Universität Hamburg
DER FORSCHUNG | DER LEHRE | DER BILDUNG

Influence of a Selenium Biofortification of Apples (*Malus domestica* BORKH.) on Nutritionally Important Metabolites of Primary and Secondary Plant Metabolism

Dissertation

zur Erlangung des Doktorgrades

Doctor rerum naturalium

(Dr. rer. nat.)

Universität Hamburg

Fakultät für Mathematik, Informatik und Naturwissenschaften

Fachbereich Chemie

Institut für Lebensmittelchemie

Vorgelegt von

Sabrina Groth

Hamburg, 2024

The present work was carried out under the supervision of Prof. Dr. Sascha Rohn at the Institute of Food Chemistry, University of Hamburg, in the period from 01.06.2017 to 31.08.2021.

First referee: Prof. Dr. Sascha Rohn

Second referee: Prof. Dr. Bernward Bisping

Date of Disputation: June 21st, 2024

Date of print release: June 16th, 2025

This work was carried out in parts within the framework of the project *BiofortiSe*

„*Biofortifikation von Äpfeln mit Selen zur Verbesserung der Fruchtqualität, der Lagerfähigkeit und des gesundheitlichen Wertes“.*

This project was funded by the German Federal Ministry of Education and Research (Bundesministerium für Bildung und Forschung, BMBF) within the framework of the idea competition "New Products for the Bioeconomy".

(FKZ 031B0299A, B, C)

TABLE OF CONTENTS

List of Publications

Publications in Professional Journals *(peer-reviewed)*

Groth, S.; Budke, C.; Weber, T.; Neugart, S.; Brockmann, S.; Holz, Sawadski, B. C.; Daum, D.; Rohn, S. Relationship between Phenolic Compounds, Antioxidant Properties, and the Allergenic Protein Mal d 1 in Different Selenium-Biofortified Apple Cultivars *(Malus domestica). Molecules* **2021**, *26*(9), 2647. doi: 10.3390/molecules26092647.

Groth, S.; Budke, C.; Weber, T.; Oest, M.; Brockmann, S.; Holz, M.; Daum, D.; Rohn, S. Selenium biofortification of different varieties of apples (*Malus domestica*) – Influence on protein content and the allergenic proteins Mal d 1 and Mal d 3. *Food Chemistry* **2021,** *362*, 130134. doi: 10.1016/j.foodchem.2021.130134.

Groth, S.; Budke, C.; Neugart, S.; Ackermann, S.; Kappenstein, F.-S.; Daum, D.; Rohn, S. Influence of a selenium biofortification on antioxidant properties and phenolic compounds of apples (*Malus domestica*). *Antioxidants* **2020**, *9*, 187. doi: 10.3390/antiox9020187.

Groth, S.; Budke, C.; Neugart, S.; Ackermann, S.; Kappenstein, F.-S.; Daum, D.; Rohn, S. Biofortifikation von Äpfeln mit Selen. Einfluss auf phenolische Verbindungen und antioxidative Eigenschaften. *FOOD-Lab* **2020**, *2*, 12-18.

Groth, S.; Wittmann, R.; Longin, C. F. H.; Böhm, V. Influence of variety and growing location on carotenoid and vitamin E contents of 184 different durum wheat varieties (*Triticum turgidum ssp. durum*) in Germany. *European Food Research Technology* **2020**, *246*, 2079-2092. doi: 10.1007/s00217-020-03557-1.

Halagarda, M.; **Groth, S.;** Popek, S.; Rohn, S.; Pedan, V. Antioxidant Activity and Phenolic Profile of Selected Organic and Conventional Honeys from Poland. *Antioxidants* **2020**, *9*, 44. doi: 10.3390/antiox9010044.

Lectures

Groth, S. Zusammenhang zwischen phenolischen Verbindungen, antioxidativen Eigenschaften und dem allergenen Protein Mal d 1 in verschiedenen Selen-biofortifizierten Apfelsorten. 54. Vortragstagung der Deutschen Gesellschaft für Qualitätsforschung (Pflanzliche Nahrungsmittel) e.V. (DGQ), 23. März 2021, digital.

Groth, S. Zusammenhang zwischen phenolischen Verbindungen, antioxidativen Eigenschaften und dem allergenen Protein Mal d 1 in verschiedenen Selen-biofortifizierten Apfelsorten. GDCh-Arbeitstagung des Regionalverbandes Nord, 18. März 2021, digital. *Lebensmittelchemie* **75**, 51. doi: 10.1002/lemi.202152004.

Groth, S. Selen-Biofortifikation von Äpfeln unterschiedlicher Sorten – Einfluss auf die allergenen Proteine Mal d 1 und Mal d 3. 58. Wissenschaftlicher Kongress der Deutschen Gesellschaft für Ernährung (DGE), 17.-19. Februar 2021, digital.

Groth, S. Selenium Biofortification of Different Varieties of Apples – Influence on the Allergenic Proteins Mal d 1 and Mal d 3. 15. Dreiländertagung DGE/ÖGE/SGE, 19.-20. November 2020, digital.

Groth, S. Einfluss einer Selen-Biofortifikation von Äpfeln unterschiedlicher Sorten auf die allergenen Proteine Mal d 1 und Mal d 3. 54. Vortragstagung der Deutschen Gesellschaft für Qualitätsforschung (Pflanzliche Nahrungsmittel) e.V. (DGQ), 04.-06. März 2020, Hohenheim; Tagung wurde aufgrund der Covid-19-Pandemie abgesagt, ein Tagungsband wurde jedoch publiziert.

Groth, S. Einfluss einer Selen-Biofortifikation sowie weiterer Faktoren auf antioxidative Eigenschaften verschiedener Apfelsorten. Posterflashtalk; 48. Deutscher Lebensmittelchemikertag, 16.-18. September 2019, Dresden.

Groth, S. Einfluss einer Selen-Biofortifikation auf antioxidative Eigenschaften von Äpfeln. GDCh-Arbeitstagung des Regionalverbandes Nord, 25.-26. März 2019, Hamburg.

Groth, S. Einfluss einer Selen-Biofortifikation auf antioxidative Eigenschaften von Äpfeln. 56. Wissenschaftlicher Kongress der Deutschen Gesellschaft für Ernährung (DGE), 19.-21. März 2019, Gießen.

Groth, S. Analyse qualitätsbestimmender Parameter in Selen-biofortifizierten Äpfeln. 52. Vortragstagung der Deutschen Gesellschaft für Qualitätsforschung (Pflanzliche Nahrungsmittel) e.V. (DGQ), 26.-27.März 2018, Gießen.

Poster

Groth, S. Influence of selenium biofortification on the antioxidant properties of apples. 12th World Congress on Polyphenols Applications, 26.-28. September 2018, Bonn.

Groth, S. Einfluss einer Selen-Biofortifikation auf antioxidative Eigenschaften von Äpfeln. 47. Deutscher Lebensmittelchemikertag, 17.-19. September 2018, Berlin.

Groth, S. Analyse qualitätsbestimmender Parameter in Selen-biofortifizierten Äpfeln. GDCh-Arbeitstagung des Regionalverbandes Nord, 14. März 2018, Hannover.

Groth, S. Analyse qualitätsbestimmender Parameter in Selen-biofortifizierten Äpfeln. 55. Wissenschaftlicher Kongress der Deutschen Gesellschaft für Ernährung (DGE), 07.-09. März 2018, Stuttgart-Hohenheim.

Groth, S.; Böhm, V. Optimierung der Probenaufarbeitung für die Analyse von Carotinoiden und Tocopherolen in Hartweizen mittels HPLC.54. Wissenschaftlicher Kongress der Deutschen Gesellschaft für Ernährung (DGE), 01.-03. März 2017, Kiel.

List of Abbreviations

AAPH	2,2′-azobis(2-methylamidinopropane)dihydrochloride
ABTS	2,2′-azino-di(3-ethylbenzthiazoline-6-sulfonic acid)
AOA	antioxidant activity
AUC	area under the curve
BC	before Christ
Bet v (1-2)	*Betula verrucosa* 1
bidest.	bidestilled
BMBF	*Bundesministerium für Bildung und Forschung*; Federal Ministry of Education and Research
BMEL	*Bundesministerium für Ernährung und Landwirtschaft*; Federal Ministry of Food and Agriculture
BS	backpack sprayer
BSA	bovine serum albumin
BUND	*Bund für Umwelt und Naturschutz*; federation for environment and nature conservation
°C	degree Celsius
C	catechin
CA	chlorogenic acid
Ca	calcium
CH	canopy height
CMR	cancerogen mutagen reprotoxic
CO	catecholoxidase
CoA	coenzyme A
CQA	*p*-coumaroylquinic acid
C ring	carbon ring
Cu	*cuprum*; copper
cv	cultivar
D-A-CH	*Deutschland-Österreich-Schweiz*; Germany-Austria-Suisse
DGE	*Deutsche Gesellschaft für Ernährung;* German Nutrition Society

DGQ	*Deutsche Gesellschaft für Qualitätsforschung (Pflanzliche Nahrungsmittel) e.V.*; German Society for Quality Research (vegetable food)
DIECA	diethyldithiocarbamate
Disc-SDS-PAGE	discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis
DNA	desoxyribonucleinacid
DPPH	2,2-diphenyl-1-picrylhydrazyl
d.w.	dry weight
EC	epicatechin
EDTA	ethylenediaminetetraacetic acid
ELISA	Enzyme-linked Immunosorbent Assay
ET	electron transfer
FCεRI	high-affinity IgE receptor
FKZ	*Förderkennzeichen*; funding code
FRAP	fluorescence recovery after photobleaching
f.w.	fresh weight
g	gram
GAE	gallic acid equivalents
GDCh	*Gesellschaft Deutscher Chemiker*; Society of German Chemists
GF-AAS	graphite furnace atomic absorption spectroscopy
GHS	globally harmonised system
Glu	glucose
GSH	glutathione
GSH-Px	glutathione peroxidase
GSSG	glutathione disulfide
H	hydrogen
h	hours
ha	hectare
HAT	hydrogen atom transfer
HepG2	hepatoma G2

HOCl	hypochlorous acid
H phrases	hazard phrases
HPLC	high performance liquid chromatography
HPLC-DAD-ESI-MS^n	high performance liquid chromatography equipped with diode array detector coupled with electrospray ionization mass spectrometry
HRP	horseradish peroxidase
HS	hand-held sprayer
H_2O	water
H_2O_2	hydrogen peroxide
IgE	immunoglobulin E
Ile	isoleucine
ITP	immuno-tissue-print-assay
kDa	kilo Dalton
Kg	kilogram
K_2SO_4	potassium peroxodisulfate
L	liter
L•	lipid radical
LC-ESI-qTOF-MS	liquid chromatography electrospray ionization quadrupole time-of-flight mass spectrometry
Leu	leucin
LH	fatty acid
LO•	lipid hydroxy radical
LOO•	lipid peroxyl radical
LOOH	lipid hydroperoxide
m	meter
m^2	square meter
Mal d (1-4)	*Malus domestica* BORKH. (1-4)
mil	million
mL	milliliter
mmol	millimole

Mo	molybdophosphoric acid
M_W	molecular weight
µg	microgram
µmol	micromole
N	nitrogen
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
$NADP^+$	nicotinamide adenine dinucleotide phosphate, oxidized form
nanoLC-MS/MS	nanoscale liquid chromatography coupled to tandem mass spectrometry
NH_3	ammonia
nm	nanometer
NO•	nitric oxide
nsLTP	non-specific lipid transfer protein
OAS	oral allergy syndrome
ÖGE	*Österreichische Gesellschaft für Ernährung;* Austrian Society for Nutrition
•OH	hydroxyl radical
O-O	peroxo
ORAC	oxygen radical absorbance capacity
OS	trailed orchard sprayer
$^1O_2$	singlet oxygen
O_2	molecular oxygen
O_2•	superoxide radical
O_2^-	superoxide anion
O_3	ozone
PAL	phenylalanine ammonia lyase
PBS	phosphate buffered saline
PC B1	procyanidin B1
PC B2	procyanidin B2
pH	*potentia hydrogenii*

PHZ	phloridzin
P phrases	precautionary phrases
PPO	polyphenoloxidase
PR	pathogenesis-related
PUFA	polyunsaturated fatty acid
PVPP	polyvinylpolypyrrolidone
PXG	phloretin xyloglucoside
QGal	quercetin-3-galactoside
QGlu	quercetin-3-glucoside
QR	quercetin-3-rhamnosid
R	quercetin-3-rutinosid/rutin
R•	radical
RNS	reactive nitrogen species
ROO•	peroxyl radical
ROS	reactive oxygen species
R^2	correlation coefficient
SDS	sodium dodecyl sulfate
Se	selenium
sec	second
SGE	*Schweizerische Gesellschaft für Ernährung*; Swiss Society for Nutrition
SH	sulfhydryl
SOD	superoxide dismutase
T	temperature
t	tons
TE	Trolox equivalents
TEAC	Trolox equivalent antioxidant capacity
TEMED	tetramethylethylendiamine
Thr	threonine
TLP	thaumatin-like protein

TMB	3,3',5,5'-tetramethylbenzidine
TPA	tungstophosphoric acid
TPC	total phenolic content
TRIS	tris(hydroxymethyl)aminomethane
Ty	tyrosinase
U	unit
UV	ultraviolet
vs.	versus
v/v	volume/volume
WHO	World Health Organization

List of Illustrations

List of Tables

1. ABSTRACT

In the present work, the influence of a selenium biofortification of apples on nutrionally important metabolites of primary and secondary plant metabolism was analyzed.

Of particular importance were the content of the trace element selenium, the qualitative and quantitative composition of phenolic compounds, and the properties associated with the latter. These include the activity of polyphenoloxidase (PPO), which plays a major catalytic role in the oxidative degradation of phenolic compounds and their antioxidant properties. In terms of primary plant metabolism, allergenic proteins, which are categorized as antinutritional constituents, were relevant, as well. Due to the nutritional significances and effects, a high content of selenium and phenolic compounds and a low content of allergenic proteins are desirable in apples. It was hypothesized that this could be achieved by applying agronomic biofortification, a targeted fertilization method, with selenium.

For this purpose, apple samples of seven different cultivars were cultivated and biofortified with selenium in different application forms and levels via foliar fertilization. This allows the selection of the optimal fertilization method and it can be verified which varieties are more or less suitable for achieving the objectives. Furthermore, by cultivating in three consecutive years at two growing sites, ecophysiological factors could be identified as a further influencing factor on the apples‘ constituents.

For the analysis of the activity of PPO, the total phenolic content (TPC) according to FOLIN-CIOCALTEU, the antioxidant activity (AOA) estimated by the Trolox equivalent antioxidant capacity (TEAC) and the oxygen radical absorbance capacity (ORAC) assay, and the measurement of the protein content according to BRADFORD, photometric methods were established and validated for the matrix apple and apple extracts. Furthermore, an extraction method for phenolic compounds from apples was developed and optimized. For the extraction of the proteins as well as for the gel electrophoretic separation of the obtained extracts by discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis (disc-SDS-PAGE), a method was also developed and optimized. A direct Enzyme-linked Immunosorbent Assay (ELISA) was developed for the quantitative determination of the content of the main allergen Mal d 1 in the fruits. High performance liquid chromatography equipped with diode array detector coupled with electrospray ionization mass spectrometry (HPLC-DAD-ESI-MS^n) was applied to identify and quantify the phenolic compounds, and

identification of allergenic proteins was performed after in-gel digestion by liquid chromatography electrospray ionization quadrupole time-of-flight mass spectrometry (LC-ESI-qTOF-MS) and nanoscale liquid chromatography coupled to tandem mass spectrometry (nanoLC-MS/MS). Analysis of selenium content was performed by a project partner using graphite furnace atomic absorption spectroscopy (GF-AAS). The selenium content in the apples was significantly increased by a factor between 10 and 40 by biofortification to contents ranging from 2.1 to 23.2 µg/100 g fresh weight (f.w.). The consumption of selenium biofortified apples can therefore contribute significantly to a better selenium supply for the population. An apple of medium size can provide about a quarter of the daily selenium requirement of an adult, which is 70 µg for men and 60 µg for women.

The applied selenium form did not play a significant role with regard to the selenium content in the apples. However, due to observed fruit damages when selenite was applied, the more plant-tolerant selenate is favored for future applications.

Biofortification resulted in lower variations of the PPO activity. Furthermore, it was shown that the level of applied selenium influenced the enzyme activity. Here, higher application levels were associated with higher PPO activities. PPO activity was related to TPC, as this enzyme catalyzes the degradation of phenolic compounds. A lower TPC was measured with higher selenium application, which is explained by the higher PPO activity, through which more phenolic compounds are oxidized.

With respect to antioxidant activity, biofortification did not show clear trends. This is probably related to the different antioxidant activities of the individual phenolic compounds. The apples analyzed in the present work contain mainly chlorogenic acid, with a content of up to 40%, as well as epicatechin, and high amounts of various quercetin glycosides and phloretin glucosides. Biofortification showed partially different effects in the cultivars 'Fiesta', 'Jonica', 'Golden Delicious', and 'Jonagold' with regard to the content and proportion of the individual phenolic compounds. In most cases, a significant increase in epicatechin and caffeeoylglucoside and a reduction in procyanidin trimer were observed.

The four allergenic proteins Mal d 1, Mal d 2, Mal d 3, and Mal d 4 were identified in the apples by disc-SDS-PAGE and nanoLC-MS/MS. The protein patterns of the different apple samples are basically similar, but differ in the intensity of the protein bands, which indicates different levels of the individual allergenic proteins. Separate analyses of peel and fruit flesh were used to

determine the localization of the different allergenic proteins. Thus, the peel appeared to be particularly rich in Mal d 3, whereas Mal d 2 was mainly found in the fruit flesh. Mal d 1 was found in both the peel and the fruit flesh.

With regard to primary metabolism, biofortification had an influence on protein content and allergenic proteins. In most cultivars, the application of selenate resulted in higher protein contents compared to the controls or to the administration of selenite. Based on more intense bands in the SDS gel, it was assumed that the biofortification leads to an increase in the synthesis of Mal d 3. In contrast, the content of Mal d 1 was reduced. In particular, statistically significant reductions in Mal d 1 content were observed in many cultivars during the application of selenate. Differences were observed between the peel and the flesh, especially the Mal d 1 content in the flesh was reduced resulting from the biofortification.

In relation to all measured parameters, the apple variety and ecophysiological conditions such as sunshine duration were identified as further influencing factors.

Correlation analyses were carried out to analyze the relationship between phenolic compounds and the Mal d 1 content. It was found that PPO activity and the content of individual phenolic compounds correlated with the Mal d 1 content. The relationship between PPO activity and Mal d 1 content appeared to be cultivar dependent as well as influenced by ecophysiological conditions, as both positive and negative correlations were found. Lower Mal d 1 contents were observed in apples with comparatively high chlorogenic acid content and low procyanidin trimer and epicatechin content. In contrast, the total phenolic content and the level of antioxidant activity seem to play only a subordinate role.

There was also a correlation between selenium and Mal d 1 content and thus, an influence of the agronomic fertilization method. A high selenium content was associated with a low Mal d 1 content. In addition, the above-mentioned changes in phenolic profile were frequently observed in the biofortified apples, which were associated with low allergenicity. Thus, this measure seems to be suitable for a reduction of the allergenic potential in apples.

2. ZUSAMMENFASSUNG

In der vorliegenden Arbeit wurde der Einfluss einer Selen-Biofortifikation von Äpfeln auf ernährungsphysiologisch wichtige Metaboliten des primären und sekundären Pflanzenstoffwechsels analysiert.

Von besonderer Bedeutung waren dabei der Gehalt an dem Spurenelement Selen, die qualitative und quantitative Zusammensetzung der phenolischen Verbindungen und die mit letzteren verbundenen Eigenschaften. Dazu gehören die Aktivität der Polyphenoloxidase (PPO), die eine wichtige katalytische Rolle beim oxidativen Abbau von phenolischen Verbindungen und deren antioxidativen Eigenschaften spielt. Im Hinblick auf den primären Pflanzenstoffwechsel waren auch allergene Proteine von Bedeutung, die als antinutritive Bestandteile eingestuft werden. Aufgrund der ernährungsphysiologischen Bedeutung und Wirkung ist ein hoher Gehalt an Selen und phenolischen Verbindungen und ein geringer Gehalt an allergenen Proteinen in Äpfeln wünschenswert. Es wurde die Hypothese aufgestellt, dass dies durch die Anwendung der agronomischen Biofortifikation, einer gezielten Düngungsmethode, mit Selen erreicht werden könnte.

Zu diesem Zweck wurden Apfelproben von sieben verschiedenen Sorten angebaut und über eine Blattdüngung mit Selen in verschiedenen Applikationsformen und -mengen biofortifiziert. Dies ermöglicht die Auswahl der optimalen Düngungsmethode und es kann überprüft werden, welche Sorten mehr oder weniger geeignet sind, um die Ziele zu erreichen. Darüber hinaus konnten durch den Anbau in drei aufeinanderfolgenden Jahren an zwei Standorten ökophysiologische Faktoren als weiterer Einflussfaktor auf die Inhaltsstoffe der Äpfel identifiziert werden.

Für die Analyse der Polyphenoloxidase-Aktivität, des Gesamtphenolgehaltes nach FOLIN-CIOCALTEU (TPC), der antioxidativen Aktivität (AOA) mittels *Trolox equivalent antioxidant capacity* (TEAC) und *oxygen radical absorbance capacity* (ORAC) Test sowie der Messung des Proteingehaltes nach BRADFORD wurden photometrische Methoden für die Matrix Apfel und die daraus erhaltenen Apfelextrakte etabliert und validiert. Außerdem wurde eine Extraktionsmethode für phenolische Verbindungen aus Äpfeln entwickelt und optimiert. Für die Extraktion der Proteine sowie für die gelelektrophoretische Auftrennung der gewonnenen Extrakte mittels diskontinuierlicher Natriumdodecylsulfat-Polyacrylamid-Gelelektrophorese

disk-SDS-PAGE) wurde ebenfalls eine Methode entwickelt und optimiert. Für die quantitative Bestimmung des Gehalts des Hauptallergens Mal d 1 in den Früchten wurde ein direkter *Enzyme-linked Immunosorbent Assay* (ELISA) entwickelt. Hochleistungsflüssigkeits-chromatographie mit Diodenarray-Detektor gekoppelt mit Elektrospray-Ionisations-Massenspektrometrie (HPLC-DAD-ESI-MS^n) wurde zur Identifizierung und Quantifizierung der phenolischen Verbindungen eingesetzt, und die Identifizierung der allergenen Proteine erfolgte nach einem In-Gel-Verdau durch Flüssigchromatographie-Elektrospray-Ionisierungs-Quadrupol-Flugzeit-Massenspektrometrie (LC-ESI-qTOF-MS) und Flüssigchromatographie im Nanomaßstab gekoppelt mit Tandem-Massenspektrometrie (nanoLC-MS/MS). Die Analyse des Selengehalts wurde von einem Projektpartner mittels Graphitrohr-Atomabsorptions-spektroskopie (GF-AAS) durchgeführt. Der Selengehalt in den Äpfeln wurde durch die Biofortifikation signifikant um einen Faktor zwischen 10 und 40 auf Gehalte von 2,1 bis 23,2 µg/100 g Frischmasse erhöht. Somit kann der Verzehr von selenbiofortifizierten Äpfeln wesentlich zu einer besseren Selenversorgung der Bevölkerung beitragen. Ein mittelgroßer Apfel kann etwa ein Viertel des täglichen Selenbedarfs eines Erwachsenen decken, dieser beträgt 70 µg für Männer und 60 µg für Frauen.

Die eingesetzte Selenform spielte für den Selengehalt in den Äpfeln keine wesentliche Rolle. Aufgrund der beobachteten Fruchtschäden bei der Anwendung von Selenit wird jedoch das pflanzenverträglichere Selenat für zukünftige Anwendungen bevorzugt.

Die Biofortifikation führte zu geringeren Schwankungen in der PPO-Aktivität. Darüber hinaus wurde gezeigt, dass die Höhe der Selenanwendung die Enzymaktivität beeinflusst. Hier wurden höhere Anwendungsmengen mit höheren PPO-Aktivitäten in Verbindung gebracht. Die PPO-Aktivität stand im Zusammenhang mit dem TPC, da dieses Enzym den Abbau von Phenolverbindungen katalysiert. Bei höherem Seleneinsatz wurde ein geringerer TPC gemessen, was sich durch die höhere PPO-Aktivität erklären lässt, durch die mehr phenolische Verbindungen oxidiert werden.

In Bezug auf die AOA zeigte die Biofortifikation keine klaren Trends. Dies hängt wahrscheinlich mit den unterschiedlichen antioxidativen Aktivitäten der einzelnen Phenolverbindungen zusammen. Die in der vorliegenden Arbeit analysierten Äpfel enthalten vor allem Chlorogensäure mit einem Gehalt von bis zu 40 % sowie Epicatechin und hohe Mengen verschiedener Quercetin-Glycoside und Phloretin-Glucoside. Die Biofortifikation zeigte bei

den Sorten 'Fiesta', 'Jonica', 'Golden Delicious' und 'Jonagold' zum Teil unterschiedliche Wirkungen in Bezug auf den Gehalt und den Anteil der einzelnen phenolischen Verbindungen. In den meisten Fällen wurde ein signifikanter Anstieg von Epicatechin und Caffeeoylglucosid und eine Verringerung von Procyanidintrimer beobachtet.

Die vier allergenen Proteine Mal d 1, Mal d 2, Mal d 3 und Mal d 4 wurden in den Äpfeln durch disk. SDS-PAGE und nanoLC-MS/MS identifiziert. Die Proteinmuster der verschiedenen Apfelproben sind grundsätzlich ähnlich, unterscheiden sich aber in der Intensität der Proteinbanden, was auf unterschiedliche Gehalte der einzelnen allergenen Proteine hinweist. Zur Bestimmung der Lokalisierung der verschiedenen allergenen Proteine wurden getrennte Analysen von Schale und Fruchtfleisch durchgeführt. So zeigte sich, dass die Schale besonders reich an Mal d 3 ist, während Mal d 2 hauptsächlich im Fruchtfleisch zu finden ist. Mal d 1 wurde sowohl in der Schale als auch im Fruchtfleisch gefunden.

In Bezug auf alle gemessenen Parameter wurden die Apfelsorte und ökophysiologische Bedingungen wie die Sonnenscheindauer als weitere Einflussfaktoren identifiziert.

Korrelationsanalysen wurden durchgeführt, um die Beziehung zwischen phenolischen Verbindungen und dem Mal d 1-Gehalt zu analysieren. Es wurde festgestellt, dass die PPO-Aktivität und der Gehalt an einzelnen phenolischen Verbindungen mit dem Mal d 1-Gehalt korrelierten. Die Beziehung zwischen der PPO-Aktivität und dem Mal d 1-Gehalt schien sowohl sortenabhängig als auch von den ökophysiologischen Bedingungen beeinflusst zu sein, da sowohl positive als auch negative Korrelationen festgestellt wurden. Niedrigere Mal d 1-Gehalte wurden bei Äpfeln mit vergleichsweise hohem Chlorogensäuregehalt und niedrigem Procyanidintrimer- und Epicatechingehalt beobachtet. Der Gesamtphenolgehalt und das Niveau der antioxidativen Aktivität scheinen dagegen nur eine untergeordnete Rolle zu spielen.

Es wurde auch eine Korrelation zwischen dem Selen- und dem Mal d 1-Gehalt und damit ein Einfluss der agronomischen Düngung festgestellt. Ein hoher Selengehalt war mit einem niedrigen Mal d 1-Gehalt verbunden. Darüber hinaus wurden bei den biofortifizierten Äpfeln häufig die oben erwähnten Veränderungen im Phenolprofil beobachtet, die mit einer geringen Allergenität einhergingen. Somit scheint diese Maßnahme geeignet zu sein, das allergene Potenzial von Äpfeln zu reduzieren.

3. INTRODUCTION

Apples are the most popular fruit in Germany, with a consumption of 25 kg per capita (BMEL, 2020; Statista, 2021a). They are rich in many nutritionally important ingredients such as vitamins, minerals, trace elements, and secondary plant metabolites, especially flavonoids and phenolic acids (Hyson, 2011; Knekt et al., 1996; Kschonsek et al., 2018; Le Marchand et al., 2000; Souci et al., 2011; Tsao et al., 2005). The consumption of apples is recommended for a healthy diet. The oligomeric procyanidins contained in apples are believed to reduce the risk of stroke, other cardiovascular diseases, and lung cancer (Gerhauser, 2008; Knekt et al., 2000; Le Marchand et al., 2000). Selenium also has an important role as an essential trace element in the human diet, and high selenium status is discussed with a lower risk of developing various cancers (Rayman, 2012). This is due to the fact that selenium is an integral part of some antioxidant enzymes, which protect cells from being damaged by radicals produced during oxidative metabolic pathways (Kielliszek & Błażejak, 2016).

In Germany and other European regions, selenium is often inadequately taken up through the diet (Steinbrenner & Brieglious-Flohé, 2015), because the trace element is present in soils only in small amounts and food plants can thus hardly accumulate selenium (Poňavič & Scheib, 2014). Apples also contain very little amount s of selenium (Navarro-Alarcon & Cabrera-Vique, 2008). The current recommendation for Germany, Austria, and Switzerland (D-A-CH reference values for nutritional intake) for the daily intake of selenium is approximately 1 µg selenium per kg body weight. With consideration of the reference body weights, the resulting estimated values for selenium intake are approximately 70 µg/day for adult men and 60 µg/day for adult women (Kipp et al., 2015). Usually, the need for selenium is largely covered by animal produce, such as meat or fish. The biofortification of plant produce allows for vegetarians or vegans in particular to fulfill their needs naturally and as an alternative to food supplements (Oster & Prellwitz, 1989; Willers et al., 2015).

The agronomic biofortification of food crops with selenium, which involves targeted fertilization, offers a good opportunity to increase the selenium content. For example, biofortification in Finland, which has been carried out nationwide since 1985, has successfully improved selenium supply to the population and achieved optimal selenium status (Alfthan et al., 2015).

The project *BiofortiSe* (Biofortification of apples with selenium to improve fruit quality, storability and health value), aimed at a fertilization of apple trees with selenium-containing fertilizers was carried out via foliar fertilization. The primary objective was to increase the selenium content in the fruit and to analyze the influence of this cultural measure on the content of secondary plant metabolites.

Biofortification with selenium has already been carried out on various food crops. Here, different degrees of selenium accumulation were found, depending on the applied selenium form (sodium selenite or sodium selenate) as well as at different fertilization rates. It was shown that the treatments resulted in increased selenium contents compared to the untreated controls (D'Amato et al., 2018; Ekanayake et al., 2015; Hawrylak-Nowak, 2008; Hawrylak-Nowak, 2013; Ríos et al., 2008; Schiavon et al., 2013; Schiavon et al., 2016). In addition, an influence on secondary plant metabolites such as phenolic compounds has already been found in different vegetable crops. Biofortification resulted in higher levels of phenolic compounds, changes in phenolic profile and/or also increases in antioxidant flavonoids or antioxidant activity (D'Amato et al., 2017; D'Amato et al., 2018; Bachiega et al., 2016; Pezzarossa et al., 2012; Schiavon et al., 2013; Schiavon et al., 2016; Zhao et al., 2013).

Consumption of apples can cause allergic reactions (Botton et al., 2008; Breiteneder & Ebner, 2000; Burney et al., 2014; Grafe, 2009), especially in people with birch pollen allergy because of the homology of the chemical structure of the allergenic proteins Bet v 1 in birch and Mal d 1 in apples (Gilissen et al., 2005). Sensitization to apples affects about 4 million people in Germany alone (Bernert et al., 2012; Kschonsek et al., 2019). So far, four allergenic proteins are known in apple: Mal d 1, Mal d 2, Mal d 3, and Mal d 4 (Grafe, 2009; Kleine-Tebbe et al., 2016), which differ in various parameters such as protein family, function, and localization in the fruit, as well as in the clinical symptoms of allergy. For example, the major allergen Mal d 1 is synthesized mainly to protect the plant from pathogens and under certain environmental stress factors (Beuning et al., 2004; Botton et al., 2009; Breiteneder & Ebner, 2000; Grafe, 2009; Matthes & Schmitz-Eiberger, 2009; Puehringer et al., 2000).

So far, many factors influencing the content of allergenic proteins in apples could be identified. Here, the content mainly depends on the cultivar (Bolhaar et al., 2005; Kschonsek et al., 2019a; Matthes & Schmitz-Eiberger, 2009; Sancho et al., 2006a; Schmitz-Eiberger & Matthes, 2011; Son & Lee, 2001; Zuidmeer et al., 2006). Other influencing factors include storage duration

and storage conditions (Bolhaar et al., 2005; Kiewning et al., 2013; Kiewning & Schmitz-Eiberger, 2014; Matthes & Schmitz-Eiberger, 2009; Schmitz-Eiberger & Matthes, 2011; Sancho et al., 2006a) as well as ecophysiological and cultivation conditions at the growing sites (Schmitz-Eiberger & Matthes, 2011; Wang et al., 2017; Zuidmeer et al., 2006) or organically cultivated apples (Fernández-Rivas et al., 2006).

It has already been shown that the primary metabolism, which includes protein biosynthesis, is also significantly affected by selenium biofortification (D'Amato et al., 2018; Jing et al., 2017; Poblaciones et al., 2013; Poblaciones et al., 2014a; Poblaciones et al., 2014b; Reis et al., 2018).

Also, a relationship of allergenicity (especially in relation to Mal d 1) with phenolic compounds and polyphenoloxidase activity, which in turn has a significant influence on polyphenol content and composition, has been previously described (Björksten et al., 1980; Bernert et al., 2012; Chung & Champagne, 2009; Kiewning et al., 2013; Kiewning & Schmitz-Eiberger, 2014; Kschonsek et al., 2019a; Kschonsek et al., 2019b; Rudeschko et al., 1995a; Rudeschko et al., 1995b; Schmitz-Eiberger & Matthes, 2011; Singh et al., 2011). In this context, it is further assumed that the interactions between the polyphenols and the allergenic protein Mal d 1 play an important role in reducing allergenic potential (Bergmann et al., 2020; Bernert et al., 2012).

4. THEORETICAL BACKGROUND

4.1. Biofortification with selenium

4.1.1. Agronomic biofortification with selenium

Biofortification is an agronomic practice used in plant breeding to selectively increase one or more nutrient contents in plant foods. This is done through the application of fertilizers. In most cases, the aim is to increase mineral nutrients such as zinc, iodine, or selenium, as these are not found in sufficient quantities in the soil (Cakmak, 2009; Gupta & Gupta, 2017; Hirschi, 2009; Lawson et al., 2016). In practical application, it is a simple and inexpensive process. Here, the incorporation of the applied nutrients depends on many factors such as the method of application of the fertilizer, the soil composition, and the nutrient mobility in the plant (Hierschi, 2009). In the *BiofortiSe* project, liquid fertilizers containing sodium selenite or sodium selenate were used for foliar fertilization of apple trees. This is intended to increase the trees' uptake of the mineral, which is only available in limited quantities in the soil, and to accumulate it naturally in the fruit (Bañuelos et al., 2017; Broadley et al., 2006; Ros et al. 2016). Selenium can be taken up via roots and above-ground plant parts and integrated into plant metabolism by incorporation into amino acids such as selenocysteine and selenomethionine (Winkel et al., 2015). The soils in Germany and other European regions are poor in selenium, which means that the selenium content of the food plants growing there is also low. This in turn results in an undersupply or malnutrition of the population in this trace element (Poňavič & Scheib, 2014).

In Finland, since the mid-1980s, all mineral fertilizers for use in food and food crop production have been mandatorily fortified with selenium. This has resulted in a significant increase in the selenium content of plant and animal foods, which led to a sustainable selenium supply for the population. The average selenium intake here is within the recommended and thus optimal range (Alfthan et al., 2015).

However, the soil fertilization practiced there, especially for cereals, does not have sufficient efficiency in fruit production. Studies on Chinese dates have shown that only a relatively small proportion of the fertilized selenium is transferred to the fruit when soil fertilization is applied (Zhao et al., 2013). In addition, there is a risk that the fertilized selenium will be fixed in the soil by sorption or lost by leaching. In apples, soil fertilization of trees also resulted in little

accumulation of selenium from applied selenium-rich fertilizers in fruits (Liu et al., 2020). A comparison between soil and foliar fertilization showed that only a fivefold repetition of soil fertilization resulted in a comparably high selenium storage in apple leaves and fruits as foliar fertilization (Jakovljevic et al., 1996). Studies of the three different agronomic techniques soil fertilization, trunk injection, and foliar fertilization on pear-jujube showed that spray treatment is the most effective method (Zhao et al., 2013). Selenium form is also a factor influencing uptake by the plant. For example, the administration of selenate usually leads to higher selenium accumulation than selenite at the same selenium application rate (D'Amato et al., 2018; Ekanayake et al., 2015; Hawrylak-Nowak, 2008; Ríos et al., 2008). Further advantage of selenate is its better plant tolerance (Ros et al., 2016; Puccinelli et al., 2017).

Biofortification can also be easily integrated in apple production as an additive in calcium-containing fertilizers, as its use for prevention of speckle, lentil cell spot, flesh browning, and storage rot is established and common practice.

4.1.2. Relevance of selenium in human nutrition

Selenium is an essential trace element in human nutrition and is mainly important as a component of proteins and enzymes such as glutathione peroxidase, thyroxine 5-deiodinase, and selenoprotein P (Kielliszek, 2019). Selenium exerts its nutritional functions in the form of 25 selenoproteins that have the amino acid selenocysteine in their active center (Rayman, 2012). Via the enzyme thyroxine-5-deiodinase, selenium is involved in the production of the active thyroid hormone and thus in the regulation of the immune system (Kielliszek & Błażejak, 2016). Furthermore, selenium is essential for reproduction and has antioxidant, anti-inflammatory, and antiviral effects (Kielliszek & Błażejak, 2016). As an integral component of some antioxidant enzymes that protect cells from radical damage, selenium is also important (Kielliszek, 2019). Optimal selenium supply can minimize selenium-dependent diseases such as reduced immune function, cardiovascular system degeneration, and cognitive decline (Gupta & Gupta, 2017; Kielliszek, 2019; Rayman, 2012). However, if selenium supply is inadequate, the risk of certain diseases such as colorectal and liver cancer and thyroid disease increases (Ambroziak et al., 2017; Combs & Yan 2016; Hughes et al., 2015; Hughes et al., 2016; Schomburg & Arnér, 2017).

Currently, the selenium supply of the population in Germany and other European countries is insufficient. With a daily intake in Germany for women averaging 38 µg selenium and for men

47 µg selenium, the actual intake in each case is only about two-thirds of the German Nutrition Society (DGE) reference values (Kipp et al., 2015). With the exception of Finland, the daily intake of the population of other countries in Europe is in similarly low ranges (European Commission, 2000). The recommendations for the daily intake of selenium for Germany, Austria, and Switzerland (D-A-CH reference values for nutritional intake) are about 1 µg selenium per kg body weight. Taking into account the reference body weights, the estimated values for selenium intake are about 70 µg/day for adult men, 60 µg/day for adult women, and 75 µg for lactating women (Kipp et al., 2015).

The intake of selenium is mainly covered by animal products such as meat and fish. Animal foods also contain higher levels of selenium due to selenium supplements in animal feed. Therefore, vegetarians and vegans are more at risk of selenium deficiency than omnivores (Fallon & Dillon, 2020). As an alternative to selenium-rich supplements, biofortification of plant-based products is highly suitable for meeting selenium requirements naturally (Oster & Prellwitz, 1989; Willers et al., 2015). A consumer survey also showed that German consumers would prefer selenium-rich apples instead of dietary supplements (Wortmann et al., 2018). Another advantage is the varying bioavailability of the selenium forms in selenium-enriched foods and in dietary supplements. The latter often contain inorganic selenium forms that are hardly bioavailable. In contrast, selenium-enriched foods transform the supplied inorganic selenium into organic compounds such as selenocysteine and selenomethionine, which can be absorbed more easily in the intestinal tract (Kielliszek & Błażejak, 2016; Kielliszek, 2019).

4.1.3. Effect of selenium on the quality of plant-based foods

In addition to an increase in the selenium content of apples, it is to be expected, based on numerous studies already published, that other fruit characteristics relevant to marketing will be positively influenced. Studies on pears and peaches showed that even a single administration of selenium can lead to a significant increase in sugar content and flesh firmness and can extend the shelf life of the fruit after storage (Pezzarossa et al., 2012). In pear-jujube (*Ziziphus jujuba* MILL. cv. 'Lizao'), a significant increase in vitamin C content was observed (Zhao et al., 2013). An effect on the content of phytochemicals has also been widely observed in different fruit and vegetable crops: for example, a significant increase in phenolic compounds was shown in olives, rice, radish, and broccoli (Bachiega et al., 2016; D'Amato et al., 2014; D'Amato et al., 2017; D'Amato et al. 2018; Pezzarossa et al., 2012; Schiavon et al.,

2013; Schiavon et al., 2016). Changes in the profile of phenolic compounds with an increase of antioxidant phenolics or increased antioxidant activity have also been previously observed in olives, tomatoes, and broccoli (Bachiega et al., 2016; D'Amato et al., 2017; Pezzarossa et al., 2012; Schiavon et al. 2016).

An influence by biofortification with selenium on the primary metabolism of food crops has also been reported. Here, selenium application led to changes in protein content in cereal and vegetable crops such as rice, jujube, peas, bread wheat, and durum wheat (D'Amato et al., 2018; Jing et al., 2017; Poblaciones et al., 2013; Poblaciones et al., 2014a; Poblaciones et al., 2014b; Reis et al. 2018).

4.2. Cultivated apple (*Malus domestica* BORKH.)

4.2.1. Origin and botanical basis

The origin of the apple dates back several million years, and the original apple varieties grew in tropical and subtropical mountain valleys in Southeast Asia (Aas, 2013; Hanke & Flachowsky, 2017; März, 2012). Figure 1 shows the origin of the apple and their evolutionary history. The original forms of cultivated apple can be traced back to the wild apple *Malus siversii*, which was cultivated in Asia more than 8,000 years ago. They spread via consumption and subsequent excretion of seeds by animals and humans along the trade route from Central Asia to the Black Sea. In the 9th century BC, the apple reached Europe. Crossing with locally native *Malus* species such as the wild European crab apple *M. sylvestris*, *M. baccata*, and *M. orientalis* resulted in new cultivars (Duan et al., 2017; Industrieverband Agrar, 2018; Juniper et al., 1998; Lieberei & Reisdorff, 2012).

The first cultivation of the apple was in Greece in the year 1,000 BC. The apple did not gain economic importance through the planting of apple trees for the sale of the fruit until the 16th century (März, 2012). The more than 30,000 apple varieties that exist today were created through selection and selective crossing (Cornille et al., 2014; Lieberei & Reisdorff, 2012). However, only about 70 of these varieties are in commercial cultivation in Europe (März, 2012). Among them, the most important varieties in cultivation in the European Union are 'Golden Delicious', 'Gala', 'Red Delicious', 'Jonagold', and 'Elstar' (Figure 2) (März, 2012; Statista, 2021b).

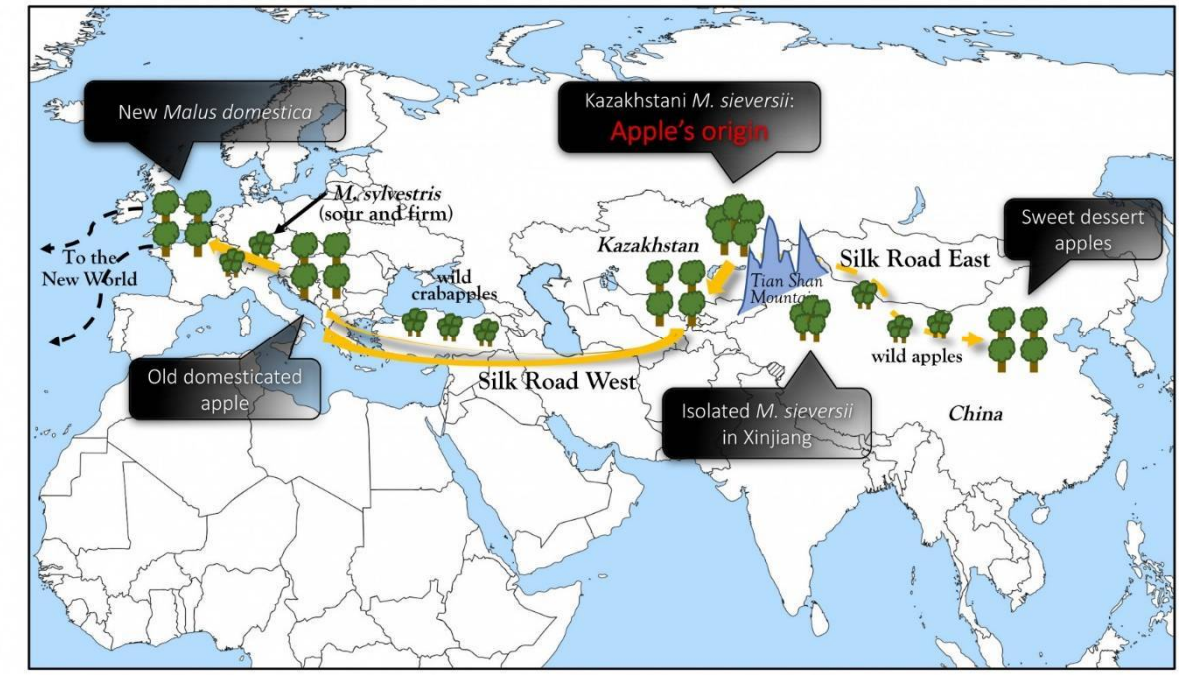


Figure 1: Origin of the apple and their evolutionary history (Industrieverband Agrar, 2018).

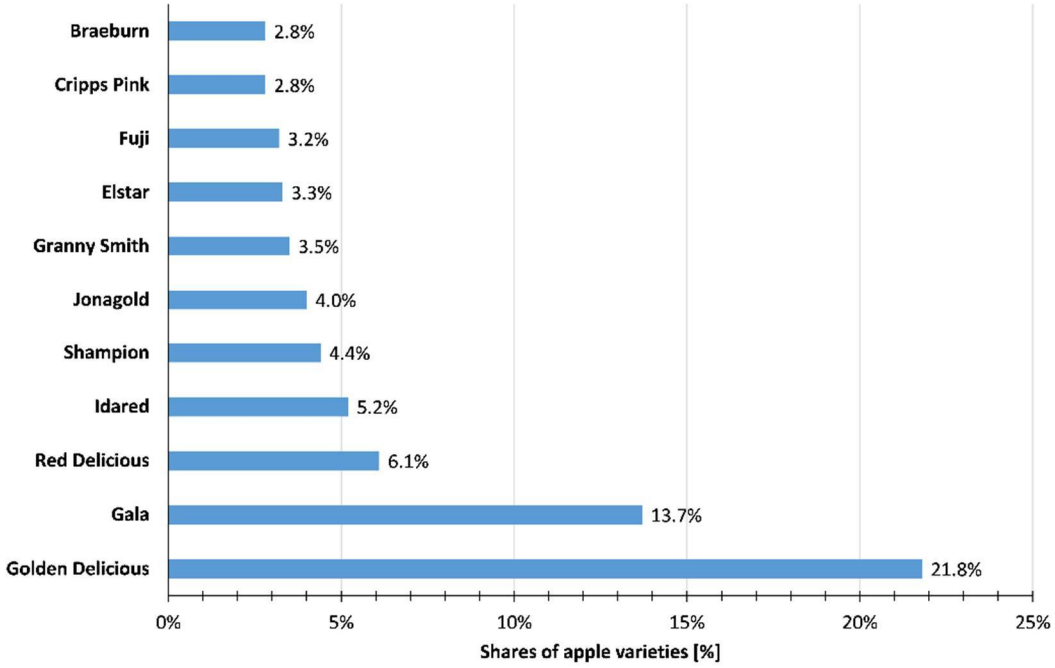


Figure 2: Shares of apple varieties in the European Union in 2019/2020 (own illustration according to Statista, 2021b).

