J. E. (1994). Inhibition of *Staphylococcus aureus* by olive phenolics in broth and in a model food system. *Journal of Food Protection,7*, 120-124.

Teixeira, J., McNeil, V., & Gänzle, M. G. (2012). Levansucrase and sucrose phoshorylase contribute to raffinose, stachyose, and verbascose metabolism by lactobacilli. *Food Microbiology, 31*, 278-284.

Teixeira, J. S., Seeras, A., Sánchez-Maldonado, A. F., Zhang, C., Su, M. S. & Gänzle, M. G. (2014). Glutamine, glutamate, and arginine-based acid resistance in *Lactobacillus reuteri*. *Food Microbiology 42,* 172-180.

Terao, J., Nagao, A., Yuki, H., & Itoh, Y. (1993). Reduction of fatty acid hydroperoxides by human parotid saliva. *Lipids, 28,* 121-124.

Thipyapong, P., Hunt, M. D., & Steffens, J. C. (2004). Antisense downregulation of polyphenol oxidase results in enhanced disease susceptibility. *Planta, 220*, 105-117.

Thygesen, P. W., Dry, I. B., & Robinson, S. P. (1995). Polyphenol oxidase in potato a multigene family that exhibits differential expression patterns. *Plant Physiology, 109*, 525-531.

Tian, F., Li, B., Ji, B., Yanga, J., Zhang, G., Chen, Y., & Luo, Y. (2009). Antioxidant and antimicrobial activities of consecutive extracts from *Galla chinensis*: The polarity affects the bioactivities. *Food Chemistry, 113*, 173-179.

Tian, Q., Rosselot, R. A., & Schwartz, S. J. (2005). Quantitative determination of intact glucosinolates in broccoli, broccoli sprouts, brussels sprouts, and cauliflower by high-performance liquid chromatography–electrospray ionization–tandem mass spectrometry. *Analytical Biochemistry, 343*, 93-99.

Tiwari, B., Valdramidis, V. P., O'Donnell, C. P., Muthukumarappan, K., Bourke, P., & Cullen, P. J. (2009). Application of natural antimicrobials for food preservation. *Journal of Agricultural and Food Chemistry, 57*, 5987-6000.

Tsao, R., Peterson, C. J., & Coats, J. R. (2002). Glucosinolate breakdown products as insect fumigants and their effect on carbon dioxide emission of insects. *BMC Ecology, 2*, 1-7.

Tsukiyama, R., Katsura, H., Tokuriki, N., & Kobayash, M. (2002). Antibacterial activity of licochalcone A against spore-forming bacteria. *Antimicrobial Agents and Chemotherapy, 6*, 1226-1230.

Tubtimdee, C., & Shotipruk, A. (2011). Extraction of phenolics from *Terminalia chebula* retz with water-ethanol and water propylene glycol and sugaring-out concentrations of extracts. *Separation and Purification Technology, 77*, 339-346.

Tunç, A., & Duman, O. (2011). Preparation of active antimicrobial methyl cellulose/carvacrol/montmorillonite nanocomposite films and investigation of carvacrol release. *LWT - Food Science and Technology, 44*, 465-472.

Tuncel, G. & Nergiz, C. (1993). Antimicrobial effect of some olive phenols in a laboratory medium. *Letters in Appiedl Microbiology, 17*, 300-302.

REFERENCES

Ulmer, H. M., Gänzle, M. G. & Voguel, R. F. (2000). Effects of high pressure on survival and metabolic activity of *Lactobacillus plantarum* TMW1.460. *Applied and Environmental Microbiology 66*, 3966–3973.

Ulmer, H. M., Herberhold, H., Fahsel, S., Gänzle, M. G., Winter, R., & Vogel, R. F. (2002). Effects of pressure-induced membrane phase transitions on inactivation of HorA, an ATP-dependent multidrug resistance transporter, in *Lactobacillus plantarum*. *Applied and Environmental Microbiology, 68*, 1088-1095.

Valcheva, R., Korakli, M., Onno, B., Prevost, H., Ivanova, I., Ehrmann, M. A., Dousset, X., Gänzle, M. G. & Vogel, R. F. (2006). *Lactobacillus hammesii* sp. nov., isolated from French sourdough. *International Journal of Systematic and Evolutionary Microbiology 55*, 763–767.

Van Beek, S. & Priest, F. G. (2000). Decarboxylation of substituted cinnamic acids by lactic acid bacteria isolated during malt whisky fermentation. *Applied and Environmental Microbiology 66*, 5322-5328.

VanEtten, H. D., Mansfield, J. W., Bailey, J. A., & Farmer, E. E. (1994). Two classes of plant antibiotics: Phytoalexins versus ‘‘phytoanticipins”. *Plant Cell, 6*, 1191-1192.

Vattem, D. A., Lin, Y. T., Ghaedian, R., & Shetty, K. (2005). Cranberry synergies for dietary management of *Helicobacter pylori* infections. *Process Biochemistry,40*, 1583-1592.

Vaughn, K. C., Lax, A. R., & Duke, S. O. (1988). Polyphenol oxidase: The chloroplast oxidase with no established function. *Physiologia Plantarum, 72*, 659-665.