The cultivated apple *Malus domestica* BORKH. belongs to the rose family (Rosaceae) and is a species of the pome fruit subfamily (Maloideae). Botanically, it belongs to the group of lower bellows fruits. The schematic structure of an apple is shown in Figure 3. The apple tree is self-incompatible, so pollination of the five-petaled white to pale pink flower occurs by cross-

pollination. In this process, a fleshy fruit with five carpels, each containing two ovules, is formed from the flower base by thickness growth. Since the flesh is thus formed from the receptacle rather than the ovary, the apple is a pseudo-fruit. The fleshy receptacle is firmly fused with the ovules, so the seeds can only be released by removing the flesh (exocarp) (Lewitzki, 2020; Lieberei & Reisdorff, 2012).

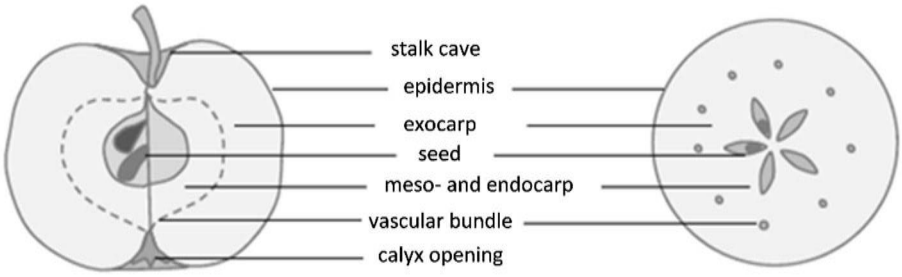


Figure 3: Schematic representation of an apple in longitudinal and cross section (according to Kadereit et al., 2014).

4.2.2. Distribution and economic importance

Cultivated apples are mainly distributed in the temperate climate zone, in the northern areas of Europe, Asia, and North America (März, 2012). In cultivation and consumption, apples are one of the most important and popular fruits. In this regard, China, the USA, Turkey, Poland, and India are the largest producers and exporting countries for apples. In Europe, Poland, Italy, France, and Germany are the main growing countries in descending order (Statista, 2021c). In 2019, the total global harvest was about 87.2 mio. t, with about 1 mil. t harvested on a cultivation area around 34,000 hectares in Germany (Statista, 2021d; Statista, 2021e). In Germany, the apple harvest accounted for about 75% of the total fruit harvest in 2019. This is followed by strawberries with a share of around 11% (Statista, 2021f). In this context, 73.6 kg of fruit were consumed per capita in Germany in 2018/2019, whereby apples formed the largest incentive with an amount of 25.5 kg (Statista, 2021g; Statista, 2021a). Thus, apples were the most popular fruits.

4.2.3. Ingredients

The apple fruit consists of the ingredients listed in Table 1. These are average values, as variations occur within the different varieties, growing regions, and growing years. The degree of ripeness also plays a role (Lieberei & Reisdorff, 2012).

Table 1: Ingredients of the apple fruit (Lieberei & Reisdorff, 2012).

Ingredient	Percentage [%]
Water	85.3
Carbohydrates	11.4
Dietary fiber	2.02
Fats	0.58
Organic acids	0.46
Proteins	0.34
Minerals	0.32

The main component of the apple fruit is water, which accounts for 85%. The sweet taste of the fruit is due to the carbohydrates it contains, which are mainly soluble sugars in the form of fructose, glucose, and saccharose. The dietary fiber content is 2.0%. It also contains small amounts of lipids (0.6%) and proteins (0.3%). Organic acids, mainly malic, quinic and citric acids, contribute to the flavor in addition to soluble sugars. In addition to the primary constituents that determine the nutrient and energy content of the fruit, a large number of nutritionally important vitamins, minerals, and secondary plant metabolites are also present. For example, apples contain mainly vitamin C (12 mg/100 g), but also a number of B vitamins (B1, B2, nicotinamide), potassium (144 mg/100 g), sodium (3 mg/100 g), iodine (2 µg/100 g) and zinc (120 µg/100 g) (Lieberei & Reisdorff, 2012; Souci et al., 2011).

The secondary plant metabolites of carotenoids and the large number of different phenolic compounds contained in apple fruit serve the plant as defenses against pests and diseases and are involved in the coloration and flavor of the fruit (März, 2012).

4.3. Phenolic compounds

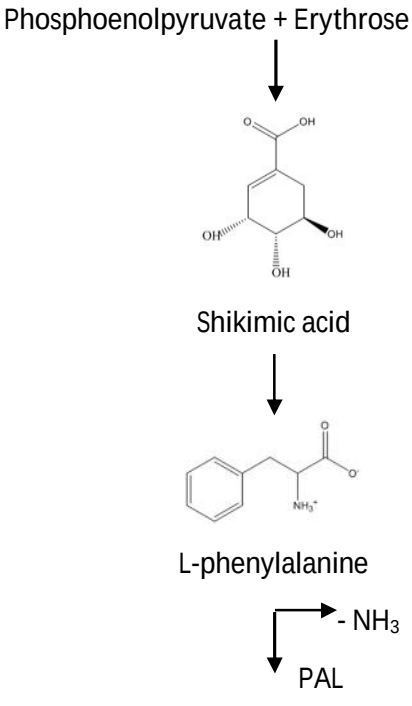
4.3.1. Functions in the plant, biosynthesis, structure, and classification

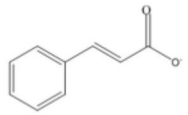
In addition to the primary metabolism of the plant, in which vital substances such as amino acids, sugars, and lipids are formed, plants synthesize other chemical compounds that are not directly vital to the plant. These are referred to as secondary plant metabolites (Munk, 2008). They occur naturally in all plant materials and their products, where they help determine color, appearance, and flavor (Lattanzio, 2003).

The phenolic compounds are among the secondary plant metabolites, and here they constitute the largest group of substances among the secondary metabolites. In general, phenols in plants function as protective substances against rot, pest or animal damage, regulation of the plant hormone balance, and use as signal substances. For example, there are substances that serve to attract insects to pollinate flowers because of their color or scent. The group of phenols includes various colorants such as anthocyanins (orange-red, red, purple, blue) and other flavonoids (yellow, orange, brown, black), which give grapes, fruits, and flowers their attractive color and thus attract insects and birds, ensuring seed dispersal (Wendelin & Eder, 2002). Particularly in outer tissues, phenolic compounds are also formed by the plant to protect its cell organelles from harmful UV radiation (Matern & Grimmig, 1993; Munk, 2008; Rhodes, 1998; Schmid & Amrhein, 1995).

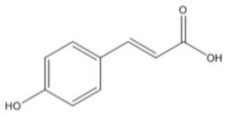
The starting substance for the biosynthesis of phenolic compounds is considered to be the amino acid L-phenylalanine, which can be synthesized via phosphoenolpyruvate and other compounds during the shikimic acid pathway (primary metabolic pathway) (Heldt & Piechulla, 2015; Munk, 2008).

Figure 4 shows a schematic representation of polyphenol biosynthesis and the individual classes. In the first step, trans-cinnamic acid is formed by deamination of L-phenylalanine by the enzyme phenylalanine ammonia lyase (PAL) in the phenylpropane pathway. In this process, ammonia is cleaved (Dixon & Paiva, 1995; Vogt, 2010).





trans-cinnamic acid

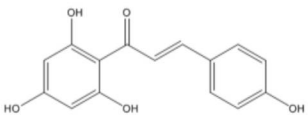


p-coumaric acid

HYDROXYCINNAMIC ACIDS → Caffeic acid → Ferulic acid

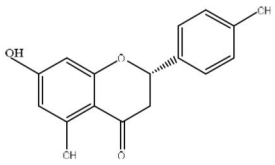
p-coumaroyl-CoA

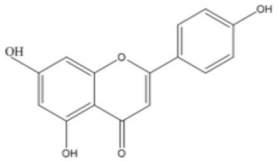
← 3 malonyl-CoA



Naringenin chalcone

CHALCONE → **DIHYDROCHALCONE**

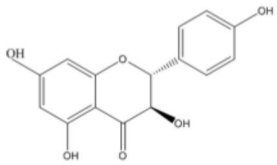


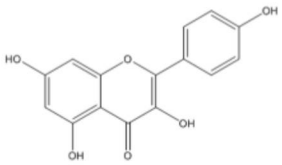


Naringenin

Apigenin

ISOFLAVONE ← **FLAVANONE** → **FLAVONE**





Dihydrokaempferol

Kaempferol

DIHYDROFLAVONOLE → **FLAVONOLE**

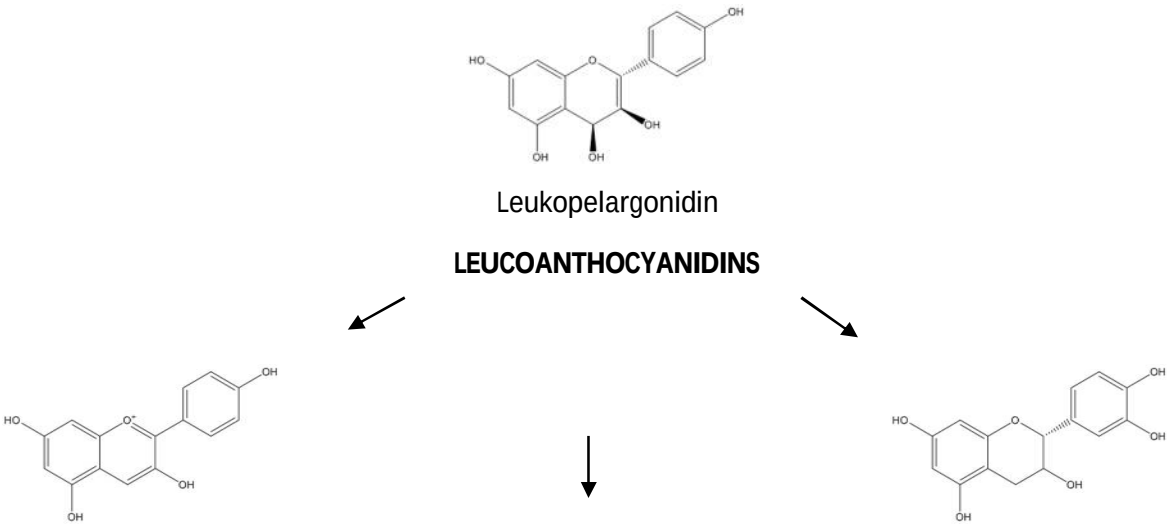


Figure 4: Schematic representation of polyphenol biosynthesis and the individual classes (own illustration according to Treutter, 2001; Vogt, 2010).

After methylation and hydroxylation by further enzymes, a large number of hydroxycinnamic acid derivatives such as coumarins, flavonoids, isoflavonoids, catechins, and proanthocyanidins are formed (Vogt, 2010).

Initially, the *p*-coumaric acid, which belongs to the hydroxycinnamic acids, is formed here by oxidation reactions. Further oxidation as well as hydroxylation and methylation reactions lead to the synthesis of caffeic and ferulic acids. The activated hydroxycinnamic acid *p*-coumaroyl-CoA, 4-coumaroyl-coenzyme A and three malonyl-CoA units initiate the formation of flavonoids. Naringenin chalcone is formed, which by condensation and with the participation of chalcone synthase is the basic structure for the formation of flavonoids. Further condensation, with the participation of the enzyme chalcone flavanone isomerase, gives rise to a flavanone (naringenin) from the chalcone. Subsequent cyclization reactions led to the formation of isoflavones and dihydroflavonols. From the dihydroflavonols (dihydrokaempferol), the leucoanthocyanidins are formed with the help of dihydroflavonol reductase, which further react to anthocyanidins by anthocyanidin synthase (Belitz et al., 2008; Gunzler et al., 1997; Tsao, 2010).

Chemically, phenols are compounds bearing one or more hydroxyl groups or derivatives thereof on an aromatic ring system (Robards et al., 1999). More than 8,000 different phenolic compounds are already known (Bravo, 1998). Phenols can be divided into the two major

groups of simple phenols and polyphenols, with the major class defined according to the nature of the basic structures of the carbon skeleton (Vermerris & Nicholson, 2006). Table 2 shows the major classes of phenolic compounds in plants (Harborne, 1980; O'Connell & Fox, 2001).

Table 2: Major classes of phenolic compounds in plants (according to Harborne, 1980; O'Connell & Fox, 2001).

C_6	C_6-C_n	C_6-$C_n$$C_6$	$(C_6$-$C_3)_n$	Tannins
Simple phenols	C_6-C_1 Phenolic acids	C_6-C_1-C_6 Xanthones	$(C_6$-$C_3)_2$ Lignan	Condensed
Benzoquinones	C_6-C_2 Acetophenones	C_6-C_2-C_6 Stilbenes	$(C_6$-$C_3)_{2+n}$ Lignin	Hydrolysible
	C_6-C_2 Phenylacetic acids	C_6-C_2-C_6 Anthraquinones		
	C_6-C_3 Hydroxycinnamates	C_6-C_3-C_6 Flavonoids		
	C_6-C_3 Coumarins			
	C_6-C_3 Chromones			
	C_6-C_4 Naphthoquinones			

The most important polyphenols in fruits can be divided into three groups: Phenolic carboxylic acid and its derivatives (non-flavonoids), flavonoids, and low molecular weight phenols (aroma compounds), see Figure 5 (Herrmann, 1992; Rechner, 2000).

The phenolic carboxylic acids can be further subdivided into the hydroxycinnamic acid and hydroxybenzoic acid compounds. Hydroxycinnamic acids, which include, for example, *p*-coumaric acid and ferulic acid, have a C3-C6 backbone (Belitz et al., 2008).

The largest class of phenolic compounds here are the flavonoids, which include the flavonols, flavanones, flavones, isoflavones, and anthocyanins (Bernhard & Gerhard, 2001; Ebermann & Elmadfa, 2008). About 6,500 different structures of flavonoids are known. Based on structural differences on the C-ring, flavonoids can be classified into the following six groups: Flavonols, Flavones, Flavanols, Anthocyanidins, Flavanones, and Isoflavones (Bernhard & Gerhard, 2001; Engelhardt, 1998,).

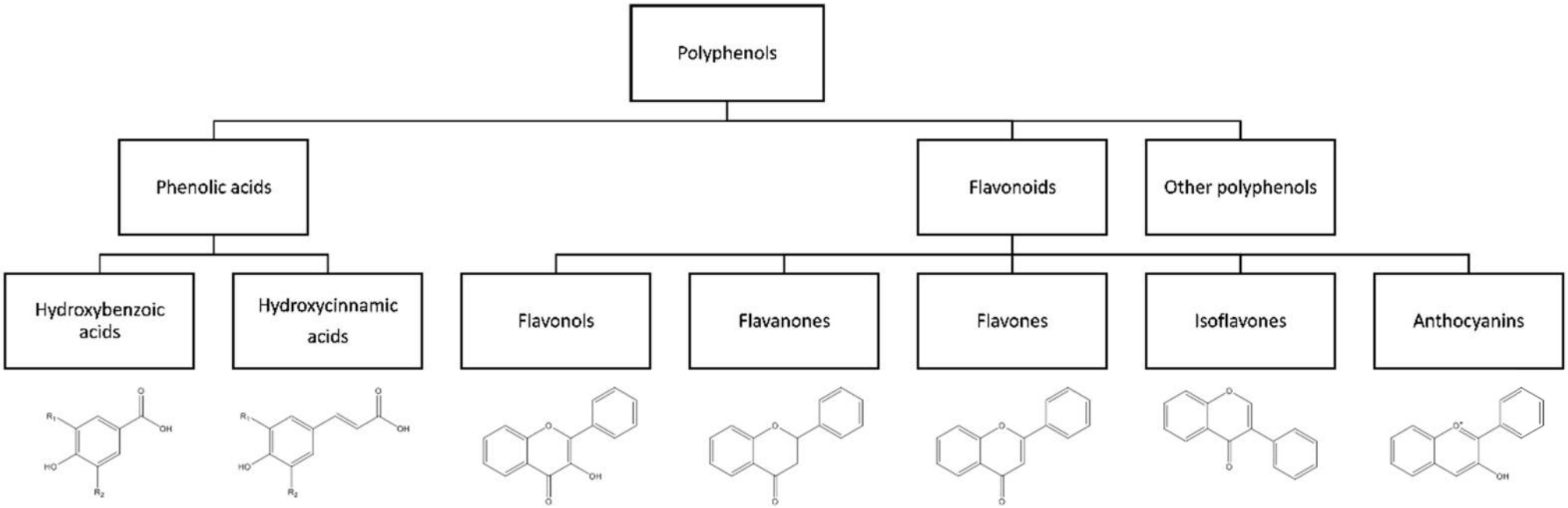


Figure 5: Tree chart for overview about the most important phenolic classes and the structural formulars (own illustration according to Crozier et al. 2009; Iriti & Faoro, 2009; Manach et al., 2004).

The flavan-3-ols form the hydrogenated flavones as colorless compounds, which have a saturated bond between C2 and C3. Flavanols have two asymmetric C atoms, therefore two diastereomeric compounds are formed. Flavanols include catechin and epicatechin (Frede, 2010). Flavonols are yellow pigments that have an unsaturated bond between C2 and C3. One flavonol is quercetin (Frede, 2010). The anthocyanidins form a group of red, purple, and blue dyes that have conjugated double bonds and a C6-C3-C6 ring system (Wendelin & Eder, 2002). Chalcones belong to the group of flavonoids, from which they can also be produced synthetically. Examples of dihydrochalcones are phloretin xyloglucosides (2'-O-β-xyloglucoside) and phloridzin (2'-O-β-glucoside), which occur specifically in the *Malus* genus (Wendelin & Eder, 2002). The proanthocyanidins represent the colorless precursor of the colored anthocyanins and are formed from the different degrees of polymerization of flavan-3-ols or flavan-3,4-diols. (+)-Catechin and (-)-epicatechin are considered to be the basic structure. An example is procyanidin B2 (Wendelin & Eder, 2002; Belitz et al., 2008).

4.3.2. Phenolic compounds in apples

The five classes of polyphenols found in apples are flavan-3-ols, phenolic acids, dihydrochalcones, flavonols, and anthocyanidins (Kschonsek et al., 2018; van der Sluis & Dekker, 2001).

Flavan-3-ols are the most abundant group of polyphenols in apples. They can be divided into monomers, oligomers, and polymers. The only two monomers of flavan-3-ols in apples are catechin and epicatechin, with the amount of epicatechin being higher on average. The condensation of these two monomers leads to oligomers called procyanidins. The most important procyanidins in apples are procyanidin B1 and procyanidin B2. Monomeric and oligomeric flavan-3-ols occur in higher amounts in the peel than in the flesh (Tsao et al., 2003). Flavan-3-ols are responsible for the astringent taste and enzymatic browning of apples by PPO (Renard & Dupont, 2007). The higher the degree of polymerization, the more astringent the sensation (Vidal & Francis, 2003).

The two subclasses of phenolic acids are hydroxycinnamic acids and hydroxybenzoic acids. In apples, only the hydroxycinnamic acids are relevant. The main phenolic acid in apples is chlorogenic acid. Chlorogenic acid is an ester of caffeic acid and quinic acid. As a hydrocinnamic acid derivative, *p*-coumaroylquinic acid occurs in small amounts in apples (Treutter, 2001). Hydroxycinnamic acid and its derivatives are found in apple peels as well as

apple pulp. The peeling of an apple consequently does not reduce the phenolic acid concentration per unit weight. In addition to flavan-3-ols, chlorogenic acid is also a substrate of PPO and also contributes to the enzymatic browning of apples (Treutter, 2001).

Dihydrochalcones are found exclusively in apples and apple products. The main dihydrochalcones in apples are phloridzin and phloretin xyloglucoside (Vrhovsek et al., 2004). They are phloretin derivatives substituted with carbohydrates at the C2-position. While phloridzin is substituted with glucose, phloretin xyloglucoside is linked with xyloglucose. Dihydrochalcones occur in higher amounts in apple peel than in apple pulp. In addition, dihydrochalcones account for up to 60% of the polyphenols in seeds. The dihydrochalcones content of apples is reduced by peeling and coring. Dihydrochalcones are also substrates of PPO in apples (Tomás-Barberán & Clifford, 2000).

Flavonols are a group of polyphenols in apples that are of minor importance. The main flavonols in apples are quercetin-3-galactoside, quercetin-3-glucoside, quercetin-3-rhamnoside, and quercetin-3-rutinoside, which is also known as rutin. These are glycosylated quercetin derivatives in which glycosylation occurs in the immediate vicinity of the ketone group (Treutter et al., 2001). Quercetin derivatives are mainly found in apple peels (Tsao et al., 2003), where their biosynthesis is induced by light (D'Archivio & Filesi, 2007). Consequently, the content of quercetin derivatives found in individual fruits of a tree and even within a fruit can vary significantly depending on sun exposure (Manach et al., 2004). Peeling an apple removes most of the quercetin glycosides, and therefore the intake of apple flavonols depends on the method of consumption. Quercetin derivatives are sterically hindered for oxidation by PPO due to glycosylation at the C3-position (van der Sluis & Dekker, 2002).

Anthocyanidins occur in small amounts in apples and are responsible for the red color of apple peels. In plants, anthocyanidins occur only glycosidically bound to various sugars. The glycosides of anthocyanidins are called anthocyanins. Glycosylation with glucose, galactose, rhamnose, arabinose, and xylulose occurs preferentially at the C3 atom, and the sugars can occur as mono-, di-, and trisaccharides (Mazza & Miniati, 1993; Prior & Wu, 2006; Rechner, 2000). Depending on pH, strong color changes occur in anthocyanins (Harborne, 1988; Jurd, 1972; Mazza & Miniati, 1993; Schütt, 2014). In red apples, anthocyanins can account for 1 to 3% of total polyphenols. The concentration is highly variable within each cultivar. The major anthocyanins in apple peels are cyanidin-3-galactoside, cyanidin-3-glucoside, cyanidin-3-

arabinoside, cyanidin-3-xyloside, and 7-arabinoside (Vrhovsek et al., 2004), although cyanidin-3-galactoside has been detected exclusively in red apple peel (Tsao et al., 2003).

Apple cultivars differ greatly in their polyphenol contents (Guyot & Marnet, 2003; Sanoner & Guyot, 1999). Table 3 summarizes the mean values of polyphenol contents obtained in five experiments with dessert apples and compares them with the data reported by Polyphenol Explorer (Ceymann, 2013; Neveu & Perez-Jiménez, 2010). The Polyphenol Explorer database contains data on all relevant polyphenols in apples but does not list variety-specific data (Neveu & Perez-Jiménez, 2010).

Data are reported in mg per 100 g of fresh weight (f.w.). Polyphenol profiles are typical for the apple cultivar and are influenced by breeding, culture, growth factors, harvest year, ripeness at harvest, and postharvest storage (McRae & Lidster 1990; Schmitz-Eiberger & Matthes, 2011; van der Sluis & Dekker, 2002).

In addition, the occurrence and distribution of the main polyphenol classes differed in apple peels and apple flesh (Kschonsek et al., 2018). In Table 4, the mean values of polyphenol contents in peels and flesh of apples of the four cultivars (cv.): 'Golden', 'Granny', 'Braeburn', and 'Jonagold' from the literature are given. Data are presented in mg per 100 g of fresh weight (Guyot & Le Bourvellec, 2002; Neveu & Perez-Jiménez, 2010; van der Sluis & Dekker, 2001).

Table 3: Mean values of the content of individual phenolic compounds in apples in mg per 100 g of fresh weight (Lee et al., 2003; Neveu & Perez-Jiménez, 2010; Podsedek & Wilska-Jeszka, 2000; Valavandis & Vlochogianni, 2009; Vrhovsek et al., 2004; Wojdylo et al., 2008).

Reference/ Phenolic compounds	Neveu et al., 2010	Valavanis et al., 2009	Wojdylo et al., 2008	Vrhovsek et al., 2004	Lee et al., 2003	Podsedek et al., 2000
Number of cultivars	10-36	4	67	5	6	10
Flavan-3-ols						
C	1.2	1.8	11.7	1.0	--	1.0
EC	8.3	9.3	58.5	6.6	8.7	6.0
PC B1	--	2.3	14.3	--	--	--
PC B2	14.6	7.4	60.4	6.5	9.4	2.3
Phenolic acids						
CA	13.3	7.1	46.9	8.4	9.0	17.3
CQA	2.3	--	5.1	1.7	--	--
Dihydrochalcones						
PHZ	2.8	3.8	10.1	1.6	5.6	--
PXG	2.6	--	--	1.0	--	--
Flavonols						
QGal	2.4	2.7	17.1	2.7	--	--
QGlu	0.6	--	4.6	0.6	--	--
QR	1.3	--	12.0	0.8	--	--
R	0.2	--	0.9	0.2	--	--

C: catechin; EC: epicatechin; PC B1: procyanidin B1; PC B2: procyanidin B2; CA: chlorogenic acid; CQA: *p*-coumaroylquinic acid; PHZ: phloridzin; PXG: phloretin xyloglucoside; QGal: quercetin-3-galactoside; QGlu: quercetin-3-glucoside; QR: quercetin-3-rhamnosid; R: quercetin-3-rutinosid/rutin.

Table 4: Mean values of the content of individual phenolic compounds in peel and fruit flesh of four different apple varieties. Data are given in mg per 100 g fresh weight (Guyot & Le Bourvellec, 2002; Neveu & Perez-Jiménez, 2010; van der Sluis & Dekker, 2001).

Reference/ Phenolic compounds	**Neveu et al., 2010**	**Neveu et al., 2010**	**Guyot et al., 2002**	**Guyot et al., 2002**	**Guyot et al., 2002**	**Guyot et al., 2002**	**Guyot et al., 2002**	**Guyot et al., 2002**	**Van der Sluis et al., 2001**	**Van der Sluis et al., 2001**
Cultivar; segment	dessert apples; flesh	Cider apples; flesh	Golden Delicious; flesh	Golden Delicious; Peel	Granny; flesh	Granny; peel	Braeburn; flesh	Braeburn; peel	Jonagold; flesh	Jonagold; peel
Flavan-3-ols										
C	1.6	5.6	0.5	0.8	2.3	4.0	0.8	0.8	--	--
EC	6.7	28.7	5.9	12.4	9.6	17.0	12.4	13.2	--	--
PC B1	5.7	6.4	--	--	--	--	--	--	--	--
PC B2	10.0	19.6	7.2	15.0	13.4	24.1	6.7	24.5	--	--
Phenolic acids										
CA	18.2	49.3	8.4	5.5	3.5	0.9	7.1	4.3	1.4	14.8
CQA	1.4	3.0	1.4	0.9	0.3	0.3	2.0	0.9	--	--
Dihydrochalcones										
PHZ	1.5	2.5	1.1	4.0	0.6	1.3	0.7	3.4	17.0	6.6
PXG	0.5	3.3	1.1	4.2	1.3	4.1	0.9	4.2	--	--
Flavonols										
QGal	0.2	0.01	--	--	--	--	--	--	0.0	12.6
QGlu	0.04	0.06	--	--	--	--	--	--	0.0	2.0
QR	0.2	0.2	--	--	--	--	--	--	0.7	14.5

C: catechin; EC: epicatechin; PC B1: procyanidin B1; PC B2: procyanidin B2; CA: chlorogenic acid; CQA: *p*-coumaroylquinic acid; PHZ: phloridzin; PXG: phloretin xyloglucoside; QGal: quercetin-3-galactoside; QGlu: quercetin-3-glucoside; QR: quercetin-3-rhamnosid.

4.3.3. Nutritional importance of phenolic compounds

In addition to the tasks that secondary plant metabolites perform in plants to defend against pests or to attract insects, they play an important role in human nutrition due to their pharmacological effects (Lieberei & Reisdorff, 2012).

According to the DGE a balanced diet provides about 1.5 g of secondary plant metabolites per day (DGE, 2004). In general, phytochemicals are believed to have positive effects on human health by reducing the risk of developing diseases such as diabetes, cardiovascular diseases, asthma, Alzheimer's disease, and cancer (Boyer & Liu, 2004; Hyson, 2011).

The multitude of beneficial effects of phenolic compounds is primarily due to their antioxidant activity. The delocalized electron system allows radicals to be scavenged and stabilized. Phenolic compounds have anticarcinogenic, antioxidant, antimicrobial, immunomodulatory, antithrombotic, blood pressure-lowering, and blood sugar-lowering effects (Belitz et al., 2008; Ebermann & Elmadfa, 2008; Hyson, 2011; Watzl & Leitzmann, 2005). Many diseases are thought to arise as a result of oxidative reactions. For example, cardiovascular disease and cancer are among the leading causes of death in Western industrialized countries (WHO, 2009).

In a consumption study, the connection between the consumption of apples and the risk of developing cancer was established. Even the consumption of more than one apple per day reduces the risk of developing cancer. This was also shown in animal experiments on rats. After the intake of procyanidins from apples, about 50% fewer cancer cells were detected in induced colon damage than in control groups. The anti-carcinogenic effect of polyphenols is based on inhibition of tumor formation by preventing the binding of carcinogenic substances to the genetic material or protecting membrane lipids and DNA from oxidation (Boyer & Liu, 2004; Bravo, 2009).

Flavonoids are able to inhibit the action of certain oxidizing enzymes in their action. By inhibiting the enzyme cyclooxygenase, flavonoids prevent the aggregation of platelets, have an anti-inflammatory effect and thus prevent heart attacks. Likewise, flavonoids have an influence on the growth of blood vessels and reduce the possibility of tumor proliferation. Through the lipid peroxidation, flavonoids counteract diseases such as arteriosclerosis (Ebermann & Elmadfa, 2008).

In a scientific work by Liu et al. it was found by means of different apple extracts from different varieties that they are able to inhibit the tumor cell growth of the liver cancer HepG2. In this case, apple with skin causes higher growth inhibition than apple without skin and there are also differences between the different varieties (Liu et al., 2001). In another experiment, it was shown that an even higher antiproliferative effect on tumor cells was achieved using the peel alone (Wolfe et al., 2003). In a comparison among several different fruits, the third highest antiproliferative effect was attributed to apple behind cranberries and lemon (Sun et al., 2002). Similarly, it has been shown that daily apple consumption in combination with the addition of the trace element selenium reduces the risk of developing asthma in adulthood. The exact connection to which substances this can be attributed has not yet been clarified. It is assumed that specific substances in apples, such as anthocyanins or dihydrochalcones, are responsible for the effect. In the same study, the experiment was carried out with onions, which did not show any correlation with the risk reduction of asthma. Thus, the flavonols or flavans contained in the onion could be excluded for the effect. Increased selenium supplementation is thought to increase the activity of glutathione peroxidase, which is present in erythrocytes and is able to reduce oxidative stress (Shaheen et al., 2001).

The positive effect of phenolic compounds on human health is limited by the bioavailability of the substances. Phenolic compounds in plants can be divided into low molecular weight extractable and high molecular weight non-extractable groups. The extractable compounds are better absorbed in the gastrointestinal tract than the non-extractable compounds. In animal studies on rats, these were shown to be excreted in large quantities. The ability to absorb phenolic compounds in the intestinal tract depends on the basic structure and the degree of conjugation (Bravo, 2009; Shahkhalili et al., 1990).

In addition to the beneficial effects on health, phenolic compounds also exhibit antinutritional properties. Especially high molecular weight tannins can bind and precipitate macromolecules such as proteins, enzymes, but also carbohydrates or lipids, making them unavailable as nutrients (Shahkhalili et al., 1990). In animal studies, it was shown in rats that phenolic compounds can decrease the bioavailability of iron, zinc, sodium, and aluminum by adsorption, but not manganese, calcium, and magnesium (Bravo, 2009). However, the ability to bind substances or ions can also be advantageous, for example to neutralize heavy metals in poisoning by complex formation (Belitz et al., 2008).

4.4. Antioxidants and antioxidant mechanisms

By definition, antioxidants are protective substances that can delay, slow down or prevent oxidation of molecules present in the body (Halliwell, 1990; Watzl & Leitzmann, 2005). In this context, antioxidants are able to donate electrons on the one hand and to accept hydrogen ions on the other hand without reacting to a radical themselves (Fiedeler & Nentwich, 2006; Watzl & Leitzmann, 2005). Molecules that can trigger oxidation are reactive oxygen species (ROS) and reactive nitrogen species (RNS). The superoxide anion (O_2^-), the hydroxyl radical (•OH) and the peroxyl radical (ROO•) are the most important radical ROS. Hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) are among the most important non-radical ROS. Of great importance in the group of RNS is nitric oxide (NO) (Somogyi et al, 2007; Biesalski, 2000; Elmadfa & Leitzmann, 2015). The different compounds are considered as precursors for the formation of free radicals such as singlet oxygen, hydrogen peroxide, hypochlorous acid (HOCl) and ozone (O_3).

The formation of free radicals is a natural process in the human body. On the one hand, they are generated endogenously, for example, by the mitochondrial respiratory chain, by oxidative enzymes, or by phagocytosing cells as part of the immune defense system. Exogenously, free radicals are supplied by food, air pollution, cigarette smoke, pharmaceuticals, and UV radiation, among others (Berger, 2003; Biesalski, 2000; Grune, 2002; Watzl & Leitzmann, 2005).

Free radicals are very reactive because they have one or two unpaired electrons in their electron shell. When a radical reacts with another - non-radical - atom or molecule, a single electron is accepted or emitted. This leads to the formation of another radical, which in turn also emits an electron. Since this reaction can be continued indefinitely, it is also called a radical chain reaction (Watzl & Leitzmann, 2005). It is subdivided into the following three partial reactions: Chain start (initiation), chain growth (propagation), and chain termination (termination) (Shahidi & Zhong, 2010). This is illustrated below using the example of the peroxidation of polyunsaturated fatty acids (PUFA), they are easily attacked by these radicals due to their double bonds and are therefore preferred reaction partners of ROS and RNS.

1. Initiation

   Reaction of a PUFA (LH) with a radical (R•), in which hydrogen is transferred to the radical and a lipid radical is formed (L•).

   R• + LH → L• + RH

2. Propagation

   Reaction of the resulting lipid radical with molecular oxygen (O_2) to form a lipid peroxyl radical. (LOO•)

   L• + O_2 → LOO•

   Lipid peroxyl radical forms a lipid hydroperoxide (LOOH) with another fatty acid and a new fatty acid radical.

   LOO• + LH → LOOH + L•

3. Termination

   Reaction of the lipid radical with another lipid radical, a lipid hydroxy radical (LO•) or a lipid peroxy radical to form stable nonradicals.

   L• + L• → L-L

   L• + LO• → L-O-L

   L• + LOO• → L-O-O-L

Chain termination also occurs by reaction with an antioxidant (Belitz et al., 2008).

Free radicals are of health importance because they can damage a variety of biologically significant macromolecules such as DNA, proteins, and lipids, and play a role in the pathogenesis of various diseases such as cancer and cardiovascular disease (Elmadfa & Leitzmann, 2015; Fiedeler & Nentwich, 2006; Finaud et al., 2006; König, 2011; Watzl & Leitzmann, 2005).

To protect against oxidative damage to important compounds, the body has various mechanisms that can be divided into endogenous and exogenous factors. Endogenous factors are protective mechanisms that originate from substances already present in the human body. These include enzymatic mechanisms in which the body's own enzymes catalyze reactions that render reactive oxygen-rich compounds or radicals harmless. Examples include glutathione peroxidase (GSH-Px), catalase, and superoxide dismutase.

Glutathione peroxidase plays a very important role in catalyzing the regeneration of water and glutathione from hydrogen peroxide. It is a selenium-dependent enzyme, which has selenocysteine bound in its active site. Figure 6 shows the reaction course of glutathioperoxidase. During the reaction, hydrogen is transferred from the enzyme to the hydrogen peroxide, forming the dimer glutathione disulfide (GSSG) and additionally water. Subsequently, reduction to the tripeptide glutathione (GSH) occurs with the participation of NADPH-dependent glutathione reductase (Halliwell & Gutteridge, 2015; Watzl & Leitzmann, 2005).

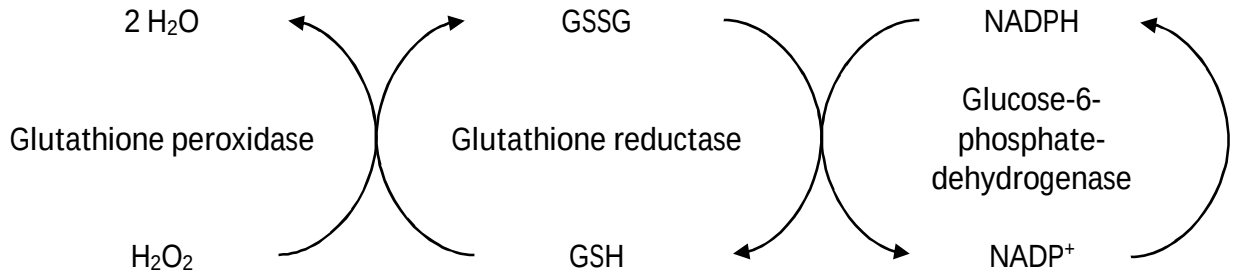


Figure 6: Reaction course of glutathioperoxidase (own illustration according to Elmadfa & Leitzmann, 2015).

The enzyme superoxide dismutase (SOD) provides primary protection against superoxide anoins (O_2^-) by catalyzing the conversion of a generated superoxide anion to hydrogen peroxide (Frei, 1994; Li Li, 1995; Nieß et al., 2002; Watzl & Leitzmann, 2005). Another enzyme, catalase, which occurs in the peroxisomes of red blood cells and in liver cells, is able to convert H_2O_2 into water and oxygen and thus render it harmless (Watzl & Leitzmann, 2005).

Exogenous protective mechanisms include antioxidants, which include some essential nutrients such as vitamins and minerals, as well as certain secondary plant metabolites. Table 5 provides an overview of the antioxidants found in food.

The antioxidants react with the radicals and thus absorb their reactivity. In the process, they are often consumed themselves. They can also be divided into watersoluble and lipidsoluble antioxidants and, according to their solubility, cause protection of the various cellular compartments (Grune, 2002).

Table 5: Essential nutrients and secondary plant metabolites with antioxidant activity (Grune, 2002; Watzl & Leitzmann, 2005).

Essential nutrients	**Secondary plant metabolites**
Vitamin E (tocopherols, tocotrienols)	Carotenoids
Vitamin C	Polyphenols
Iron, zinc, copper, manganese, selenium (as cofactors of enzymatic mechanisms)	Phytoestrogens
	Protease inhibitors
	Sulfides

As a lipidsoluble antioxidant, vitamin E can inhibit lipid peroxidation processes. It acts as a radical scavenger and leads to the termination of the radical chain reaction by reacting with hydroxyl radicals and releasing a hydrogen atom of its hydroxyl group. The resulting tocopherol radical can be regenerated by vitamin C to form vitamin E again. This interaction of the two vitamins is called synergism, whereby vitamin C itself becomes a radical that is regenerated again by the glutathione enzyme system. Vitamin C, as a radical scavenger, also reacts with watersoluble radicals. Minerals such as iron, zinc, copper, manganese, and selenium play a role as cofactors of enzymatic protective mechanisms (Biesalski, 2000; Grune, 2002; Watzl & Leitzmann, 2005).

The antioxidant mechanisms of action of polyphenols, which belong to the watersoluble secondary plant metabolites, are very diverse. Some act as radical scavengers, as chelating agents for metal ions, as protection against oxidation by singlet oxygen or prevent the formation of carcinogenic nitrosamines (Watzl & Leitzmann, 2005). An important prerequisite for the antioxidant activity of a polyphenol is the presence of an alkyl group in the ortho- or para-position on the phenol ring. When a radical reacts with an antioxidant active polyphenol, a phenoxy radical is formed, which can be stabilized by the migration of the unpaired electron. The flavonoid quercetin is able to inhibit the autoxidation of polyunsaturated fatty acids. However, its main task is to scavenge superoxide anions, see Figure 7 (Watzl & Leitzmann, 2005).

4.5. Polyphenoloxidases

Polyphenoloxidases are copper-containing enzymes that catalyze redox reactions. Polyphenoloxidases, which belong to the enzyme class of oxidoreductases (EC 1.14.18.1), include the enzymes tyrosinase (EC 1.14.18.1), catecholoxidase (EC 1.10.3.2), and laccase (EC 1.10.3.2) (Gerdemann et al., 2002). In the literature, the generic term PPO has become established for the three enzymes (Jeske et al., 2019; Marusek et al., 2006; Rescigno et al., 2002; Salzbrunn, 2007).

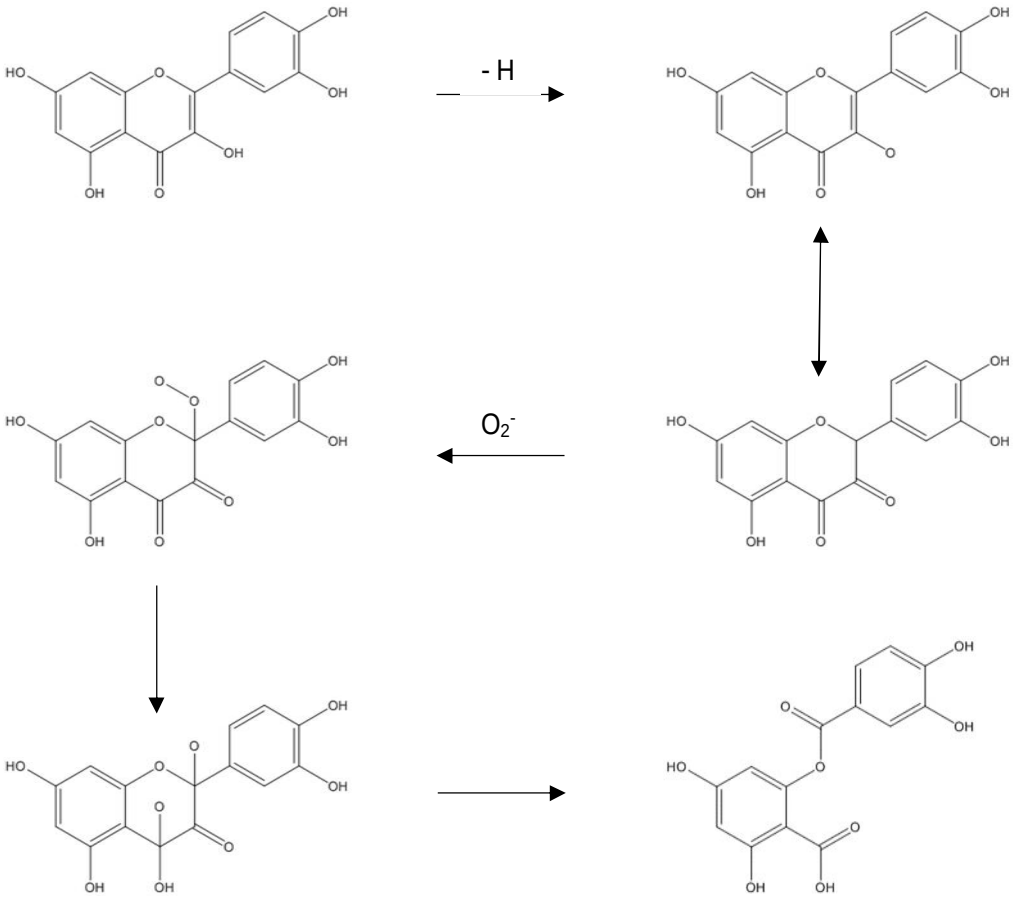


Figure 7: Reaction mechanism for scavenging superoxide anions using quercetin as an example (own illustration according to Nimse & Pal, 2015).

They occur both membrane-bound in chloroplasts and non-membrane-bound in mitochondria, whereas their substrates, the phenolic compounds, are localized in the vacuole. After rupture of the cell by wounding, infection or crushing, the membrane is lysed and only then enzyme and substrate come into contact (Carbonaro & Mattera, 2001; Pizzocaro et al., 1993; Song et al., 2007). In the presence of oxygen, the enzyme catalyzes the *o*-hydroxylation of monophenols (monophenolase or cresolase activity) to catechols and the two-electron

oxidation of *o*-diphenols to the corresponding *o*-quinones (diphenolase or catecholase activity) (Figure 8) (Belle, 2013; Burton, 1994; Gerdemann et al., 2002; Mayer, 2006).

In a non-enzymatic reaction, the *o*-quinones polymerize to melanins. These polymeric pigments are perceived as brown areas of fruit flesh. Oxidation of other phenolic compounds by *o*-quinones is also possible, resulting in further browning of the flesh. As a result of the biochemical reactions in the cells, in addition to optical and sensory changes, there is above all a loss of the phenolic compounds as nutrients (Amiot et al., 1992; Pizzocaro et al., 1993).

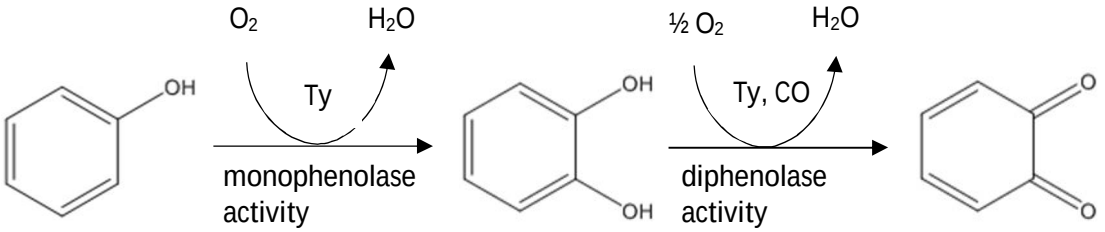


Figure 8: Reaction scheme of the catalyzed oxidation of phenolic compounds by the PPOs tyrosinase (Ty) and catecholoxidase (CO) (own illustration according to Belle, 2013).

Figure 8 shows the reaction pathway of the catalyzed oxidation of phenolic compounds by tyrosinase and catecholoxidase. Here, tyrosinase can catalyze the reaction of both monophenols and *o*-diphenols, whereas catecholoxidase can only oxidize *o*-diphenols as substrate (Belle 2013, Gerdemann et al., 2002). The enzyme activities are pH-dependent, with pH optima ranging from 5 to 7.5 depending on the substrate. Under basic conditions, none of the enzymes shows significant activity (Belle 2013). Laccases can oxidize various aromatic and non-aromatic compounds by a radical-catalyzed reaction mechanism such as *o*-dichinones, *p*-diphenols, acyrlamines, and aminophenols, as well as catalyze other reactions of phenolic compounds such as demethylations and (de)polymerizations (Claus, 2004; Mayer & Harel, 1979; Mayer, 1987; Tron, 2013; Walker & Ferrar, 1998).

PPOs consist of several subunits with two copper-binding sites in their active site. Here, copper is bound by six or seven histidine residues and one cysteine residue. Depending on the position of the copper atoms, the active site is called the CuA atom (left) and the CuB atom (right). During the catalytic cycle, the dinuclear copper center goes through three different forms at different stages (Fig. 9): a native met state (CuII-CuII), a reduced deoxy state (CuI CuI), and an oxy state with a dicopper center bound disoxygen (CuII-O_2-CuII) (Belle 2013). Figure 9 shows

the mechanisms of enzymatic oxidation by monophenolase (cresolase) and diphenolase (catecholase) activity (Belle 2013; Rolff et al., 2011; Solomon et al., 1996; Tepper et al., 2010).

The monophenolase cycle begins with the reduced deoxy form of the copper center. Through the addition of molecular oxygen, a conversion to the reactive oxy form takes place. Subsequently, the hydroxyl group of a monophenol binds to one of the two copper atoms, here CuB atom. Here, the aromatic ring is aligned with the bound peroxide. In this step, the phenol releases a proton, and the binding of the phenol occurs. This is followed by selective *o*-hydroxylation by electrophilic aromatic substitution, the rate of which is determined by the electron donor ability of the para substituents of the phenol substrate. The electrophilic property of the peroxide ligand is due to the strong charge orientation of the peroxo group (O-O) into the copper centers. Simultaneously with the *o*-hydroxylation, the peroxo bond is cleaved. Finally, the complexed *o*-diphenol is directly oxidized to the *o*-quinone, releasing a water molecule and regenerating the deoxy form of the copper (Cu) center (Belle 2013; Rolff et al., 2011; Solomon et al., 1996; Tepper et al., 2010).

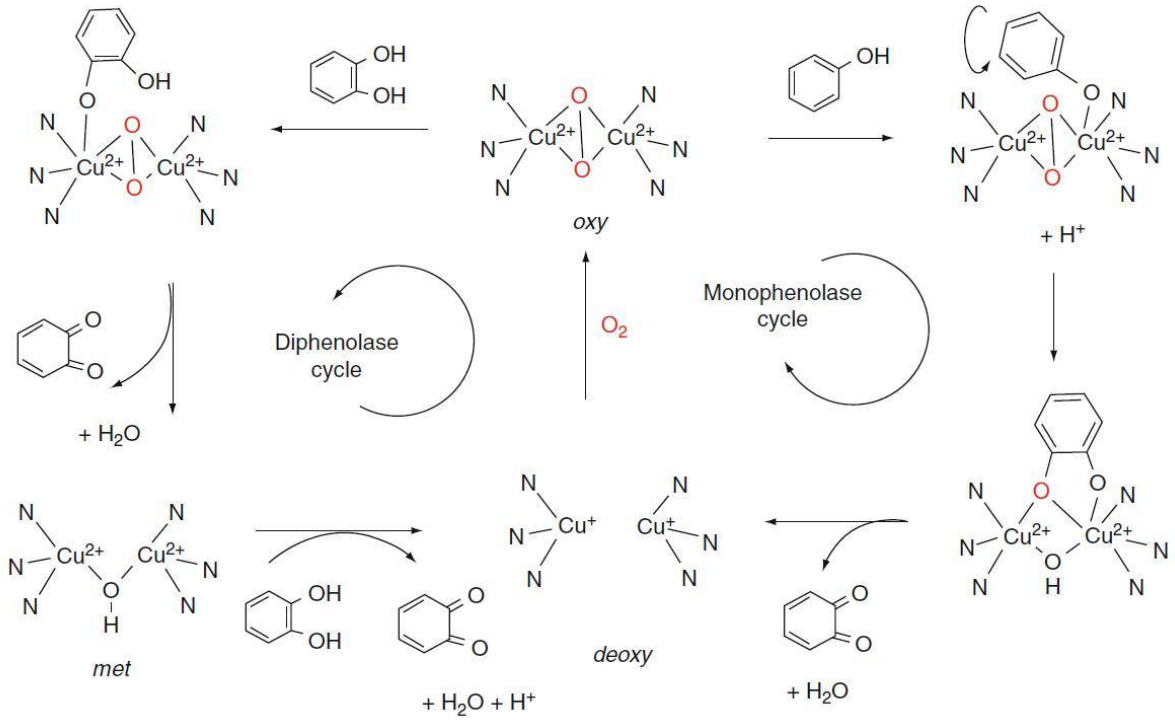


Figure 9: Schematic representation of the enzymatic oxidation of phenolic compounds by monophenolase and diphenolase activity (Belle 2013; Rolff et al., 2011; Solomon et al., 1996; Tepper et al., 2010).

The diphenolase cycle describes the conversion of two diphenol molecules into the corresponding quinones. Analogous to the monophenolase cycle, the diphenolase cycle also

starts with the conversion of the deoxy form into the oxy form by oxygen uptake. Subsequently, a diphenol molecule binds to the oxy-form and transfers two electrons to the peroxide of the deoxy-form to convert it to water. After dissociation of the quinone, the enzyme remains in the met form. The second diphenol molecule then binds to the met form and donates electrons to the Cu (II) ions, producing the reduced deoxy form. Binding of the diphenol to the met form results in the transfer of a proton from the diphenol to the hydroxide ligand of the met form and the concomitant dissociation of the water molecule of its bridging position (Rolff et al., 2011; Solomon et al., 1996; Tepper et al., 2010).

PPOs occur ubiquitously in animals, plants, fungi, and bacteria. In plants, they are probably part of the defense mechanism, as a positive correlation between PPO levels and resistance to pathogens and herbivores has often been observed (Mayer 2006). In apples, the effect of harvest maturity and storage conditions on PPO activity has been studied (Schmitz-Eiberger & Matthes, 2011). Other proposed functions of PPOs in plants include participation in photosynthesis (Gerdemann et al., 2002; van Gelder et al., 1997; Walker & Ferrar, 1998) and flower pigment formation (Gerdemann et al., 2002; Nakayama et al., 2000).

4.6. Apple allergy

4.6.1. Role of pathogenesis-related (PR) proteins in the plant

Due to the lack of an immune system in plants, they have to protect themselves from pathogens as well as herbivorous insects by different strategies. To this end, plants firstly establish a physical barrier by strengthening their cell walls and produce antibiotics, so-called phytoalexins, and accelerate cell death to suppress the spread of infectious pathogens (Hoffmann-Sommergruber, 2000).

In addition, a number of proteins are encoded by the host and induced by various types of pathogens (viruses, bacteria, and fungi) or by chemicals such as ethylene and salicylic acid, mimicking the effect of pathogen infection and thus inducing stress (Stintzi et al., 1993). These proteins are referred to as pathogenesis-related proteins. The biochemical characteristics of PR proteins are low molecular weight, stability at low pH, and resistance to proteases (Hoffmann-Sommergruber, 2000). The biological functions of PR proteins in plants are versatile, ranging from antifungal, antibacterial, antiviral, insecticidal, and nematocidal (Edreva, 2005; Hejgaard et al., 1991).

The family of PR-10 proteins, also known as Bet v 1 homologs, is ubiquitously distributed in plants (Fernandes et al., 2013). Different stresses specifically induce gene expression so that plant defense function can be enabled. Bet v 1-related members include diverse pollen and food allergens and are particularly abundant in the plant families Rosaceae, Fabaceae, Fagales, and Apiaceae. They are found in pome and stone fruits (apples, cherries, apricots, peaches), nuts (hazelnuts), and legumes (soybeans, peanuts) (Hauser et al., 2008).

4.6.2. Reactions of immune system in apple allergy

Apple allergy is classified as an immunoglobulin E (IgE)-mediated food allergy (Kleine-Tebbe & Jakob, 2015; Kleine-Tebbe et al., 2016; von Baehr, 2017). Food allergies are based on a misdirected immune response and hypersensitivity of the adaptive immune system to food proteins. IgE-mediated food allergies are divided into primary and secondary groups. While primary food allergies are based on sensitization of IgE to animal or plant proteins, secondary food allergies are based on cross-reactions between IgE primarily sensitized to pollen proteins and homologous allergen structures in plant foods. Thus, apple allergy is a secondary IgE-mediated food allergy (Kleine-Tebbe et al., 2016).

This falls under the classification of pathogenic immune reactions established by Gell and Coombs in 1963, type I reaction and is also referred to as immediate type. Upon initial contact, allergen-specific IgE antibodies are formed in type I allergy, which bind to the surface of mast cells or basophilic granulocytes via the so-called Fc receptors (Ring 2007; von Baehr 2017). When bridging of at least two IgE molecules on the surface of mast cells or basophils occurs by binding of the antigen or allergen, mast cells or basophils are activated. This leads to degranulation of mast cells and basophilic leukocytes and subsequent release of preformed vasoactive inflammatory mediators such as histamine (Fig. 10) (Gallin et al., 1992; Ring, 2007). The classic symptoms, such as wheal, redness, and itching, occur within seconds to minutes upon dual contact with allergens (Gell & Coombs, 1963; von Baehr, 2017).

4.6.3. Allergenic proteins in apples

In addition to the nutritive ingredients, apples also contain various allergenic proteins. Table 6 shows an overview of the four, so far known, groups of allergenic proteins in apples: Mal d 1, Mal d 2, Mal d 3, and Mal d 4 (Grafe, 2009; Kleine-Tebbe et al., 2016). The nomenclature of

the immediate-type allergens indicates the botanical name (genus and species) and the order of their first description based on the numbering (Kleine-Tebbe & Jakob, 2015).

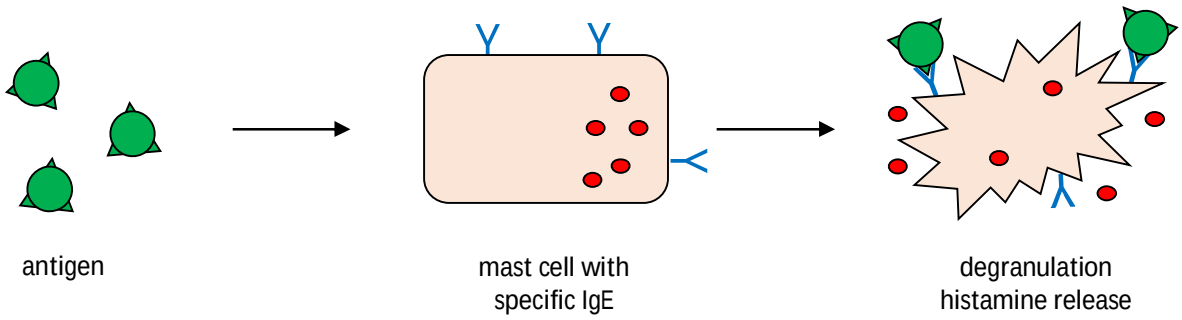


Figure 10: Reaction mechanism of type I allergy/immediate reaction (own illustration according to Gallin et al., 1992; Ring, 2007).

	Mal d 1	**Mal d 2**	**Mal d 3**	**Mal d 4**
Molecular weight	17,65 kDa	25,68 kDa	11,41 kDa	13,96 kDa
Clinical symptoms	oral allergy syndrome (OAS)	--	generalized symptoms	--
Protein family	PR-10	PR-5	PR-14	Profilin
Homology/ Cross-reactivity	Bet v 1	thaumatin-like protein (TLP)	non-specific lipid transfer protein (nsLTP)	Bet v 2
Function in the fruit	fruit ripening; defense against pathogens	antifungal	transport of fatty acids and cell wall components	--
Localization in the fruit	in pulp and peel	in ripe fruit	mainly in the peel	mainly in fruit pulp
Properties	sensitive to acid, heat, enzymes	--	resistant to acid and heat	--
Occurrance of allergy	Northern and Central Europe	--	Mediterranean region	--

Table 6: Properties of the four groups of Mal d family allergens (Ballmer-Weber & Hoffmann-Sommergruber 2011; Gomez et al., 2014; Kleine-Tebbe & Jakob, 2015).

Among them, the major allergen Mal d 1 is most important in Northern and Central Europe and leads to oral allergy syndrome in apple allergic patients (Gomez et al., 2014). Mal d 1 has a molecular weight of 17.65 kDa and is a Bet v 1 homologous protein belonging to the PR-10

family. Bet v 1 homologs are proteins that have molecular similarity to Bet v 1, causing allergenic cross-reactions. Bet v 1 is the major allergen of warty birch (*Betula verrucosa*) and induces more than 95% of IgE binding to birch pollen allergens and represents the most common cause of pollen-associated food allergies in Northern and Central Europe (Ballmer-Weber & Hoffmann-Sommergruber, 2011; Kleine-Tebbe & Jakob, 2015). The two allergens Mal d 1 and Bet v 1 are recognized by the same antibody. Thus, apple allergy often develops on the basis of sensitization by birch pollen (Grafe, 2009; Paschke, 2008). Mal d 1 shows high homology to the sequence and structure of Bet v 1, so that cross-reactions are common (Gilissen et al., 2005; Gomez et al., 2014; Hoffmann-Sommergruber, 2005; Jenkins et al., 2005). The amino acid sequence identity of Mal d 1 and Bet v 1 is 64.5% (Krath et al., 2009). Figure 11 illustrates the structures of Mal d 1 and Bet v 1, explaining the cross-reactivities. More than 70% of patients with birch allergy develop allergic cross-reactivity to Mal d 1 (Ahammer et al., 2017).

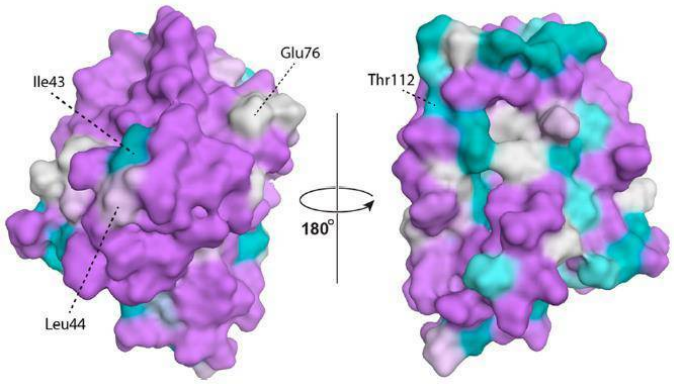


Figure 11: Amino acid similarities between Bet v 1.0101 and Mal d 1.0101 using a color gradient from purple (very similar) to blue-green (very different). Epitope residues that differ between Bet v 1.0101 and Mal d 1.0101 are labeled (Ahammer et al., 2017).

The protein is produced during fruit ripening and to defend against pathogens. Mal d 1 is expressed in equal amounts in the fruit flesh and peel of apples and is both acid and heat sensitive (Marzban et al., 2005). Due to denaturation of the protein in the acidic environment of the stomach after consumption, the accompanying allergic reactions in the form of the oral allergy syndrome (OAS) are weak. There are mainly oral symptoms such as irritation of the mouth and throat, which manifest themselves as tingling or swelling. Heated apple products (above 60°C), such as pasteurized juice, are usually harmless to allergy sufferers from Northern and Central Europe and are well tolerated because the protein has been denatured (Grafe, 2009; Kleine-Tebbe et al., 2016; Marzban et al., 2005; Paschke, 2008; von Baehr, 2017;

Worm et al., 2014). Southern Europeans show almost no allergic reactions to the protein Mal d 1, because birch trees are scarcely native in these regions and therefore hardly any sensitization by birch pollen can occur. In these regions, allergic individuals react predominantly to Mal d 3, often experiencing severe reactions, also referred to as generalized symptoms (Gomez et al., 2014).

Mal d 3 is a protein weighing 11.41 kDa and belongs to the superfamily of prolamins, specifically PR-14 proteins, and belongs to the subgroup of nsLTP proteins. These are acid and heat resistant and insensitive to enzymes, therefore allergic reactions such as gastric, intestinal or circulatory complaints up to anaphylactic shock occur, which are much stronger than with Mal d 1 (Grafe, 2009; Marzban et al., 2005; Paschke, 2008). Thus, severe allergic symptoms can also occur when processed apples are consumed (van Ree et al., 1997). The protein is mainly localized in the peel of the fruit, where it performs a function in the transport of fatty acids and cell wall components (Marzban et al., 2005).

Compared to Mal d 1 and Mal d 3, allergic reactions to Mal d 2 and Mal d 4 occur extremely rarely and are of lesser importance for the allergenic potential of apples (Paschke, 2008). Mal d 2 is a thaumatin-like protein and belongs to the PR-5 family (Krebitz et al., 2003). It is characterized as an antifungal protein and is abundant in ripe apple fruit (Botton et al., 2008). Furthermore, it is particularly resistant to protease and heat treatments due to its eight disulfide bridges (Oh et al., 2000; Breiteneder & Ebner, 2000).

With respect to Mal d 4-related allergies, a prevalence similar to that of Mal d 3 has been frequently reported (van Ree et al., 1995), occurring predominantly in the Mediterranean region. These allergens include to the profilin protein family and are also involved in allergic reactions to fruits of other species (Asero et al., 2003; Scheurer et al., 2001; Wensing et al., 2002; Westphal et al., 2004), with strong cross-activity with the birch pollen profilin Bet v 2 (van Ree et al., 1995).

The extent of the allergic reaction is determined by various factors. The decisive factor is the concentration of the allergen in the consumed product. Allergy sufferers also have varying degrees of sensitivity, so that the threshold concentration that leads to an allergic reaction can vary greatly from individual to individual. Cross-allergy also contributes to the overall allergic potential of a food. Another factor that influences allergic potential is the presence of different isoforms, which have different allergenic potential. This is due to the fact that these

proteins are sometimes encoded by different genes, with slight differences in molecular structure. The body reacts differently to the different types (Grafe, 2009; Paschke, 2008).

It has been shown that the Mal d 1 content varies greatly in different varieties (Marzban et al., 2005; Sancho et al., 2006). Therefore, a distinction is made between allergy-tolerant and allergy-intolerant varieties. The federation for environment and nature conservation (BUND) Lemgo has published a corresponding overview with recommendations of individual varieties for apple allergy sufferers. The old apple varieties, which include the 'Gravensteiner', 'Roter Boskoop', and 'Berlepsch', are considered to be particularly tolerable. These usually have only a low content of the allergenic protein Mal d 1. Apples of the new varieties, on the other hand, such as 'Golden Delicious', 'Jonagold', 'Elstar', or 'Granny Smith' trigger reactions in a large number of allergy sufferers (BUND-Ortsgruppe Lemgo, 2021). Apples of the "new" varieties, in which the polyphenol content has been reduced by breeding for taste reasons, often have a higher Mal d 1 content in connection with this and are thus considered to be allergy provoking. A direct correlation between decreasing polyphenol content and increasing Mal d 1 content is therefore suspected (Lewitzki, 2020).

However, variations also occur within a variety, mainly due to different site-related environmental conditions, the stage of ripeness and storage types or durations (Matthes & Schmitz-Eiberger, 2009; Sancho et al., 2006; Zuidmeer et al., 2006). This is due to the fact that the protein is synthesized mainly in stress situations, which can be caused by environmental and cultivation conditions, storage types, or the duration of storage. Thus, a variety considered to be not allergy provoking may also have a higher Mal d 1 content. It has already been demonstrated that the Mal d 1 content in the fruit increases during the storage period after harvesting. This is due to degradation processes, which represent a stress situation in the apple (Matthes & Schmitz-Eiberger, 2009; Sancho et al., 2006).

In addition, the influence of the phenolic content in apple on the Mal d 1 content is also discussed. An enzymatic conversion of the polyphenols to quinones could lead to the reaction of these with a part of the proteins and thus also of the allergens to form polymeric structures. These are no longer recognized by the immune system as foreign substances, which means that an allergic reaction does not occur and allergenicity is reduced. However, polyphenols and Mal d 1 proteins are located separately in the cell. Only during the chewing process the allergens and polyphenols or quinones come into contact, so that reactions can occur. It is

questionable whether a corresponding reaction between quinones and allergens can still take place effectively in the short time available (Grafe, 2009; Neumüller, 2021).

4.7. Phenol-protein interactions

Interactions can occur between phenolic compounds and proteins, which are divided into non-covalent or reversible bonds and covalent or irreversible bonds. Figure 12 shows an overview of reversible interactions, which can occur via hydrogen bonds, electrostatic interactions, hydrophobic interactions, van der Waals forces, and ionic bonds (Haslam, 1996). Here, hydrophobic interactions occur between aromatic rings of polyphenols and hydrophobic sites of proteins such as alanine, valine, isoleucine, leucine, methionine, phenylalanine, tyrosine, tryptophan, proline, and glycine residues. Amino acids such as lysine, arginine, histidine, asparagine, glutamine, serine, threonine, aspartic acid, glutamic acid, tyrosine, cysteine, and tryptophan, on the other hand, can be bound to phenolic compounds by hydrogen bonds that can occur between their nitrogen or oxygen and hydroxyl groups of phenols (Prigent, 2005; Rawel & Rohn, 2010). The other non-covalent interactions play only a minor role (Le Bourvellec & Renard, 2012). Non-covalent binding of the phenolic compounds chlorogenic, ferulic, and gallic acids, as well as quercetin, rutin, and isoquercetin to various proteins is influenced by the protein structure, i.e., amino acid sequence, and the resulting structural conformation, as well as by external conditions such as pH, temperature, and ionic strength (Le Bourvellec & Renard, 2012; Rawel et al., 2005; Rawel et al., 2006).

Polyphenols and especially their oxidation products, quinones, are among the most reactive ingredients in apples. Irreversible interactions between phenols and proteins occur due to the reaction of phenols as phenoxy radical, quinone, or semiquinone radical (Kroll et al., 2003; Selinheimo et al., 2007). This is influenced by the redox capacity of the phenolic compounds (Alu'datt et al., 2013; Brudzynski et al., 2013; Han & Koh, 2011; Liu et al., 2015; Swieca et al., 2013).

The oxidative degradation of phenolic compounds catalyzed by PPO leads to the formation of *o*-quinones. Since these are reactive, they can subsequently form dimers with other phenolic compounds as well as adducts with proteins. The dimers can be reoxidized and covalently cross-link to proteins. Adduct formation occurs via a Michael reaction with nucleophilic centers of proteins such as primary amino groups (lysine residues), thiol groups (cysteine

residues), thioether groups (methionine residues), and indole rings (tryptophan residues) due to the eletrophilic nature of quinones (Kroll et al., 2003; Le Bourvellec & Renard, 2012; Machholz & Lewerenz, 1989; Rawel & Rohn, 2010). Figure 13 shows the mechanism of reaction of *o*-quinones with functional side chains of proteins.

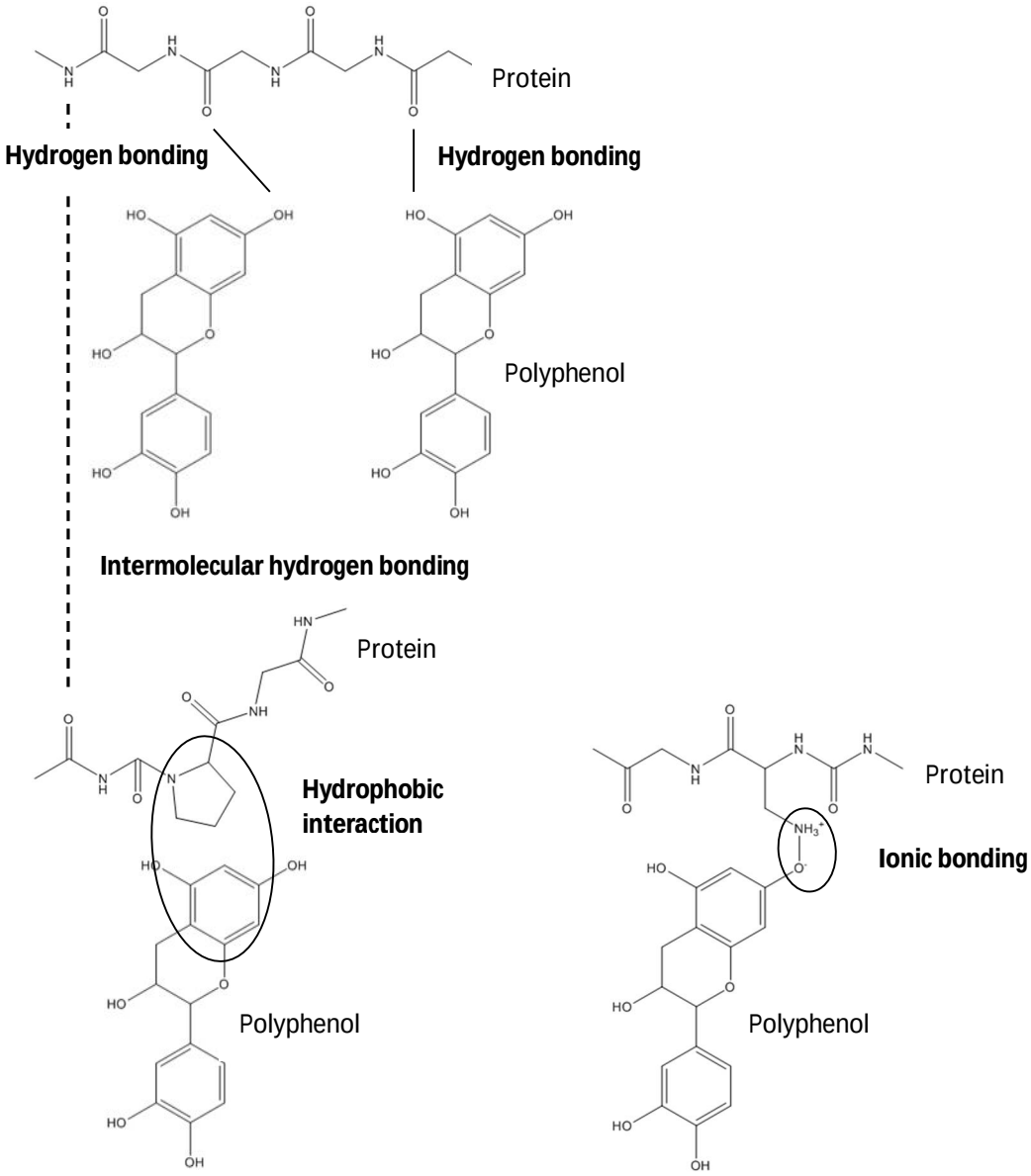


Figure 12: Overview of reversible interactions between phenolic compounds and proteins (own illustration according to Assano et al., 1982; Le Bourvellec & Renard, 2012).

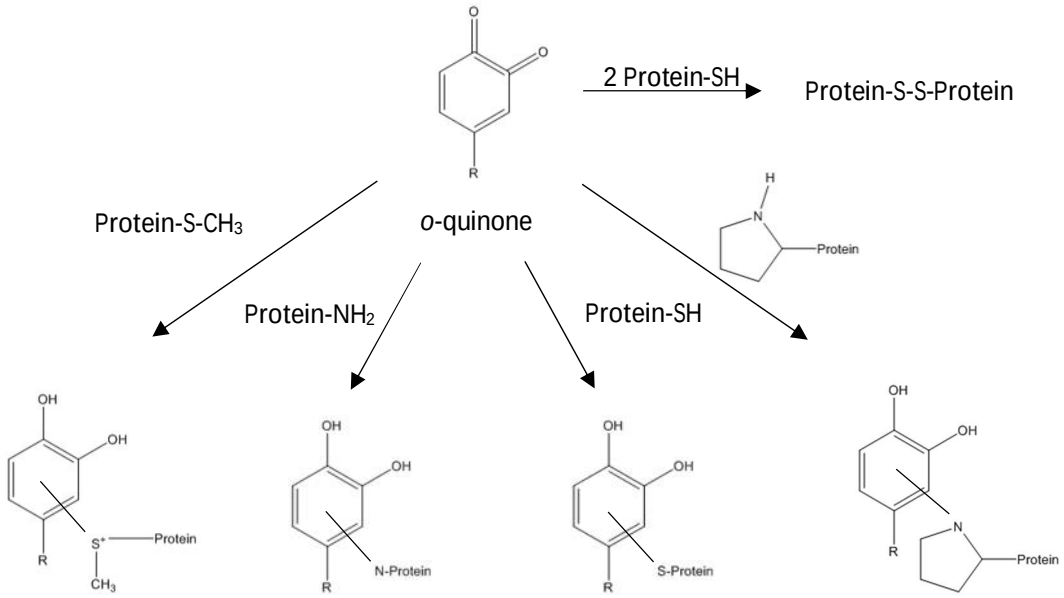


Figure 13: Reaction mechanism of *o*-quinones with functional side chains of proteins (own illustration according to Machholz & Lewerenz, 1989).

After addition to a protein side chain, the phenolic compounds remain reactive. Therefore, there is the possibility of reoxidation, which can lead to binding of another protein. This reaction, called protein cross-linking (cross-links), leads to the formation of dark-colored protein complexes with high molecular weight (Rohn, 2014). Figure 14 shows the reaction mechanism of protein cross-linking.

Phenol-protein interactions can lead to the formation of the following three types of products: (A) simple reaction products in which individual phenolic compounds are bound to a specific amino acid chain, (B) mixed reaction products consisting of monomers and bound oligomers, (C) complex cross-linked proteins and variations thereof (Fig. 15) (Rohn, 2014). Since covalent interactions are irreversible, they can lead to changes in secondary and tertiary protein structures as well as alter protein solubility and thermal stability (Dunn et al., 2015; Kroll et al., 2003; Ozdal et al., 2013).

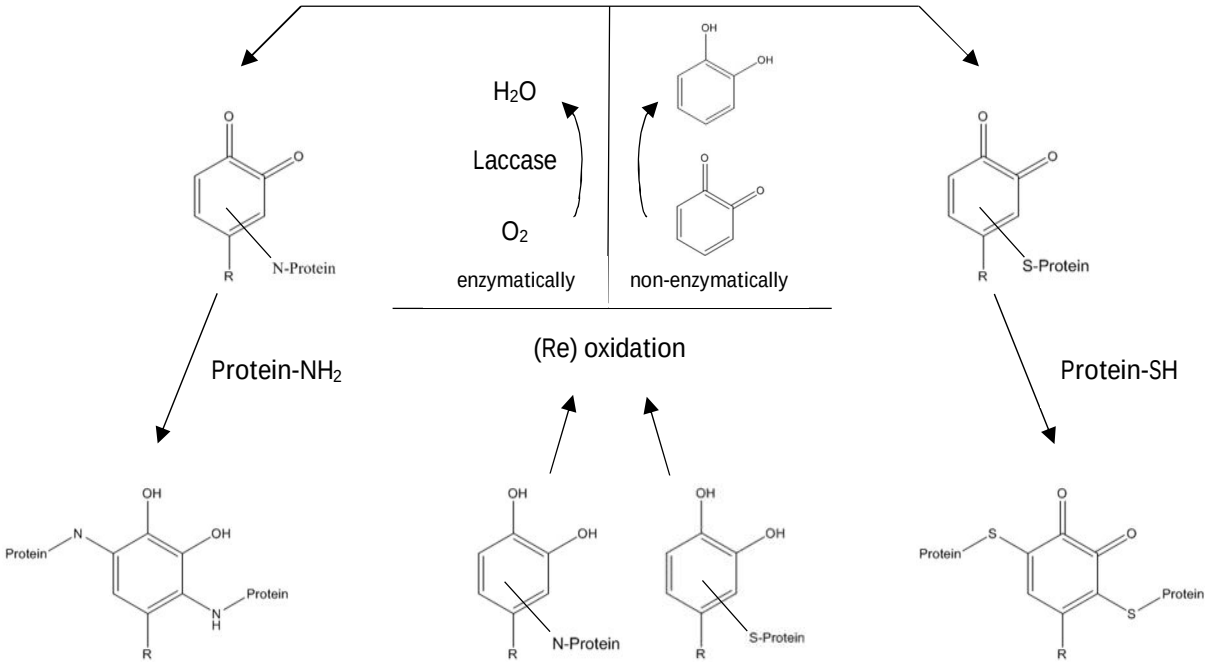


Figure 14: Reaction of *o*-quinones with functional side chains to form protein crosslinks (cross left) (own illustration according to Rohn, 2014).

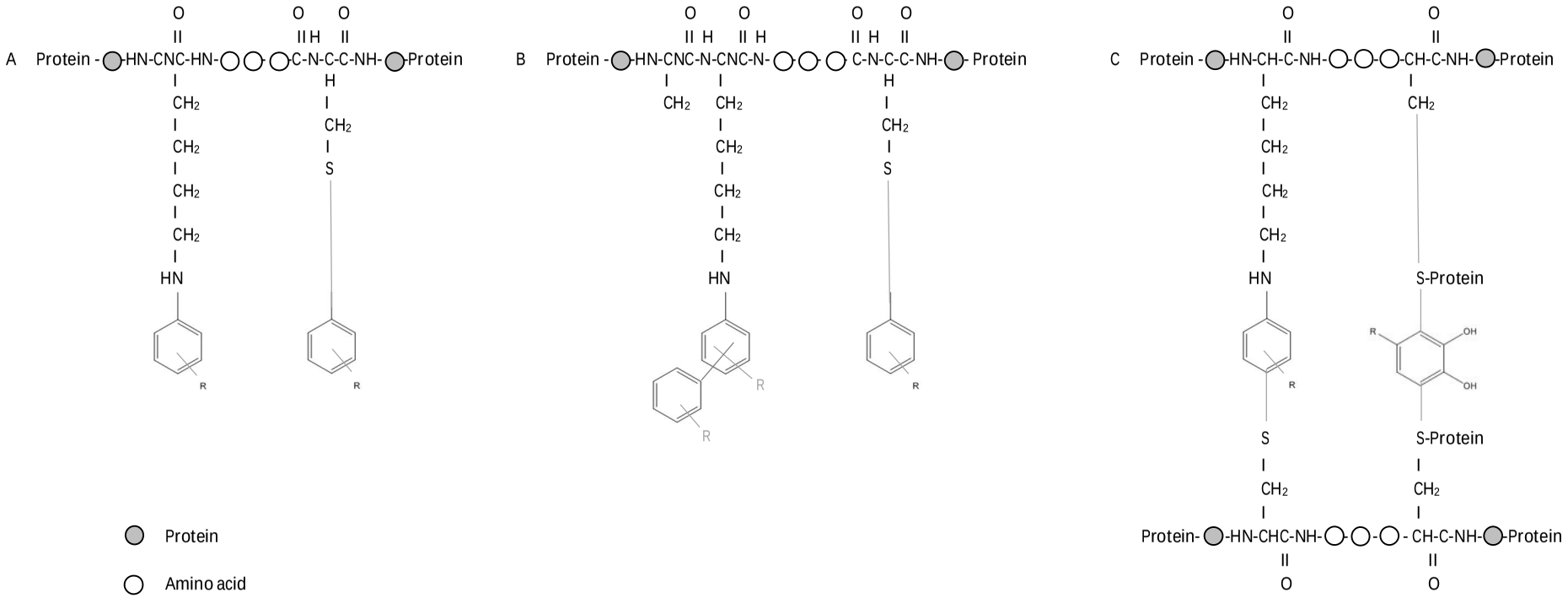


Figure 15: Reaction products of phenol-protein interactions via covalent interactions (own illustration according to Buitimea-Cantúa et al., 2018; Rohn, 2014).

4.8. Analytical methods

4.8.1. Determination of polyphenoloxidase activity

Quantitative determination of the catalytic activity of PPO is performed by measuring the conversion rate of the substrate used, catechol, by the enzyme PPO. Catechol is oxidized to quinone by the activity of PPO, see Figure 16. Quinone absorbs at the wavelength λ = 420 nm compared to catechol (λ = 470 nm). Therefore, by changing the extinction maxima of substrate and product, photometry can be used to determine enzyme activity.

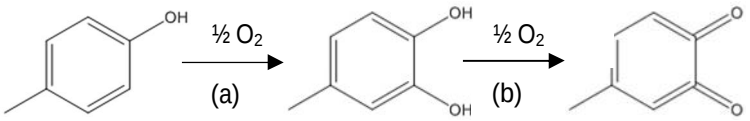


Figure 16: Reactions of PPO as (a) monophenolase: in the presence of oxygen, the hydroxylation of phenols to catechols is catalyzed. And as (b) *o*-diphenolase: the catechols are oxidized to *o*-quinones by the activity of PPO (Burton, 1994; Mayer, 2006).

Figure 17 shows an example of the reaction course of the PPO. Due to the activity of the PPO, the concentration of quinones increased steadily and reached a maximum after about ten minutes. The PPO was then probably inhibited by the high concentration of the products formed, so that the concentration slowly decreased. Within the first minute, the increase in concentration followed a linear function, so the change in absorbance within the first minute could be used to determine the activity of the PPO.

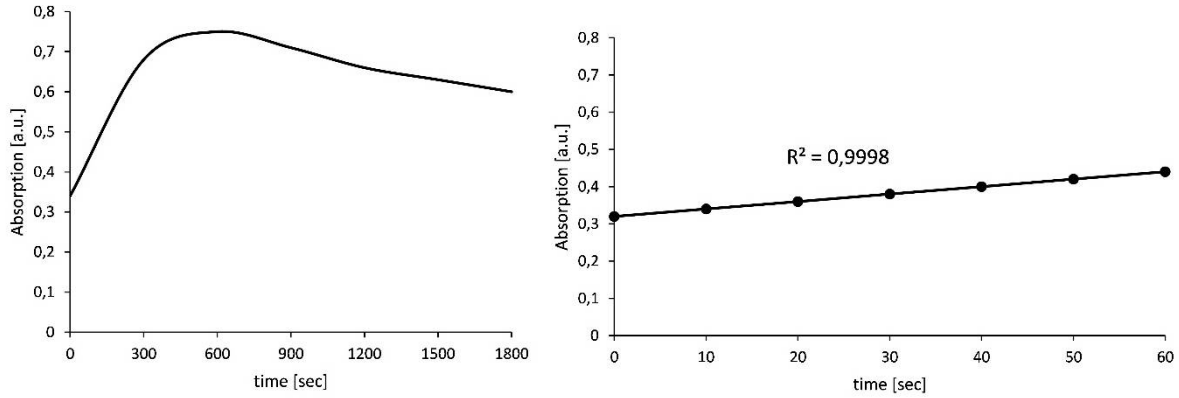


Figure 17: (A) Reaction course of PPO over 30 min by formation of quinones. (B) Determination of the linear section of the reaction course of PPO.

The activity of the PPO was calculated from the difference in the increase in absorbance, taking into account dilution factors, sample volume and initial weight. The catalytic activity is expressed in units (U). One unit corresponds to one µmol substrate turnover per minute and

describes the reaction rate at which an enzymatic reaction proceeds (Bisswanger, 2015; Kolodziejcyk et al., 2010). In enzyme reactions, the general conditions specific to the enzyme must be maintained. These include the pH, temperature, and concentration of substrates and cosubstrates. The amino acids in the active site of the enzyme have functional groups that are dissociated depending on the pH; only then the substrate can bind to the active site. If the pH is too acidic, denaturation can lead to a change in the tertiary structure of the proteins, resulting in inactivation of the enzyme. Therefore, buffer solutions must be used for extraction (Bisswanger, 2015). The PPO has two pH optima at pH 5.1 and pH 7.3, and the buffer solution can stabilize the pH of a solution even when the hydroxyl or oxonium ion concentration changes (Gey, 2012; Janovitz-Klapp et al., 1989).

4.8.2. Determination of the total phenolic content according to FOLIN-CIOCALTEU

Total phenolic content can be determined using the FOLIN-CIOCALTEU assay and is based on the redox reaction of phenolic compounds with tungstophosphoric acid (TPA) and molybdophosphoric acid (Mo) and contained in the FOLIN-CIOCALTEU reagent. The reagent has a yellow color in acidic conditions. Phenols exhibit reducing properties and can reduce the ions, and the phenolic compounds are oxidized in the process. The partial reduction of the oxidation state leads to the formation of a complex and to a color change from yellow to blue, see Figure 18 (Al-Duais et al., 2009; Singleton & Rossi, 1965).

$$\underset{\textit{(light yellow)}}{TPA^{+VI} / Mo^{+VI}} \xrightarrow{\text{Reduction}} \underset{\textit{(dark blue)}}{TPA^{+V} / Mo^{+V}}$$

Figure 18: Color change in the FOLIN-CIOCALTEU reagent due to a reduction.

The absorbance can be measured photometrically at 765 nm. The color intensity here depends on the polyphenol content; the higher the content of phenolic compounds, the more ions of the reagent are reduced and the stronger the blue coloration appears. Since the phenolic compounds are only present in their dissociated form in an alkaline environment, the reaction is pH-dependent and can occur under alkaline conditions. Gallic acid, an aromatic compound of the hydroxybenzoic acid class, is used for calibration and the results are expressed as gallic acid equivalents (GAE). When using THE FOLIN-CIOCALTEU method, it must be taken into account that not only phenolic compounds but also other substances with reducing properties are recorded. These include, for example, ascorbic acid or reducing sugars, which are also found

in plant foods. These also lead to the reduction of molybdate and tungstate ions and thus to a color change (Al-Duais et al., 2009; Singleton & Rossi, 1965).

4.8.3. Photometric determination of antioxidant activity

Antioxidant activity is the extent to which substrates are protected from oxidative attack. The determination of this parameter is important in nutritional research as well as for the food industry. Oxidative processes of cellular components play an important role in the development of various diseases. Therefore the determination of AOA of tissues of the organism is also important. As antioxidants from food have a protective influence on the pathogenesis of various diseases, the AOA of foodstuffs as well as of individual ingredients is determined (Güngör et al., 2011; Ou et al., 2002; Özyürek et al., 2011). The determination of the AOA is performed using some established rapid tests, but there are significant differences in radical sources, substrates, reaction conditions, and quantification methods (Frankel & Meyer, 2000), so that the results are only comparable to a limited extent. A standardized method does not exist yet.

Antioxidant activity can be determined using various photometric methods, these differ in the chemical reactions on which the methods are based. In principle, there are two different approaches for measuring the AOA: a distinction is made between electron transfer (ET) and hydrogen atom transfer (HAT). ET-based methods, which are based on a redox reaction with an oxidizing agent as the detection of the endpoint of the reaction, include the TEAC assay and also the FOLIN-CIOCALTEU determination of total phenolic content. HAT-based methods are based on the interaction of a synthetic free radical with an oxidizable sample and the antioxidants it contains. These include the ORAC assay (Huang et al., 2005).

The Trolox Equivalent Antioxidant Capacity assay is one of the electron transfer methods. The 2,2′-Azino-di(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radical used as reagent is first generated by oxidation with the addition of potassium peroxodisulfate (K_2SO_4). In this process, a coloration of the colorless ABTS to the blue-green radical takes place. The presence of antioxidant substances such as phenolic compounds leads to the reduction of the radical and the associated loss of color, see Figure 19. The attenuation of the absorbance can be measured photometrically at 734 nm. This is an endpoint measurement. Trolox, a synthetic hydrophilic vitamin E analog, is used for calibration. The antioxidant activity of a substance is expressed as Trolox equivalents (TE) (Huang et al., 2005; Re et al., 1999).

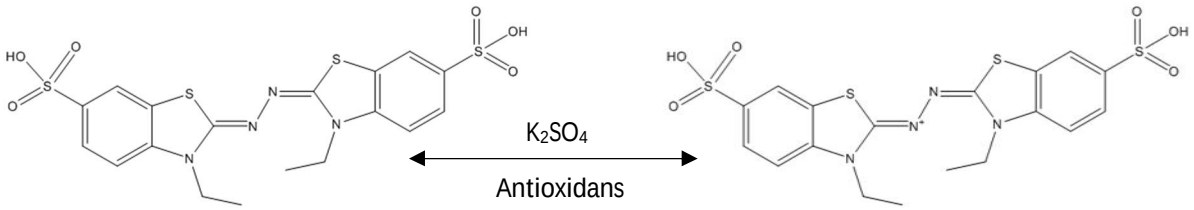


Figure 19: Reaction mechanism of ABTS (own illustration according to Huang et al. 2005).

The principle of the Oxygen Radical Absorbance Capacity Test method is based on the reaction of the artificial radical generator 2,2′-azobis(2-methylamidinopropane)dihydrochloride (AAPH) with the fluorescent dye fluorescein. Fluorescein exhibits nearly constant fluorescence over time at neutral pH, and is used as a negative control to measure fluorescence. In the presence of oxygen, oxidation of AAPH to peroxyl radicals occurs at temperatures above 30°C. The radicals generated oxidize fluorescein resulting in a loss of fluorescence. In the presence of antioxidants, the reaction of the radicals with the antioxidants occurs due to the property of the antioxidants as radical scavengers or reducing agents (see Fig. 20). The reaction with fluorescein is delayed depending on the type and amount of antioxidants present. Since this is a progression measurement, the reaction is measured until all antioxidants and the fluorescein have been oxidized by the peroxyl radicals, therefore fluorescence is no longer measurable (Huang et al., 2002; Ou et al., 2001).

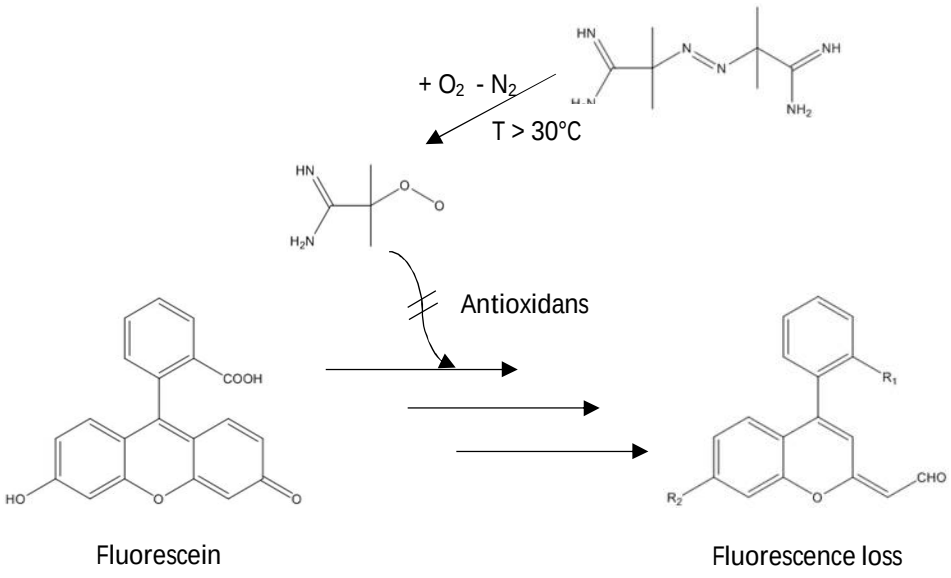


Figure 20: Reaction scheme of fluorescein with the radical generator AAPH (own illustration according to Huang et al., 2002).

The fluorescence measured over time is plotted as a sigmoid curve. The area under the curve (AUC) corresponds to the AOA. The delay in the decrease in fluorescence caused by the

antioxidants is used as a measure of the AOA of the sample. The ORAC value is expressed in Trolox equivalents per volume or unit weight of the sample, e.g., mmol TE/100 g or mmol TE/100 mL. Unlike other methods of measuring AOA, ORAC is a progression measurement, meaning that the antioxidant reaction is measured throughout its course. This takes into account antioxidants that slow down the reaction rate as well as antioxidants that prolong the reaction time. Time point measurements such as TEAC only allow determination of AOA at one time point (Huang et al., 2002).

4.8.4. Total protein determination according to BRADFORD

Total protein determination according to BRADFORD is used to quantify the protein content. This photometric method is based on a group of blue acid dyes, Coomassie Brilliant Blue G250 (Fig. 21) and can be performed in 96-well microtiter plates. Thus, it is fast and easy to handle and also provides good reproducibility and sensitivity. Coomassie-Brilliant Blue binds non-specifically to the cationic and non-polar hydrophobic side chains of proteins in an acidic environment. This leads to a shift of the absorption maximum of the dye from 465 nm (red) to 595 nm (blue). It is assumed that here the unprotonated, anionic sulfonate form is stabilized in a complex. With increasing protein concentration, the absorbance of the solution increases accordingly. When determining a protein mixture, standard proteins such as bovine serum albumin (BSA), chymotrypsin, or lysozyme are used for calibration (Bradford, 1976; Lottspeich & Engels, 2006).