Vermorel, M., Davicco, M. J., & Evrard, J. (1987). Valorization of rapeseed meal. 3. effects of glucosinolate content on food intake, weight gain, liver weight and plasma thyroid hormone levels in growing rats. *Reproduction Nutrition Development, 27*, 57-66.

Walters, D., Raynor, L., Mitchell, A., Walker, R., & Walker, K. (2004). Antifungal activities of four fatty acids against plant pathogenic fungi. *Mycopathologia,157*, 87-90.

Wansi, J. D., Chiozen, D. D., Tcho, A. T., Toze, F. A. A., Devkota, K. P., Ndjakou, L. B. & Sewald, N. (2010). Antimicrobial and antioxidant effects of phenolic constitutens from *Klainedoxa gabonensis*. *Pharmaceutical Biology, 48*, 1124-1129.

Ward, S. M., Delaquis, P. J., Holley, R. A., & Mazza, G. (1998). Inhibition of spoilage and pathogenic bacteria on agar and pre-cooked roast beef by volatile horseradish distillates. *Food Research, 31*, 19-26.

Wätcher, G. A., Homann, J. J., Furbacher, T., Blake, M. E., & Timmermann, B. N. (1999). Antibacterial and antifungal favanones from *Eysenhardtia texana*. *Phytochemistry, 52*, 1469-1471.

REFERENCES

Weete, J. W., Abril, M., & Blackwell, M. (2010). Phylogenic distribution of fungal sterols. *Plos One, 5*, e10899.

Weltring, K. M., Wessels, J., & Geyert, R. (1997). Metabolism of the potato saponins α-chaconine and α-solanine by *Gibberella pilicaris*. *Phytochemistry, 46*, 1005-1009.

Wen, A., Delaquis, P., Stanich, K. & Toivonen, P. (2003). Antilisterial activity of selected phenolic acids. *Food Microbiology, 20,* 305-311.

Wershaw, R. L. (2004). Evaluation of conceptual models of natural organic matter (humus) from a consideration of the chemical and biochemical processes of humification. In U.S. Geological Survey (Ed.), (pp. 11). Reston, Virginia: U.S. Geological Survey, Information Services.

Wink, M. (2003). Evolution of secondary metabolites from an ecological and molecular phylogenetic perspective. *Phytochemistry, 64*, 3-19.

Wu, H., Zhang, G. A., Zenga, S., & Lina, K. (2009). Extraction of allyl isothiocyanate from horseradish (*Armoracia rusticana*) and its fumigant insecticidal activity on four stored-product pests of paddy. *Pest Managment Science, 65,* 1003-1008.

Wu, Z.-G.; Xu, H.-Y.; Ma, Q.; Cao, Y.; Ma, J.-N.; Ma, C.-M. (2012). Isolation, identification and quantification of unsaturated fatty acids, amides, phenolic compounds and glycoalkaloids from potato peel. *Food Chemistry, 135*, 2425-2429.

Xia, D., Wu, Z., Shi, J., Yang, Q. & Zhang, Y. (2010) Phenolic compounds from the edible seeds extract of Chinese Mei (*Prunus mume* Sieb, et Zucc) & their antimicrobial activity. *LWT-Food Science and Technology, 44*, 347-349.

Yencho, G. C., Kowalski, S. P., Kennedy, G. G., & Sanford, L. L. (2000). Segregation of leptine glycoalkaloids and resistance to colorado potato beetle (*Leptinotarsa decemlineata* (say) in F2 *Solanum tuberosum* (4x) x S. chacoense (4x) potato progenies. *American Journal of Potato Research, 77*, 167-178.

Yeung, Y., Hong, S., & Corey, E. J. (2006). A short enantioselective pathway for the synthesis of the anti-influenza neuramidase inhibitor oseltamivir from 1,3-butadiene and acrylic acid. *Journal of the American Chemical Society, 128*, 6310-6311.

Yoruk, R., & Marshall, M. R. (2006). Physicochemical properties and function of plant polyphenol oxidase: A review. *Phytochemistry, 67*, 2318-2331.

Zangerl, A. R., & Bazzaz, F. A. (1993). Theory and pattern in plant defense allocation. In R. S. Fritz, & E. L. Simms (Eds.), *Plant resistance to hervibores and pathogens* (pp. 363-361). Chicago: University of Chicago Press.

REFERENCES

Zhang, C., Brandt, M. J., Schwab, C., & Gänzle, M. G. (2010). Propionic acid production by cofermentation of *Lactobacillus buchneri* and *Lactobacillus diolivorans* in sourdough. *Food Microbiology, 27*, 390-395.

Zhang, Y., & Talalay, P. (1994). Anticarcinogenic activities of organic isothiocyanates: Chemistry and mechanisms. *Cancer Research, 54*, 1976s-1981s.

Zúñiga, G. E., & Corcuera, L. J. (1986). Effect of gramine in the resistance of barley seedlings to the aphid *Rhopalosiphum padi Entomologia Experimentalis et Applicata, 40 (3)*, 259-262.