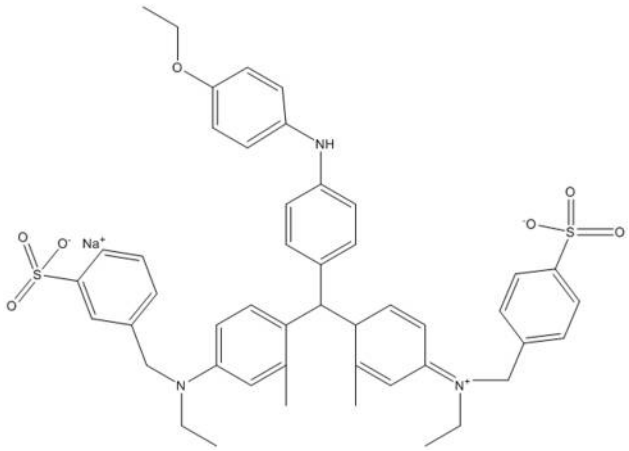


Figure 21: Chemical structural formula of Coomassie Brilliant Blue G250 as sodium salt (own illustration according to Bradford, 1976; Lottspeich & Engels, 2006).

4.8.5. Discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Discontinuous SDS-PAGE is an analytical method for the separation of proteins in a protein mixture, which mainly allows qualitative statements about the proteins. The separation is based on the molecular weights of the individual proteins. An acrylamide-based discontinuous gel is used as the separation medium, which is composed of a wide-mesh collection gel and a narrow-mesh separation gel. The gels are prepared in a free-radical polymerization of acrylamide and N,N'-methylenebisacrylamide, which serves as a crosslinker. The ratio between acrylamide and crosslinker determines the pore size of the three-dimensional crosslinked gel. To initiate the radical polymerization, a radical initiator, such as ammonium peroxodisulfate, which is homolytically cleaved in solution and thus readily decomposes into free radicals, is required. In addition to the reagents described, a tetramethylethylendiamine (TEMED) solution is required to act as a polymerization catalyst to accelerate the reaction. The use of a collecting gel or a separating gel buffer, which contain tris(hydroxymethyl)-aminomethane (TRIS) and SDS in different compositions, is used to adjust the desired pH in the respective gels. The collection gel has a pH of 6.8, while the separation gel has a pH of 8.8.

The gels are prepared in a gel cassette in which the gels are layered on top of each other. First, the narrow-pore release gel is poured. After the polymerization time, the collection gel is then added. Plastic combs are inserted into the still liquid gel to form pockets into which the samples are pipetted for electrophoretic separation.

A reduction buffer consisting of TRIS, glycerol, SDS, 2-mercaptoethanol, and bromophenol blue was added to the protein extracts to be applied and heated to 95°C. Here, the anionic surfactant SDS serves to mask the intrinsic charges of the proteins by attaching its hydrophobic moiety to the hydrophobic side chains of the proteins. On average, 1.5 molecules of SDS bind per peptide bond. The attachment causes the intrinsic charge of the proteins to be masked by the negative charge of the SDS, creating a uniform overall charge in them. This is dependent on the molecular weights of the proteins, since more molecules of SDS can attach as the size of the proteins increases. The use of 2-mercaptoethanol in combination with heating leads to the cleavage of disulfide bridges and thus to the denaturation of the proteins. At the same time, renaturation, which would occur with heating alone, is prevented. Therefore, by using the listed chemicals, separation of proteins is possible regardless of their secondary or tertiary structure as well as intrinsic charge (Gey, 2008; Matissek et al., 2014).

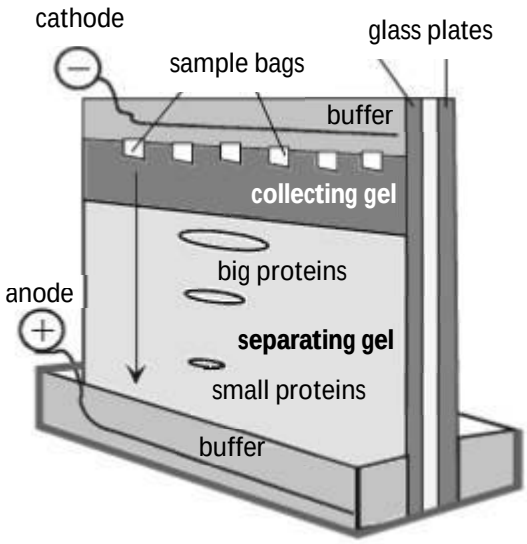


Figure 22: Set-up of a vertical electrophoresis apparatus for SDS-PAGE (according to Gey, 2008).

Figure 22 shows the schematic structure of a gel electrophoresis apparatus consisting of collection and separation gels arranged vertically on top of each other. The protein extracts mixed with reduction buffer are first applied to the sample pockets of the collection gel and overlaid with running buffer. The collecting gel has the task of focusing the proteins in protein stacks in order to obtain increased resolution and band sharpness with the aid of the chloride ions and glycine contained in the running buffer. To identify the protein bands, a molecular weight marker is also used and pipetted into a sample pocket.

By applying an electric voltage, the SDS-protein complexes migrate in the electric field towards the anode. The chloride ions in the collection gel buffer have a high electrophoretic mobility and are therefore referred to as conducting ions. In contrast, glycine has almost no electrophoretic mobility because the pH of the collection gel buffer is very similar to the isoelectric point of glycine (6.06). It is therefore also referred to as a subsequent ion. In the collection gel, a field strength gradient finally builds up between glycine as the following ion and chloride as the conducting ion when an electrical voltage is applied, which ensures that the proteins are focused. The proteins arrange themselves according to their isoelectric point, leading to the formation of protein stacks. Once the glycine reaches the separation gel, the electrophoretic mobility increases due to the increase in pH. Glycine is present deprotonated, correspondingly rushing ahead of the proteins and overtaking the chloride ions. This causes the protein stacks to dissolve. In this case, gel electrophoretic separation at constant field

strength depends on the molecular weight of the individual proteins (Gey, 2008; Matissek et al., 2014).

After electrophoresis, the protein bands must be fixed and stained. This is very often done using a Coomassie Brilliant Blue solution with a methanol additive. In this case, the staining principle corresponds to that of the total protein determination according to BRADFORD. Alternatively, silver staining can also be performed. This is characterized by a higher sensitivity, but is associated with a significantly higher workload and the use of hazardous chemicals (Holtzhauer, 1996). After staining and fixation, the unknown protein bands of the samples can be compared with the known molecular weights of the recombinant proteins of the marker and thus identified (Gey, 2008; Matissek et al., 2014).

4.8.6. Enzyme-linked Immunosorbent Assay

ELISA is a frequently used method for the identification and quantification of allergens in food and is characterized by a high sample throughput combined with a high specificity and sensitivity. It is an immunochemical method and is based on an enzymatic color reaction using antibodies that interact with antigens in a specific antigen-antibody manner. The binding works according to the lock-and-key principle. The antigen binding site of the antibody is called "paratope". The corresponding binding site on the allergen is called "epitope". With the help of enzyme-labeled antibodies, a dye can be produced by conversion of a substrate, allowing the measurement to be detected photometrically (Matissek et al., 2014).

Antibodies are glycoproteins secreted in the human organism to defend against foreign substances such as viruses or microorganisms. In doing so, they can specifically distinguish between endogenous and exogenous substances. Also known as immunoglobulins (Ig), they are divided into the five classes: Immunoglobulins A, D, E, G, and M. They are all present in the organism at the same time, although their composition differs in the tissues. IgG is the main component in plasma and therefore has the greatest importance for immunochemistry (Lottspeich & Engels, 2006). Antibodies for analytical purposes are obtained using laboratory animals. An appropriate allergen is introduced into the animal body. Through the subsequent immune response, the organism forms corresponding antibodies, which are isolated from the animal's blood by purification. In this way, polyclonal antibodies, a mixture of different antibodies, are obtained. For the synthesis of monoclonal antibodies, after immunization of the test animal, B lymphocytes, which secrete the monoclonal antibody, are isolated from its

spleen cells and fused in vitro with cancer cells. The resulting hybridoma cell line, which arises from the fusion of two original cells, retains their properties. Thus, this one has the capabilities to divide indefinitely and produce the desired monoclonal antibody (Matissek et al., 2014).

Both polyclonal and monoclonal antibodies can be used in analytics. The former can react with multiple epitopes of an allergen and therefore offer the advantage of obtaining amplified measurement signals due to the possibility of binding with different epitopes of an antigen. However, this decreases the specificity of binding (Lottspeich & Engels, 2006; Matissek et al., 2014). Monoclonal antibodies, on the other hand, react only with one epitope of the allergen and are accordingly more specific.

There are different variants of ELISA, which can basically be divided into direct and indirect methods. The simplest and usually also most cost-effective method is the direct ELISA. Here, the allergen to be analyzed is first bound to a microtiter plate by hydrophobic adsorption. Usually, plates made of polystyrene are used, as this plastic has a high protein binding capacity. The binding of the antigen to the microtiter plate is subject to an equilibrium reaction. Thus, in addition to the bound antigen, unbound antigen is also present in solution. In order for a sufficient amount of antigen to bind to the microtiter plate, an appropriate incubation time is required. This varies depending on the material to be bound and the pH and temperature of the solution during the process and can be up to 24 hours. To remove unbound antigen and other non-binding matrix components, a wash step is then performed with buffer solutions containing surfactants. To ensure that the antibody reacts only with the bound antigen in the later course, possible free binding sites on the microtiter plate must be occupied. The use of BSA or casein is suitable for this purpose. To remove excess unbound blocking reagent, another washing step is performed. This is followed by the addition of a detector antibody that specifically binds to the antigen. The blocking reagent is thus not detected. For detection, an enzyme-labeled antibody is used that converts a substrate into a colored product, the absorbance of which can subsequently be determined photometrically (Ganten & Ruckpaul, 1997; Kaufmann, 2014; Luttmann et al., 2014).

Another variant is the indirect ELISA. Here, the principle corresponds fundamentally to that of the direct ELISA. In contrast to direct ELISA, in which only an enzyme-labeled detector antibody is used, indirect ELISA first uses a primary antibody that binds to the target antigen. In a second step, the enzyme-labeled antibody designated as secondary then binds for

detection. This gives a higher specificity of the measurement (Ganten & Ruckpaul, 1997; Kaufmann, 2014; Kemeny, 1994; Luttmann et al., 2014; ThermoFisher Scientific, 2021).

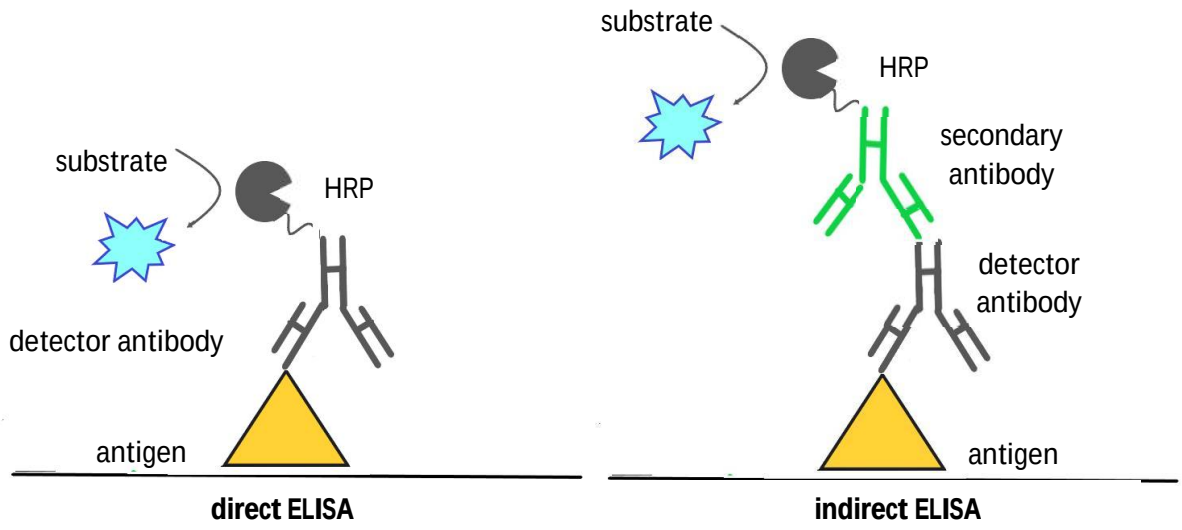


Figure 23: Basic principles of a direct and indirect ELISA (own illustration according to according to ThermoFisher Scientific, 2021).

Figure 23 shows the schematic structure of the direct and indirect ELISA. The use of a polyclonal antibody is recommended as a detector antibody, as this can bind to several epitopes of the antigen, thus intensifying the signal (Gey, 2008; Kemeny, 1994). The enzyme horseradish peroxidase (HRP) is frequently used for labeling. It belongs to the class of oxidoreductases and catalyzes the reduction of peroxides such as hydrogen peroxide so that a chromogen can be oxidized, forming a colored product. 3,3',5,5'-tetramethylbenzidine (TMB), which forms a blue dye upon oxidation and has an absorption maximum at 450 nm, is often used in this process. The coloration of the oxidation product becomes more intense with increasing incubation time. To stop the enzymatic reaction, sulfuric acid is added after a fixed incubation time, which destroys the enzyme and no further conversion occurs (Ganten & Ruckpaul, 1997; Kaufmann, 2014; Luttmann et al., 2014).

After activation by peroxidase, TMB is blue at an absorption maximum of 650 nm, see Figure 24. When sulfuric acid is added, TMB turns yellow at 450 nm. Sulfuric acid is used to induce the color change.

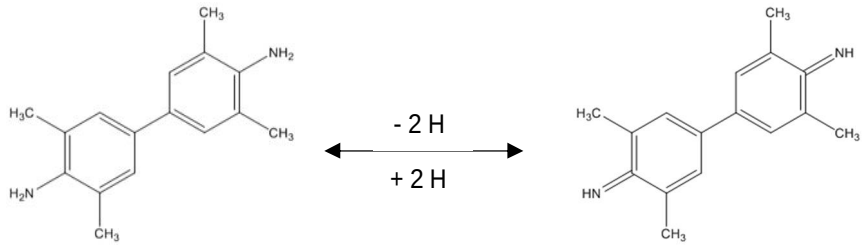


Figure 24: Structural formula of TMB; oxidation to blue dye.

From the properties of the oxidation of TMB to the colored end product, various explanations for the more intense absorption result. On the one hand, the oxidation proceeds via a charge-transfer complex as an intermediate and is also temperature-, pH-, and time-dependent. This is an electron-donor-acceptor complex in which charge transfer occurs. By lowering the pH during measurement, it is believed that autoxidation of the charge-transfer complex to the final product is achieved. At the same time, a shift of the reaction equilibrium in favor of the products could also play a role. An influence on the linear absorption of the product is also conceivable (Bally & Gribnau, 1989).

The intensity of the coloration that occurs is proportional to the concentration of the oxidation product formed and thus also proportional to the concentration of the antigen (Gey, 2008). The absorbance can be determined photometrically using a microplate reader. Based on a calibration with defined different concentrations of the recombinant antigen identical to the target allergen, the content of allergen in a sample can be quantified.

Another widely used variant of the ELISA is the sandwich ELISA, which is characterized above all by its high specificity. Direct and indirect detection is possible. An illustration of the basic principles can be found in Figure 25. In general, the sandwich ELISA is frequently used when side reactions of the antibodies with sample components other than the target antigen are to be expected. For this purpose, microtiter plates are usually used that are already coated with an antibody, also referred to as primary antibody. Here, monoclonal antibodies are particularly well suited to achieve high specificity, as the binding of non-specific matrix components, which could sometimes also bind to the detector antibody, is avoided. After addition of the sample, the contained allergen binds to the antibody. Unbound matrix components are removed by a washing step with buffer solutions containing surfactants. In the case of direct detection, this is followed by the addition of a detector antibody, which simultaneously represents the secondary enzyme-labeled antibody. This also binds to the

antigen, but to a different epitope. As in direct ELISA, a substrate (usually TMB) suitable for the enzyme (usually HRP) is used. The absorbance of the conversion into a colored product is subsequently determined photometrically for quantification of the allergen contained in the sample. If detection is indirect, a detector antibody first binds to the antigen. In a second step, the secondary, enzyme-labeled antibody binds, resulting in higher specificity.

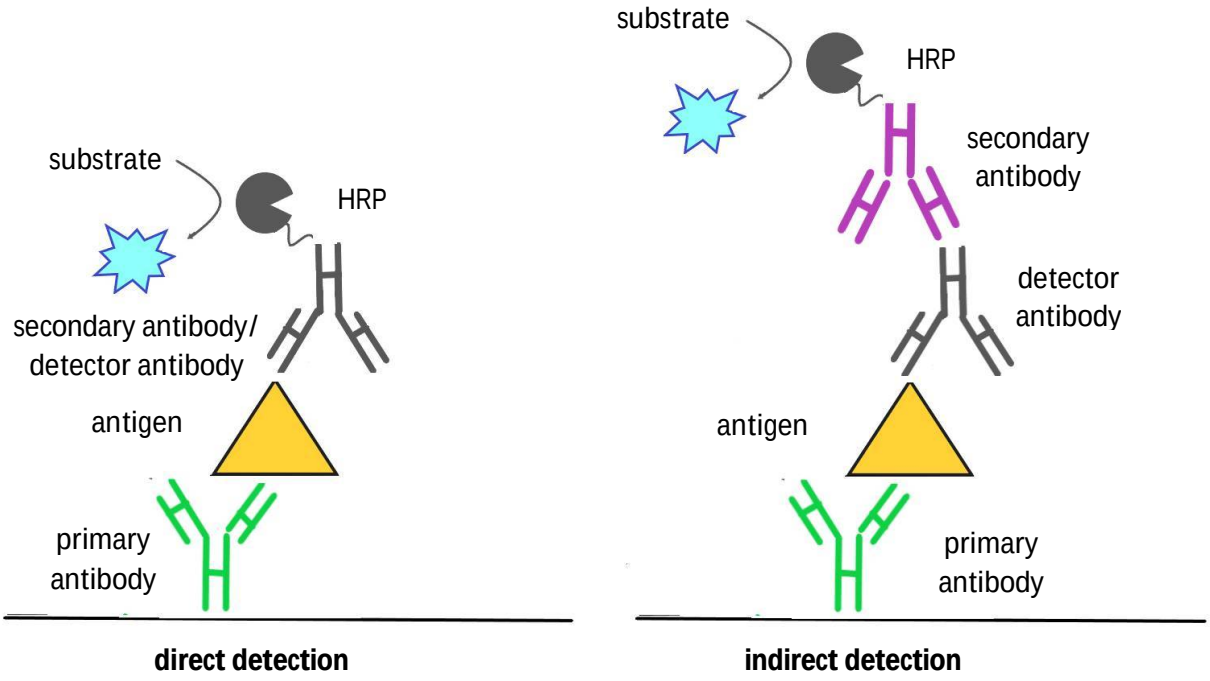


Figure 25: Basic principle of the Sandwich-ELISA ((own illustration according to according to ThermoFisher Scientific, 2021).

Compared to the simple ELISA, the sandwich ELISA offers several advantages. For example, it is characterized by a higher specificity, since the analyte is specifically bound to the primary antibody, while the other components of the samples are removed by the subsequent washing step. In the direct method, other components of the sample can also adsorb on the microtiter plate, although it cannot be ruled out that they may later interact with the antibody. There is also a higher sensitivity with the sandwich ELISA. The antigen to be determined is aligned by binding to the primary antibody. This avoids partial non-detection due to misalignment, as occurs with direct ELISA. Here, the antigen is non-specifically bound to the microtiter plate, so that it cannot be ensured that the antigen is available in the required configuration for the antibody.

The disadvantages of the sandwich ELISA are the time required and the costs. Since several different antibodies are used in this method, a sandwich ELISA is much more expensive to perform and requires a significantly longer period of time to measure due to the incubation of each added antibody (Ganten & Ruckpaul, 1997; Luttmann et al., 2014). Therefore, performing a simple direct ELISA may be preferred due to cost and time constraints. Besides, its application is advantageous when the analyte is very small, so that the formation of two antibodies is spatially impossible (Cell Signaling Technology, 2021).

All ELISA variants described so far are non-competitive methods. However, it is also possible to perform a competitive ELISA, which is used especially for the quantification of very small antigenes. Figure 26 shows the basic principle of the competitive ELISA. In this case, a defined concentration of antibody as pure as possible is bound to the microtiter plate at the beginning. After blocking still unoccupied formation sites with, for example, BSA, the microtiter plate is incubated with antigen-containing samples and detector antigenes. This causes the antigen contained in the sample and the detector antigene to enter into a competitive reaction for the binding sites of the antibody. In a subsequent washing step, among other things, the complex of free sample antigen and detector antibody is removed so that only the antibody that binds to the antigen of the microtiter plate is available for detection. Analogous to direct ELISA, a substrate is then used for detection using the enzyme-labeled antibody. After conversion to the colored product, photometric detection is performed (Ganten & Ruckpaul, 1997; Kaufmann, 2014; Luttmann et al., 2014).

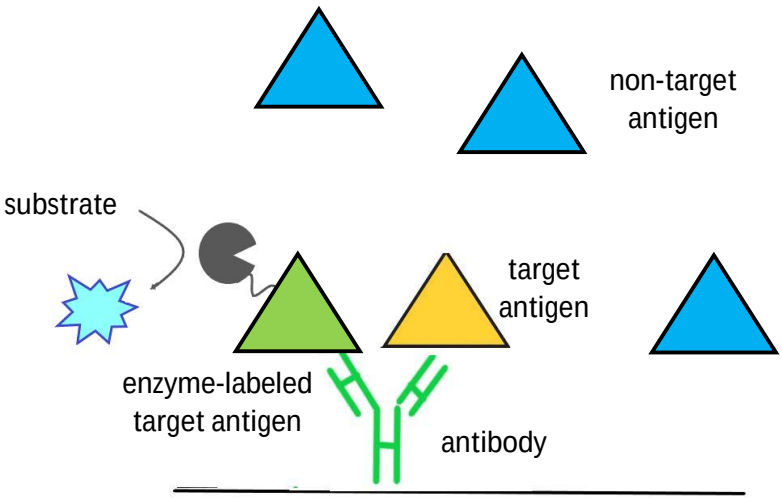


Figure 26: Basic principle of the competitive ELISA (own illustration according to ThermoFisher Scientific, 2021).

The reaction between antigen and antibody is an equilibrium reaction. It follows that a lot of antibody will bind to the free antigen if there is a lot of antigen in the sample, which is removed in the washing step. Consequently, less antibody will bind to the antigen in the microtiter plate, so a high antigen concentration in the sample will ultimately result in a lower signal from the assay. Conversely, a high amount of detector antibody binds to the microplate antigen when little sample antigen is present. This produces a stronger signal (Luttmann et al., 2014).

Competitive ELISA can be performed by direct or indirect detection, which is analogous to the detection described for direct ELISA. In the direct variant, an enzyme-labeled antigen is used as a competitor to the sample antigen. The indirect variant is based on the use of the target antigen and another, unlabeled antigen, both of which can bind to the bound antibody. A subsequently added secondary enzyme-labeled antibody specifically binds the sample antigen and can be measured photometrically by addition of the substrate, as described earlier. In general, washing and blocking steps are also used in competitive ELISA to avoid extraneous binding and adulteration by excess antibodies and antigens (Ganten & Ruckpaul, 1997; Luttmann et al., 2014).

5. OBJECTIVE OF THE WORK

In the present work, the influence of selenium biofortification of apples on nutritionally important metabolites of primary and secondary plant metabolism was investigated. The primary objective of the overalls project *BiofortiSe* was to increase the selenium content in the fruits. Furthermore, phenolic compounds and their properties, such as the activity of the polyphenoloxidases, which catalyze the oxidation of phenolic compounds, and the antioxidant activity were the main focus of this dissertation. Based on previously conducted studies on other various fruit and vegetable crops, an increase in antioxidant-active phenolic compounds was hypothesized. Furthermore, it was also investigated if, and to which extent, the biofortification also affects the allergenic profile and content of the major allergen Mal d 1 in the apples. In addition, a correlation between phenolic compounds and the content of allergenic proteins was suspected, which was examined in further analysis.

For this purpose, apple samples of seven different cultivars, grown in three consecutive years at two different growing locations, were analyzed. In 2017, apples of the varieties 'Fiesta', 'Golden Delicious', 'Idared', 'Jonagold', and 'Jonica' were cultivated at the Horticultural Research Station of the Osnabrück University of Applied Sciences, Germany. The varieties 'Boskoop', 'Golden Delicious', Jonagold', and 'Jonica' were cultivated in 2018 also in Osnabrück. In the following year, apples of the cultivar 'Elstar' were cultivated in an orchard of a commercial fruit farm in the "Alte Land" region, Jork, Germany. The biofortification with selenium was performed by foliar fertilization. Different field trials, which differed in the form of applied selenium (sodium selenite and sodium selenate) as well as in the application level, were conducted to investigate the influence of different methodical approaches of selenium fertilization on important quality parameters of the fruits. Due to the experimental conditions, other possible influencing factors on the above parameters, such as ecophysiological conditions, could thus also be analyzed.

6. CUMULATIVE PART OF THE DISSERTATION

The results of the dissertation are presented in the form of scientific manuscripts. They have been published in the journals *Antioxidants*, *Food Chemistry,* and *Molecules*. The releases of the publishers for the use of the articles in the present work are available. Supplementary information on the publications can be found in the appendix under section B.

The publications describe the influence of selenium biofortification of apples of different varieties on value-giving metabolites of primary and secondary plant metabolism. Here, the focus is on phenolic compounds and their associated properties as well as on the allergenic proteins of the Mal d family.

First, the photometric methods for the analysis of polyphenoloxidase activity, the total phenolic content according to FOLIN-CIOCALTEU, and the antioxidant properties as estimated by TEAC and ORAC assay, were established and validated for the matrix apples and apple extracts. Subsequently, the extraction method for phenolic compounds from apples was developed and optimized by applying different solvent concentrations or using different equipment. An HPLC-DAD-ESI-MS^n was established and validated for the qualitative and quantitative analysis of phenolic compounds. With respect to primary plant metabolism, the focus was on the analysis of allergens. Here, the method for the extraction of proteins from the apples as well as the method for the gel electrophoretic separation of the obtained protein extracts via disc-SDS-PAGE were first developed and optimized, and the method for the determination of the total protein content according to BRADFORD was established and validated. Furthermore, a direct ELISA for the quantitative determination of the Mal d 1 content in apples was developed. In-gel digestion of the bands from the SDS gels was also performed for mass spectrometric identification of allergenic proteins by LC-ESI-qTOF-MS and nanoLC-MS/MS.

Based on the above methods, the following parameters were subsequently determined in the selenium-biofortified apples:

- Polyphenoloxidase activity
- Total phenolic content
- Antioxidant activity
- Phenolic profile

- Total protein content
- Protein pattern with focus on allergenic proteins
- Mal d 1 content
- Mass spectrometric identification of apple proteins.

By comparing the results of the selenium-biofortified apples with the controls, the influence of the agronomic fertilization method was then analyzed, and possible relationships between individual constituents were investigated using correlation analyses.

6.1. Analysis of antioxidant properties and phenolic compounds in selenium biofortified apples

Influence of a Selenium Biofortification on Antioxidant Properties and Phenolic Compounds of Apples *(Malus domestica)*

Sabrina Groth [1], Christoph Budke [2], Susanne Neugart [3], Sofia Ackermann [1], Fenja-Sarah Kappenstein [1], Diemo Daum [2] and Sascha Rohn [1]*

[1] Hamburg School of Food Science, Institute of Food Chemistry, University of Hamburg, Grindelallee 117, 20146 Hamburg, Germany

[2] Department of Plant Nutrition, Osnabrück University of Applied Sciences, 49090 Osnabrück, Germany

[3] Department of Crop Sciences, Division Quality and Sensory of Plant Products, Georg-August-Universität Göttingen, 37075 Göttingen, Germany

Antioxidants **2020**, *9*, 187

doi: 10.3390/antiox9020187

Accepted: 22 February 2020

In this work, apples of the cultivars 'Golden Delicious' and 'Jonagold' were biofortified with selenium via foliar fertilization in the growing years 2017 and 2018. Different selenium forms as well as application levels were used.

The aim of the biofortification was to increase the selenium content of the apples and to analyze the influence on value-giving secondary plant metabolites, especially phenolic compounds, as well as their antioxidant properties. Here, the total phenolic content was determined by FOLIN-CIOCALTEU, the activity of the enzyme polyphenoloxidase, which plays a catalyzing role in the oxidation of phenolic compounds, and the antioxidant activity by TEAC and ORAC assays. HPLC-DAD-ESI-MS^n analysis was also performed to determine differences in the content of individual phenolic compounds.

Significant increases in selenium content were found in the fruits. Thus, an apple of the cultivar 'Golden Delicious' can cover the daily selenium requirement by 17-20%, and an apple of the

cultivar 'Jonagold' even by 20-25%. Biofortification also resulted in lower fluctuations of PPO activities and varied depending on the level of selenium application. Higher levels of application were associated with higher PPO activities. This also explains the relationship with TPC. With higher selenium application, lower TPC were measured, as more phenolic compounds were degraded by the higher PPO activity. With respect to AOA, no clear trends were observed by selenium biofortification. This is probably related to the different antioxidant activities of the individual phenolic compounds. Apples contained mainly chlorogenic acid and were rich in epicatechin, caffeeoylglucoside, and a procyanidin trimer, probably C1. Biofortification showed different effects on the two analyzed cultivars: the phenolic profile of 'Golden Delicious' was comparatively insensitive, whereas especially the application of selenate to 'Jonagold' led to significant changes in individual phenolic compounds. In relation to all measured parameters, the apple variety and ecophysiological conditions such as sunshine duration could also be identified as further influencing factors.

Work shares

Sabrina Groth:	Establishment and validation of methods for the determination of polyphenoloxidase activity, total phenolic content, and antioxidant properties by TEAC and ORAC assay in apples; measurement of PPO activity, TPC as well as AOA; experimental and statistical evaluations; preparation of the manuscript
	Total contribution: 35%

antioxidants

MDPI

Article

Influence of a Selenium Biofortification on Antioxidant Properties and Phenolic Compounds of Apples (*Malus domestica*)

Sabrina Groth [1], Christoph Budke [2], Susanne Neugart [3], Sofia Ackermann [1], Fenja-Sarah Kappenstein [1], Diemo Daum [2] and Sascha Rohn [1,*]

1 Hamburg School of Food Science, Institute of Food Chemistry, University of Hamburg, Grindelallee 117, 20146 Hamburg, Germany; sabrina.groth@chemie.uni-hamburg.de (S.G.); ackermannsofia@aol.com (S.A.); nudel5555@web.de (F.-S.K.)

2 Department of Plant Nutrition, Osnabrück University of Applied Sciences, 49090 Osnabrück, Germany; c.budke@hs-osnabrueck.de (C.B.); d.daum@hs-osnabrueck.de (D.D.)

3 Department of Crop Sciences, Division Quality and Sensory of Plant Products, Georg-August-Universität Göttingen, 37075 Göttingen, Germany; susanne.neugart@uni-goettingen.de

* Correspondence: rohn@chemie.uni-hamburg.de; Tel.: +49-40-42838-7979

Received: 27 January 2020; Accepted: 22 February 2020; Published: 24 February 2020

Abstract: Biofortified apples seem to be a suitable produce. In this study, different selenium forms and application levels were applied to the two apple varieties 'Golden Delicious' and 'Jonagold', grown in the years 2017 and 2018 in order to increase the selenium uptake within a typical Western diet. It was shown that the biofortification, which was performed as a foliar application implemented in usual calcium fertilization, led to significantly increased selenium contents in the fruits. Furthermore, biofortification affected the total phenolic content (TPC), the polyphenol oxidase activity (PPO), as well as the antioxidant activity (AOA), the latter measured with the two well-known assays Trolox Equivalent Antioxidant Capacity Assay (TEAC) and Oxygen Radical Absorbance Capacity Assays (ORAC). The varying selenium forms and application levels showed a differing influence on the parameters mentioned before. Higher fertilizer levels resulted in higher selenium accumulation. It was found that PPO activity fluctuates less in biofortified apples. With regard to TPC, selenate led to higher amounts when compared to the untreated controls and selenite resulted in lower TPC. AOA analysis showed no clear tendencies as a result of the selenium biofortification. In the case of 'Jonagold', a higher AOA was generally measured when being biofortified, whereas, in the case of 'Golden Delicious', only one form of application led to higher AOA. Additionally, differences in the amount of major phenolic compounds, measured with High Performance Liquid Chromatography Mass Spectrometry (HPLC-DAD-ESI-MS^n), were observed, depending on the conditions of the biofortification and the variety.

Keywords: apple; selenium; agronomic biofortification; antioxidant activity; phenolic compounds; TEAC; Total Phenolic Content; phenoloxidase

1. Introduction

Biofortification is an agronomic practice for specifically enriching food crops with certain nutrients. In most cases, it is aimed at increasing the content of minerals, such as zinc or selenium, because soil conditions often do not allow for a natural presence of adequate amounts of these compounds [1–4]. Especially in Germany and other European regions, selenium is only present in small amounts in the soils, which means that the selenium content of plant produce is correspondingly low [5]. As a result of targeted applications of selenium-containing fertilizers, the plant increasingly absorbs the mineral,

which is then integrated into the endogenous plant metabolism by incorporation into amino acids, such as selenocysteine and selenomethionine [6].

Since 1985, foods, such as cereals, have been successfully biofortified with selenium in Finland. There, it was shown that the selenium supply of the mean population steadily improved [7]. Biofortification seems to be suitable for addressing selenium deficiency that many people are suffering from worldwide. Consequently, selenium deficiency-related diseases, such as reduced immune function, degeneration of the cardiovascular system, and cognitive decline, could be minimized [4,8,9]. A prolonged deficiency of selenium is associated with the endemic diseases Keshan and Keshin-Beck [9].

Selenium is an essential element in human nutrition and therefore plays an important role in the human organism, especially as a component of proteins and enzymes such as glutathione peroxidase, thyroxine 5-deiodinase, and selenoprotein P [9]. Selenium is involved in the production of active thyroid hormones and the regulation of the immune system. It is essential for reproduction and has antioxidant, anti-inflammatory, and antiviral effects [10]. Furthermore, selenium is an integral part of some antioxidant enzymes, which protect cells from being damaged by radicals produced during oxidative metabolic pathways [9]. Some benefits of higher selenium status on the risk of prostate, lung, colorectal, and bladder cancers have already been described [8].

The recommendations for the daily selenium intake for Germany, Austria, and Switzerland (D-A-CH reference values for nutritional intake) are approximately 1 µg selenium per kg body weight. With consideration of the reference body weights, the resulting estimated values for selenium intake are approximately 70 µg/day for adult men and 60 µg/day for adult women [11]. Usually, the need for selenium is largely covered by animal produce, such as meat or fish. The biofortification of plant produce allows for vegetarians or vegans in particular to fulfill their needs naturally and as an alternative to food supplements [12,13]. A previous consumer survey clearly indicated that German consumers would prefer selenium-rich apples instead of food supplements for improving their selenium supply [14]. Another advantage of the intake of selenium-containing food as compared to the intake of supplements is the different bioavailability of the varying selenium forms. The organic forms (when anorganic selenium has been already transformed to organic compounds, such as selenocysteine and selenomethionine in plants) can be more easily absorbed in the intestinal tract when compared to the inorganic forms, being often present in dietary supplementation products [9,10].

In Germany, apple is the most popular fruit with a consumption of 21.0 kg per capita [15] and therefore particularly suitable for reaching a broad proportion of the population with a chance for improving the selenium supply for many people.

Other research groups already carried out studies on various crops for selenium biofortification, while using foliar fertilization with sodium selenite or sodium selenate. It has been shown that those treatments led to increased selenium levels in the plants when compared to the untreated controls. A higher accumulation with selenate was observed when compared to selenite [16–19]. Furthermore, the influence of different fertilizer levels was investigated, where it was observed that the selenium content in the plants increased with increasing selenium level [16,18–22]. However, Hawrylak-Nowak found a decline in the biomass of hydroponically cultivated butterhead lettuce (*L. sativa* L. var. *capitata*, cv. 'Justyna') at the highest tested level of 15 µM Se that was contained in the nutrient solution [20].

In addition to the selenium content, the influence of a biofortification on other parameters, such as fruit quality, and the content of secondary plant substances, such as phenolic compounds, were also investigated in several studies: Zhao et al. found a significant increase of vitamin C content in selenium biofortified pear-jujube (*Ziziphus jujuba* M. cv. 'Lizao') [23]. D'Amato et al. conducted a study on olive oil in which significantly higher levels of phenolic compounds and changes in the phenolic profile were observed, with the contents of certain antioxidant phenolic compounds increasing, as a result of the biofortification [24]. Further analyses on rice (*Oryza sativa* L., cv. 'Selenio') showed that moderate doses of selenite up to 45 mg/L were the best compromise between high selenium levels and an increase of phenolic acid concentration [16]. Schiavon et al. also found elevated phenolic compound contents in tomatoes (*Solanum lycopersicum* L., cv. 'Margoble'), resulting from a selenium biofortification [21]. Especially, they

found elevated levels of the antioxidant flavonoids naringenin chalcone, and kaempferol. However, a simultaneous decrease of cinnamic acid derivatives was observed [21]. In a subsequent study, the same research group investigated the influence of a selenium biofortification on the leaves and roots of radish (*Raphanus sativus* L., cv. 'Saxa'). The total phenolic content (TPC) in the roots was reduced by 40–60%, whereas an increase of 10% of the TPC was observed in the leaves when compared to leaves of the control plants [22]. Bachiega et al. investigated the relationship between phenolic compounds and the antioxidant activity (AOA) in selenium biofortified broccoli (*Brassica oleracea*, cv. 'Italica'). They found a significant increase in phenolic compounds as well as a higher AOA [25]. Pezzarossa et al. performed studies on peaches (*Prunus persica*) and pears (*Pyrus communis* L.) that were biofortified with sodium selenite: There, an extended shelf life of the fruits after removal from the storage was shown, being hypothesized to be related to increased TPC [26].

So far, only few data exist on the selenium biofortification of apples. In 2019, Babalar et al. investigated the influence of a selenium biofortification with sodium selenate on the apple variety 'Starking Delicious' and various quality parameters, also including the TPC. However, it was only analyzed as a function of the storage time of the fruit; a direct comparison between biofortified and untreated apples was not done [27]. The aims of the present study were to increase the selenium concentration in the apples and identify the appropriate dosage form and application level. A further focus was on the increase of value-added phytochemicals, especially on substances related to AOA. The relationship between selenium biofortification with phenolic compounds and antioxidant properties in apples has not yet been investigated. Within the scope of this work, this was analyzed at hand of a large number of different applications, in which the selenium form and the level of application were varied. The two apple varieties 'Golden Delicious' and 'Jonagold' were studied in two consecutive years because of being very important cultivars for the German market.

2. Materials and Methods

2.1. Chemicals

Disodium hydrogen phosphate dodecahydrate was purchased from Bernd Kraft GmbH (Duisburg, Germany). Sodium dihydrogen phosphate monohydrate was from AppliChem GmbH (Darmstadt, Germany) and catechol was from ThermoFisher GmbH (Kandel, Germany). Aceton was used from VWR International LLC (Fontenay-sous-Bois, France). The standards for the HPLC analysis (chlorogenic acid, catechin, epicatechin, phloretin-2-glucoside, and quercetin 3-glucoside) and hydrochloric acid (25%) were from Carl Roth GmbH & Co. KG (Karlsruhe, Germany). Sodium carbonate was purchased from Grüssing GmbH (Filsum, Germany) and potassium peroxodisulphate was from Fisher Scientific UK Ltd. (Loughborough, UK). Folin-Ciocalteu's phenol reagent, potassium dihydrogen phosphate, and nitric acid (65%) were purchased from Merck KgaA (Darmstadt, Germany). Galllic acid and 2,2'-azobis(2-methylpropionamidine) dihydrochloride were from Fisher Scientific GmbH (Schwerte, Germany). 2,2'-Azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt, trolox, and fluorescein were purchased from Sigma-Aldrich Chemie GmbH (Deisenhofen, Germany). All of the chemicals were of analytical grade. Water was purified through a Milli-Q water system (PURELAB®, Elga LabWater, Veolia Water Technologies GmbH, Celle, Germany) and was used for buffers, the extracting agents, and dilution of sample extracts.

2.2. Sample Material

The apple cultivars 'Golden Delicious' and 'Jonagold' were evaluated. Fruits were grown in 2017 and 2018 at the Horticultural Research Station of the Osnabrück University of Applied Sciences, Germany (N52.310654°, E008.02844°; 69 m a.s.l.).

The apple trees were grown on Plaggen soil, the topsoil, and subsoil were loamy sand. The pH value of the soil was 5.5 and the organic matter content in the topsoil amounted 2.4% and in the subsoil 1.8%. On an adjacent, the horticulturally used area (also loamy sand), the soil Se content determined by

extraction with aqua regia amounted to 0.25 mg/kg d.w. and by extraction with 0.1 M K_2HPO_4/KH_2PO_4 (pH 7.0) was below the detection limit (< 0.1 mg/kg d.w.). This indicates that a relatively low content of phytoavailable Se was present at the test site. The very low Se contents of the untreated control apples that ranged between 0.2 and 0.4 µg Se/100 g d.w. confirm that the selenium content in the soil was very low.

The experimental plant was a randomized block plant design with four repetitions, whereby one tree corresponded to one repetition. The trees were treated and harvested from both sides. The rows were aligned in north-south direction. The apples were biofortified with selenium while using a foliar fertilization approach and the selenium forms sodium selenate and sodium selenite were used in analytical grade. Foliar fertilization is advantageous over soil application, as plants are directly treated and, thus, compounds might easily enter fruits. Only the selenium that reaches the fruit is relevant for biofortification, since selenium is not significantly shifted from the leaves into the fruit. Furthermore, application can be done in parallel with the traditionally performed calcium sprays [28]. For improving wetting properties, all of the solutions used for foliar sprays additionally contained 0.02% (*v*/*v*) of the nonionic organosilicone adjuvant Break-Thru® S 240 (AlzChem AG, Trostberg, Germany).

In 2017, the apples were biofortified with 0.15 kg Se per hectare and meter canopy height (Se/ha x m CH), divided into six applications during the season (beginning in July until the end of September) with a hand-held spray system (model Easy-Sprayer Plus, Lehnartz GmbH, Remscheid, Germany). Pure water was sprayed on the trees for the control treatments. During sampling, ten well-developed medium size apples from well exposed middle parts of the trees were harvested for subsequent analysis.

The apples were processed at the Osnabrück University of Applied Sciences after two weeks of storage at 2 °C. First, the fruits were divided into eight segments and the stalk while using an apple slicer. The stalk segment was discarded. The segment samples were directly shock frozen with liquid nitrogen and stored at −27 °C.

In 2018, the application rate of the fertilizer was reduced to 0.075 kg Se/ha x m CH and only sodium selenate was applied. The application rate was reduced in 2018 due to slight fruit damages occurring in the year 2017. The selenium fertilizer was applied together with the calcium-containing foliar fertilizer WUXAL®Ascofol Ca (5 L/ha; Aglukon Spezialdünger GmbH &Co. KG, Düsseldorf, Germany). For the control treatments, pure water and WUXAL®Ascofol Ca were sprayed on the trees. In that season (end of June until the end of August), a backpack sprayer (REB 15 AZ2, Birchmeier Sprühtechnik AG, Stetten, Switzerland) was used for application. Sampling was analogous to the previous year.

With the exception of the enzyme activity determination of the polyphenol oxidase, which was done from thawed apple samples, all other samples were lyophilized to prevent the degradation of the phenolic compounds. A frozen sample was placed in a knife mill (Blixer® 4-3000; robot coupe S.N.C., Vincennes Cedex, France) with the addition of dry ice and homogenized for 60 s at 3000 rpm. After homogenization, the sample was freeze dried for 48 h. The dried samples were then filled into 50 mL tubes and then stored at −20 °C until further analysis. A sample set of four randomly chosen apples per variety were analyzed.

2.3. Determination of the Selenium Content

For the determination of the selenium content, the apples were pre-prepared at the Osnabrück University of Applied Sciences, as described in 2.2. After the subdivision into eight segments, the samples were directly dried at 60 °C in a fresh air drying oven until their weight remained constant. After drying, the samples were ground in an ultracentrifugal mill (Retsch ZM 200, Retsch GmbH, Haan, Germany) at 14,000 rpm to a particle size ≤ 0.5 mm. The powder was stored in plastic tubes until further sample preparation. A sample digestion was carried out according to the standardized method DIN EN 13805 [29]. For this purpose, 0.5 g of the ground plant material was digested while using microwave pressure digestion in quartz glass vessels with 65% nitric acid at 190 °C. The digestion solution was measured with a graphite tube atomic absorption spectrometer (Thermo

Scientific UNICAM SOLAAR M Series AA, Thermo Fisher Scientific Inc., Waltham, MA, USA). Internal and external certified reference material was used to ensure the quality of the analysis [ERM-BB422 fish muscle and NIST-1849a infant/adult nutritional (milk) powder]. For samples with low selenium concentrations (< 2.5 µg/L), selenium analysis was alternatively carried out while using the hydride technique in accordance with DIN 38405-23 [30].

2.4. Determination of the Polyphenol Oxidase (PPO) Activity

The determination of the PPO activity of the apple samples was done according to Kolodziejczyk et al. [31] and González et al. [32], with an adaption to a miniaturized procedure. About 10 g of the frozen sample was weighed and crushed in a mortar. Subsequently, 25 mL of a phosphate buffer (0.05 M, pH 7.0) were added and then mixed. The sample was incubated for 120 min. at 4 °C in the dark, centrifuged (15 min., 4 °C, 3225 g), and the supernatant used to determine PPO activity. First, 30 µL of the sample extract were given in a 96-well microtiter plate and either 270 µL of the phosphate buffer (0.2 M, pH 5.5) as blank value or 270 µL of a catechol solution (0.1 M in 0,2 M phosphate buffer, pH 5.5) were added. The enzyme activity was immediately determined at 25 °C by measuring the change in absorption over 10 min. at a wavelength of λ = 420 nm with a BioTek Synergy HT microplatereader (BioTek Instruments Inc., Winooski, VT, USA), whereby the change in absorption was recorded every 60 s. The enzyme activity was given as activity units per 100 g of fresh weight (f.w.) of a fruit sample. One unit is defined as the change of 0.01 in the absorbance value per minute [31,32].

2.5. Method for Extracting Phenolic Compounds

Sixty miligrams of the lyophilized apple sample were weighed into a 2 mL tube. One milliliter of the extraction agent 50% aqueous acetone and 0.1% HCl (v/v) was added and treated in an ultrasonic bath for 5 min. at 30 °C. Subsequently, four glass beads (i.D. 4 ± 0.3 mm) were added to each sample and the sample was ground and mixed in a ball mill (5 min., 25 Hz). The samples were then centrifuged for 5 min. at 20,817 g and the supernatant transferred into a 15 mL tube. The extraction with the ball mill was repeated twice and supernatants were combined. The total volume was filled to 4 mL.

2.6. Determination of the Total Phenolic Content (TPC) according to Folin-Ciocalteu

The TPC of the apple samples was determined while using a modified Folin-Ciocalteu methodology, according to Müller et al. [33]. Twenty microliters of the sample extract were given in a 96-well microtiter plate, 100 µL of the Folin-Ciocalteu phenol reagent (1:10; *v*/*v*), and 80 µL of an aqueous 7.5% (*w*/*v*) sodium carbonate solution were added. Subsequently, incubation was performed for 2 h at room temperature in the dark. The absorption was measured at a wavelength of 765 nm at 30 °C while using the BioTek Synergy HT microplatereader. TPC is given in gallic acid equivalents per 100 g of dry weight (mg GA/100 g d.w.) [33,34].

2.7. Analysis of the Antioxidant Activity (AOA) using the Trolox Equivalent Antioxidant Capacity Assay (TEAC)

The determination of the AOA using the TEAC assay was performed according to Müller et al. [33]. A 75 mM phosphate buffer (pH 7.4) as well as a 7 mM 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) stock solution and a 2.45 mM potassium peroxodisulphate solution were prepared. Both of the solutions were mixed, transferred to an amber glass bottle, and stored for 24 h at room temperature, until the $ABTS^{\cdot+}$ radical was completely formed. The reagent, known as ABTS working solution I, was then stored in a refrigerator. Two hours before starting a determination, the ABTS working solution I was diluted with phosphate buffer (75 mM, pH 7.4) to an absorbance of E_{730} = 0.700 ± 0.050. This ABTS working solution II was left at room temperature until measurement. For calibration, a 2.5 mM trolox stock solution was prepared and diluted 1:10 (*v*/*v*) with water. A dilution series was prepared from this. Twenty microliters of various dilutions of the samples, trolox, or water (blank value) were given in a 96-well microtiter plate and 200 µL of ABTS working solution II were

added. The adsorption was measured after 6 min. incubation at 30 °C at a wavelength of λ = 730 nm with the BioTek Syngergy HT microplatereader. AOA is calculated as trolox equivalent 100 g dry weight per (mmol TE/100 g d.w.) [33,35].

2.8. Analysis of the AOA using the Oxygen Radical Absorbance Capacity Assays (ORAC)

For the ORAC assay, which was also done according to Müller et al. [33], a 0.12 mM fluorescein solution was prepared from fluorescein and phosphate buffer (75 mM, pH 7.4). From this solution, the final fluorescein working solution was freshly prepared by a 1:100 dilution with phosphate buffer, before each analysis. For the 2,2'-azobis(2-methylpropionamidine) dihydrochloride (AAPH) stock solution (c = 129 mM), AAPH was dissolved in phosphate buffer. Calibration was also done with trolox. Ten microliters of each sample in the different dilutions, trolox, or water were given in a 96-well microtiter plate. Subsequently, 100 µL phosphate buffer (75 mM, pH 7.4) or 250 µL for the negative control were added. After a 10 min. incubation period in the BioTek Synergy HT microplatereader at 37 °C, 150 µL of the AAPH stock solution were added to the blank value, standards, and samples. The measurement, which was based on fluorescence quenching, was performed at an excitation wavelength of λ = 485 nm and an emission wavelength of λ = 528 nm at 37 °C. The course of the reaction was recorded for 120 min., with one measurement every two minutes. AOA is also calculated as trolox equivalent per 100 g dry weight (mmol TE/100 g d.w.) [33,36].

2.9. Qualitative and Quantitative Determination of Phenolic Compounds Using High Performance Liquid Chromatography Mass Spectrometry (HPLC-MS)

The phenolic compounds were extracted from the lyophilized apple samples (0.01 g) in a triple extraction with 60% aqueous methanol, according to Neugart et al. [37]. Phenolic compound identification and quantification were determined while using an 1100 series HPLC system (Agilent Technologies GmbH, Waldbronn, Germany) equipped with a degasser, binary pump, autosampler, column oven, and photodiode array detector. An Ascentis® Express F5 column (150 mm × 4.6 mm, 5 µm, Sigma-Aldrich Chemical Co., St. Louis, USA) was used to separate the compounds at a temperature of 25 °C. Eluent A was 0.5% acetic acid, and eluent B was 100% acetonitrile. The gradient used for eluent B was 5–12% (0–3 min.), 12–25% (3–46 min.), 25–90% (46–49.5 min.), 90% isocratic (49.5–52 min.), 90–5% (52–52.7 min.), and 5% isocratic (52.7–59 min.). The determination was conducted at a flow rate of 0.85 mL/min. and wavelengths of 280 nm, 320 nm, and 370 nm for phloretin glycosides and flavanols, hydroxycinnamic acid derivatives, and non-acylated flavonol glycosides, respectively. The hydroxycinnamic acid derivatives and glycosides of flavonols were identified as deprotonated molecular ions and characteristic mass fragment ions according to Schmidt et al. [38] by HPLC-DAD-ESI-MS^n while using an Agilent ion trap mass spectrometer in negative ionization mode. Nitrogen was used as the dry gas (10 L/min, 325 °C) and the nebulizer gas (40 psi) with a capillary voltage of −3500 V. Helium was used as the collision gas in the ion trap. The mass optimization for the ion optics of the mass spectrometer for quercetin was performed at *m/z* 301 or arbitrarily at *m/z* 1000. The MS^n experiments were performed up to HPLC-DAD-ESI-MS^3 in a scan mode from *m/z* 200–2000. The standards (chlorogenic acid, catechin, epicatechin, phloretin-2-*O*-glucoside, and quercetin-3-*O*-glucoside) were used for external calibration curves. The results are presented as mg/100 g dry weight.

2.10. Statistical Analysis

The number of analyses per application with selenium fertilizer or control was $n = 4$. All of the analyses were done twice. The data are given in mean ± standard deviation and further evaluated while using Microsoft Excel (Microsoft Office Professional Plus 2016, Redmond, WA, USA). The statistical analyses were carried out using SPSS (Version 25, IBM® Corporation, Armonk, NY, USA) and the data were further evaluated with a two-way analysis of variance (ANOVA). The means were compared while using the Bonferroni post-hoc test at $p < 0.05$.

3. Results and Discussion

Table 1 gives an overview of the results of the determination of the selenium content, the polyphenol oxidase activity, the total phenolic content, and the antioxidant activity that was determined with both assays - TEAC and ORAC in the biofortified apples of the varieties 'Golden Delicious' and 'Jonagold' with the various selenium applications, as well as the corresponding untreated controls of the years 2017 and 2018.

3.1. Selenium Content

Biofortification significantly increased in the selenium content of apples in general as compared to the untreated controls (Table 1). This increase was 10 to 14-fold. The highest content was achieved in both varieties, which was 5.6 µg Se/100 g f.w when applying 0.15 kg Se per hectare and meter canopy height in the form of selenite in 2017. The application of selenate at the same dosage level also led to an identical Se level in 'Golden Delicious' and a slightly lower content of 4.5 µg Se/100 g f.w. in 'Jonagold'. However, these genotypic differences were statistically not significant. The application of the lower levels of selenium in 2018 resulted in significantly lower selenium contents for both varieties. Again, the influence of the variety on the selenium content was not significant. The results of the present study are in line with published data. A significant increase of the selenium content resulting from biofortification with foliar fertilization has already been observed by other research groups in a variety of plant foods, especially on vegetables [16–19,21,22,25]. In those studies, the dosage form and the fertilizer level played a significant role. It was found that selenate leads to higher selenium accumulations than selenite [16–19] and the selenium content in the plants increased with increasing application level [16,18–21]. In the present experiments, no significant difference was found between the two forms of the selenium that were applied.

An increase in selenium concentration was also observed in different fruits. Pezzarossa et al. carried out a biofortification with 1.0 mg Se/L in the form of sodium selenate on peach (*Prunus persica* Batch. cv. Flavorcrest) and pear (*Pyrus communis* L. cv. 'Conference') and increased the selenium concentration in the fruits from < 0.1 µg Se/100 g f.w. to 0.9 µg Se/100 g f.w. and 3.6 µg Se/100 g f.w., respectively [26]. With regard to phytotoxicity resulting from fertilization with selenium, only slight damages on the fruits were observed in the year 2017. With reduced selenium levels in the follow-up year, there was no damage, anymore. However, moderate damages on the leaves were present in both years.

The use of selenium in a long-term cultivation program already showed good experience in Finland. Here, selenium fertilization has been carried out for many years on a national and compulsory basis. No corresponding ecological problems have been identified [39,40].

3.2. PPO Activity

PPO are very important enzymes, especially in apples, as quick browning of freshly cut apples is not accepted by the consumer [41]. Further, formation of the brown colored melanins has not yet been investigated with regard health risks. Usually, PPO substrates, small phenolic compounds, are still regarded being more health-beneficial [42–45].

The results show that the application of a higher amount of selenium (0.15 kg Se/ha x m CH), regardless of the form of selenium used, led, on average, to a higher PPO activity than those of the untreated controls for the two varieties 'Golden Delicious' and 'Jonagold' (Table 1, Figure 1; Figure 2). When on the other hand, the amount of selenium applied was lower (0.075 kg Se/ha x m CH), a lower PPO activity when compared to the controls was observed, being also valid for both cultivars. However, these differences between biofortified apples and the corresponding controls were not statistically significant. Smoleń et al. found increased PPO activities in comparison to the untreated controls, when performing a biofortification of potatoes (*Solanum tuberosum* L., cv. 'Vineta') with selenium (6.3 µM in the form of sodium selenite) and iodine, with being also not significant [46].

Furthermore, it was observed that the standard deviation of the measurements was - except for 'Golden Delicious' in 2018 - lower in the Se treatments as compared to the corresponding controls. The coefficient of variation ranged between 25.2 and 73.1% (mean 47.7%) in the controls, whereas, in the selenium biofortified apples, the values were between 4.8 and 59.4% (mean 38.5%). Holderbaum et al. also observed high variation coefficients of PPO in four apple cultivars at initial, intermediary, and final fruit development stages [47]. Reinkensmeier et al. measured low variation coefficients in selected varieties (e.g., 'Golden Delicious' and 'Jonagold') [48]. Smoleń et al. also found a high variation in PPO activity in potatoes as compared to the control with regard to the influence of a biofortification with selenium [46].

Table 1. Results of the determination of the selenium content, polyphenol oxidase activity, total phenolic content, and antioxidant activity in all apple samples. Data are given as mean value ± standard deviation (n = 4). In each column, means followed by different letters are significantly different ($p < 0.05$).

Variety and Year o Cultivation	Application *	Se [µg/100 g f.w.]	PPO [units/100 g f.w.]	Total Phenolic Compound [mg GAE/100 g d.w.]	TEAC [mmol TE/100 g d.w.]	ORAC [mmol TE/100 g d.w.]
Golden Delicious 2017	control	0.4 ± 0.2 ^{a}	12.50 ± 4.05 ^{,b}	858.4 ± 92.5 ^{b,c,d}	6.76 ± 0.87 ^{a}	5.53 ± 1.32 ^{a,b}
	0.15 kg selenite	5.6 ± 0.6 ^{b}	17.85 ± 0.85 ^{ab}	788.3 ± 37.3 ^{b}	5.89 ± 0.25 ^{a}	4.82 ± 3.50 ^{a}
	0.15 kg selenate	5.6 ± 0.8 ^{b}	23.67 ± 2.66^{bc}	859.6 ± 23.0 ^{b,c,d}	7.37 ± 2.01 ^{a}	6.36 ± 1.04 ^{a,b}
Golden Delicious 2018	control	< 0.2 ^{a}	37.69 ± 9.50 ^{d}	663.0 ± 102.8 ^{a}	10.98 ± 1.93 ^{b}	9.60 ± 3.23 ^{bc}
	0.075 kg selenate	2.7 ± 0.8 ^{c}	35.07 ± 20.84 ^{c,d}	761.3 ± 25.1 ^{a,b}	10.83 ± 0.84 ^{b}	9.03 ± 4.19 ^{a,b,c}
'Jonagold' 2017	control	0.4 ± 0.2 ^{a}	3.90 ± 2.34 ^{a}	954.7 ± 58.1 ^{d}	7.58 ± 0.75 ^{a}	9.56 ± 2.26 ^{b,c}
	0.15 kg selenite	5.6 ± 1.1 ^{b}	3.09 ± 1.50 ^{a}	928.4 ± 37.9 ^{c,d}	10.51 ± 3.78 ^{b}	9.76 ± 2.51 ^{b,c}
	0.15 kg selenate	4.5 ± 1.6 ^{b}	7.11 ± 3.92 ^{a}	889.1 ± 54.6 ^{c,d}	13.24 ± 1.10 ^{b}	9.15 ± 1.62 ^{b,c}
'Jonagold' 2018	control	< 0.2 ^{a}	9.19 ± 6.72 ^{a}	867.7 ± 105.5 ^{b,c,d}	12.42 ± 1.33 ^{b}	11.24 ± 1.93 ^{c,d}
	0.075 kg selenate	2.0 ± 0.3 ^{c}	6.39 ± 3.29 ^{a}	826.5 ± 72.1 ^{b,c}	11.27 ± 0.44 ^{b}	14.55 ± 0.98 ^{d}

* Foliar spray rate per hectare and meter canopy height.

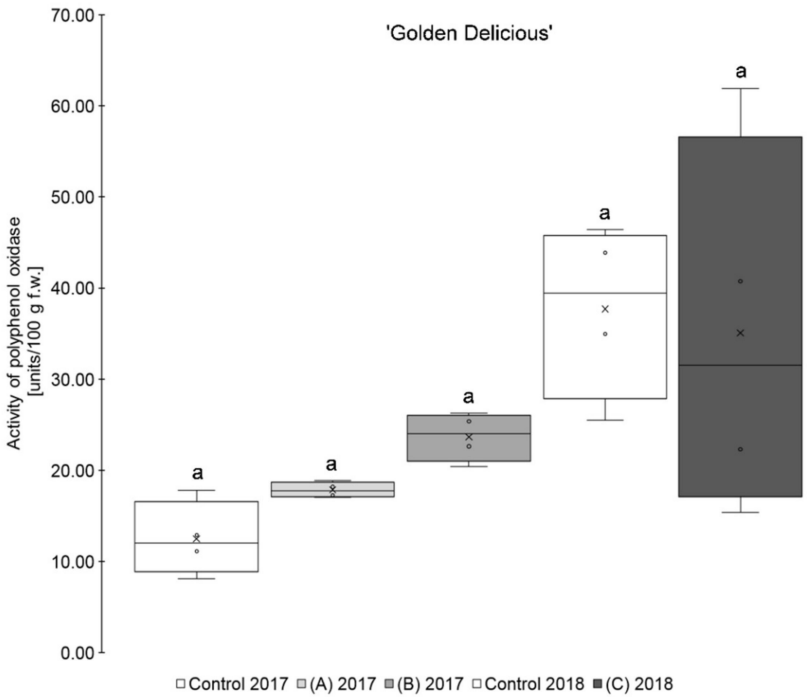


Figure 1. Polyphenol oxidase activity in units/100 g fresh weight (f.w.) for the apple samples of the variety 'Golden Delicious'. Foliar Se application per hectare and meter canopy height: 0.15 kg as selenite (A), or selenate (B), 0.075 kg as selenate (C) (n = 4). Different letters are significantly different ($p < 0.05$).

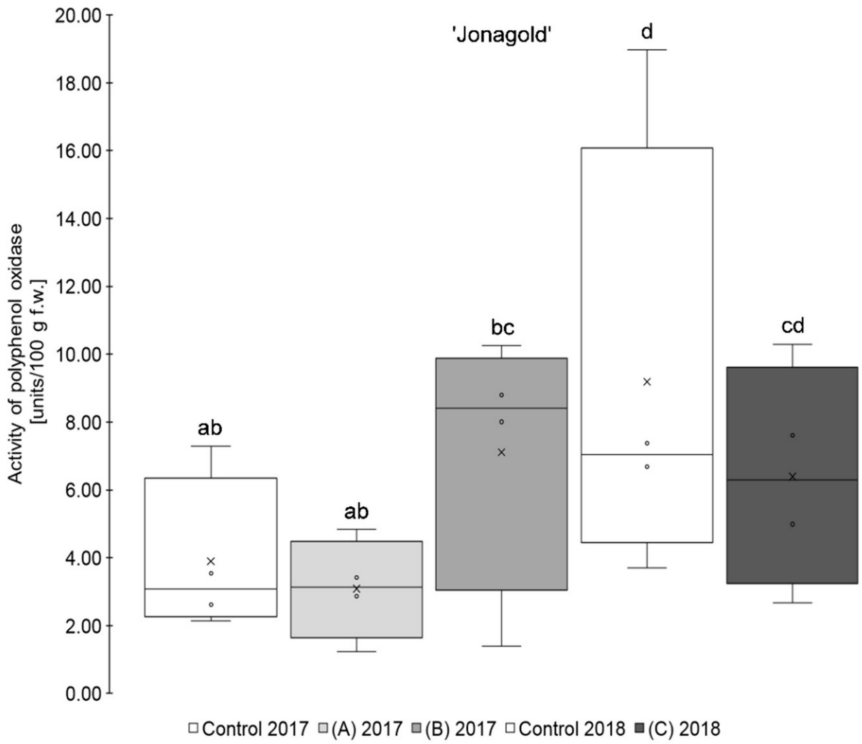


Figure 2. Results of polyphenol oxidase activity in units/100 g fresh weight for the apple samples of the variety 'Jonagold'. Foliar Se application per hectare and meter canopy height: 0.15 kg as selenite (A), or selenate (B), 0.075 kg as selenate (C) ($n = 4$). Different letters are significantly different ($p < 0.05$).

In addition, variety-specific differences in the PPO activity were observed. Significantly higher PPO activity was measured for 'Golden Delicious' as compared to 'Jonagold' in 2018 and partly also in 2017. Thus, 'Golden Delicious''s PPO activity was in a range between 12.50 and 37.69 units/100 g fw, whereas 'Jonagold's' PPO activity was significantly lower and between 3.09 and 9.19 units/100 g f.w. Variety-specific differences of PPO activity were also observed by Holderbaum et al. [47], Kolodziejczyk et al. [31], and Kschonsek et al. [49]. The latter investigated various apple varieties, including 'Golden Delicious', which had the highest PPO activity of all the tested varieties [49]. This is in line with the results that were obtained here.

In addition to the variety influence, there were also differences in the PPO activity between both growth seasons, which can be explained by an influence of the different ecophysiological conditions of the crop years, like the sunshine duration and the resulting UV radiation [50]. This difference was significant for 'Golden Delicious', but not for 'Jonagold'. Kolodziejczyk et al. have already observed differences in PPO activity within one variety in two consecutive years on a number of different apple varieties harvested in 2007 and 2008 [31]. Other research groups already investigated the influence of a UV-C treatment, which is an important postharvest treatment and influence on the PPO activity. For example, Manzocco et al. observed an inactivation of PPO and the prevention of enzymatic browning in 'Golden Delicious' apples by UV-C radiation [51]. Müller et al. also found a reduction of PPO activity in apple juices, when apples have been treated with UV-C light. In contrast, treatment with UV-B radiation did not show any effects [52]. Additionally, reduced PPO activities after UV-C treatments were observed in other vegetable crops [53,54].

3.3. Total Phenolic Content (TPC)

The results of the TPC determination showed the following trends for the two varieties 'Golden Delicious' and 'Jonagold' (Table 1 and Figure 3). The application of selenite led on average to lower TPC values when compared to the untreated controls. The tendencies in the application of selenate were different, depending on the amount of fertilizer. Higher levels led to lower or constant TPC values,

whereas lower levels of selenate tended to higher TPC values as compared to the untreated controls. However, the differences resulting from a biofortification with selenium were not statistically significant.

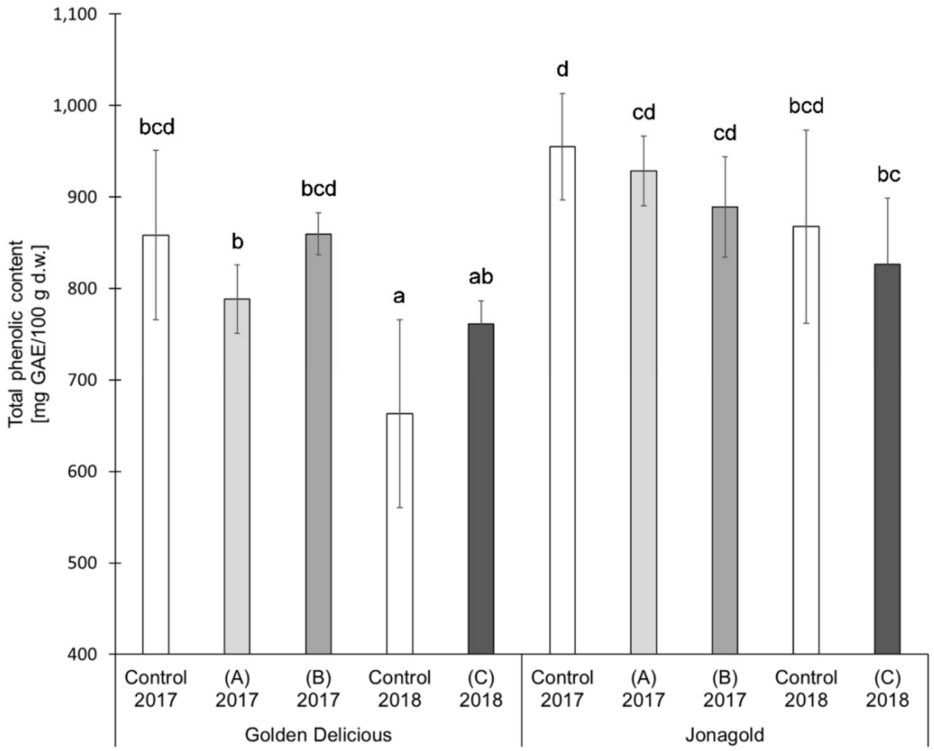


Figure 3. Total phenolic content (TPC) in mg GAE/100 g d.w. for the apple samples, depending on harvest year, apple variety, and form of selenium. Foliar Se application per hectare and meter canopy height: 0.15 kg as selenite (A), or selenate (B), 0.075 kg as selenate (C) (n = 4). Different letters are significantly different ($p < 0.05$).

An increasing TPC of selenium biofortified produce has already been found by other research groups: Bachiega et al. performed an application of 50 µM selenate to broccoli, which led to a significant increase in TPC [25]. In onion (*Allium cepa* L., cv. 'Hercules'), Põldma et al. observed that an application of 50 µg/mL selenate via foliar treatment led to increased TPC when compared to the untreated controls, whereas a higher level with 100 µg/mL resulted in lower TPC [55]. In tomatoes, Schiavon et al. found that selenate in low concentrations also led to an increase in TPC, when performing foliar fertilization of up to 20 mg Se/plant [21]. In a follow-up study on radish in 2016, an increase in TPC of 10% in the leaves when compared to the controls was recorded [22]. Hawrylak-Nowak found that the application of a moderate level of selenite (63.3 µM) applied via foliar fertilization led to enhanced TPC with a maximum increase of 43.9% in basil leaves *(Ocimum basilicum* L.) [18].

TPC also shows variety-specific differences between 'Golden Delicious' and 'Jonagold', with 'Jonagold' having higher values. The mean values of the controls were 858.4 mg GAE/100 g d.w. for the season 2017 and 663.0 mg GAE/100 g d.w. for the season 2018 for 'Golden Delicious', while the values for 'Jonagold' were 954.7 mg GAE/100 g d.w. and 867.7 mg GAE/100 g d.w., respectively. Variety-specific differences in TPC have already been described by other research groups. In the studies that were described by Kschonsek et al. and Xu et al., the TPC varied in the different apple varieties [49,56,57]. In 2018, Kschonsek et al. measured the TPC in 15 different apple cultivars and studied the peel and the fruit flesh, separately. In the peel, TPC was in a range between 521.9 mg GAE/100 g and 1590.5 mg GAE/100 g d.w. 'Golden Delicious' had the lowest TPC with a content of 521.9 mg GAE/100 g d.w., whereas the TPC of 'Jonagold' was 1224.2 mg GAE/100 g d.w. The amounts in the flesh were 136.5 mg GAE/100 g d.w. for 'Golden Delicious' and 177.5 mg GAE/100 g d.w. for 'Jonagold' [49]. When comparing the results of the selenium biofortified apples of the present

study with those that were obtained by Kschonsek et al. [49], the TPC values of 'Golden Delicious' were in a comparable range, whereas the TPC of 'Jonagold' apples was somehow much lower in the present study.

Besides a genotypic influence, it is also obvious that seasonal influences that result from a differing ecophysiology might lead to differences. In 2018, lower TPC were measured for both varieties when compared to the previous season. For the summer 2018, a sunshine duration of 756 h, an average rainfall of 88.5 L/m^2, and an average temperature of 19.8 °C were determined in the area of Osnabrück, Germany, where apples were grown. In comparison, the sunshine duration of the previous year 2017 was only 549.4 h with an average rainfall of 224.3 L/m^2 and an average temperature of 18.2 °C [50]. The difference in the amount of rain only plays a marginal role because of the use of artificial irrigation. Consequently, sunshine duration in particular seems to be the dominant influence on the level of the TPC. Moreover, it is not really the sunshine duration, but its direct correlation to UV radiation. Eichholz et al showed that light intensity and quality are some of the most effective factors on the biosynthesis of phenolic compounds in white asparagus (*Asparagus officinnalis* L., cv. 'Gijnlim') on the basis of UV-B treatments [58]. The influence of UV-B has been recently reviewed by Neugart and Schreiner [59]. Scattino et al. could also demonstrate that a postharvest UV-B irradiation induced changes of TPC in peaches (*Prunus persica* L., cv. 'Suncrest') and nectarines (*Prunus persica* var. *nucipersica*, cv. 'Big Top') [60]. A higher TPC was expected for the apples in comparison to the previous year based on these results and the present results of year 2018, in which UV-B radiation was more intensive. However, the TPC of samples from 2017 was higher (Table 1 and Figure 3).

There is an inverse correlation between PPO activity and TPC, because the enzyme catalyzes the oxidation reaction of phenolic compounds to quinones, which further react to brown colored polymeric melanins [31,42]. This could explain the influence of the selenium biofortification as well as the variety-specific differences, where lower PPO activities are associated with higher TPC and higher enzyme activities with lower TPC.

At an application level of 0.15 kg Se/ha (selenite or selenate), higher PPO activities and lower TPC were measured as compared to the untreated control. PPO activity and TPC showed that biofortification at an application level of 0.075 kg Se/ha in the form of sodium selenate—when compared to the untreated controls—resulted in significant lower enzyme activities on the one hand and a significant increase of the TPC on the other hand. With regard to genotype, 'Jonagold' showed significantly lower enzyme activity than 'Golden Delicious', which resulted in a lower degradation of phenolic compounds.

An increased PPO activity is undesirable, because the enzymatically induced reaction of phenolic compounds leads to a degradation of the phenolic compounds and, thus, reduces the nutritional value of apples and apple products and has a negative influence on sensory properties. The consumer does not accept fast brown of apples and polymeric polyphenolic melanins might contribute to a certain astringency of food products [58]. Smaller phenolic compounds have positive effects on human health, due to their antioxidant, anti-inflammatory, and antimicrobial properties [42–45]. However, high PPO activities in the context of allergenicity of different apple varieties are negatively associated. Kschonsek et al. found that in some apple varieties high PPO activity are accompanied with lower concentrations of Mal d 1 [49]. This might be desirable in order to provide consumers with apple varieties of low allergenic potential.

There are further different data available in the literature regarding the correlation of PPO activity and TPC. Song et al. found positive correlations between PPO and TPC based on studies of ten apple varieties [61]. In contrast, Kolodziejczyk et al. found no correlation between these two parameters on the basis of 22 apple varieties [31]. Allahveran et al. performed biofortification with ascorbic acid and citric acid on apples of the variety 'Red Spur' and determined the PPO activity and TPC among other parameters. There, biofortification led to a significant increase in TPC and a decrease in PPO activity [62].

3.4. Antioxidant Activity (AOA)

The AOA of the apple samples was determined while using the two well-known assays TEAC and ORAC, which are based on different reaction mechanisms and, thus, different evaluations of AOA can be done. The ORAC assay bases on hydrogen transfer and measures the antioxidant inhibition being induced by peroxyl radicals. It represents a biologically relevant mechanism and the antioxidant activity is determined over time, so that the potential effects of secondary antioxidant compounds can also be measured and an underestimation can be prevented. The TEAC assay is easy to perform. Therefore, it is often used and there are many comparative values in the literature. Its mechanism bases on electron transfer reactions. It is comparatively insensitive to pH and determines both hydrophilic and lipophilic antioxidants. The TEAC is well suited for the determination of antioxidant activity in phenolic rich samples, such as apples, as the $ABTS^{\bullet+}$ reacts quickly with antioxidants and many phenolic compounds of low redox potential [63].

In the present study, selenium biofortification did not reveal any clear tendencies of an influence on AOA. The two studied varieties were differently affected (Table 1, Figure 4, Figure 5): 'Jonagold' mostly showed a higher AOA (measured with TEAC) resulting from the biofortification and independently of the selenium form and the level of application, whereas the AOA of 'Golden Delicious' was only slightly influenced by the biofortification. The increase in AOA was significant for 'Jonagold' in 2017 with the application of 0.15 kg Se/ha in the form of selenite and in the form of selenate. Related to the dose of selenium, the evaluation of the AOA with the TEAC assay provided the following results: The application of 0.15 kg selenate in the season 2017 led to higher AOA in both varieties. The treatment with 0.15 kg selenite resulted in lower AOA for 'Golden Delicious' and higher AOA for 'Jonagold' compared to the corresponding controls (Table 1 and Figure 4). When 0.075 kg Se/ha in the form of selenate were applied in 2018, a slight, non-significant reduction in AOA was observed for both varieties.

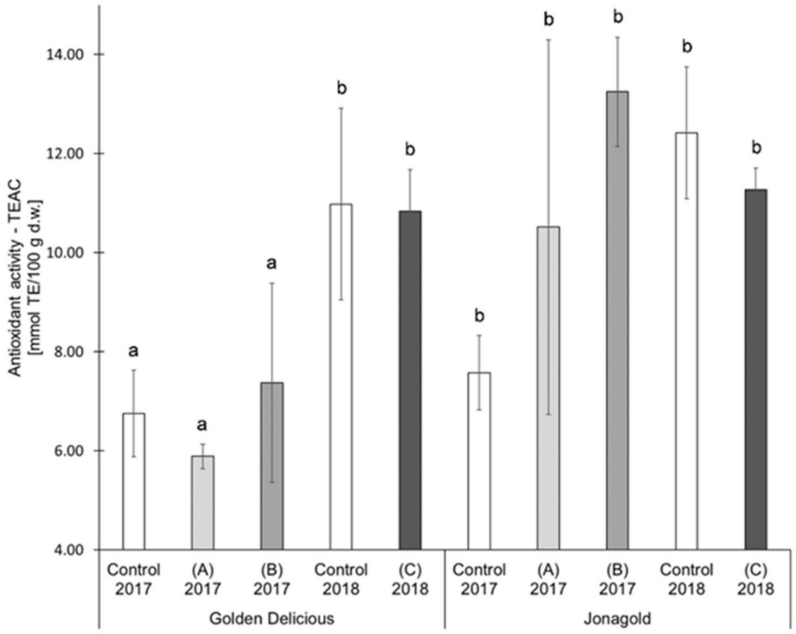


Figure 4. Results of the antioxidant activity (Trolox Equivalent Antioxidant Capacity Assay (TEAC) assay) in mmol TE/100 g d.w. for the apple samples. Foliar Se application per hectare and meter canopy height: 0.15 kg as selenite (A), or selenate (B), 0.075 kg as selenate (C) (n = 4). Different letters are significantly different ($p < 0.05$).

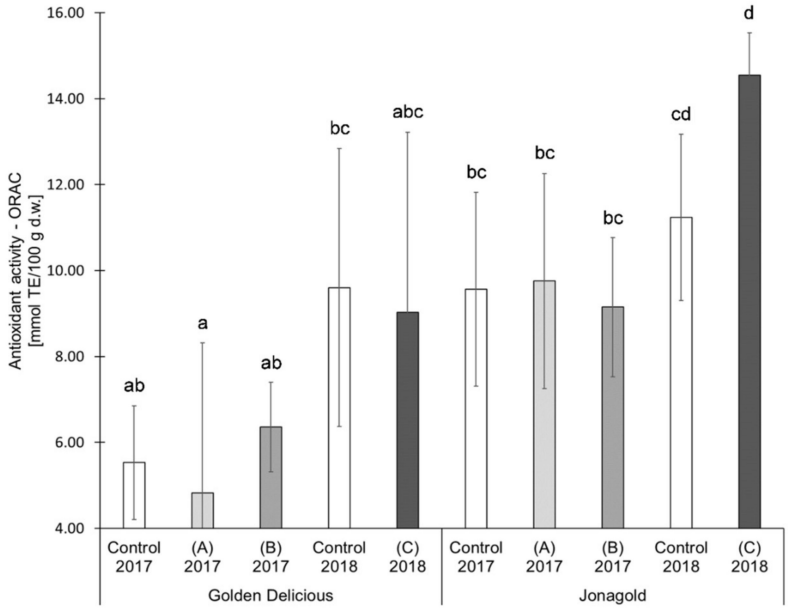


Figure 5. Results of the antioxidant activity (Oxygen Radical Absorbance Capacity Assays (ORAC) assay) in mmol TE/100 g d.w. for the apple samples. Foliar Se application per hectare and meter canopy height: 0.15 kg as selenite (A), or selenate (B), 0.075 kg as selenate (C) (n = 4). Different letters are significantly different ($p < 0.05$).

For 'Golden Delicious, the determination of the AOA with the ORAC assay showed the same tendencies as the TEAC values. However, differences were found for 'Jonagold': While the TEAC value for an application level of 0.15 kg selenate was significantly higher compared to the control, the ORAC value was lower when compared to the control. The treatment with 0.075 kg selenate led to an increase of the ORAC value in comparison to the decrease of the TEAC value.

An increase in AOA due to biofortification with selenium has also been noted by other researchers: Ríos et al. were able to show that increasing doses of selenite and selenate lead to an increase in AOA, as measured by FRAP and DPPH assay in lettuce plants (*Lactuca sativa* L. cv 'Philipus'). Selenate showed higher AOA when compared to selenite [19]. Põldma et al. determined an increase in AOA, as measured by TEAC, in onions (*Allium cepa* L. cv. 'Hercules') at a dose of 50 µg/mL Se [55]. Bachiega et al. found a significant increase in AOA as a result of a biofortifiaction of broccoli in addition to a significantly higher TPC. There, 50 µM selenate was used as fertilizer. The positive correlation can be explained by the fact that phenolic compounds represent the largest group of antioxidant active substances in broccoli [25]. Additionally, Ekanayake et al. observed an increase of the AOA of lentils (*Lens culinaris* cv. 'Medikus'), due to a biofortification with selenium [17].

Variety-specific differences occurred in the present study. AOA of 'Jonagold' was higher in both years of cultivation than for 'Golden Delicious'. Variety-specific differences in the AOA of apples have already been described in the literature by Xu et al., Kschonsek et al., and Wojdylo et al. [56,57,64]. In those studies, the higher AOA of the variety 'Jonagold' as compared to 'Golden Delicious' were found. Kschonsek et al. determined the AOA in the skin and the flesh of different apple varieties: TEAC values of 2.4 mmol TE/100 g d.w. for 'Golden Delicious' and 9.1 mmol TE/100 g d.w. for 'Jonagold' were measured for the peel, ORAC values were 8.6 mmol TE/100 g d.w. and 24.6 mmol TE/100 g d.w., respectively [56], and are therefore comparable with the results that were obtained in the present study. Wojdylo et al. determined the AOA using the TEAC assay and measured an AOA of 88.6 ± 6.7 mmol TE/100 g d.w. for 'Golden Delicious' and 181.9 ± 0.9 mmol TE/100 g d.w. for 'Jonagold' [64].

Xu et al., Kschonsek et al., and Wojdylo et al. were able to show that there are significant positive correlations between TPC (measured according to Folin-Ciocalteu or via HPLC) and AOA [56,57,64].

Kschonsek et al. and Wojdylo et al. also determined that the TPC was different, both between the individual substance groups of the polyphenols and between the individual compounds [56,64]. There, Kschonsek et al measured the highest positive correlation between flavanols and ORAC. Those compounds are the major contributors to AOA. Within the flavanols, epicatechin had the strongest influence on the intensity of the AOA [56]. Wojdylo et al. found the highest correlations between AOA and procyanidins and hydroxycinnamic acids, while using the TEAC, FRAP, and DPPH assay. The different AOA of the individual varieties are, therefore, due to the different composition of the phenolic compounds, as these show different antioxidant capacities and potentials [64].

An influence of the weather can also be deduced when comparing the controls from the years 2017 and 2018, similarly to the TPC values. AOA measured as TEAC of the untreated 'Golden Delicious' apples from the season 2017 was 6.76 mmol TE/100 g d.w. In the following year, an increase of 62.4% was observed with a value of 10.98 mmol TE/100 g d.w. For 'Jonagold', AOA of 7.58 mmol TE/100 g d.w., and 12.42 mmol TE/100 g d.w. were measured, corresponding to an increase of 63.9%. With regard to ORAC values, an increase of the AOA could also be observed for 'Golden Delicious', being 73.6% (5.53 mmol TE/100 g d.w. in 2017 and 9.60 mmol TE/100 g d.w. in 2018). For 'Jonagold', on the other hand, only a moderate increase of 17.6% was observed (9.56 mmol TE/100 g d.w. in 2017 and 11.24 mmol TE/100 g d.w. in 2018).

3.5. Qualitative and Quantitative Determination of Phenolic Compounds Using HPLC-MS^n

The following major phenolic compounds could be identified and quantitatively determined in the apple samples of the varieties 'Golden Delicious' and 'Jonagold' while using HPLC-MS^n analysis: the dihydrochalcones phloretin-2-xylosyl-glucoside and phloretin-2-glucoside, the flavan-3-ol epicatechin, and a procyanidin dimer, and a procyanidin trimer, the hydrocinnamic acid derivatives caffeoyl glucoside and chlorogenic acid, as well as the flavonols quercetin-3-*O*-galactoside, quercetin-3-*O*-xyloside and quercetin-3-*O*-glucoside. Chlorogenic acid, epicatechin, caffeoyl glucoside, and the procyanidin trimer were the main compounds in the samples.

Figure 6 shows an exemplary HPLC-chromatogram at 280 nm of an apple sample of the cultivar 'Jonagold', biofortified with 0.075 kg Se/ha in the form of selenate, produced in the year 2018. In Table 2, the total phenolic content as the sum of all individual phenolic compounds, the content of the four main phenolic compounds and their respective shares of the total phenolic content of the biofortified apples of the varieties 'Golden Delicious' and 'Jonagold' with the various selenium applications, as well as the corresponding untreated controls of the years 2017 and 2018 are listed. For 'Golden Delicious', no data were available from the season 2018.

In the apple varieties 'Golden Delicious' and 'Jonagold' hydroxycinnamic acid derivatives could be identified, in particular. The main phenolic compound was chlorogenic acid, with shares between 22.3% and 31.6% of the total sum of individual phenolic compounds. Furthermore, the apples were rich in epicatechin, caffeoyl glucoside, and a procyanidin trimer. Based on tentative structure elucidation in the present study and literature descriptions, this trimer is suggested being procyanidin C1 [65].

In a recent review, Rana and Bhushan compiled and evaluated a large number of data of the analysis of phenolic compounds in apples [66]. It was found that the phenolic compounds of the subclasses flavonols, dihydrochalcones, flavan-3-ols, and phenolic acids have already been identified in apples of various varieties. Epicatechin, procyanidin B2, chlorogenic acid, phloridzin, caffeic acid, and quercetin derivatives are the major components. Dhyani et al. and Zardo et al. identified chlorogenic acid and epicatechin as major components in 'Golden Delicious' [67,68]. In 2005 and 2006, Wojdylo et al. determined the phenolic compounds in 69 apple cultivars, including 'Golden Delicious' and 'Jonagold'. In both varieties, most of all oligomeric procyanidins, and chlorogenic acid were found, whereas 'Jonagold' contained more epicatechin and chlorogenic acid in comparison [64]. Deviating results were obtained in the study that was described by Kschonsek et al. In those apple samples from the varieties 'Golden Delicious' and 'Jonagold', mainly flavonols were determined, whereas quercetin derivatives, especially hyperosides, were the main components. Only 5.3% of chlorogenic

acid ('Golden Delicious') and 1.6% ('Jonagold') were present in the peel. The flesh only contained very small amounts of phenolic compounds [56].

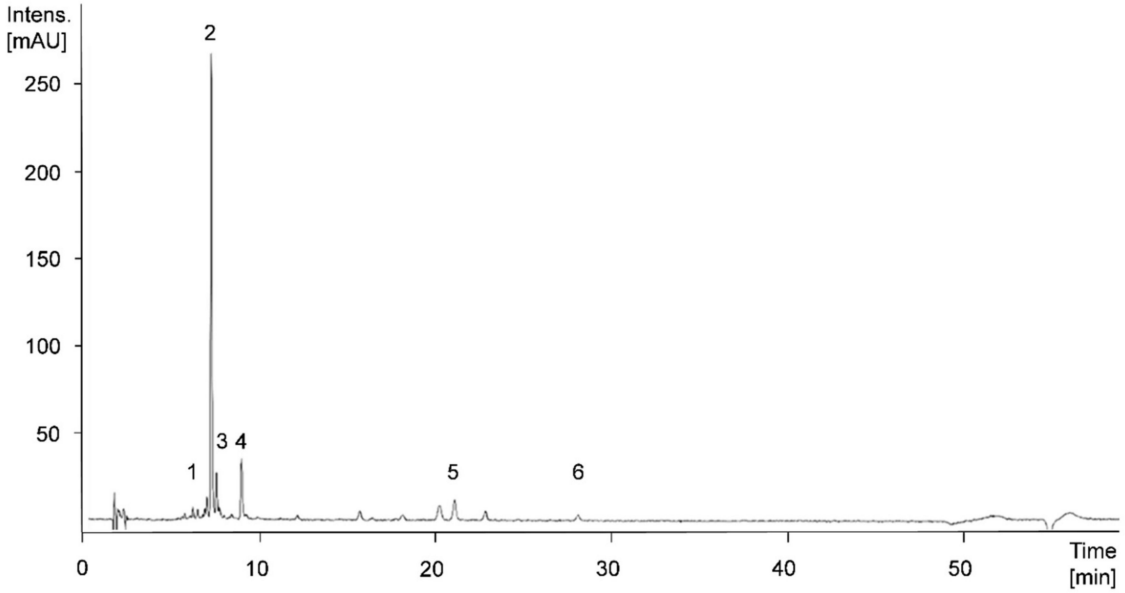


Figure 6. High Performance Liquid Chromatography Mass Spectrometry (HPLC-MS)^n-chromatogram at 280 nm of apple cultivar 'Jonagold', biofortified with 0.075 kg Se/ha in the form of selenate in 2018. Peak numbers: 1, procyanidin dimer; 2, chlorogenic acid; 3, epicatechin; 4, procyanidin trimer; 5, phloretin-2-xylosyl-glucoside; 6, phloretin-2-glucoside.

In the present study, biofortification with selenium showed different effects for 'Golden Delicious' and 'Jonagold' with regard to the content and proportion of the individual phenolic compounds. In particular, the four phenolic compounds that are listed in Table 2 have been influenced resulting from the biofortification. Further phenolic compounds were not significantly affected.

The phenolic profile of 'Golden Delicious' was comparatively insensitive, whereas, in the case of 'Jonagold', the application of selenate, in particular, led to changes in the proportions of the individual phenolic compounds. Here, significant differences in the content of the procyanidin trimer and caffeoyl glucoside occurred in the samples from 2017, when the higher amount of selenate was applied.

Lower total contents of individual phenolic compounds were measured when compared to the corresponding controls in all selenium applications on the 'Jonagold' variety. These results correspond to the TPC results. The application of 0.15 kg selenate/ha in 2017 resulted in a significantly lower concentration and proportion of the procyanidin trimer in 'Jonagold' when compared to the untreated control, whereas the concentration and proportion of caffeoyl glucoside significantly increased from 3.4% to 9.2%. AOA measured with the TEAC assay was highest in the apples of the selenate applications, which suggests that, due to the high proportion, caffeoyl glucoside is mainly responsible for AOA in 'Jonagold'. This trend - the increase of caffeoyl glucoside and the TEAC-value - was also observed at the biofortification of 0.15 kg selenite in 2017, but to a lower extent compared to the selenate treatments.

The application of the lower level of selenate (0.075 kg/ha) in 2018 did not confirm the observations from the previous year, as no significant changes between control and selenium-biofortified apple samples have been observed. This observation might be related to the lower amount of selenium applied. With regard to the AOA, very similar values were also measured in the control and biofortified samples. Based on the results of the 'Jonagold' samples from 2017, correlations between the individual phenolic compounds and their AOA can be concluded. With a high content of the procyanidin trimer, low AOA with TEAC and high AOA with ORAC were measured. On the other hand, high concentrations of caffeoyl glucoside were associated with high AOA by TEAC and low AOA by ORAC. These results further suggest that these two phenolic compounds have different AOA and -

due to the different reaction mechanisms of both antioxidant assays - the AOA of different phenolic compounds were determined and secondary antioxidant products were additionally measured when using the ORAC assay [69]. The individual contribution of the phenolic compounds should be analyzed by HPLC-online TEAC because of the different antioxidant capacities and potentials of the phenolic compounds.

Various research groups have already observed a change in the phenolic profile that results from a biofortification with selenium: D'Amato et al. found an increase of oleacein, ligustroside aglycone, and oleocanthal in olive oil, whose contents increased by 32% to 57% compared to the untreated control [24]. In a follow-up study on rice in 2018, hydroxybenzoic acids and hydroxycinnamic acids were identified, with an increase in ferulic acid and salicylic acid, whereas the concentrations of gallic acid decreased [16]. Schiavon et al. carried out experiments with the biofortification of radish leaves and roots. In roots, the antioxidant flavonoids naringenin chalcone and kaempferol showed enhanced concentrations and a decrease of cinnamic acid derivatives was observed. In leaves, the hydroxycinnamic acids, especially kaempferol derivatives, were increased, caffeic acid did not increase, and other identified phenolic compounds did not show any variation in concentration or decreased [21]. Pezzarossa et al. performed an application of 1 mg Se/L (as sodium selenate) in tomatoes (*Solanum lycopersion* cv. 'Red Bunch'), in which a significant increase of quercetin was observed in addition to a decrease of β-carotene and lycopene. Rutin was not influenced [70].

Table 2. Results of the determination of phenolic compounds using HPLC-MS^n. Data are in average ± standard deviation. The total phenolic content in mg/100 g d.w. was calculated by the sum of all quantitative determined phenolic compounds. For the four main phenolic compounds the content in mg/100 g d.w. and the percentage share is given. In each column, means followed by different letters are significantly different ($p < 0.05$).

Variety and Year of Cultivation	Application *	Σ [mg/100 g d.w.]	Chlorogenic Acid [mg/100 g d.w.]	%	Epicatechin [mg/100 g d.w.]	%	Procyanidin Trimer [mg/100 g d.w.]	%	Caffeoyl glucoside [mg/100 g d.w.]	%
Golden Delicious' 2017	control	126.86 ± 14.04 ^{a,b}	32.73 ± 2.92 ^{a}	25.9	13.05 ± 3.82 ^{a,b}	10.2	9.78 ± 2.30 ^{a}	7.6	14.07 ± 1.03 ^{d}	11.2
	0.15 kg selenite	119.73 ± 42.13 ^{a}	33.62 ± 3.24 ^{a}	31.6	12.15 ± 4.75 ^{a,b}	10.0	9.07 ± 3.46 ^{a}	7.5	14.27 ± 0.63 ^{d}	13.8
	0.15 kg selenate	123.66 ± 12.96 ^{a,b}	33.49 ± 2.22 ^{a}	28.4	13.18 ± 1.64 ^{a,b}	10.7	8.33 ± 1.37 ^{a}	6.7	13.90 ± 1.72 ^{c,d}	11.3
Golden Delicious' 2018	control	-	-	-	-	-	-	-	-	-
	0.075 kg selenate	-	-	-	-	-	-	-	-	-
'Jonagold' 2017	control	152.52 ± 27.88 ^{b}	36.42 ± 13.30 ^{a}	24.0	19.50 ± 9.81 ^{b}	12.2	18.61 ± 7.48 ^{b}	11.8	5.11 ± 0.17 ^{a}	3.4
	0.15 kg selenite	130.88 ± 4.38 ^{a,b}	29.30 ± 4.22 ^{a}	22.3	17.11 ± 2.87 ^{a,b}	13.1	14.74 ± 4.11 ^{a,b}	11.3	7.68 ± 1.46 ^{a}	5.9
	0.15 kg selenate	124.74 ± 11.94 ^{a,b}	29.41 ± 2.82 ^{a}	23.5	12.10 ± 1.40 ^{a,b}	9.7	9.87 ± 1.87 ^{a,}	7.9	11.23 ± 2.72 ^{b,c,d}	9.2
'Jonagold' 2018	control	107.39 ± 8.22 ^{a}	31.31 ± 0.91 ^{a}	29.2	10.74 ± 1.66 ^{a,b}	10.0	8.45 ± 0.32 ^{a}	7.9	11.47 ± 1.20 ^{b,c,d}	10.7
	0.075 kg selenate	104.15 ± 2.97 ^{a}	27.07 ± 0.23 ^{a}	26.0	9.60 ± 8.39 ^{a}	9.1	8.09 ± 5.40 ^{a}	7.7	11.28 ± 2.65 ^{b,c,d}	10.8

* Foliar spray rate per hectare and meter canopy height.

4. Conclusions

The aim of the present study was to investigate the biofortification of apples with selenium and its influence on the selenium content, phenolic compounds, and the properties associated with these substances.

The selenium content, PPO activity, TPC, AOA, and the composition of the phenolic compounds were influenced in different ways, depending on the conditions of biofortification. Here, the level of application and the form of selenium used played a major role. Furthermore, variety-specific differences in the level of the parameters could be identified. 'Golden Delicious' and 'Jonagold' behaved differently in some cases. The influence of ecophysiological conditions, especially the different sunshine duration, was also identified.

Biofortification led to a significant increase in the selenium content in the apples. Here, the level of selenium accumulation in the fruits mainly depended on the level of fertilizer. The form of selenium used only played a minor role.

When increasing the selenium content in the apples, the selenium supply with meeting the nutritional recommendations, can be improved. An apple of the variety 'Golden Delicious' with an average weight of 220 g (± 16,5 g) in 2017 and 213 g (± 15,0 g) in 2018 can, therefore, cover the daily requirement by approximately 17–20%. Taking into account the higher average weight of 'Jonagold' with values of 273 g (± 31,5 g) in 2017 and 255 g (± 25,3 g) in 2018, the consumption of one apple can cover 20–25% of the daily requirement of selenium.

Further, it can be stated that selenium biofortification has a stabilizing effect on the activity of PPO, as the values between the apples varied less. The PPO activity was also related to the amount of selenium fertilizer used—higher levels led to increased activities. This can also explain the TPC, as higher selenium levels resulted in constant or lower values, because more phenolic compounds are potentially degraded. A stabilized PPO activity will enable stable browning reactions when focusing on processed apple products in the future. A quick browning of freshly cut apples is not accepted by the consumer. Further, the formation of the brown colored melanins has not yet been investigated with regard health risks. Usually, PPO substrates—small phenolic compounds—are still regarded being health-beneficial principles.

In further studies, HPLC-onlineTEAC coupling should be used to investigate the AOA of individual phenolic compounds, as the results that were obtained by HPLC-DAD-ESI-MS^n indicate a certain variability of the phenolic compounds, providing different AOA. Based on the results, it can be concluded for the present study that the phenolic compounds contained, especially the procyanidin trimer (suggested to be procyanidin C1) and caffeoyl glucoside, have different AOA, which may also be different, depending on the variety. These should be further analyzed by HPLC-onlineTEAC. It has already been described in the literature that different phenolic compounds contribute differently to the total AOA. Riehle et al. determined the AOA of the individual phenolic compounds in *Cistus incanus* herbal tea infusions while using HPLC-onlineTEAC and found that the individual phenolic compounds had different AOA and different proportions of the total AOA of the samples [71]. Zietz et al. and Fiol et al. analyzed kale (*Brassica oleraceae* var. *Sabellica*) and found different AOA of the flavonoid glycosides and hydroxycinnamic acid derivatives contained in the samples [72,73].

When the phenolic profiles with their corresponding single antioxidant capacities are evaluated, it is possible to conclude also for their bioavailability and even bioactivity of the polyphenols.

Author Contributions: Conceptualization, D.D., S.R.; methodology, S.G.; software, S.G.; validation, S.G.; formal analysis, S.G., C.B., S.N.; investigation, S.G., S.A., F.-S.K.; field experiment conduction and preliminary fruit sample preparation, C.B.; resources, D.D., S.R.; data curation, C.B., S.N., S.R.; writing—original draft preparation, S.G.; writing—review and editing, C.B., S.N., D.D., S.R.; visualization S.G.; supervision, D.D., S.R.; project administration, D.D., S.R.; funding acquisition, D.D., S.R. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Federal Ministry of Education and Research, grant number FKZ: 031B0299B.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

References

1. Hirschi, K.D. Nutrient biofortification of food crops. *Annu. Rev. Nutr.* **2009**, *29*, 401–421. [CrossRef]
2. Lawson, P.G.; Daum, D.; Czauderna, R.; Vorsatz, C. Factors influencing the efficacy of iodine foliar sprays used for biofortifying butterhead lettuce (*Lactuca sativa*). *J. Plant Nutr. Sci.* **2016**, *179*, 661–669. [CrossRef]
3. Cakmak, I. *Agronomic Approaches in Biofortification of Food Crops with Micronutrients*; UC Davis: Department of Plant Sciences: Davis, CA, USA, 2009.
4. Gupta, M.; Gupta, S. An overview of selenium uptake, metabolism, and toxicity in plants. *Front. Plant Sci.* **2017**, *7*, 2074. [CrossRef]
5. Poňavič, M.; Scheib, A. Distribution of selenium in European agricultural and grazing land soil. In *Chemistry of Europe's Agricultural Soils*; Reimann, C., Birke, M., Demetriades, A., Filzmoser, P., O'Connor, P., Eds.; Geologisches Jahrbuch (Reihe B), Schweizerbart: Stuttgart, Germany, 2014; Volume 103, pp. 131–144.
6. Winkel, L.H.; Vriens, B.; Jones, G.D.; Schneider, L.S.; Pilon-Smits, E.; Bañuelos, G.S. Selenium cycling across soil-plant-atmosphere interfaces: A critical review. *Nutrients* **2015**, *7*, 4199–4239. [CrossRef]
7. Alfthan, G.; Eurola, M.; Ekholm, P.; Venäläinen, E.; Root, T.; Korkalainen, K.; Hartikainen, H.; Salminen, P.; Hietaniemi, V.; Aspila, P.; et al. Effects of nationwide addition of selenium to fertilizers on foods, and animal and human health in Finland: From deficiency to optimal selenium status of the population. *J. Trace Elem. Med. Biol.* **2015**, *31*, 142–147. [CrossRef]
8. Rayman, M. Selenium and human health. *Lancet* **2012**, *379*, 1256–1268. [CrossRef]
9. Kielliszek, M. Selenium-fascinating microelement, properties and sources in food. *Molecules* **2019**, *24*, 1298. [CrossRef]
10. Kielliszek, M.; Błażejak, S. Current knowledge on the importance of selenium in food for living organisms: A review. *Molecules* **2016**, *21*, 609. [CrossRef]
11. Kipp, A.P.; Strohm, D.; Brigelius-Flohé, R.; Schomburg, L.; Bechthold, A.; Leschik-Bonnet, E.; Heseker, H. German Nutrition Society (DGE) Revised reference values for selenium intake. *J. Trace Elem. Med. Biol.* **2015**, *32*, 195–199. [CrossRef]
12. Oster, O.; Prellwitz, W. The daily dietary selenium intake of West German adults. *Biol. Trace Elem. Res.* **1989**, *20*, 1–14. [CrossRef]
13. Willers, J.; Heinemann, M.; Bitterlich, N.; Hahn, A. Intake of minerals from food supplements in a German population—A nationwide survey. *Food Nutr. Sci.* **2015**, *6*, 205–215. [CrossRef]
14. Wortmann, L.; Enneking, U.; Daum, D. German consumers' attitude towards selenium-biofortified apples and acceptance of related nutrition and health claims. *Nutrients* **2018**, *10*, 190. [CrossRef]
15. Statista. Pro-Kopf-Konsum von Obst in Deutschland nach Art in den Jahren 2010/11 bis 2017/18 (in Kilogramm). Available online: https://de.statista.com/statistik/daten/studie/6300/umfrage/pro-kopf-verbrauch-von-obst-in-deutschland/ (accessed on 8 July 2019).
16. D'Amato, R.; Fontanella, M.C.; Falcinelli, B.; Beone, G.M.; Bravi, E.; Marconi, O.; Benincasa, P.; Businelli, D. Selelium biofortification in rice (*Oryza sativa* L.) sprouting: Effects on Se yield and nutritional traits with focus on phenolic acid profile. *J. Agric. Food Chem.* **2018**, *66*, 4082–4090. [CrossRef]
17. Ekanayake, L.J.; Thavarajah, D.; Vial, E.; Schatz, B.; McGee, R.; Thavarajah, P. Selenium fertilization on lentil (*Lens culinaris* Medikus) grain yield, seed delinium concentration, and antioxidant activity. *Field Crop. Res.* **2015**, *177*, 9–14. [CrossRef]
18. Hawrylak-Nowak, B. Enhanced selenium content in sweet basil (*Ocimum basilicum* L.) by foliar fertilization. *Veg. Crop. Res. Bull.* **2008**, *69*, 63–72. [CrossRef]
19. Ríos, J.J.; Rosales, M.A.; Blasco, B.; Cervilla, L.M.; Romero, L.; Ruiz, J.M. Biofortification of Se and induction of the antioxidant capacity in lettuce plants. *Sci. Hort.* **2008**, *116*, 248–255. [CrossRef]
20. Hawrylak-Nowak, B. Comparative effects of selenite and selenate on growth and selenium accumulation in lettuce plants under hydroponic conditions. *Plant Growth Regul.* **2013**, *70*, 149–157. [CrossRef]

21. Schiavon, M.; dall'Acqua, S.; Mietto, A.; Pilon-Smits, E.A.H.; Sambo, P.; Masi, A.; Malagoli, M. Selenium fertilization alters the chemical composition and antioxidant constituents of tomato (*Solanum lycopersicon* L.). *J. Agric. Food Chem.* **2013**, *61*, 10542–10554. [CrossRef]
22. Schiavon, M.; Berto, C.; Malagoli, M.; Trentin, A.; Sambo, P.; Dall'Acqua, S.; Pilon-Smits, E.A.H. Selenium biofortification in radish enhances nutritional quality via accumulation of methyl-selenocystein and promotion of transcripts and metabolites related to glucosinolates, phenolics, and amino acids. *Front. Plant Sci.* **2016**, *7*, 1371. [CrossRef]
23. Zhao, Y.; Wu, P.; Wang, Y.; Feng, H. Different approaches for selenium biofortification of pear-jujube (*Zizyphus jujuba* M. cv. Lizao) and associated effects on fruit quality. *J. Food Agric. Environ.* **2013**, *11*, 529–534.
24. D'Amato, R.; Proietti, P.; Onofri, A.; Regni, L.; Esposto, S.; Servili, M.; Businelli, D.; Selvaggini, R. Biofortification (Se): Does it increase the content of phenolic compounds in virgin olive oil (VOO)? *PLoS ONE* **2017**, 12. [CrossRef]
25. Bachiega, P.; Salgado, J.M.; de Carvalho, J.E.; Ruiz, A.L.; Schwarz, K.; Tezotto, T.; Morzelle, M.C. Antioxidant and antiproliferative activities in different maturation stages of broccoli (*Brassica oleracea* var. *italica*) biofortified with selenium. *Food Chem.* **2016**, *190*, 771–776. [CrossRef]
26. Pezzarossa, B.; Remorini, D.; Gentile, M.L.; Massai, R. Effects of foliar and fruit addition of sodium selenate on selenium accumulation and fruit quality. *J. Sci. Food Agric.* **2012**, *92*, 781–786. [CrossRef]
27. Babalar, M.; Mohebbi, S.; Zamani, Z.; Askari, M.A. Effect of foliar application with sodium selenate on selenium biofortification and fruit quality maintenance of 'Starking Delicious' apple during storage. *J. Sci. Food Agric.* **2019**, *99*, 5149–5156. [CrossRef]
28. Lawson, P.G.; Daum, D.; Czauderna, R.; Meuser, H.; Härtling, J.W. Soil versus foliar iodine fertilization as a biofortification strategy for field-grown vegetables. *Front. Plant Sci.* **2015**, *6*, 450. [CrossRef]
29. *DIN EN 13805: Foodstuffs—Determination of Trace Elements—Pressure Digestion, German Version DIN EN 13805:2014-12*; Beuth Verlag: Berlin, Germany, 2014. [CrossRef]
30. *DIN 38405-23: German standard methods for the examination of water, waste water and sludge—Anions (Group D)—Part 23: Determination of Selenium by Atomic Absorption Spectrometry (D 23)*; Beuth Verlag: Berlin, Germany, 1994. [CrossRef]
31. Kolodziejczyk, K.; Milala, J.; Sojka, M.; Kosmala, M.; Markowski, J. Polyphenol oxidase activity in selected apple cultivars. *J. Fruit Ornam. Plant Res.* **2010**, *18*, 51–61.
32. Gonzalez, E.M.; de Ancos, B.; Pilar Cano, M. Partial characterization of polyphenol oxidase activity in raspberry fruits. *J. Agric. Food Chem.* **1999**, *473*, 4068–4072. [CrossRef]
33. Müller, L.; Gnoyke, S.; Popken, A.M.; Böhm, V. Antioxidant capacity and related parameters of different fruit formulations. *LWT Food Sci. Technol.* **2010**, *43*, 992–999.
34. Singleton, V.L.; Rossi, J.A., Jr. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic aid reagents. *Am. J. Enol. Viticult.* **1965**, *16*, 144–158.
35. Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans, C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Rad. Biol. Med.* **1999**, *26*, 1231–1237. [CrossRef]
36. Ou, B.; Hampsch-Woodill, M.; Prior, R.L. Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. *J. Agric. Food Chem.* **2001**, *49*, 4619–4626. [CrossRef]
37. Neugart, S.; Baldermann, S.; Ngwene, B.; Wesonga, J.; Schreiner, M. Indigenous leafy vegetables of Eastern Africa—A source of extraordinary secondary plant metabolites. *Food Res. Int.* **2017**, *100*, 411–422. [CrossRef]
38. Schmidt, S.; Zietz, M.; Schreiner, M.; Rohn, S.; Kroh, L.W.; Krumbein, A. Identification of complex, naturally occurring flavonoid glycosides in kale (*Brassica oleracea* var. *sabellica*) by high-performance liquid chromatography diode-array detection/electrospray ionization multistage mass spectrometry. *Rapid Commun. Mass Spectrom* **2010**, *24*, 2009–2022. [CrossRef]
39. Smoleń, S.; Kowalska, I.; Czernicka, M.; Halka, M.; Keska, K.; Sady, W. Iodine and selenium biofortification with additional application of salicylic acid affects yield, selected molecular parameters and chemical composition of lettuce plants (*Lactuca sativa* L. var. *capitata*). *Front. Plant Sci.* **2016**, *7*, 1553. [CrossRef]
40. Eurola, M.; Alfthan, G.; Aro, A.; Ekholm, P.; Hietaniemi, V.; Rainio, H.; Rankanen, R.; Venäläinen, E. *Results of the Finnish Selenium Monitoring Program 2000–2001*; MTT Agrifood Research Finland: Jokioinen, Finland, 2003.
41. Nicolas, J.J.; Richard-Forget, F.C.; Goupy, P.M.; Amoit, M.; Aubert, S.Y. Enzymatic browning reactions in apple and apple products. *Crit. Rev. Food Sci. Nutr.* **1994**, *34*, 109–157. [CrossRef]

42. Bravo, L. Polyphenols: Chemistry, dietary sources, metabolism, and nutritional significance. *Nutr. Rev.* **1998**, *56*, 317–333. [CrossRef]
43. Kroon, P.; Williamson, G. Polyphenols: Dietary components with established benefits to health? *J. Sci. Food Agric.* **2005**, *85*, 1239–1240. [CrossRef]
44. Del Rio, D.; Costa, L.G.; Lean, M.E.J.; Crozier, A. Polyphenols and health: What compounds are involved? *Nutr. Metab. Cardiovasc. Dis.* **2010**, *20*, 1–6. [CrossRef]
45. Tomás-Barberán, F.A.; Andrés-Lacueva, C. Polyphenols and health: Current state and progress. *J. Agric. Food Chem.* **2012**, *60*, 8773–8775. [CrossRef]
46. Smoleń, S.; Kowalska, I.; Skoczylas, L.; Liszka-Skoczylas, M.; Grzanka, M.; Halka, M.; Sady, W. The effect of salicylic acid on biofortification with iodine and selenium and the quality of potato cultivated in the NFT system. *Sci. Hortic.* **2018**, *240*, 530–543. [CrossRef]
47. Holderbaum, D.F.; Kon, T.; Kudo, T.; Guerra, M.P. Enzymatic browning, polyphenol oxidase activity, and polyphenols on four apple cultivars: Dynamics during fruit development. *HortScience* **2010**, *45*, 1150–1154. [CrossRef]
48. Reinkensmeier, A.; Steinbrenner, K.; Homann, T.; Bußler, S.; Rohn, S.; Rawel, H.M. Monitoring the apple polyphenol oxidase-modulated adduct formation of phenolic and amino compounds. *Food Chem.* **2016**, *194*, 76–85. [CrossRef] [PubMed]
49. Kschonsek, J.; Dietz, A.; Wiegand, C.; Hipler, U.; Böhm, V. Allergenicity of apple allergen Mal d 1 as effected by polyphenols and polyphenol oxidase due to enzymatic browning. *Lebens. Wiss. Technol.* **2019**, 113. [CrossRef]
50. WetterKontor GmbH. Monats-Und Jahreswerte Für Deutschland. Available online: https://www.wetterkontor.de/de/wetter/deutschland/monatswerte.asp?y=2018&m=15 (accessed on 18 September 2019).
51. Manzocco, L.; Quarta, B.; Dri, A. Polyphenoloxidase inactivation by light exposure in model systems and apple derivatives. *Innov. Food Sci. Emerg. Technol.* **2009**, *10*, 506–511. [CrossRef]
52. Müller, A.; Noack, L.; Greiner, R.; Stahl, M.R.; Posten, C. Effect of UV-C and UV-B treatment on polyphenol oxidase acitvity and shelf life of apple and grape juice. *Innov. Food Sci. Emerg. Technol.* **2014**. [CrossRef]
53. Lei, J.; Li, B.; Zhang, N.; Yan, R.; Guan, W.; Brennan, C.S.; Gao, H.; Peng, B. Effects of UV-C treatment on browning and the expression of polyphenol oxidase (PPO) genes in different tissues of *Agaricus bisporus* during cold storage. *Postharvest Biol. Technol.* **2018**, *139*, 99–105. [CrossRef]
54. Cirilli, M.; Caruso, G.; Gennai, C.; Urbani, S.; Frioni, E.; Ruzzi, M.; Servili, M.; Gucci, R.; Poerio, E.; Muleo, R. The role of polyphenoloxidase, peroxidase, and ß-glucosidase in phenolics accumulation in *Olea europeaea* L. fruits under different water regimes. *Front. Plant Sci.* **2017**, *8*, 717. [CrossRef]
55. Põldma, P.; Moor, U.; Tõnutare, T.; Herodes, K.; Rebane, R. Selenium treatment under field conditions affects mineral nutrition, yield and antioxidant properties of bulb onion (*Allium cepa* L.). *Acta Sci. Pol. Hortorum Cultus* **2013**, *12*, 167–181.
56. Kschonsek, J.; Wolfram, T.; Stöckl, A.; Böhm, V. Polyphenolic compounds analysis of old and new apple cultivars and contribution pf polyphenolic profile to the in vitro antioxidant capacity. *Antioxidants* **2018**, *7*, 20. [CrossRef]
57. Xu, Y.; Fan, M.; Ran, J.; Zhang, T.; Sun, H.; Dong, M.; Zhang, Z.; Zheng, H. Variation in phenolic compounds and antioxidant activity in apple seeds of seven cultivars. *Saudi J. Biol. Sci.* **2016**, *23*, 379–388. [CrossRef]
58. Eichholz, I.; Rohn, S.; Gamm, A.; Beesk, N.; Herppich, W.B.; Kroh, L.W.; Ulrichs, C.; Huyskens-Keil, S. UV-B-mediated flavonoid synthesis in white asparagus (*Asparagus officinalis* L.). *Food Res. Int.* **2012**, *48*, 196–201. [CrossRef]
59. Neugart, S.; Schreiner, M. UVB and UVA as eustressors in horticultural and agricultural crops. *Sci. Hortic.* **2018**, *234*, 370–381. [CrossRef]
60. Scattino, C.; Castagna, A.; Neugart, S.; Chan, H.M.; Schreiner, M.; Crisosto, C.H.; Tonutti, P.; Ranieri, A. Post-harvest UV-B irradiation induces changes of phenol contents and corresponding biosynthetic gene expression ion peaches and nectarines. *Food Chem.* **2014**, *163*, 51–60. [CrossRef] [PubMed]
61. Song, Y.; Yao, Y.; Zhai, H.; Du, Y.; Chen, F.; Wei, S. Polyphenolic compound and the degree of browning in processing apple varieties. *Agric. Sci. China* **2007**, *6*, 607–612. [CrossRef]
62. Allahveran, A.; Farokhzad, A.; Asghari, M.; Sarkhosh, A. Foliar application of ascorbic and citric acids enhanced 'Red Spur' apple fruit quality, bioactive compounds and antioxidant activity. *Physiol. Mol. Biol. Plants* **2018**, *24*, 433–440. [CrossRef]

63. Prior, R.L.; Wu, X.; Schaich, K. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *J. Agric. Food Chem.* **2005**, *53*, 4290–4302. [CrossRef]
64. Wojdylo, A.; Oszmianski, J.; Laskowski, P. Polyphenolic compounds and antioxidant activity of new and old apple varieties. *J. Agric. Food Chem.* **2008**, *56*, 6520–6530. [CrossRef]
65. Masuda, I.; Koike, M.; Nakashima, S.; Mizutani, Y.; Ozawa, Y.; Watanabe, K.; Sawada, Y.; Sugiyama, H.; Sugimoto, A.; Nojiri, H.; et al. Apple procyanidins promote mitochnondrial biogenesis and proteoglycan biosynthesis in chondrocytes. *Nature* **2018**, *8*, 7729. [CrossRef]
66. Rana, S.; Bhushan, S. Apple phenolics as nutraceuticals: Assessment, analysis and application. *J. Food Sci. Technol.* **2016**, *53*, 1727–1738. [CrossRef]
67. Dhyani, P.; Bahukhandi, A.; Rawat, S.; Bhatt, I.D.; Rawal, R.S. Diversity of bioactive compounds and antioxidant activity in Delicious group in Western Himalaya. *J. Food Sci. Technol.* **2018**, *55*, 2587–2599. [CrossRef]
68. Zardo, D.M.; Silva, K.M.; Guyot, S.; Nogueira, A. Phenolic profile and antioxidant capacity of the principal apples produced in Brazil. *Int. J. Food Sci. Nutr.* **2013**, *64*, 611–620. [CrossRef] [PubMed]
69. Csepregi, K.; Neugart, S.; Schreiner, M.; Hideg, E. Comparative evaluation of total antioxidant capacities of plant polyphenols. *Molecules* **2016**, *21*, 208. [CrossRef] [PubMed]
70. Pezzarossa, B.; Rosellini, I.; Malorgio, F.; Borghesi, E.; Tonutti, P. Effects of selenium enrichment of tomato plants on ripe fruit metabolism and composition. In Proceedings of the 7th International Postharvest Symposium, Kuala Lumpur, Malaysia, 25–29 June 2013; pp. 247–251.
71. Riehle, P.; Vollmer, M.; Rohn, S. Phenolic compounds in *Cistus incanus* herbal infusions—Antioxidant capacity and thermal stability during the brewing process. *Food Res. Int.* **2013**, *53*, 891–899. [CrossRef]
72. Zietz, M.; Weckmüller, A.; Schmidt, S.; Rohn, S.; Schreiner, M.; Krumbein, A.; Kroh, L.W. Genotypic and climatic influence on the antioxidant activity of flavonoids in kale (*Brassica oleracea* var. *sabellica*). *J. Agric. Food Chem.* **2010**, *58*, 2123–2130. [CrossRef] [PubMed]
73. Fiol, M.; Weckmüller, A.; Neugart, S.; Schreiner, M.; Rohn, S.; Krumbein, A.; Kroh, L.W. Thermal-induced changes of kale's antioxidant activity analyzed by HPLC-UV/Vis-online-TEAC detection. *Food Chem.* **2013**, *138*, 857–865. [CrossRef] [PubMed]

6.2. Influence of selenium biofortification of apples on the protein content and the allergenic proteins Mal d 1 and Mal d 3

Selenium Biofortification of Different Varieties of Apples *(Malus domestica)* – Influence on Protein Content and the Allergenic Proteins Mal d 1 and Mal d 3

Sabrina Groth [1], Christoph Budke [2], Timo Weber [2], Marie Oest [1], Sven Brockmann [1], Martina Holz [1], Diemo Daum [2] and Sascha Rohn [1,3]*

[1] Hamburg School of Food Science, Institute of Food Chemistry, University of Hamburg, Grindelallee 117, 20146 Hamburg, Germany

[2] Department of Plant Nutrition, Osnabrück University of Applied Sciences, 49090 Osnabrück, Germany

[3] Technische Universität Berlin, Institute of Food Technology and Food Chemistry, Department of Food Chemistry and Analysis, TIB 4/3-1, Gustav-Meyer-Allee 25, 13355 Berlin, Germany

Food Chemistry **2021**, *362,* 130134

doi: 10.1016/.j.foodchem.2021.130134

Accepted: 15 May 2021

In this work, the influence of selenium biofortification of seven different apple cultivars, grown in three consecutive years at two different cultivation sites in northern Germany, on the main allergenic proteins Mal d 1 and Mal d 3 was investigated. Biofortification was performed by foliar fertilization. Different forms of selenium and different application levels were used. The aim of this work was to find out whether, to which extent, and under which conditions the biofortification of apples with selenium is suitable to reduce the allergenic potential and – in the long term – to provide affected consumers with low allergenic fruits.

First, the proteins were extracted from the apples and the total protein content was determined according to BRADFORD. Subsequently, the protein pattern with focus on the allergenic proteins was analyzed gel electrophoretically by disc-SDS-PAGE and the content of

Mal d 1 was quantitatively determined by a direct ELISA. Detection of the allergenic proteins was performed after in-gel digestion of the obtained bands from the SDS gels by mass spectrometry.

By means of SDS-PAGE and nano-MS/MS the four allergenic proteins Mal d 1, Mal d 2, Mal d 3, and Mal d 4 could be detected in the apples. Here, the different samples basically showed a similar protein pattern, but showed differences in the intensity of the bands and thus different contents of the individual allergens. The separate analysis of peel and fruit flesh also allowed the determination of the localization of the individual apple allergens. Thus, the peel appears to be particularly rich in Mal d 3, whereas Mal d 2 is mainly found in the fruit flesh. Mal d 1 is found in both the peel and the fruit flesh. Biofortification seems to increase the synthesis of Mal d 3, as more intense bands were observed here. In most cases, the content of Mal d 1 in the apples was reduced by biofortification. In particular, the application of selenate resulted in statistically significant reductions in Mal d 1 content in many cultivars, although different fruit compartments were affected to varying degrees by biofortification. Thus, especially in the fruit flesh, the Mal d 1 content was significantly reduced. In addition to biofortification, apple variety and climatic conditions at the growing location were identified as further factors influencing allergenic proteins.

Work shares

Sabrina Groth:	Performance of the in-gel digestion of bands from SDS gels for mass spectrometric analysis of apple proteins; experimental and statistical evaluations; preparation of the manuscript
	Total contribution: 40%

Food Chemistry 362 (2021) 130134

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Selenium biofortification of different varieties of apples (*Malus domestica*) – Influence on protein content and the allergenic proteins Mal d 1 and Mal d 3

Sabrina Groth [a], Christoph Budke [b], Timo Weber [b], Marie Oest [a], Sven Brockmann [a], Martina Holz [a], Diemo Daum [b], Sascha Rohn [a,c,*]

[a] University of Hamburg, Hamburg School of Food Science, Institute of Food Chemistry, Grindelallee 117, 20146 Hamburg, Germany
[b] Osnabrück University of Applied Sciences, Department of Plant Nutrition, 49090 Osnabrück, Germany
[c] Technische Universität Berlin, Institute of Food Technology and Food Chemistry, Department of Food Chemistry and Analysis, TIB 4/3-1, Gustav-Meyer-Allee 25, 13355 Berlin, Germany

ARTICLE INFO

Keywords:
Apple
Selenium
Agronomic biofortification
Mal d 1
Mal d 3
SDS-PAGE
ELISA
Allergy

ABSTRACT

As allergy towards apples is widespread, the evaluation of various cultivation and postharvest influences on the allergenic potential is of great importance. Therefore, the analysis of the Mal d 1 content was the focus of this study, originally dealing with investigating the influence of a selenium biofortification on apple quality. The Mal d 1 content of apples was in most cases reduced when the fruits were biofortified with selenium. Apple variety and climatic conditions were identified as further influencing factors for the Mal d 1 content of the fruits. The separate analysis of the peel and the fruit flesh showed that the content of Mal d 1 in the fruit flesh was significantly lower in the biofortified samples than in the controls. In conclusion, the results indicate that the selenium biofortification of apples and biochemical mechanism behind can reduce the allergenic potential regarding the content of Mal d 1.

1. Introduction

In terms of cultivation and *per capita* consumption, apple is the most popular type of fruit in Germany. In 2019, 69% of the total fruit harvest were apples, per capita consumption was about 25 kg per year (Bundesministerium für Ernährung und Landwirtschaft (BMEL), 2020; Statistisches Bundesamt, 2020). The fruits contain nutritionally important vitamins as well as minerals and are an important source of secondary plant metabolites such as carotenoids and polyphenols (Groth et al., 2020; Kschonsek, Wolfram, Stöckl, & Böhm, 2018; Souci, Fachmann, & Kraut, 2011; Tsao, Yang, Xie, Sockovie, & Khanizadeh, 2005). However, apples can also cause allergic reactions in sensible people, because of the presence of allergenic proteins. Across Europe, 6.5% of the population are suffering from an apple allergy (Burney et al., 2014). In Northern and Central Europe sensitization to the allergenic protein Mal d 1 is particularly common and affects 50–75% of people that are also sensible to birch pollen (Ahammer, Grutsch, Kamenik, Liedl, & Tollinger, 2017; Grafe, 2009). The allergenic protein Bet v 1 present in birch (*Betulaceae*) and Mal d 1 present in apple are homologous molecules, recognized by the same IgE antibodies, and can therefore cause a cross allergy. Mal d 1 belongs to the group of *pathogenesis-related proteins* (PR 10 family) that are mainly synthesized to defend plants against pathogens and under certain environmental stress conditions (Breiteneder & Ebner, 2000; Grafe, 2009; Matthes & Schmitz-Eiberger, 2009). Mal d 1 is expressed in the fruit as a response to biotic and abiotic stress factors and the protein is involved in the binding and the transport of plant steroids (Beuning et al., 2004; Botton et al., 2009; Pühringer et al., 2000). Birch trees are not very common in Southern Europe. Accordingly, allergic reactions to apples caused by Mal d 1 are rather rare in this region, but sensitization towards Mal d 3 is more frequent. It belongs to the *non-specific lipid transfer proteins* (nsLPTs). Reactions towards Mal d 2 and Mal d 4 are very rare and therefore of only minor importance (Grafe, 2009; Hed, 2008; Ma et al., 2006).

Various factors influencing Mal d 1 content in apples have been

* Corresponding author at: Technische Universität Berlin, Institute of Food Technology and Food Chemistry, Department of Food Chemistry and Analysis, TIB 4/3-1, Gustav-Meyer-Allee 25, 13355 Berlin, Germany.
E-mail addresses: sabrina.groth@chemie.uni-hamburg.de (S. Groth), budke@hs-osnabrueck.de (C. Budke), marie.oest@chemie.uni-hamburg.de (M. Oest), daum@hs-osnabrueck.de (D. Daum), rohn@tu-berlin.de (S. Rohn).

https://doi.org/10.1016/j.foodchem.2021.130134
Received 2 March 2021; Received in revised form 10 May 2021; Accepted 13 May 2021
Available online 16 May 2021

already identified. Its content is primarily depending on the apple variety (Bolhaar et al., 2005; Kschonsek, Wiegand, Hipler, & Böhm, 2019a; Matthes & Schmitz-Eiberger, 2009; Sancho et al., 2006a; Schmitz-Eiberger & Matthes, 2011; Son & Lee, 2001; Zuidmeer et al., 2006). Storage time and storage conditions are further influencing factors (Bolhaar et al., 2005; Kiewning & Schmitz-Eiberger, 2014; Kiewning, Baab, & Schmitz-Eiberger, 2013; Matthes & Schmitz-Eiberger, 2009; Schmitz-Eiberger & Matthes, 2011; Sancho et al., 2006a). Furthermore, differences were observed when cultivating the same variety at different locations (Zuidmeer et al., 2006). Schmitz-Eiberger & Matthes (2011) found that especially the cultivation system leads to differences in the Mal d 1 content. Organically cultivated apples showed significantly higher levels of Mal d 1 than those from conventional production. Organically cultivated trees that are more susceptible to environmental stress factors such as fungi, viruses, and bacterial attack synthesized higher Mal d 1 contents (Fernández-Rivas et al., 2006). Consequently, varying ecophysiological and cultivation conditions (e.g., climatic factors, water and nitrogen supply of the soil) can influence the allergen content of several fruits (Wang, Vanga, & Raghavan, 2017).

With regard to the Mal d 3 content, another important allergenic protein in apple, also various influencing factors have been already described, such as the variety, the position of the fruit on the tree, and the associated intensity of solar radiation, as well as storage conditions (Sancho et al, 2006b). However, the synthesis of the two allergenic proteins Mal d 1 and Mal d 3 is not influenced by external factors in the same way. For example, during fruit storage the formation of Mal d 1 is promoted by cold temperatures and under controlled atmosphere conditions (Bolhaar et al., 2005; Hsieh, Moos jr., & Lin, 1995; Sancho et al., 2006a), whereas for Mal d 3 the opposite was observed (Sancho et al, 2006b).

The influence of agronomic biofortification with minerals on the allergens in apples has not yet been the subject of research. This approach is used for the targeted enrichment of food crops with specific nutrients. The focus is mainly on trace elements such as zinc, selenium, or iodine, which are only scarcely phytoavailable from the soils, wherein the plants are growing (Budke, thor Straten, Mühling, Broll, & Daum, 2020a; Budke, Mühling, & Daum, 2020b; Cakmak, 2009; Gupta & Gupta, 2017; Hirschi, 2009). In Germany and other European countries, soils are often relatively low in selenium, so that the selenium content of plant produce is correspondingly low (Poňavič & Scheib, 2014). The manifold influence of selenium biofortification on secondary plant metabolites in apples (Groth et al., 2020) and other crops (Bachiega et al., 2016; D'Amato et al., 2017, 2018; Pezzarossa, Remorini, Gentile, & Massai, 2012; Schiavon et al., 2013, 2016; Zhao, Wu, Wang, & Feng, 2013) has already been reported. The primary metabolism of plants, which includes protein biosynthesis, is significantly affected by selenium (D'Amato et al., 2018; Jing et al., 2017; Poblaciones, Rodrigo, & Santamaría, 2013; Poblaciones, Santamaría, García-White, & Rodrigo, 2014a; Poblaciones, Rodrigo, Santamaría, Chen, & McGrath, 2014b; Reis et al., 2018).

The aim of the present study was to investigate the relationship between selenium biofortification and the occurrence of the main allergenic proteins Mal d 1 and Mal d 3 in apples. Within the scope of this work, different aerial applications with variations in the selenium form and the level of application were studied. Seven different apple varieties from three consecutive growth seasons, harvested on two different locations in Northern Germany were characterized. The long-term goal is to reduce the allergenic potential of apples by selecting low-allergen varieties in combination with selected cultivation techniques.

2. Materials and methods

2.1. Sample material

Seven different apple varieties, grown in three subsequent years on two different locations were characterized. In 2017, apples of the varieties 'Fiesta', 'Golden Delicious', 'Idared', 'Jonagold', and 'Jonica' were cultivated at the Horticultural Research Station of the Osnabrück University of Applied Sciences, Germany (52°31′06.5′'N 8°02′84.4′'E; 69 m a.s.l.). The varieties 'Boskoop', 'Golden Delicious', Jonagold', and 'Jonica'' were cultivated in 2018 also in Osnabrück. In the following year, apples of the cultivar 'Elstar' were cultivated in an orchard of a commercial fruit farm in the "Alte Land" region, Jork, Germany (53°30'37.4"N 9°44'44.6"E; 4 m a.s.l.).

The location conditions in Osnabrück have been already described recently (Groth et al., 2020). In Jork, the apple trees were grown in a sandy loam podzolic soil. The selenium (Se) content of the soil determined by extraction with aqua regia was 0.51 mg/kg d.w. in the upper soil layer and 0.52 mg/kg d.w. in lower soil layer. The experiments were performed in a randomized block design with four repetitions. The apple trees were treated with a total of 0.075 – 0.450 kg selenium per hectare and meter canopy height (Se/ha × m CH) by applying foliar sprays. The apple variety 'Fiesta' was sprayed once two weeks before the harvest. All other varieties were treated repeatedly (2–7 times) between mid-June and the end of September. The last application always took place at least two weeks before the harvest. Biofortification by foliar fertilization proved to be more efficient in fruit crops than soil fertilization. Using this approach, the aboveground plant parts are directly wetted and thus, the target compounds can easily reach and enter the fruits (Budke et al., 2020a; Pezzarossa et al., 2012). Furthermore, aerial applications can be realized in one operation together with calcium sprays (Lawson, Daum, Czauderna, & Vorsatz, 2016), being common in apple cultivation to improve fruit quality and storage ability (Dris & Niskanen, 1999; Raese & Drake, 1993). In 2017, both sodium selenate and sodium selenite were applied. For improving wetting properties, the spray solutions, and the water treatment in the control plots additionally contained 0.02% (v/v) of the non-ionic organosilicone adjuvant Break-Thru® S 240 (AlzChem AG, Trostberg, Germany). In 2018 and 2019, sodium selenate fertilizer was applied together with the calcium-containing foliar fertilizer WUXAL® Ascofol Ca (5 l/ha; AGLUKON Spezialdünger GmbH &Co. KG, Düsseldorf, Germany).

Foliar applications carried out in 2017 with a hand-held spray system (model Easy-Sprayer Plus, Lehnartz GmbH, Remscheid, Germany). In 2018, the treatments of the varieties 'Golden Delicious' and 'Jonagold' were performed with a backpack sprayer (model REB 15 AZ2, Birchmeier Sprühtechnik AG, Stetten, Switzerland), and the treatment of the varieties 'Boskoop' and 'Jonica' by using a trailed orchard sprayer with an axial-cross-flow blower (model SZA32/1000–100, Hans Wanner GmbH, Wangen, Germany). For the field trials conducted in 2019, a trailed, air-assisted parcel tunnel sprayer was in use (model TSG-N1, LIPCO GmbH, Sasbach, Germany).

All analytical parameters, except the selenium content of the fruits, were determined in lyophilized sample material. Freeze-drying was performed after prior homogenization as described in Groth et al. (2020). All chemicals were of analytical grade.

2.2. Selenium content

The determination of the selenium content was done at the Osnabrück University of Applied Sciences, as also described in Groth et al. (2020) using a graphite tube atomic absorption spectrometer (Thermo Scientific UNICAM SOLAAR M Series AA, Thermo Fisher Scientific Inc., Waltham, MA, USA).

2.3. Extraction of proteins

For the determination of the content of Mal d 1, the proteins were extracted from the lyophilized apples samples. 1.0 g of the whole apple or 1.0 g of the fruit flesh or 0.5 g in the case of the peel were weighed into grinding bowls. In total, 15 mL of a Björksten extraction buffer with some modifications were added (Björksten, Halmepuro, Hannuksela, & Lahti, 1980). A buffer consisting of eidetic acid, diethyldithiocarbamate,

and polyvinylpyrrolidon was used; no sodium azide was added, as, in comparison with different buffer compositions, it provided the highest extraction yield. After adding the buffer, 15 stainless steel beads (Ø 2.778 mm) were added to each sample and the sample was ground and mixed in a ball mill (10 min., 25 Hz; RETSCH® MM 400, Retsch GmbH, Haan, Germany). The samples were centrifuged for 10 min at 20,817 × g and the supernatant was transferred into a 15-mL tube. The extraction with the ball mill was repeated twice and supernatants were combined. Subsequently, samples were concentrated to a volume of 3–4 mL using a gaseous nitrogen stream and filled up to 5 mL in a volumetric flask. A double determination was carried out with each sample.

2.4. SDS-PAGE analysis

For the extraction of proteins, a triple extraction with a PBS buffer (sodium chloride 0.034 mmol/L, potassium hydrogen phosphate 0.016 mmol/L; pH 7.0) was carried out using a ball mill as described in section 2.3 and a smaller scale was chosen. For this purpose, 0.2 g sample was mixed with a total of 6 mL PBS buffer and was extracted. 1 mL of the supernatant obtained was concentrated under nitrogen flow to dryness, made up with 100 µL PBS buffer and then used for the gel electrophoretic separation.

First, 75 µL of each sample extract were mixed with 25 µL of a reduction buffer (12.11 g/L tris(hydroxymethyl)-aminomethane, 200 mL glycerol, 39.99 g SDS, 40 mL 2-mercaptoethanol, 5.02 g bromophenol blue, make up with bidist. water) and heated up to 95 °C for 5 min. A 4% collection gel and an 18% separation gel were used according to Matthes and Schmitz-Eiberger (2009) and Laemmli (1970). A mixture of ten recombinant proteins with molecular weights between 10 and 250 k_D was used as molecular weight marker (Precision Plus Protein Kaleidoscope, Bio-Rad Laboratories GmbH, Feldkirchen, Germany). In the remaining positions, 10 µL each of the reduced sample extract were applied. A constant voltage of 200 V, a maximum current of 100 mA, and a maximum power of 25 W was applied. The running time was 1 h. The gels were stained with a Coomassie Brilliant Blue G 250 solution consisting of 5 mL phosphoric acid (85%), 25.231 g ammonium sulfate and 251 mg Coomassie Blue G 250 in bidist. water at a total volume of 250 mL. For staining, 5 mL methanol was added to 20 mL of the staining solution per gel. The staining was carried out overnight on the shaker. For subsequent discoloration, the gel was left on the shaker three times with 25 mL bidist. water for 30 min.

2.5. In-gel digestion procedure for mass spectrometric characterization of apple proteins

For the identification of apple allergens by liquid chromatography-tandem mass spectrometry (LC-MS/MS), an in-gel digestion of the protein bands obtained during SDS-PAGE analysis was performed, using a modified method described by Shevchenko et al. (Shevchenko, Wilm, Vorm, & Mann, 1986; Shevchenko, Tomas, Havlis, Olsen, & Mann, 2006). First, the bands were cut out of the gel, transferred to 1 mL of a wash solution (acetonitrile/50 mM ammonium hydrogen carbonate; 4:6; v/v) in a tube and shaken for 30 min at 37 °C. The washing solution was then discarded until the gel pieces were discolored. The gel pieces dried in a rotary vacuum concentrator (RVC 2–25CD plus, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) for 30 min. Reduction was achieved by adding 60 µL reduction buffer (dithiothreitol 10 mM in buffer; buffer solution consisted of ammonium hydrogen carbonate 100 mM in bidist. water) each for 30 min at 56 °C in a shaker. Alkylation was performed by adding 60 µL of an alkylation buffer (55 mM iodoacetamide in buffer) to each gel piece. The gel pieces were dried in the vacuum centrifuge for 15 min.

For proteolytic digestion, 10 µL trypsin digestion solution (0.03 µg trypsin/µL 50 mM ammonium hydrogen, sequencing grade modified trypsin, Promega GmbH, Walldorf, Germany) were added to the gel pieces and the samples were incubated overnight at 37 °C. Trypsin stock solution was done as follows: dissolve 25 µg in 250 µL 1 mM HCl. For extracting the peptides from the gel pieces, 20 µL of water, 60 µL of acetonitrile and 60 µL of 5% formic acid were added in three steps and shaken for 15 min (1,000 rpm, 37 °C), respectively.

Prior to the mass spectrometric analysis, samples were desalted using Zip Tips® C18 (Pierce™ C18 Tips, 100 µL, Thermo Fisher Scientific Inc., Waltham, MA, USA) as described in the user guides (User Guide for Reversed Phase ZipTip® Pipette, 2005). For protein identification, peptides were analyzed using nanoLC-MS/MS. 5 µL of the samples were injected into a nano-liquid chromatography system (Dionex Ultimate 3000 RSLCnano, Thermo Scientific, Bremen, Germany) equipped with a trapping column (Acclaim™ PepMap™ µ-precolumn, C18, 300 µm × 5 mm, 5 µm, 100 Å, Thermo Fisher Scientific GmbH, Bremen, Germany) and a separation column (Acclaim™ PepMap™ 100, C18, 75 µm × 250 mm, 2 µm, 100 Å, Thermo Fisher Scientific GmbH, Bremen, Germany). The nano-LC system was coupled with an electrospray-ionization (ESI) mass spectrometer equipped with a quadrupole, a linear trap, and an orbitrap (Orbitrap Fusion, Thermo Fisher Scientific GmbH, Bremen, Germany). The flow rate was 200 nL/min (buffer A: 0.1% FA in HPLC-water, B: 0.1% FA in ACN. Peptides were separated by gradient elution (2–30% B in 30 min) and detected by MS in positive ion mode and a capillary voltage of 1,650 V.

NanoLC-MS/MS data were processed with Proteome Discoverer™ (Thermo Fisher Scientific GmbH, Bremen, Germany). For protein identification, the search engine Sequest HT was used. The database search was performed against the uniport.fasta database (with special regard to the organism *Malus*) and a contaminant database. The following parameters were applied: Precursor mass tolerance: 10 ppm, fragment mass tolerance: 0.02 Da, missed cleavages: two, fixed modifications: carbamidomethylation on cysteine residues, variable modifications: oxidation on methionine residues. Peptides with a false discovery rate of 1% were identified.

2.6. Determination of the Mal d 1 content using a direct ELISA

Following the methodology of an indirect competitive ELISA according to Sancho et al. (2005) (Sancho et al., 2005), a direct ELISA was developed. For calibration, recombinant Mal d 1 (2 µg/mL, Biomay AG, Vienna, Austria) in a range of 0.1 to 2.0 µg/mL was used and 200 µL each was pipetted into the wells of a 96-well microtiter plate. From the sample extracts, a 1:10 dilution was prepared. 10 µL of the diluted sample extracts were given into the microtiter plate and diluted further with 190 µL buffer. It was incubated for 22 h at 4 °C. After washing five times with 300 µL PBS-T buffer (PBS buffer + 0.5% Tween 20), a 1% BSA solution was used as blocking agent and incubated another 2 h at room temperature. Washing was repeated five times with 300 µL PBS-T solution each. Subsequently, 200 µL HRP-labelled goat anti-mouse antibody (goat anti-mouse IgG antibody, peroxidase conjugated, H + L, Merck KGaA, Darmstadt, Germany) were added and incubated for 18 h at 4 °C.

Afterwards, the reaction solution was prepared. For this purpose, 10 mL of a citric acid buffer (6.327 g/L citric acid monohydrate in bidist. water, pH 4.1) were mixed with 0.5 mL of a TMB reagent (2.410 g/L 3,3′,5,5′-tertamethylbenzidine, 0.5 mL hydrogen peroxide (30%), 100 mL acetone, and 900 mL ethanol) (63). 200 µL of the reaction solution were pipetted into the wells and the microtiter plate was incubated for 90 min in the dark at room temperature. After adding 50 µL sulphuric acid (2 M) as stop solution, the photometric measurement was performed at λ = 450 nm at 30 °C in a microplate reader (BioTek Syngergy HT, BioTek Instruments Inc., Winooski, VT, USA). The Mal d 1 content was given in µg/100 g f. w.

2.7. Statistical analysis

The number of analyses per application with selenium fertilizer or control was $n = 4$ for the selenium content, and $n = 2$ for the Mal d 1

content. Exceptionally in the experiment with the variety 'Idared' in 2017, only one sample per treatment was included in the analyses ($n = 1$). All of the analyses were done twice. The data are given in mean ± standard deviation and further evaluated while using Microsoft Excel (Microsoft Office Professional Plus 2016, Redmond, WA, USA). The statistical analyses were also carried out using Excel. In order to check for significant differences, Mann-Whitney-U-tests for the Mal d 1-content were used at $p < 0.05$.

3. Results and discussion

3.1. Selenium content

By means of foliar fertilization, apples of various varieties could be significantly biofortified with selenium like previously reported for 'Golden Delicious' and 'Jonagold' (Groth et al., 2020). While the selenium content in the control apples ranged between 0.1 and 0.7 μg/100 g f. w., selenium- biofortified fruits reached 3.1 – 23.2 μg/100 g f. w. (Table 1). Thus, the selenium content of apples increased up to a factor of about 40.

Table 1
Selenium content and content of the allergenic protein Mal d 1 in all apple samples. Data are given as mean value ± standard deviation (n = 4 for selenium content; n = 2 for Mal d 1 content; n = 1 for 'Idared').

Variety and Year of Cultivation	Application*	Se(μg/100 g f.w.)	Mal d 1(μg/100 g d.w.)
'Fiesta' 2017	Control (HS)	0.1 ± 0.1	51.0 ± 19.2
	0.1 kg selenite (HS)	3.1 ± 1.5	68.8 ± 18.7
	0.1 kg selenate (HS)	3.1 ± 1.4	24.8 ± 4.8
'Idared' 2017	0.15 kg selenite (HS)	3.3	24.7
	0.15 kg selenate (HS)	3.4	49.9
'Jonica' 2017	Control (HS)	0.7 ± 0.2	43.1 ± 4.5
	0.15 kg selenite (HS)	13.9 ± 1.3	37.3 ± 8.9
'Golden Delicious'2017	Control (HS)	0.4 ± 0.2	44.5 ± 3.2
	0.15 kg selenite (HS)	5.6 ± 0.5	36.5 ± 3.9
	0.15 kg selenate (HS)	5.6 ± 0.8	35.0 ± 3.2
'Jonagold' 2017	Control (HS)	0.4 ± 0.2	107.5 ± 4.9
	0.15 kg selenite (HS)	5.6 ± 1.2	24.1 ± 4.2
	0.15 kg selenate (HS)	4.5 ± 1.6	53.3 ± 19.0
'Golden Delicious'2018	Control (BS)	0.3 ± 0.0	40.3 ± 5.7
	0.075 kg selenate (BS)	3.7 ± 0.4	29.9 ± 4.0
'Jonagold' 2018	Control (BS)	0.2 ± 0.0	25.9 ± 3.5
	0.075 kg selenate (BS)	2.1 ± 0.7	39.0 ± 5.7
'Boskoop' 2018	Control (OS)	0.4 ± 0.1	43.1 ± 6.5
	0.075 kg selenate (OS)	5.3 ± 0.3	30.9 ± 0.1
'Jonica' 2018	Control (OS)	0.3 ± 0.1	38.0 ± 7.7
	0.075 kg selenate (OS)	3.9 ± 0.7	32.6 ± 5.2
'Elstar' 2019	Control (OS)	0.6 ± 0.0	46.9 ± 1.0
	0.15 kg selenate (OS)	8.7 ± 1.4	39.0 ± 3.3
	0.45 kg selenate (OS)	23.2 ± 2.7	53.4 ± 13.6
'Elstar' – peel2019	Control (OS)	1.2 ± 0.7	78.9 ± 3.0
	0.45 kg selenate (OS)	98.2 ± 31.6	58.9 ± 1.3
'Elstar' – fruit flesh2019	Control (OS)	0.6 ± 0.1	29.9 ± 9.9
	0.45 kg selenate (OS)	13.4 ± 2.0	17.1 ± 2.7

* Foliar spray rate of selenium per hectare and meter canopy height applied with a hand-held sprayer (HS), a backpack sprayer (BS) or a trailed orchard sprayer (OS).

3.2. Protein pattern analysis

In general, bands in the range of 18 kDa were detected, where the well-known apple allergen Mal d 1 ($M_w = 17.65$ kDa) can potentially be found in the SDS-PAGE gels. Further bands were detected in the range of approx. 9 kDa, indicating the potential presence of the Mal d 3 ($M_w = 11.41$ kDa). Slightly above the band close to 25 kDa of the molecular weight marker, broader bands were additionally visible in many samples. These can be attributed to the apple allergen Mal d 2, which has a molecular weight of 25.68 kDa. Mal d 4, which has a molecular weight of 13.96 kDa was not detected.

Fig. 1A, B, 2, and 3 show the results of the gel electrophoretic separation of the apple protein extracts from the cultivation years 2017 and 2018 (Osnabrück) and from 2019 (Jork). Apple samples of the different cultivars and the differing selenium application forms or controls were also applied for a direct visual comparison. The cultivars investigated showed a similar protein pattern with regard to Mal d 1, Mal d 3, and Mal d 2 and intensity differences between the varieties, the years of cultivation, and due to the biofortification were found (Supplementary material, Table 1).

Due to the corresponding different intensity of the band coloring, it is assumed that the Mal d 1 content is reduced by selenate fertilization compared to the control or the selenite application in most cases. The determination of the Mal d 1 content using SDS-PAGE allows only a semi-quantitative evaluation. As the Mal d 1 content was further determined more precisely by ELISA, the results are discussed in section 3.4. Mal d 3, on the other hand, seems to be synthesized to a higher extent. Consequently, it can be concluded that the biofortification leads to an increase in the Mal d 3 content. However, this was not observed for 'Jonica' apples (Fig. 1B, lanes 10 and 11; Fig. 2, lanes 2 and 8) and 'Elstar' showed only slight differences (Fig. 3, lanes 5–7). When comparing the cultivars from 2017, 'Fiesta', and 'Idared' showed the most intensively colored bands at a molecular weight of 9 kDa. It can therefore be assumed that Mal d 3 contents of these varieties are higher than those of 'Jonagold', 'Golden Delicious', and 'Jonica'. On the other hand, the bands of Mal d 1 were significantly pronounced in 'Jonagold' apples. The comparison of all varieties from 2018 showed the highest band intensities and thus, the highest Mal d 3 content in 'Golden Delicious'.

Other research groups also identified the different apple allergens and determined differences between varieties. Marzban et al. (2005) performed an immuno-tissue-print-assay (ITP) and found variety-specific differences in the content of Mal d 1, Mal d 2, and Mal d 3. In contrast to the present work, they found higher Mal d 3 contents in 'Idared' than in 'Golden Delicious' (Marzban et al, 2005). A follow-up study a gel electrophoretic separation of protein extracts from different apple varieties confirmed the results of variety-specific differences. Here, the Mal d 1 content was higher in 'Golden Delicious', 'Granny Smith', 'Fuji', and 'Pink Lady' than in 'Topaz' and 'Braeburn' (Marzban et al., 2014). Sancho et al. (2006b) studied apple extracts of the varieties 'Cox orchard 3′ and 'Jonagored' using SDS-PAGE and special regard to the allergen Mal d 3. Further, they used only the peel, as Mal d 3 is mainly located there. In that study, SDS-PAGE showed an identical protein pattern for the two varieties. The content of Mal d 3, which was subsequently determined by an indirect competitive ELISA, differed depending on the variety: 'Cox orchard 3′ had the highest content with 70.2 μg Mal d 3/g peel, while 'Jonagored' and 'Gala' contained only 31 μg/g. That study also showed that the content of Mal d 3 depends on the position of the fruit on the tree (sunny *vs.* shady positions): Mal d 3 levels increased 2-fold in apples harvested from the shady site. Furthermore, differences in the content were dependent on lower and upper part of the tree (Sancho et al, 2006b).

It has been previously described that the expression of nsLTPs is

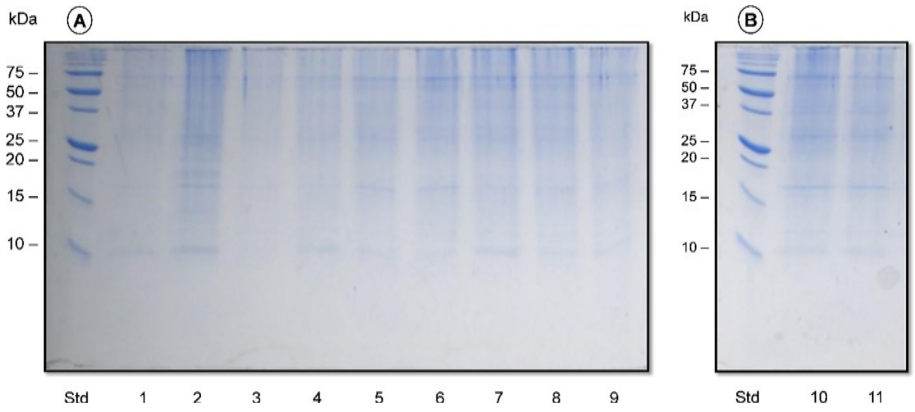


Fig. 1. SDS-PAGE of apple protein extracts of different varieties and treatments. Fruits of four different cultivars, harvested in Osnabrück, Germany in 2017. Std. indicates molecular weight marker; (A) 1: 'Fiesta', 0.10 kg Se/ha × m CH as sodium selenate; 2: 'Fiesta', 0.10 kg Se/ha × m CH as sodium selenite; 3: 'Fiesta', control; 4: 'Jonagold', 0.15 kg Se/ha × m CH as sodium selenate; 5: 'Jonagold', 0.15 kg Se/ha × m CH as sodium selenite; 6: 'Jonagold', control; 7: 'Golden Delicious', 0.15 kg Se/ha × m CH as sodium selenate; 8: 'Golden Delicious', 0.15 kg Se/ha × m CH as sodium selenite; 9: 'Golden Delicious', control; (B) 10: 'Jonica', 0.15 kg Se/ha × m CH as sodium selenite; 11: 'Jonica', control.

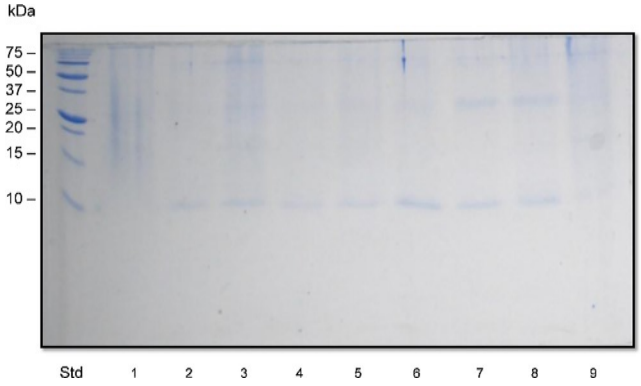


Fig. 2. SDS-PAGE of apple protein extracts of different varieties and treatments. 1: 'Elstar', control peel (for other tests); 2: 'Jonica', 0.075 kg Se/ha × m CH as sodium selenate; 3: 'Boskoop', 0.075 kg Se/ha × m CH as sodium selenate; 4: 'Jonagold', 0.075 kg Se/ha × m CH as sodium selenate; 5: 'Jonagold', control; 6: 'Golden Delicious', 0.075 kg Se/ha × m CH as sodium selenate; 7: 'Golden Delicious', control; 8: 'Jonica', control; 9: 'Boskoop', control.

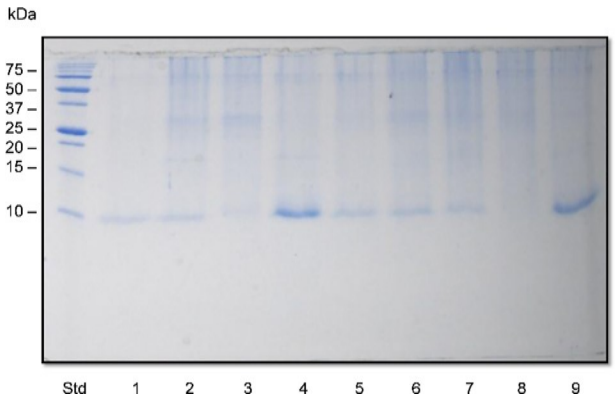


Fig. 3. SDS-PAGE of apple protein extracts of the cultivar 'Elstar', harvested in Jork, Germany in 2019 and the cultivar 'Idared', harvested in Osnabrück, Germany in 2017. Std indicates molecular weight marker; 1: 'Idared', 0.15 kg Se/ha × m CH as sodium selenate; 2: 'Idared', 0.15 kg Se/ha × m CH as sodium selenate; 3: 'Elstar', control fruit flesh; 4: 'Elstar', control peel; 5: 'Elstar', control whole fruit; 6: 'Elstar', 0.15 kg Se/ha × m CH as sodium selenate, whole fruit; 7: 'Elstar', 0.45 kg Se/ha × m CH as sodium selenate, whole fruit; 8: 'Elstar', 0.45 kg Se/ha × m CH as sodium selenate, fruit flesh; 9: 'Elstar', 0.45 kg Se/ha × m CH as sodium selenate, peel.

affected by different abiotic stress factors. However in this context, differences have been identified in a bunch of selected crops such as tomato, pepper, and barley. For example, it was observed that salinity led to an induction of gene expression in the three crops mentioned before, while tomato and pepper responded to drought and cold, and only pepper responded to wounding (Jung, Kim, & Wang, 2003; Molina & Garcia-Olmedo, 1993; Torres-Schumann, Godoy, & Pintor-Toro, 1992; Treviño & O'Connell, 1998). With regard to apples, the application of selenium-containing fertilizers also seems to be a stress factor for the plant to a certain extent, as the content of Mal d 3 increased.

Protein extracts of the fruit flesh and the peel of the apple samples of the 'Elstar' variety were applied separately using gel electrophoretic separation. Hereby, it can be assessed in which fruit compartments the various allergenic proteins are located. Fig. 3 (lanes 3–4, 8–9) further shows differences in the intensities of the bands at 9 kDa, indicating different contents of Mal d 3 in the peel and the fruit flesh in both the controls and the biofortified samples. The band intensity of Mal d 3 was highest in the peels (Fig. 3, lanes 4 and 9), indicating that Mal d 3 is mainly located in the peel of the apples. The flesh, on the other hand, contained hardly any Mal d 3 (Fig. 3, lane 3 and 8). Mal d 1 is also mainly present in the peel of the fruits, but the differences in content between peel and fruit flesh were less pronounced than for Mal d 3. Another band was obvious between 25 kDa and 37 kDa, which can be assigned to Mal d 2. Differences between peel and fruit flesh were also found for this protein, with Mal d 2 being mainly present in the flesh of the fruit.

The results obtained in the present study are in line with previously published data. Marzban et al. (2014) performed a Northern Blot for the detection of the allergens Mal d 1 and Mal d 3 and an ITP to show the localization of Mal d 1, Mal d 2, and Mal d 3 within apple tissues in four different apple cultivars. They also found that Mal d 1 is present in peel as well as the fruit flesh, while Mal d 2 is mainly expressed in the fruit flesh and Mal d 3 is only present in the peel. The Northern blot analysis showed that Mal d 1 transcripts were found in the peel and in the fruit flesh, whereas Mal d 3 expression could only be measured in the peel (Marzban et al., 2014). The specific localization of Mal d 3 in the peel underlines the involvement of nsLTPs in the biosynthesis of epicuticular wax or cuticula and in plant defense mechanisms such as antimicrobial activities (Hoffmann-Sommergruber and the SAFE consortium, 2005; Marzban et al., 2014; Salcedo, Diaz-Perales, & Sanchez-Monge, 1999; Salcedo, Sanchez-Monge, Diaz-Perales, Garcia-Casado, & Barber, 2004). Botton et al. (2009) were able to show that the Mal d 1 genes are expressed at higher levels in the fruit flesh than in the peel. Mal d 3 genes are expressed mainly in the epidermal cells of the peel. It has been decribed that attack of pathogens increases the expression of *thaumatin-like proteins* (TLPs) to which Mal d 2 belongs, in apple apoplasts. Therefore, pathogen-resistance is responsible for the TLP content. Other factors influencing the expression levels of TLPs in apples are climate and environmental changes, storage, and cultivation practices (Biggs & Miller, 2005; Botton et al., 2008; Gau, Koutb, Piotrowski, & Kloppstech,

2004; Marzban et al., 2014).

3.3. Identification of the allergens apple proteins using LC-MS/MS

The apple proteins of the Mal d-family were identified by LC-MS/MS. For this, the targeted spots were cut out of the SDS-PAGE gel, in-gel-digested and injected into a LC system. The isolated fractions were further analyzed by MS/MS. Proteins were identified against the uniport.fasta database (with special regard to the organism *Malus*). As expected, the band at approximately 9 kDa was identified as the Mal d 3 allergen. Further allergens were also confirmed.

3.4. Mal d 1 content

Also with regard to the Mal d 1 content, the different parameters of the biofortification, the apple variety, and the ecophysiological conditions were identified as influencing factors. There were also differences in the separately analyzed peel and fruit flesh samples (Fig. 4).

The application of selenite, which was carried out in 2017, resulted in lower Mal d 1 contents for the varieties 'Jonica' and 'Golden Delicious' and significantly lower Mal d 1 contents for 'Jonagold' compared to the controls. However, excluded from this trend was 'Fiesta', where biofortification led to an increase in Mal d 1 content (Table 1 and Fig. 4).

In addition, the application of selenate in the same and the follow-up years of cultivation led to a reduction of the Mal d 1 content in all analyzed varieties. For 'Fiesta' and 'Jonagold' from 2017, and 'Golden Delicious' from 2017 and 2018 the differences were even statistically significant. The only exception was 'Jonagold' in 2018, where an increase in the Mal d 1 content was observed in the biofortified samples (Table 1 and Fig. 5). An increased application of 0.45 kg selenate/ha × m CH for 'Elstar' resulted in a higher Mal d 1 content, but was not statistically significant (Table 1 and Fig. 6).

When comparing the different application forms, it was observed that the application of selenate tended to lower Mal d 1 content than the application of selenite. However, the differences are not statistically significant.

A classification of the results can only be made to a limited extent, due to a lack of comparative studies, as the influence of a biofortification with certain nutrients in plant foods on allergenic proteins has not been described yet. With regard to the influence of the content of the allergenic protein Mal d 1 in apples, a selected number of other factors have already been reported in the literature. Among other aspects, the

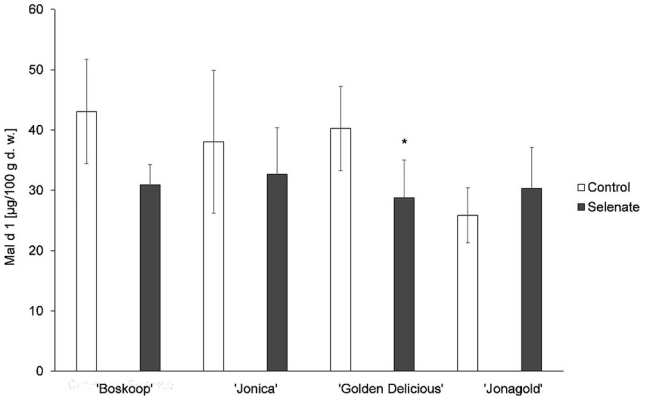


Fig. 5. Mal d 1 content (µg/100 g d. w.) in the apple samples of the varieties 'Boskoop', 'Jonica', 'Golden Delicious', and 'Jonagold', harvested in the year 2018 in Osnabrück/Germany. Foliar Se application per hectare and meter canopy height: 0.075 kg Se/ha × m CH for all varieties. Data are given as mean value ± standard deviation (n = 2). * significant difference ($p < 0.05$) between biofortified sample and control of one variety.

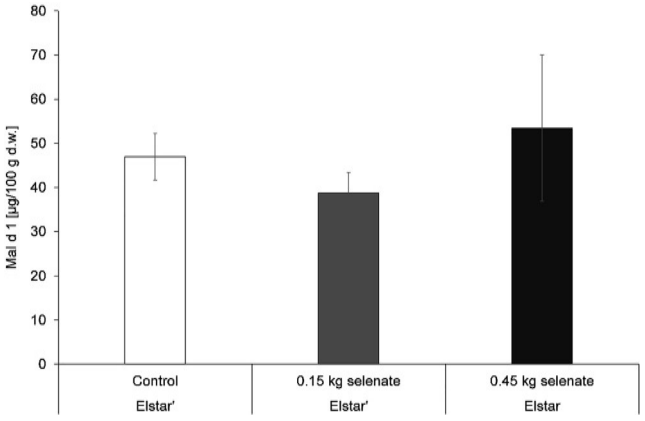


Fig. 6. Mal d 1 content (µg/100 g d. w.) in the apple samples of the variety 'Elstar', harvested in the year 2019 in Jork/Germany. Foliar Se application per hectare and meter canopy height. Data are given as mean value ± standard deviation (n = 2).

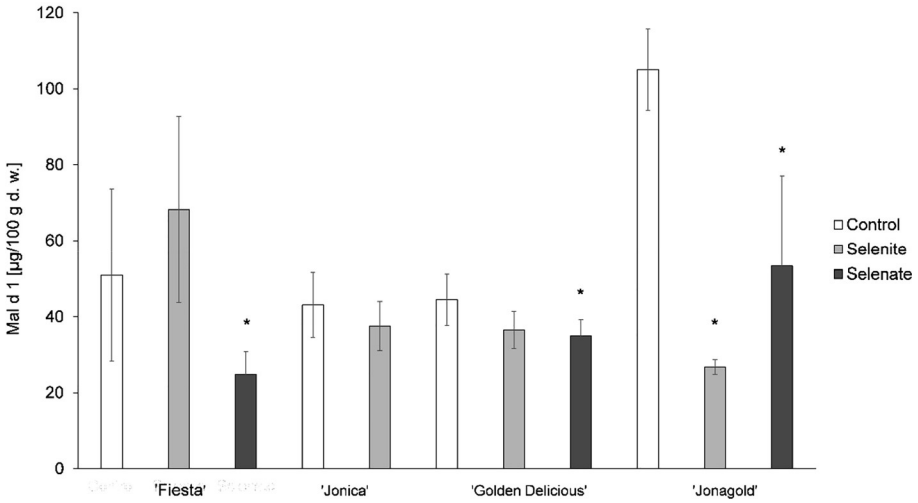


Fig. 4. Mal d 1 content (µg/100 g d. w.) of apple fruits of the varieties 'Fiesta', 'Jonica', 'Golden Delicious', and 'Jonagold', harvested in the year 2017 in Osnabrück/Germany. Foliar Se application per hectare and meter canopy height: 'Fiesta', 0.10 kg Se/ha × m CH; 'Jonica', 'Golden Delicious', and 'Jonagold', 0.15 kg Se/ha × m CH; Data are given as mean value ± standard deviation (n = 2). * significant difference ($p < 0.05$) between biofortified sample and control of one variety.

cultivation system seems to play a role. It was observed that apples grown organically had a significantly higher Mal d 1 content (Schmitz-Eiberger & Matthes, 2011) and patients showed a higher sensitivity when consuming such apples (Klockenbring et al., 2001). Fruit trees under organic cultivation seems to be more susceptible to environmental stress factors such as fungal, viral, and bacterial attack that le a higher a biosynthesis rate of Mal d 1 (Fernández-Rivas et al., 2006). Mal d 1 is a pathogenesis-related protein and mainly synthesized by the fruits as a defense agent against pathogens and under the influence of selected ecophysiological stress factors of biotic and abiotic nature (Beuning et al., 2004; Botton et al., 2009; Breiteneder & Ebner, 2000; Grafe, 2009; Matthes & Schmitz-Eiberger, 2009; Pühringer et al., 2000). The results obtained in the present work are therefore in line with the previous findings. It is hypothesized that the application of selenium-containing fertilizers leads to a better protection of the fruits against certain stress factors, whereby less Mal d 1 is synthesized. An induction of other plant-protective substances such as phenolic compounds, resulting from a biofortification with selenium in apples (Groth et al., 2020) and other crops (Bachiega et al., 2016; D'Amato et al., 2017, 2018; Pezzarossa et al., 2012: Schiavon et al., 2013, 2016; Zhao et al., 2013) has already been determined in a previous study (Groth et al., 2020). Thus, the reduction of the Mal d 1 content resulting from the selenium biofortification might be also related to the increase in total phenolic content. It has already been shown that there are dependencies between the composition of phenolic compounds, the activity of polyphenoloxidases, and the content of Mal d 1 (Bernert et al., Münstermann, Kothe, & Zapp, 2012; Kiewning & Schmitz-Eiberger, 2014; Kschonsek, Dietz, Wiegand, Hipler, & Böhm, 2019b; Schmitz-Eiberger & Matthes, 2011). Furthermore, selenium, which is also plant-protective agent, plays a role. It has already been shown that this trace element can boost plants against a plenty of abiotic stresses such as cold, drought, radiation, salinity, and heavy metals (Feng, Wei, & Tu., 2013; Gupta & Gupta, 2017). An additionally increased synthesis of the plant-protective Mal d 1 is no longer necessary and the gene expression is therefore downregulated.

The role of selenium according environmental stress has often been attributed to the regulation of reactive oxygen species and the stimulation of antioxidant systems (Feng et al., 2013; Hawrylak-Nowak, Hasanuzzaman, & Matraszek-Gawron, 2018). A connection of a selenium biofortification with the resistance to pathogens has also been shown. The treatment of tomatoes (*Lycopersicon esculentum Mill.* cv. Zhenbao) with selenium at 24 mg/L selenite led to significantly inhibited spore germination of the fungal pathogen *Botrytis cinerea.* Wu et al. (2016) hypothesized that this treatment seems to induce the generation of intracellular reactive oxygen species in the fungal spores (Wu et al., 2016).

The controls were used to identify variety-specific differences in the Mal d 1 content. Its content in the variety 'Jonagold' from the growth season 2017 with a value of 107.5 μg/100 g d. w. and from the follow-up year with a value of 25.9 μg/100 g d. w. differed significantly from the other varieties, whose contents were either significantly lower or higher. The Mal d 1 contents of the varieties 'Jonica', 'Golden Delicious', 'Boskoop', and 'Elstar' are almost in the same range (Table 1).

In the literature, data on the Mal d 1 content of a large number of varieties, including 'Jonagold', 'Golden Delicious', and 'Boskoop', have been published by different research groups (Bolhaar et al., 2005; Kschonsek et al., 2019b; Matthes & Schmitz-Eiberger, 2009; Romer et al., 2020; Sancho et al., 2006a; Schmitz-Eiberger & Matthes, 2011; Son & Lee, 2001; Zuidmeer et al., 2006;). However, different values for the Mal d 1 content for the same variety of apples were measured by Zuidmeer et al. (2006), Matthes and Schmitz-Eiberger (2009), Marzban et al. (2014), Kschonsek et al. (2019), and Romer et al. (2020). Matthes and Schmitz-Eiberger measured Mal d 1 concentrations of 'Jonagold' between 1.3 μg/g f. w. and 8.7 μg/g f. w. Converted with an approximated dry matter content of 15%, this results in values from 0.9 mg/100 g d. w. to 5.8 mg/100 g d. w. The values for 'Golden Delicious' were in a similar range. Romer et al. (2020) have also measured values for 'Golden Delicious' in this range, converted to dry matter these are from 1.9 mg/100 g d. w. to 2.3 mg/100 g d. w.. These values are higher compared to the present study. Possible causes may lie in the different cultivation locations or the use of different extraction buffer followed by dialysis by the researchers.

The almost identical Mal d 1 contents of the varieties 'Jonica', 'Golden Delicious', 'Boskoop', and 'Elstar' are assumed to be result from genetic relations (Jackson, 2003). In addition, Zuidmeer et al. (2006), Matthes and Schmitz-Eiberger (2009), and Kschonsek et al. (2019) could not find any significant differences between the varieties. In contrast, Marzban et al. (2014) found significant differences between 'Jonagold' and 'Golden Delicious' with higher values for 'Golden Delicious'. Kiewning and Schmitz-Eiberger (2014) observed higher contents at 'Elstar' compared to 'Boskoop'.

The influence of the year of cultivation on the Mal d 1 content was also determined by comparing the control samples of the 'Jonica', 'Jonagold', and 'Golden Delicious' varieties. In all three varieties, the Mal d 1 content in 2018 was lower than in the previous year; for 'Jonagold', the difference was statistically significant. The Mal d 1 content of 'Jonagold' showed a high susceptibility to different ecpophysiological influence factors, while the other varieties were only slightly affected. Thus, a decrease of 76% of the Mal d 1 content was observed in the growth season 2018 compared to 2017. The two other varieties also showed higher Mal d 1 contents in 2017 than in the follow-up year.

The influence of environmental conditions such as precipitation, temperature, and solar radiation on the Mal d 1 content has already been described in the literature. Zuidmeer et al. (2006) found significant differences in the Mal d 1 content of five different varieties, among others 'Jonagold' and 'Golden Delicious', which were grown in Italy and the Netherlands. They found no significant differences at the Italian site, whereas the cultivation of the same varieties led to significant differences at the Dutch site, with 'Golden Delicious' containing more Mal d 1 (Zuidmeer et al., 2006).

The observations made in the present study are not consistent with the results published by Matthes & Schmitz-Eiberger (2009). They investigated the influence of site conditions on 12 varieties, including 'Jonagold' and 'Golden Delicious', and found that the Mal d 1 content was significantly higher at the site with significantly lower precipitation (-44%) and higher sunshine duration (Matthes & Schmitz-Eiberger, 2009). In contrast, the present study found a lower Mal d 1 content in the growth season 2018, when precipitation was significantly lower. Botton et al. (2009) showed that shading has a significant influence the transcription of individual Mal d 1 genes. Consequently, the peel, which is more directly exposed to solar radiation, showed a higher extent of the level of transcription than in the fruit flesh. Furthermore, it was observed that water stress had only a small effect on the expression of apple-allergen-related genes, whereby a Mal d 1 gene present in the peel was strongly up-regulated under drought conditions (Botton et al, 2009).

Based on the separate analysis of the fruit flesh and the peel of 'Elstar' apples from the growth season 2019, it was found that the allergenic protein Mal d 1 is predominantly present in the peel, independent of the biofortification. The proportion of Mal d 1 in the peel was 72.6% in the control and 77.5% in the biofortified samples. Accordingly, only 27.4% and 22.5% of Mal d 1 were localized in the fruit flesh (Fig. 7).

Furthermore, it was found that the content of Mal d 1 in the fruit flesh was significantly lower in the selenium biofortified samples than in the controls. In the biofortified samples, the Mal d 1 content in the fruit flesh was reduced by 42.8% compared to the control. Whereas, the content of Mal d 1 in the peel was influenced by the biofortification only to a lesser extent – the Mal d 1 content was only 25.4% lower. The Mal d 1 content of the individual plant parts was influenced by the selenium biofortification in different ways (Fig. 8).

In the literature, it is also reported that Mal d 1 is distributed

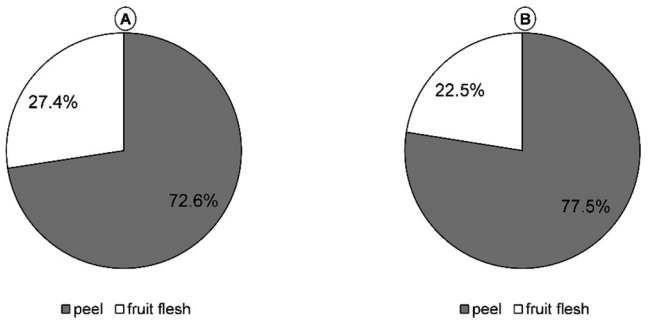


Fig. 7. Proportion of Mal d 1 in peel and fruit flesh in (A) the control and (B) in the selenium biofortified apples of the variety 'Elstar'.

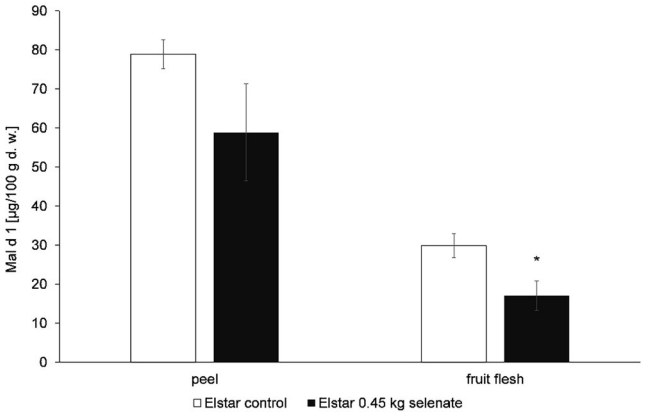


Fig. 8. Mal d 1 content (µg/100 g d. w.) in the peel and the fruit flesh of the variety 'Elstar', control samples and biofortified with 0.45 kg Se per meter canopy height as sodium selenate. Data are given as mean value ± standard deviation (n = 2). * significant difference ($p < 0.05$) between biofortified sample and control of one variety.

differently within the fruit depending on the variety and also between individual apples of one variety (Marzban et al., 2014; Savazzini, Ricci, & Tartarini, 2015). In the case of the variety 'Braeburn', six individual apples were found to contain Mal d 1 in the peel in proportions of about 20% to 80% (Marzban et al., 2005). With regard to the gene expression of Mal d 1, Botton et al. (2009) found that the genes responsible for Mal d 1 are expressed in higher levels in the fruit flesh than in the peel of the 'Golden Delicious' variety .

3.5. Correlations between protein content and Mal d 1 content

No correlation was found between protein and Mal d 1 content (results not shown). Sancho et al. (2006b) describe also a lack of a correlation, which indicates that Mal d gene expression is regulated independently of the other proteins, for example, depending on stress factors . It has been shown that the transcription of PR-10 proteins is activated upon microbial attack, certain elictors, wounding, or further stress-related stimuli (Pühringer et al., 2000).

4. Conclusions

In this study, we found out that biofortification of apples with selenium influences the content of allergenic proteins in the different cultivars partly in different extent. Therefore, not all apple varieties seems to be suitable for an improved reduction of the allergenic potential with a biofortification using selenium. This is promising for varieties such as 'Fiesta', providing already originally a low allergenicity and might therefore bear only a limited risk for sensible consumers. Although the Mal d 1 content of 'Golden Delicious' and 'Jonica', which are not tolerated by allergy sufferers, was significantly reduced, this might be not enough to reach acceptable levels for allergic consumers. Therefore, apple varieties such as 'Santana' or 'Wellant', which already show a low allergenic potential, seem to be suitable for testing a further reduction of the Mal d 1 content by applying elicitors such as the biofortification with selenium. In 'Jonagold', ecophysiological conditions appear to have a comparatively strong influence on the formation of allergenic proteins, as different effects on the Mal d 1 content were observed depending on the cultivation year. Further analysis of other varieties are necessary. During the treatment of apples with selenium, the fertilizer was applied directly to leaves and the peel of the fruits, whereupon the majority of the analyzed apple varieties responded with an increased synthesis of Mal d 3, localized in the peel. This was observed in particular with the application of selenate. The increased content of Mal d 3 in the samples biofortified with selenium indicates that this protein is synthesized to a larger extent due to the external stress factor of selenium fertilization, which is applied directly to the peel of growing fruits. Mal d 3 belongs to the nsLTPs, whose expression has been described to be mainly due to abiotic stressors. Based on the present results, selenium biofortification also seems to represent a stress factor with the consequence of an increased Mal d 3 formation. On the other hand, the content of Mal d 1 decreased in most varieties compared to the controls and selenite addition. However, in contrast to Mal d 3, the biofortification with selenate does not represent a stress factor for the synthesis of Mal d 1. Whether the different localization of the allergens Mal d 1 and Mal d 3 in the apple (peel *vs.* pulp) also plays a role should be characterized in further studies. The present results indicate that the expression of allergenic proteins in apples, especially Mal d 1 and Mal d 3, may be influenced by a mixture of genetic and ecophysiological conditions. Besides the conditions of biofortification such as form and concentration of the selenium fertilizer, the apple variety and the climatic conditions of the cultivation sites were identified as further influencing factors on both the protein and the Mal d 1 content. These must therefore be taken into account when choosing low-allergen varieties.

CRediT authorship contribution statement

Sabrina Groth: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing - original draft, Visualization. **Christoph Budke:** Conceptualization, Methodology, Formal analysis. **Timo Weber:** Investigation. **Marie Oest:** Investigation. **Sven Brockmann:** Investigation. **Martina Holz:** Investigation. **Diemo Daum:** Conceptualization, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition. **Sascha Rohn:** Conceptualization, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors gratefully acknowledge Prof. Dr. Hartmut Schlüter and his team (Department of Clinical Chemistry, Mass Spectrometric Proteomics, University Medical Center Hamburg-Eppendorf, Hamburg, Germany) for the nanoLC-MS/MS analyses.

Funding

This research was funded by the Federal Ministry of Education and Research (BMBF), grant numbers FKZ: 031B0299A, B and, C.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2021.130134.

References

Ahammer, L., Grutsch, S., Kamenik, A. S., Liedl, K. R., & Tollinger, M. (2017). Structure of the major apple allergen Mal d 1. *Journal of Agricultural and Food Chemistry, 65*, 1606–1612. https://doi.org/10.1021/acs.jafc.6b05752.

Bachiega, P., Salgado, J. M., de Carvalho, J. E., Ruiz, A. L., Schwarz, K., Tezotto, T., & Morzelle, M. C. (2016). Antioxidant and antiproliferative activities in different maturation stages of broccoli *(Brassica oleracea* var. italica) biofortified with selenium. *Food Chemistry, 190*, 771–776. https://doi.org/10.1016/j.foodchem.2015.06.024.

Bernert, T., Münstermann, J., Kothe, A., Zapp, J. (2012). Polyphenolgehalt in Alten und Neuen Apfelsorten im Bezug auf Allergene Wirkungen; Poster; Hochschule Ostwestfalen-Lippe: Lemgo, Germany. Retrieved from: https://www.bund-lemgo.de/download/POSTER_Hochschule_OWL_ 455_ LCT_2012.pdf. Accessed on Mai 5, 2021.

Beuning, L., Bowen, J., Persson, H., Barraclough, D., Bulley, S., & Macrae, E. (2004). Characterization of Mal d 1-related genes in *Malus*. *Plant Molecular Biology, 55*(3), 369–388.

Biggs, A., & Miller, S. (2005). Comparative Relative Susceptibility of NE-183 Apple Cultivars to Fruit Rot Pathogens in West Virginia. *Journal American Pomological Society, 59*, 72–77.

Björkstén, F., Halmepuro, L., Hannuksela, M., & Lahti, A. (1980). Extraction and Properties of Apple Allergens. *Allergy, 35*(8), 671–677.

Bolhaar, S. T., van de Weg, W. E., van Ree, R., Gonzalez-Mancebo, E., Zuidmeer, L., Bruijnzeel-Koomen, C. A., … Knulst, A. C. (2005). In vivo assessment with prick-to-prick testing and double-blind, placebo-controlled food challenge of allergenicity of apple cultivars. *Journal of Allergy and Clinical Immunology, 116*, 1080–1086. https://doi.org/10.1016/j.aci.2005.07.004.

Botton, A., Lezzer, P., Dorigoni, A., Barcaccia, G., Ruperti, B., & Ramina, A. (2008). Genetic and envirnonmental factors affecting allergen-related gene expression in apple fruit (*Malus domestica* L. Borkh). *Journal of Agricultural and Food Chemistry, 56*, 6707–6716. https://doi.org/10.1021/jf800813d.

Botton, A., Lezzer, P., Dorigoni, A., Rupertis, B., & Ramina, A. (2009). Environmental factors affecting the expression of apples (Malus x domestica L. Borkh) allergen-encoding genes. Journal of Horticultural Sciences and Biotechnology, Isafruit Special Issue, 182-187. doi:10.1080/ 14620316.2009.11512618.

Breiteneder, H., & Ebner, C. (2000). Molecular and biochemical classification of plant-derived food allergens. *Journal of Allergy and Clinical Immunology, 106*(1), 27–36. https://doi.org/10.1067/mai.2000.106929.

Budke, C., thor Straten, S., Mühling, K. H., Broll, G., & Daum, D. (2020a). Iodine biofortification of field-grown strawberries–Approaches and their limitations. *Scientia Horticulturae, 269*, 109317. https://doi.org/10.1016/j.scienta.2020.109317.

Budke, C., Mühling, K. H., & Daum, D. (2020b). Iodine uptake and translocation in apple trees grown under protected cultivation. *Journal of Soil Science and Plant Nutrition, 183*(4), 468–481. https://doi.org/10.1002/jpln.v183.410.1002/jpln.202000099.

Bundesministerium für Ernährung und Landwirtschaft (BMEL) (2020). Pro-Kopf-Verbrauch von Äpfeln in Deutschland in den Jahren 2007/08 bis 2018/19 (in Kilogramm). Retrieved from https://de.statista.com/statistik/daten/studie/643179/umfrage/pro-kopf-konsum-von-aepfeln-in-deutschland. Accessed September 8, 2020.

Burney, P. G. J., Potts, J., Kummeling, I., Mills, E. N. C., Clausen, M., Dubakiene, R., … van Ree, R. (2014). The prevalence and distribution of food sensitization in European adults. *Allergy, 69*(3), 365–371. https://doi.org/10.1111/all.2014.69.issue-310.1111/all.12341.

Cakmak, I. (2009). *Agronomic approaches in biofortification of food crops with micronutrients*. UC Davis: Department of Plant Sciences.

D'Amato, R., Proietti, P., Onofri, A., Regni, L., Esposto, S., Servili, M., … Gualillo, O. (2017). Biofortification (Se): Does it increase the content of phenolic compounds in virgin olive oil (VOO)? *PLoS ONE, 12*(4), e0176580. https://doi.org/10.1371/journal.pone.0176580.

D'Amato, R., Fontanella, M. C., Falcinelli, B., Beone, G. M., Bravi, E., Marconi, O., … Businelli, D. (2018). Selenium biofortification in rice (*Oryza sativa* L.) sprouting: Effects on Se yield and nutritional traits with focus on phenolic acid profile. *Journal of Agricultural and Food Chemistry, 66*(16), 4082–4090. https://doi.org/10.1021/acs.jafc.8b0012710.1021/acs.jafc.8b00127.s001.

Dris, R., & Niskanen, R. (1999). Calcium chloride sprays decrease physiological disorders following long-term cold storage of apple. *Plant Foods for Human Nutrition, 54*(2), 159–171. https://doi.org/10.1023/A:1008171909458.

Feng, R., Wei, C., & Tu, S. (2013). The roles of selenium in protecting plants against abiotic stresses. *Environmental and Experimental Botany, 87*, 58–68. https://doi.org/10.1016/j.envexpbot.2012.09.002.

Fernández-Rivas, M., Bolhaar, M., González-Mancebo, E., Asero, R., van Leeuwen, A., Bohle, B., … Sancho, A. I. (2006). Apple allergy across Europe: How allergen sensitization profiles determine the clinical expression of allergies to plant foods. *Journal of Allergy and Clinical Immunology, 118*(2), 481–488. https://doi.org/10.1016/j.aci.2006.05.012.

Gau, A. E., Koutb, M., Piotrowski, M., & Kloppstech, K. (2004). Accumulation of pathogenesis related proteins in the apoplast of a susceptible cultivar of apple (*Malus domestica* cv. Elstar) after infection by *Venturia inaequalis* and constitutive expression of PR genes in the resistant cultivar Remo. *European Journal of Plant Pathology, 110*(7), 703–711. https://doi.org/10.1023/B:EJPP.0000041552.98673.e3.

Grafe, C. (2009). Apfelallergie: Aktueller Wissensstand und Ausblick. *Obstbau, 12*.

Groth, S., Budke, C., Neugart, S., Ackermann, S., Kappenstein, F.-S., Daum, D., & Rohn, S. (2020). Influence of a selenium biofortification on antioxidant properties and phenolic compounds of apples (*Malus domestica*). *Antioxidants, 9*, 187. https://doi.org/10.3390/antiox9020187.

Gupta, M., & Gupta, S. (2017). An overview of selenium uptake, metabolism, and toxicity in plants. *Frontiers in Plant Science, 7*, 2074. https://doi.org/10.3389/fpls.2016.02074.

Hawrylak-Nowak, B., Hasanuzzaman, M., & Matraszek-Gawron, R. (2018). In *Plant Nutrients and Abiotic Stress Tolerance* (pp. 269–295). Singapore: Springer Singapore. https://doi.org/10.1007/978-981-10-9044-8_12.

Hed, J. (2008). *Kreuzreaktivität bei Allergien gegen pflanzliche Nahrungsmittel*. Uppsala, Schweden: Thermo Fisher Scientific Inc.

Hirschi, K. D. (2009). Nutrient biofortification of food crops. *Annual Review of Nutrition, 29*(1), 401–421. https://doi.org/10.1146/annurev-nutr-080508-141143.

Hoffmann-Sommergruber, K., & the SAFE consortium,. (2005). The SAFE project: Plant food allergies: Field to table strategies for reducing their incidence in Europe an EC-funded study. *Allergy, 60*, 436–442. https://doi.org/10.1111/j.1398-9995.2005.00809.x.

Hsieh, L.-S., Moos, M., & Lin, Y. (1995). Characterization of apple 18 and 31 kd allergens by microsequencing and evaluation of their content during storage and ripening. *Journal of Allergy and Clinical Immunology, 96*(6), 960–970. https://doi.org/10.1016/S0091-6749(95)70234-2.

Jackson, J. E. (2003). *Biology of Apples and Pears. Biology of Horticultural Crops* ((1st ed.), pp. 30–31). Cambridge University Bridge.

Jing, D.-W., Du, Z.-Y., Ma, H.-L., Ma, B.-Y., Liu, F.-C., Song, Y.-G., … Li, L. (2017). Selenium enrichment, fruit quality and yield of winter jujube as affected by addition of sodium selenite. *Scientia Horticulturae, 225*, 1–5. https://doi.org/10.1016/j.scienta.2017.06.036.

Jung, H. W., Kim, W., & Hwang, B. K. (2003). Three pathogen-inducible genes coding lipid transfer protein from pepper are different activated by pathogens, abiotic, and environmental stresses. *Plant, Cell and Environment, 26*, 915–928. https://doi.org/10.1046/j.1365-3040.2003.01024.x.

Kiewning, D., Baab, G., & Schmitz-Eiberger, M. (2013). Effect of 1-MCP treatment on the apple (*Malus domestica* L. Borkh.) allergen Mal d 1 during long-term storage. *Lebensmittel-Wissenschaft & Technologie, 53*, 198–203. https://doi.org/10.1016/j.lwt2013.01.025.

Kiewning, D., & Schmitz-Eiberger, M. (2014). Effects of long-term storage on Mal d 1 content of four apple cultivars with initial low Mal d 1 content. *Journal of the Science of Food and Agriculture, 94*, 798–802. https://doi.org/10.1002/jsfa.6448.

Klockenbring, T., Bez, C., Klowat, B., Liappis, N., Köpke, U., Noga, G., … Goerlich, R. (2001). Allergenic potential of apple cultivars from organic and integrated fruit production. *Allergy, 68 (suppl.), 889*.

Kschonsek, J., Wolfram, T., Stöckl, A., & Böhm, V. (2018). Polyphenolic compounds analysis of old and new apple cultivars and contribution pf polyphenolic profile to the in vitro antioxidant capacity. *Antioxidants, 7*, 20. https://doi.org/10.3390/antiox7010020.

Kschonsek, J., Wiegand, C., Hipler, U. C., & Böhm, V. (2019a). Influence of polyphenolic content on the in vitro allergenicity of old and new apple cultivars: A pilot study. *Nutrition, 58*, 30–35. https://doi.org/10.1016/j.nut.2018.07.001.

Kschonsek, J., Dietz, A., Wiegand, C., Hipler, U.-C., & Böhm, V. (2019b). Allergenicity of apple allergen Mal d 1 as effected by polyphenols and polyphenol oxidase due to enzymatic browning. *Lebensmittel-Wissenschaft & Technologie, 113*, 108289. https://doi.org/10.1016/j.lwt.2019.108289.

LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, 227*(5259), 680–685.

Lawson, P. G., Daum, D., Czauderna, R., & Vorsatz, C. (2016). Factors influencing the efficacy of iodine foliar sprays used for biofortifying butterhead lettuce (*Lactuca sativa*). *Journal of Plant Nutrition and Soil Science, 179*(5), 661–669. https://doi.org/10.1002/jpln.v179.510.1002/jpln.201600213.

Ma, Y., Zuidmeer, L., Bohle, B., Bolhaar, S. T. H., Gadermaier, G., Gonzalez-Mancebo, E., … Hoffmann-Sommergruber, K. (2006). Characterization of recombinant Mal d 4 and its application for component-resolved diagnosis of apple allergy. *Clinical &. Experimental Allergy, 36*(8), 1087–1096. https://doi.org/10.1111/cea.2006.36.issue-810.1111/j.1365-2222.2006.02541.x.

Marzban, G., Puehringer, H., Dey, R., Brynda, S., Ma, Y., Martinelli, A., … Laimer, M. (2005). Localisation and distribution of the major allergens in apple fruits. *Plant Science, 169*(2), 387–394. https://doi.org/10.1016/j.plantsci.2005.03.027.

Marzban, G., Kinaciyan, T., Maghuly, F., Brunner, R., Gruber, C., Hahn, R., … Laimer, M. (2014). Impact of sulfur and vitamin C on the allergenicity of Mal d 2 from apple (*Malus domestica*). *Journal of Agricultural and Food Chemistry, 62*(30), 7622–7630. https://doi.org/10.1021/jf5020878.

Matthes, A., & Schmitz-Eiberger, M. (2009). Apple (*Malus domestica* L. Borkh.) Allergen Mal d 1: Effect of Cultivar, Cultivation System, and Storage Conditions. *Journal of Agricultural and Food Chemistry, 57*(22), 10548–10553. https://doi.org/10.1021/jf901938q.

Molina, A., Garcia-Olmedo, F. Development and pathogen induced expression of three barley genes encoding lipid transfer proteins. The Plant Journal, 4, 983-991. doi: 10.1046/j.1365-313x.1993.04060983.x.

Pezzarossa, B., Remorini, D., Gentile, M. L., & Massai, R. (2012). Effects of foliar and fruit addition of sodium selenate on selenium accumulation and fruit quality. *Journal of the Science of Food and Agriculture, 92*(4), 781–786. https://doi.org/10.1002/jsfa.v92.410.1002/jsfa.4644.

CUMULATIVE PART OF THE DISSERTATION

Poblaciones, María. J., Rodrigo, S. M., & Santamaría, O. (2013). Evaluation of the potential of peas (*Pisum sativum* L.) to be used in selenium biofortification programs under Mediterranean conditions. *Biological Trace Element Research, 151*(1), 132–137. https://doi.org/10.1007/s12011-012-9539-x.

Poblaciones, M. J., Santamaría, O., García-White, T., & Rodrigo, S. M. (2014a). Selenium biofortification in bread-making wheat under Mediterranean conditions: Influence on grain yield and quality parameters. *Crop and Pasture Science, 65*, 362–369. https://doi.org/10.1071/CP14061.

Poblaciones, M. J., Rodrigo, S. M., Santamaría, O., Chen, Y., & McGrath, S. P. (2014b). Agronomic selenium biofortification in *Triticum durum* under Mediterranean conditions: From grain to cooked pasta. *Food Chemistry, 146*, 378–384. https://doi.org/10.1016/j.food.chem.2013.09.070.

Poňavič, M., & Scheib, A. (2014). Distribution of selenium in European agricultural and grazing land soil. In Chemistry of Europe's agricultural soils, Reimann, C., Birke, M., Demetriades, A., Filzmoser, P., & O'Connor P. (Eds.), Geologisches Jahrbuch (Reihe B), 103, Schweizerbart, Stuttgart/Germany, 131-144.

Pühringer, H., Moll, D., Hoffmann-Sommergruber, K., Watillon, B., Katinger, H., & da Câmara Machado, M. L. (2000). The promoter of an apple *Ypr10* gene, encoding the major allergen Mal d 1, is stress- and pathogen-inducible. *Plant Science, 152*(1), 35–50. https://doi.org/10.1016/S0168-9452(99)00222-8.

Raese, J. T., & Drake, S. R. (1993). Effects of preharvest calcium sprays on apple and pear quality. *Journal of Plant Nutrition, 16*(9), 1807–1819. https://doi.org/10.1080/01904169309364651.

Reis, H. P. G., Barcelos, Jéssica. P.de. Q., Junior, E. F., Santos, E. F., Silva, Vinícius. M., Moraes, M. F., … Reis, André. R.dos. (2018). Agronomic biofortification of upland rice with selenium and nitrogen and its relation to grain quality. *Journal of Cereal Science, 79*, 508–515. https://doi.org/10.1016/j.jcs.2018.01.004.

Romer, E., Chebib, S., Bergmann, K. C., Plate, K., Becker, S., Ludwig, C., … Schwab, W. (2020). Tiered approach for the identification of Mal d 1 reduced, well tolerated apple genotypes. *Nature Scientific Reports, 10*, 9144. https://doi.org/10.1038/s441598-020-66051-4.

Salcedo, G., Diaz-Perales, A., & Sanchez-Monge, R. (1999). Fruit allergy: Plant defence proteins as novel potential panallergens. *Clinical & Experimental Allergy, 29*, 1158–1160. https://doi.org/10.1046/j.1365-2222.1999.00629.x.

Salcedo, G., Sanchez-Monge, R., Diaz-Perales, A., Garcia-Casado, G., & Barber, D. (2004). Plant non-specific lipid transfer proteins as food and pollen allergens. *Clinical & Experimental Allergy, 34*, 1336–1341. https://doi.org/10.1016/j.bbalip.2007.01.001.

Sancho, A. I., Rigby, N. M., Zuidmeer, L., Asero, R., Mistrello, G., Amato, S., … Mills, E. N. C. (2005). The effect of thermal processing on the IgE reactivity of the non-specific lipid transfer protein from apple, Mal d 3. *Allergy, 60*(10), 1262–1268. https://doi.org/10.1111/all.2005.60.issue-1010.1111/j.1398-9995.2005.00876.x.

Sancho, A. I., Foxall, R., Browne, T., Dey, R., Zuidmeer, L., Marzban, G., … Mills, E. N. C. (2006a). Effect of postharvest storage on the expression of the apple allergen Mal d 1. *Journal of Agricultural and Food Chemistry, 54*(16), 5917–5923. https://doi.org/10.1021/jf060880m.

Sancho, A. I., Foxall, R., Rigby, N. M., Browne, T., Zuidmeer, L., van Ree, R., … Mills, E. N. C. (2006b). Maturity and storage influence on the apple (*Malus domestica*) allergen Mal d 3, a nonspecific lipid transfer protein. *Journal of Agricultural and Food Chemistry, 54*(14), 5098–5104. https://doi.org/10.1021/jf0530446.

Savazzini, F., Ricci, G., & Tartarini, S. (2015). Apple allergens genomics and biotechnology: Unravelling the determinants of apple allergenicity. In *Applied Plant Genomics and Biotechnology* (pp. 35–54). Amsterdam: Woodhead Publishing.

Schiavon, M., dall'Acqua, S., Mietto, A., Pilon-Smits, E. A. H., Sambo, P., Masi, A., & Malagoli, M. (2013). Selenium fertilization alters the chemical composition and antioxidant constituents of tomato (*Solanum lycopersicon* L.). *Journal of Agricultural and Food Chemistry, 61*(44), 10542–10554. https://doi.org/10.1021/jf4031822.

Schiavon, M., Berto, C., Malagoli, M., Trentin, A., Sambo, P., dall'Acqua, S., & Pilon-Smits, E. A. H. (2016). Selenium biofortification in radish enhances nutritional quality via accumulation of methyl-selenocystein and promotion of transcripts and metabolites related to glucosinolates, phenolics, and amino acids. *Frontiers in Plant Science, 7*, 1371. https://doi.org/10.3389/fpls.2016.01371.

Schmitz-Eiberger, M., & Matthes, A. (2011). Effect of harvest maturity, duration of storage and shelf life of apples on the allergen Mal d 1, polyphenoloxidase activity and polyphenol content. *Food Chemistry, 127*(4), 1459–1464. https://doi.org/10.1016/j.foodchem.2011.01.101.

Shevchenko, A., Wilm, M., Vorm, O., & Mann, M. (1996). Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels. *Analytical Chemistry, 68*(5), 850–858.

Shevchenko, A., Tomas, H., Havli, J., Olsen, J. V., & Mann, M. (2006). In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nature Protocols, 1*(6), 2856–2860. https://doi.org/10.1038/nprot.2006.468.

Son, D., & Lee, S. (2001). Comparison of the characteristics of the major allergen Mal d 1 according to apple varieties. *Food Science and Biotechnology, 10*, 132–136.

Souci, S. W., Fachmann, W., & Kraut, H. (2011). *Lebensmitteltabelle für die Praxis* ((5th ed.)., pp. 873–876). Wissenschaftliche Verlagsgesellschaft Stuttgart.

Statistisches Bundesamt (2020). Anbaufläche von Baumobst im Marktobstbau in Deutschland nach Obstsorten in den Jahren 2002 bis 2019 (in Hektar). Retrieved from https://de.statista.com/statistik/daten/studie/166874/umfrage/anbauflaeche-von-baumobst-nach-obstsorte. Accessed September 8, 2020.

Torres-Schumann, S., Godoy, J. A., Pintor-Toro, J. A. (1992). A probable lipid transfer protein gene is induced by NaCl in stems of tomato plants. Plant Molecular Biology, 18, 749-757. doi: 10.1007/BF00020016.

Treviño, M. B., & O'Connell, M. A. (1998). Three drought-responsive members of the nonspecific lipid-transfer protein gene family in *Lycopersicon pennellii* show different development patterns of expression. *Plant Physiology, 116*, 1461–1468. https://doi.org/10.1104/pp.116.4.1461.

Tsao, R., Yang, R., Xie, S., Sockovie, E., & Khanizadeh, S. (2005). Which polyphenolic compounds contribute to the total antioxidant activities of apple? *Journal of Agricultural and Food Chemistry, 53*(12), 4989–4995. https://doi.org/10.1021/jf048289h.

Wang, J., Vanga, S. K., & Raghavan, V. (2019). Effect of pre-harvest and post-harvest conditions on the fruit allergenicity: A review. *Critical Reviews in Food Science and Nutrition, 59*(7), 1027–1043. https://doi.org/10.1080/10408398.2017.1389691.

Wu, Z., Yin, X., Banuelos, G. S., Lin, Z. Q., Zhu, Z., Liu, Y., … Li, M. (2016). Effect of selenium on control of postharvest gray mold of tomate fruit and the possible mechanisms involved. *Frontiers in Microbiology, 6*, 1441. https://doi.org/10.3389/fmicb.2015.01441.

Zhao, Y., Wu, P., Wang, Y., & Feng, H. (2013). Different approaches for selenium biofortification of pear-jujube (*Zizyphus jujuba* M. cv. Lizao) and associated effects on fruit quality. *Journal of Food, Agriculture and Environment, 11*, 529–534.

Zuidmeer, L., van Leeuwen, W. A., Kleine Budde, I., Breiteneder, H., Ma, Y., Mills, C., … van Ree, R. (2006). Allergenicity Assessment of Apple Cultivars: Hurdles in Quantifying Labile Fruit Allergens. *International Archieves of Allergy and Immunology, 141*(3), 230–240. https://doi.org/10.1159/000095293.

6.3. Analysis of the relationship between phenolic compounds and the allergenic protein Mal d 1 selenium-biofortified apples

Relationship between Phenolic Compounds, Antioxidant Properties, and the Allergenic Protein Mal d 1 in Different Selenium-Biofortified Apple Cultivars *(Malus domestica)*

Sabrina Groth [1], Christoph Budke [2], Timo Weber [2], Susanne Neugart [3], Sven Brockmann [1], Martina Holz [1], Bao Chau Sawadski [1], Diemo Daum [2] and Sascha Rohn [1,4]*

[1] Hamburg School of Food Science, Institute of Food Chemistry, University of Hamburg, Grindelallee 117, 20146 Hamburg, Germany

[2] Department of Plant Nutrition, Osnabrück University of Applied Sciences, 49090 Osnabrück, Germany

[3] Department of Crop Sciences, Division Quality and Sensory of Plant Products, Georg-August-Universität Göttingen, 37075 Göttingen, Germany

[4] Technische Universität Berlin, Institute of Food Technology and Food Chemistry, Department of Food Chemistry and Analysis, TIB 4/3-1, Gustav-Meyer-Allee 25, 13355 Berlin, Germany

Molecules **2021**, *26,* 2647

doi: 10.3390/molecules26092647

Accepted: 29 April 2021

This work analyzes the relationship between the parameters determined in the two previous works. Here, the aim is to investigate the interactions between allergenic proteins with other substances such as phenolic compounds and their properties in selenium-biofortified apples. For this purpose, selenium-biofortified apples and the corresponding controls of different cultivars were analyzed and correlation analyses between total phenolic content, polyphenoloxidase activity, antioxidant activity, and phenolic profile with the content of the allergenic protein Mal d 1 were performed. Another objective is to evaluate whether the innovative agronomic method of biofortification is suitable for increasing the content of selenium and polyphenols in apples while reducing the content of allergenic proteins.

It was found that there is a correlation between PPO activity and the content of individual phenolic compounds with the Mal d 1 content. The relationship between PPO activity and Mal d 1 content appears to be cultivar dependent as well as influenced by ecophysiological conditions, as both positive and negative correlations were found. Apples high in chlorogenic acid and low in procyanidin trimer and/or epicatechin showed lower allergic potential. In contrast, the total phenolic content and the level of antioxidant activity seem to play only a minor role. In the biofortified apples, the changes in phenolic profile described above were frequently observed. Thus, this measure seems to be suitable for a reduction of the allergenic potential in apples.

Work shares

Sabrina Groth:	Establishment and validation of the methods for determination of polyphenoloxidase activity, total phenolic content, and antioxidant properties by TEAC and ORAC assay in apples; measurement of PPO activity, TPC, and AOA; performance of in-gel digestion of bands from SDS gels for mass spectrometric analysis of apple proteins; experimental and statistical evaluations; preparation of the manuscript.
	Total contribution: 35%

molecules

MDPI

Article

Relationship between Phenolic Compounds, Antioxidant Properties, and the Allergenic Protein Mal d 1 in Different Selenium-Biofortified Apple Cultivars (*Malus domestica*)

Sabrina Groth [1], Christoph Budke [2], Timo Weber [2], Susanne Neugart [3], Sven Brockmann [1], Martina Holz [1], Bao Chau Sawadski [1], Diemo Daum [2] and Sascha Rohn [1,4,*]

[1] Hamburg School of Food Science, Institute of Food Chemistry, University of Hamburg, Grindelallee 117, 20146 Hamburg, Germany; sabrina.groth@chemie.uni-hamburg.de (S.G.); svenbrockmann7@gmx.de (S.B.); martina-holz@gmx.de (M.H.); baochau2506@gmail.com (B.C.S.)
[2] Department of Plant Nutrition, Osnabrück University of Applied Sciences, 49090 Osnabrück, Germany; c.budke@hs-osnabrueck.de (C.B.); timo.weber23@gmx.de (T.W.); d.daum@hs-osnabrueck.de (D.D.)
[3] Department of Crop Sciences, Division Quality and Sensory of Plant Products, Georg-August-Universität Göttingen, 37075 Göttingen, Germany; susanne.neugart@uni-goettingen.de
[4] Department of Food Chemistry and Analysis, Institute of Food Technology and Food Chemistry, Technische Universität Berlin, TIB 4/3-1, Gustav-Meyer-Allee 25, 13355 Berlin, Germany
* Correspondence: rohn@tu-berlin.de; Tel.: +49-30-314-72583

Abstract: Notable parts of the population in Europe suffer from allergies towards apples. To address this health problem, the analysis of the interactions of relevant allergens with other substances such as phenolic compounds is of particular importance. The aim of this study was to evaluate the correlations between the total phenolic content (TPC), polyphenol oxidase (PPO) activity, antioxidant activity (AOA), and the phenolic compound profile and the content of the allergenic protein Mal d 1 in six apple cultivars. It was found that the PPO activity and the content of individual phenolic compounds had an influence on the Mal d 1 content. With regard to the important constituents, flavan-3-ols and phenolic acids, it was found that apples with a higher content of chlorogenic acid and a low content of procyanidin trimers and/or epicatechin had a lower allergenic potential. This is probably based on the reaction of phenolic compounds (when oxidized by the endogenous PPO) with proteins, thus being able to change the conformation of the (allergenic) proteins, which further corresponds to a loss of antibody recognition. When apples were additionally biofortified with selenium, the composition of the apples, with regard to TPC, phenolic profile, AOA, and PPO, was significantly affected. Consequently, this innovative agronomic practice seems to be promising for reducing the allergenic potential of apples.

Keywords: apple; biofortification; selenium; antioxidant properties; phenolic compounds; polyphenol oxidase; Mal d 1; allergy

Citation: Groth, S.; Budke, C.; Weber, T.; Neugart, S.; Brockmann, S.; Holz, M.; Sawadski, B.C.; Daum, D.; Rohn, S. Relationship between Phenolic Compounds, Antioxidant Properties, and the Allergenic Protein Mal d 1 in Different Selenium-Biofortified Apple Cultivars (*Malus domestica*). *Molecules* **2021**, *26*, 2647. https://doi.org/10.3390/molecules26092647

Academic Editors: Tuba Esatbeyoglu and Banu Bayram

Received: 8 April 2021
Accepted: 29 April 2021
Published: 30 April 2021

1. Introduction

Apples contain important compounds that are of health-beneficial relevance. Besides vitamins, a diverse set of minerals and trace elements are present in the fruits [1–4]. Furthermore, the fruits are rich in secondary plant metabolites, especially flavonoids and phenolic acids [5,6]. In in vitro studies, apple extracts and isolated compounds, especially oligomeric procyanidins, have been shown to influence several mechanisms of cancer development [7]. The consumption of apples is recommended for a healthy diet, as they are hypothesized to reduce the risk of stroke, as well as cardiovascular disease and lung cancer [5,6,8].

However, eating apples can also provoke allergic reactions [9–12]. Most commonly, there are symptoms that primarily occur in patients with hay fever [13]. About 70% of birch pollen allergy sufferers also show allergic symptoms towards apples because of

the chemical-structural homology of the allergenic proteins Bet v 1 and Mal d 1, both belonging to the protein family PR-10 [14]. In a population-based study of young adults (between 20 and 44 years) in 13 European countries, the prevalence of a type I sensitization towards apples ranged from 0% in Iceland to 10.3% in Germany (overall average for all countries: 4.2%) [11]. Type I reaction describes an immediate-type allergy and includes IgE-mediated reactions [15]. Nationwide, approximately four million Germans are impaired by an apple allergy [16,17].

The identification of hypoallergenic apple cultivars is important for dietary recommendations, especially for patients suffering from severe symptoms of apple allergy [18,19]. For this reason, various aspects of apple allergy have been highlighted in numerous scientific studies since the early 1990s [16–35]. The assessment of the allergenic potential of an apple cultivar is a complex issue, as the allergen content of apples is influenced by various factors: the content of allergenic proteins depends primarily on the genotype [17,20–26] but is also influenced by the level of maturation, postharvest conditions, as well as cultivation conditions and practices, such as the use of selected fertilizers [21,22,24–29,36,37].

However, it is hypothesized that certain apple cultivars with comparatively higher total phenolic contents (TPC) are more tolerable with regard to allergenicity. In this context, it is further assumed that the interactions between the polyphenols and the allergenic protein Mal d 1 play an important role in reducing allergenic potential [13,16]. Such interactions can be of a different nature, depending on the structure of the phenolic compounds. Similar to protein–protein interactions, hydrogen, ionic, hydrophobic, and aromatic interactions can occur, leading to a change in the conformation of the proteins [38,39].

Some studies in the literature even describe dependencies on the polyphenol composition, the allergenicity (mainly with regard to Mal d 1), and the activity of polyphenol oxidases, with the latter significantly influencing polyphenol content and composition in apples [16,17,22,28]. Bernert et al. (2012) found a statistically significant correlation between TPC and apple allergenicity [16]. The results of previous studies reported that interactions between oxidized plant polyphenols with allergenic proteins are especially believed to reduce their allergenicity [30,31,40]. During peeling, crushing, or squeezing of the fruits, *p*- and *o*-dihydroxybenzene derivatives are oxidized to quinones, forming soluble and insoluble protein–phenolic complexes with Mal d 1, and thereby "inactivating" the protein [20,30,31,41,42]. Such reactions between polyphenols and proteins can even be between far larger crosslinked melanin-like compounds [39]. A high PPO activity favors the oxidation of phenolic compounds and, consequently, the suppression of the allergenic effect of Mal d 1 [27]. The study described by Kiewning et al. (2013) showed that the activity of PPO seems to be even more important than TPC for lowering the Mal d 1 content. At a high PPO activity, Mal d 1 was reduced, even when the TPC was low [27]. This assumption is supported by the studies described by Kschonsek et al. (2019). They observed that apple cultivars with a high polyphenol content and an equally high PPO activity have a lower allergenicity [20]. Apple cultivars with a high TPC provided a better tolerance. Schmitz-Eiberger et al. (2009) evaluated the relationship between Mal d 1 content, PPO activity, TPC, and antioxidant capacity. The results showed that higher PPO activities and TPCs lead to a diminished extractability of the allergenic proteins [22].

The two phenolic compound classes, phenolic acids and flavonoids, in particular seem to exhibit a high reactivity towards proteins, as many of their chemical structures are highly susceptible to oxidation [39]. These compounds are found in apples, and in some cultivars in particularly high concentrations [43–46]. Garcia et al. (2007) showed that the addition of the flavan-3-ols catechin and epicatechin can contribute to a reduction of allergenicity. Red-fleshed apple cultivars, which can accumulate phenolic compounds from the anthocyanin class, not only in the fruit peel, but also partially in the fruit flesh, proved to be particularly low in allergenicity [32,33]. Kschonsek et al. (2019) showed that high levels of chlorogenic acid, caffeic acid, and epicatechin were associated with a low concentration of sulfidoleucotrienes, which are synthesized and released by leucocytes after a contact with allergens [17].

As mentioned above, apple composition is influenced by all kinds of physical, chemical, and biological elicitations [47]. In a previous study, the influence of the biofortification of apples with selenium by foliar fertilization was investigated. There, it was found that the content and composition of phenolic compounds were significantly influenced by selenium [48]. In other experiments it was found that even the Mal d 1 content was reduced, in most cases, when the fruits were biofortified with selenate, while apple cultivar and ecophysiological conditions (e.g., climate) were identified as further influencing factors [34].

The aim of the present study was to investigate the relationship between antioxidant properties (as a measure of a phenolic compound's reactivity), phenolic compound composition, and the allergenic protein Mal d 1, when biofortifying with selenium. Selenium might be an interesting influencing factor in this case, as it is also a redox active trace element. For this purpose, six different apple cultivars from three consecutive growth seasons, and harvested in two different locations, were characterized. With this study, it might be possible to evaluate an innovative agronomic practice for enhancing polyphenols and selenium in apples, while at the same time reducing the content of allergenic proteins.

2. Results and Discussion

To analyze the strength and direction of the linear relationship between the Mal d 1 content and the phenolic compounds, as well as the related properties, correlation analyses were performed, and the coefficient of determination was calculated. Furthermore, the influence of a selenium biofortification was also investigated. The contents of the following parameters in the different selenium-biofortified apple samples and controls are shown in Tables S1 and S2 in the Supplementary Materials: selenium content, Mal d 1 content, PPO activity, TPC, content of individual phenolic compounds, and AOA measured by TEAC and ORAC. The biofortification resulted in a significant increase of the selenium content in the fruits, by a factor of 10 to 30 compared to the corresponding controls. Furthermore, the Mal d 1 content of the biofortified apples was reduced in most cases. Regarding the other parameters in the selenium-biofortified apples, a lower variation of PPO activity, higher TPCs upon application of selenite, and changes in the concentration of the major phenolic compounds, such as chlorogenic acid, the fraction of procyanidin trimers, and caffeoylglucoside were observed.

2.1. Correlation Analysis between Selenium Content and Mal d 1 Content

The correlation analyses showed no correlation between selenium and Mal d 1 content across all selenium-biofortified apple samples of the six cultivars analyzed. However, a negative correlation was found for most of the individual cultivars, and a high selenium content was therefore associated with a low Mal d 1 content. Variety-specific differences were found. The correlations also varied in strength, with correlation coefficients between 0.0244 and 0.7673 (Table 1). Biofortification with selenium resulted in significantly lower content of the allergenic protein Mal d 1 in 'Golden Delicious' and 'Boskoop', making these cultivars particularly suitable for a further targeted reduction of the Mal d 1 content by the applied agronomic approach. In the case of 'Jonagold', differences between the two cultivation seasons were observed, and a highly significant negative correlation was found for the year 2017. In contrast, a slight positive correlation was determined in the following year. The Mal d 1 content of the cultivars 'Jonica' and 'Elstar' was only reduced or increased to a small extent by the biofortification. As there was no correlation between Mal d 1 and selenium content in 'Fiesta', the Mal d 1 content of this variety was not affected in association to the biofortification approach.

Table 1. Correlation between Selenium Content and Mal d 1 Content.

Cultivar and Year of Cultivation	Correlation Coefficient Selenium—Mal d 1
All	−0.0154
'Fiesta' 2017	0.0244
'Jonica' 2017	−0.4099
'Golden Delicious' 2017	−0.6493 *
'Jonagold' 2017	−0.7673 **
'Boskoop' 2018	−0.7463 *
'Jonica' 2018	−0.3524
'Golden Delicious' 2018	−0.7318 *
'Jonagold' 2018	0.2491
'Elstar' 2019	0.3922

* $p \leq 0.05$; ** $p \leq 0.01$.

A comparison of the results with the literature can only be made to a limited extent, due to the lack of comparable studies. To the best of our knowledge, the influence of the selenium biofortification of plant foods on allergenic proteins had not been described previously. Nevertheless, a number of other factors have been analyzed for influence on the content of allergenic proteins in apples, including the cultivation system. Schmitz-Eiberger (2011) showed that apples from organic cultivation showed significantly higher Mal d 1 contents [22]. Furthermore, allergic persons showed a higher sensitivity when consuming such apples [29]. The organic cultivation of fruit trees leads to higher susceptibilities towards environmental stress factors such as fungal, bacterial, and viral attack, which were shown to result in a higher biosynthesis rate of Mal d 1 [36]. Mal d 1 is a pathogenesis-related protein, which is synthesized by fruits mainly for defense against such pathogens and occasionally as an response against certain environmental stress conditions [10,12,21]. Therefore, the results of the present study are in line with the previous findings. It is hypothesized that the application of selenium-containing fertilizers leads to the better protection of the fruits against certain stress factors, whereby only a lower synthesis rate of the Mal d 1 protein is required. The induction of further plant-protective substances, such as phenolic compounds, resulting from biofortification with selenium in apples [48] and other crops [49–55] has also been determined in previous studies. Furthermore, it has already been shown that selenium can protect plants from a range of abiotic stresses such as cold, drought, radiation, salinity, and heavy metals [56,57]. In such cases, it seems that the synthesis of the plant protecting protein Mal d 1 is no longer necessary and therefore reduced in its expression. The role of selenium is associated with the regulation of reactive oxygen species and the stimulation of antioxidant systems [57,58].

2.2. Relationship between PPO Activity and Mal d 1 Content

The analysis of the correlation between PPO activity and the Mal d 1 content of all investigated samples showed no correlation. There were also no correlations in a separate consideration of the two groups, "controls" and "selenium-biofortified apples" (Table 2). For the analysis of the correlation of the parameters for the individual cultivars, the following was found: for the cultivars 'Jonica' and 'Golden Delicious' from the cultivation year 2017 and 'Elstar' from the year 2019, a low Mal d 1 content was associated with a higher PPO activity (Figure 1A). For 'Jonica', the correlation was significant. In contrast, a positive correlation was found for the first two cultivars and for 'Boskoop' in the following year 2018 (Figure 1B). There was a high significance for 'Boskoop'. At this point, the hypothesis was made that the correlation between PPO activity and Mal d 1 content is influenced by genotype, as well as ecophysiological conditions. 'Fiesta' and 'Jonagold' showed no correlation between PPO activity and Mal d 1 content in all cultivation seasons.

Table 2. Relationship between PPO Activity, TPC, Antioxidant Activity (TEAC and ORAC), and Mal d 1 Content.

Cultivar and Year of Cultivation	Correlation Coefficient R^2 PPO—Mal d 1	Correlation Coefficient R^2 TPC—Mal d 1	Correlation Coefficient R^2 TEAC—Mal d 1	Correlation Coefficient R^2 ORAC—Mal d 1
All	−0.1164	0.0582	−0.1676	−0.0211
All control samples	−0.1635	−0.0115	−0.3207	−0.0375
All biofortified samples	−0.1524	0.1378	0.0006	−0.1382
'Fiesta' 2017	0.1463	0.0529	−0.1343	−0.4863
'Jonica' 2017	−0.7158 *	−0.4915	−0.3110	−0.3962
'Golden Delicious' 2017	−0.5614	−0.2115	−0.3889	0.1618
'Jonagold' 2017	−0.0444	0.1980	−0.4260	0.6741 *
'Boskoop' 2018	0.8589 **	−0.2949	−0.4697	0.0013
'Jonica' 2018	0.3496	−0.0322	0.0759	0.0767
'Golden Delicious' 2018	0.3847	0.5139	0.8740 *	0.3760
'Jonagold' 2018	−0.0296	−0.6023	−0.5536	0.4483
'Elstar' 2019	−0.4324	0.3780	0.4998	−0.2930
'Fiesta' 2017 Control	0.3081	−0.6859	−0.6037	−0.4795
'Fiesta' 2017 Selenium	−0.1074	0.5634	0.0820	−0.9576 ***
'Jonica' 2017 Control	−0.9364	−0.4338	−0.3799	−0.3814
'Jonica' 2017 Selenium	−0.5521	−0.4679	−0.2717	0.1818
'Golden Delicious' 2017 Control	0.5215	−0.4503	−0.4439	−0.5066
'Golden Delicious' 2017 Selenium	−0.4871	−0.7232 *	−0.5940	0.1390
'Jonagold' 2017 Control	−0.1373	0.7581	0.8501	−0.6730
'Jonagold' 2017 Selenium	0.7316 *	0.1126	0.5074	0.7491 *
'Boskoop' 2018 Control	0.7455	−0.7328	−0.8318	−0.3367
'Boskoop' 2018 Selenium	0.2508	−0.1612	−0.2445	0.7092
'Jonica' 2018 Control	0.0281	−0.6060	−0.4973	−0.6569
'Jonica' 2018 Selenium	0.8033	0.7256	0.7631	0.4636
'Golden Delicious' 2018 Control	−0.5166	0.9170	0.7411	0.3569
'Golden Delicious' 2018 Selenium	−0.8993	0.4206	0.8222	−0.9424
'Jonagold' 2018 Control	0.6978	−0.9217	−0.8754	−0.4095
'Jonagold' 2018 Selenium	−0.9821 *	−0.7753	−0.6820	0.6942
'Elstar' 2019 Control	−0.2390	0.1797	0.1275	−0.1005
'Elstar' 2019 Selenium	−0.4857	0.3917	0.5802	−0.3532

* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Among others, 'Jonica' and 'Golden Delicious' were cultivated in 2017 and 2018. Consequently, a comparison between the years of cultivation can be made to analyze the differences in the correlation between PPO activity and the Mal d 1 content. Here, controls and selenium-biofortified samples were included. Both cultivars were found to have a significantly higher PPO activity and lower Mal d 1 content in 2018 compared to the previous year (Table 3). When the data of the individual apples were used for the correlation analysis, an inverse correlation between PPO activity and Mal d 1 content resulted.

In the two years of cultivation, there were different climatic conditions in the apple orchard in Osnabrück, Germany. Compared to the previous year, a significantly higher sunshine duration (+37%) and a significantly lower precipitation (−61%) was recorded for the year 2018 [59].

A negative correlation between PPO activity and Mal d 1 content has been reported for different apple cultivars [20,22,27,32]. In one of their studies, Garcia et al. (2007) investigated the correlation of these parameters in 'Golden Delicious' and 'Jonagold' and conducted experiments on 'Golden Delicious', where an excess of exogenous PPO was added to the apple samples. It was shown that the treatment with PPO reduced allergenicity in the form of a lower IgE-binding capacity of Mal d 1 [32]. Schmitz-Eiberger et al. (2011) also analyzed the relationship between the Mal d 1 content and the PPO activity. Fruits of

the three apple cultivars 'Braeburn', 'Topaz', and 'Golden Delicious' were used. The results of that study showed that a higher PPO activity led to a diminished extractability of Mal d 1 [22]. Kiewning et al. (2013) also performed correlation analyses between Mal d 1 content and PPO activity of different cultivars. 'Elstar' and 'Diwa' showed a high correlation, while the correlation for fruits of 'Boskoop' was only moderate [27]. Likewise, Kschonsek et al. found this type of correlation for six different apple cultivars, including 'Golden Delicious'. Determining the Mal d 1 content and PPO activity after a 60-min oxidation period of the fruits showed a strong decrease of Mal d 1 content, associated with a high PPO activity, as well [20].

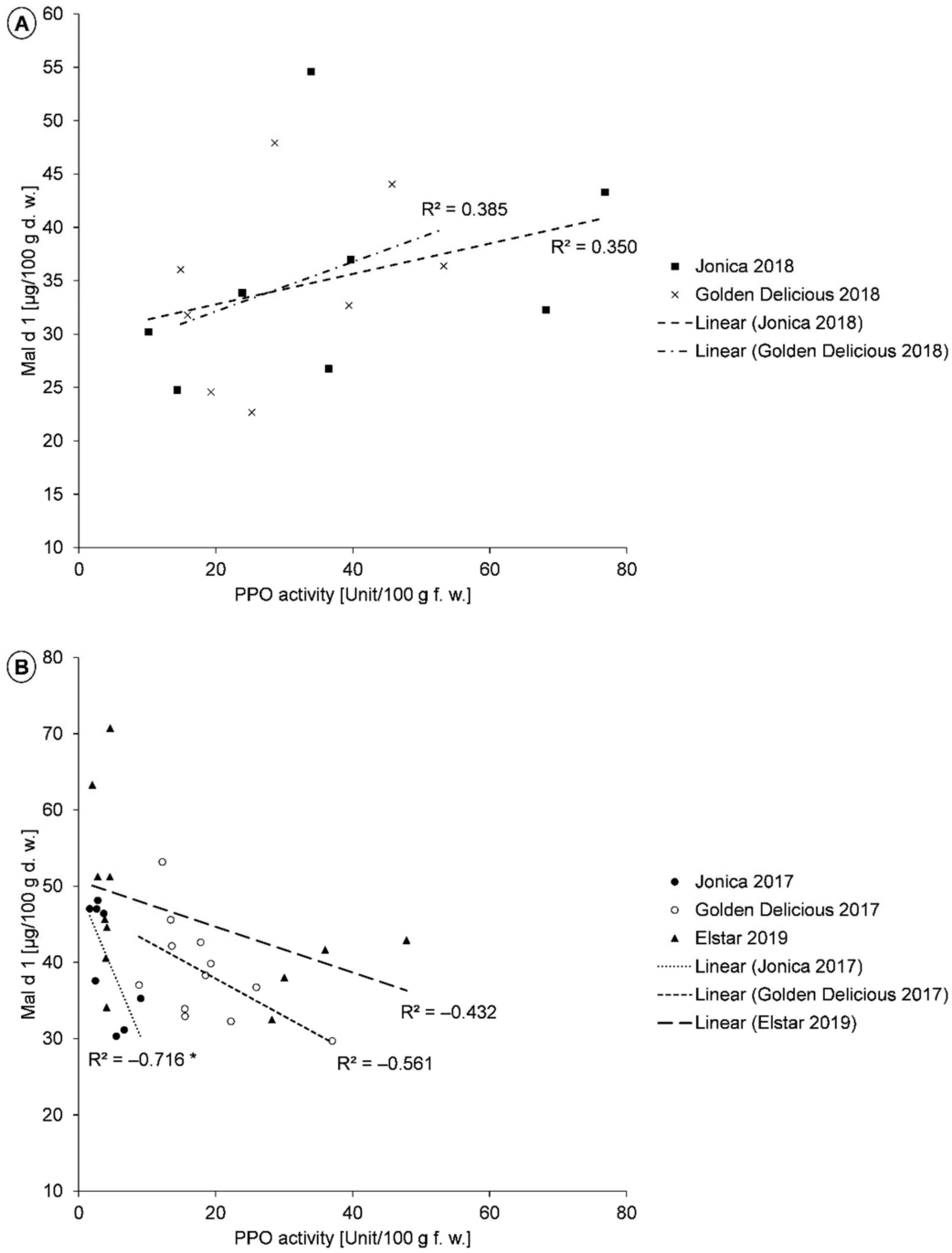


Figure 1. A,**B**. Correlation between PPO activity and Mal d 1 content of the cultivars 'Jonica' (2018), $n = 8$, 'Golden Delicious' (2018), $n = 12$, 'Jonica' (2017), $n = 8$, 'Golden Delicious' (2017), $n = 12$, and 'Elstar' (2019), $n = 12$. The controls and biofortified samples are shown. Indication of the coefficient of determination R^2 for the respective cultivars. * $p \leq 0.05$.

Table 3. Correlation coefficients between the content of Mal d 1 and the different phenolic compounds.

Cultivar and Year of Cultivation	Mal d 1	Mal d 1	Mal d 1	Mal d 1	Mal d 1	Mal d 1
	Chlorogenic Acid	Epicatechin	Procyanidin Trimers	Caffeoyl Glucosides	Σ Phloretin Glucosides	Σ Quercetin Glycosides
All 2017	−0.0379	0.2277	0.5165 ***	−0.2685	−0.0361	−0.1151
All controls 2017	−0.3064	0.1077	0.4866	−0.3484	0.3345	0.3230
'Fiesta' Control 2017	−0.9558 *	0.7474	0.9904 **	0.6394	−0.4251	−0.8979
'Jonica' Control 2017	0.4068	0.2401	−0.9204	0.0662	0.7636	−0.6081
'Golden Delicious' Control 2017	−0.5851	−0.8429	−0.6738	−0.2393	−0.0145	−0.7521
'Jonagold' Control 2017	−0.8553	0.5818	0.6812	−0.3038	0.6768	0.6710
All biofortified 2017	0.2869	0.3325	0.4929 **	−0.1586	−0.2357	−0.1782
'Fiesta' Selenium 2017	0.2344	0.8735 *	0.4806	0.1037	−0.6540	−0.6743
'Jonica' Selenium 2017	−0.0262	0.0871	0.6946	−0.6670	−0.2565	−0.0444
'Golden Delicious' Selenium 2017	0.6621	0.5236	0.6252	0.6760	0.8206 *	0.7218 *
'Jonagold' Selenium 2017	0.3544	−0.0528	0.4626	−0.0715	−0.0557	0.2322

* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

A decrease in Mal d 1 content or IgE-binding capacity and the accompanied reduced immunoreactivity seem to result from the reaction of *o*-quinones, deriving from the oxidation of phenolic compounds, with the proteins. As PPO catalyzes this reaction, a high enzyme activity leads, accordingly, to high *o*-quinone contents. These in turn can lead to an irreversible change in the tertiary structure of the allergen by modifying the nucleophilic amino acid side chains of the proteins, with the possibility of follow-up polymerizations [60]. Due to these cross-linkages, conformational epitopes of the allergen get lost, which reduces or even eliminates allergenicity [32,41,61].

To investigate the influence of selenium biofortification on allergenicity, correlation analyses of the individual cultivars were performed for the controls and the biofortified samples (Table 2). No consistent effects were found across all cultivars. For 'Fiesta' and 'Golden Delicious' from the year 2017, and 'Jonagold' from the year 2018, the biofortification led to a change in correlation towards negative values. For 'Jonagold' (2017) and 'Jonica' (2018), a change towards a positive correlation was observed for the selenium-biofortified samples. 'Golden Delicious' from the year 2018 and 'Elstar' from the year 2019 showed a stronger negative correlation for the biofortified samples compared to the controls. The correlation between PPO activity and Mal d 1 content was only significant for 'Jonagold'.

2.3. Analysis of the Relation between TPC and Mal d 1 Content

The analysis of the correlation between TPC and Mal d 1 showed no correlation, when considering all samples, and comparing "control" and "selenium biofortification" (Figure 2). A separate analysis of the individual cultivars showed only a weak negative correlation for 'Jonica' (2017) and 'Jonagold' (2018), and only a weak positive correlation for 'Golden Delicious' (2018) and 'Elstar' (2019) (Table 2). No correlation was of statistical significance. At this point, no trend was identified. It was therefore assumed that TPC alone does not, or only to a small extent, influence the content of allergenic proteins.

In line with this, Kiewning et al. (2013) and Kschonsek et al. (2019b) concluded that TPC plays only a minor role with regard to Mal d 1 content. In contrast, the activity of PPO proved to be more important for the reduction of Mal d 1. At high PPO activity, Mal d 1 activity can be reduced, even when the TPC is low [20–27].

According to the consistent results of several studies, there is an inverse relationship between TPC and the allergenicity of apples [17,22]. Bernert et al. (2012) analyzed the cultivars 'Red Boskoop' and 'Golden Delicious', among others, and found that apple cultivars with a high content of total polyphenols provided a better tolerance for apple allergy sufferers [16]. Kschonsek et al. (2019a) detected an inverse correlation between high TPC and low in vitro allergenicity of apples [17]. One of the first attempts to evaluate the relationship between Mal d 1 content and PPO, TPC, and antioxidant capacity in different

apple cultivars was reported by Schmitz-Eiberger et al. (2011). Their results showed that higher PPO activity and TPC lead to a diminished extractability of the allergenic protein Mal d 1 [22]. It is assumed that oxidative reactions between apple polyphenols and the allergen are responsible [30,31]. The reduction in allergenicity could be due to the masking of IgE-binding sites on the allergenic protein, through cross-linking of proteins induced by oxidative enzymes [39,41]. PPO is the main factor involved in these oxidative reactions in fruit [32]. A decrease in the allergenic potential of the protein Pru av 1 in the presence of polyphenols and PPO was also observed in cherries [61].

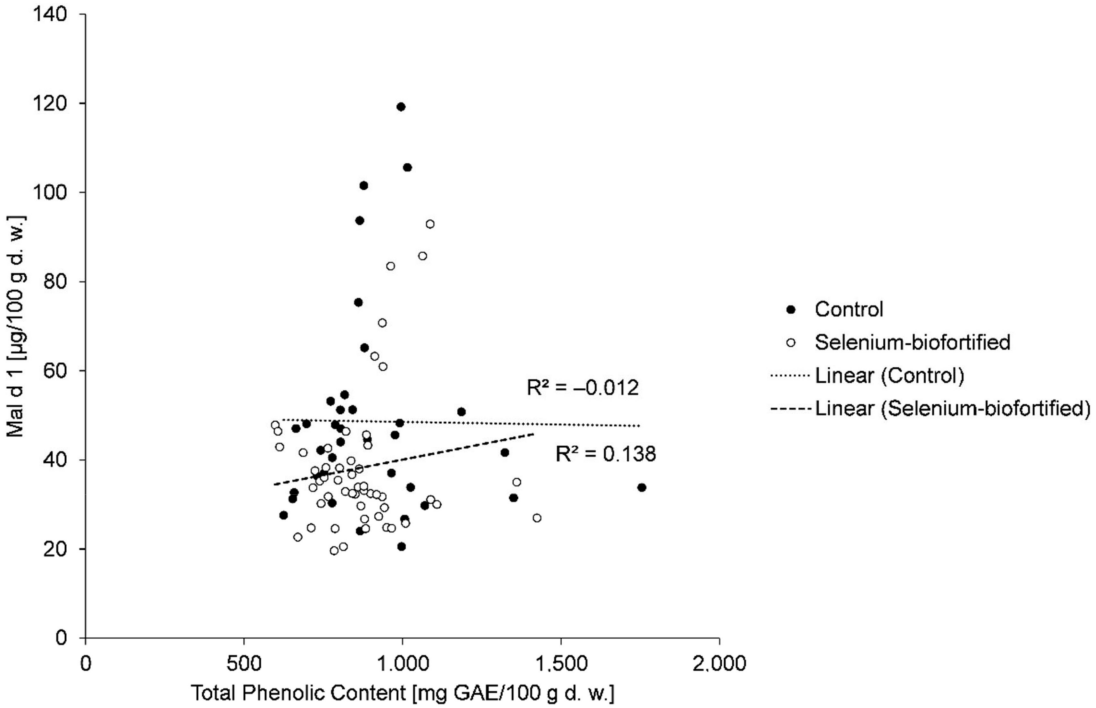


Figure 2. Correlation between TPC and Mal d 1 of all control samples (n = 36) and of all selenium-biofortified samples (n = 52).

The biofortification of apples with selenium did not result in any consistent effects across cultivars with regard to the relationship between TPC and PPO activity. For example, a change in correlation from negative values (as estimated for the controls) to a positive correlation was observed in the biofortified samples of the cultivar 'Fiesta' from the year 2017. This effect also occurred for 'Jonica' (2018). 'Golden Delicious' from the year 2017 showed a significantly higher negative correlation, while all other cultivars showed only marginal differences in correlation between the two parameters.

2.4. Individual Phenolic Compounds Influence the Content of Mal d 1

A qualitative and quantitative analysis of the phenolic compounds of the apple samples from the cultivation year 2017 was performed by HPLC-MS^n. The following compounds were detected: the dihydrochalcones phloretin-2-xylosyl-glucoside and phloretin-2-glucoside, the flavan-3-ol epicatechin, a procyanidin dimer and a fraction of procyanidin trimers, the hydroxycinnamic acid derivatives caffeoylglucoside and chlorogenic acid, as well as the flavonols quercetin-3-*O*-galactoside, quercetin-3-*O*-xyloside, and quercetin-3-*O*-glucoside.

The main compounds in apples are chlorogenic acid, the sum of the quercetin glycosides, the sum of the two phloretin glucosides, and epicatechin. Significant differences were found between the cultivars, especially in the content of chlorogenic acid and quercetin glycosides (Table S2, Supplementary Materials).

The cultivar 'Fiesta' was characterized above all by a high proportion of chlorogenic acid (40%). The other cultivars only had proportions of 21–27%. Furthermore, differences appeared in the proportion of epicatechin: 'Fiesta' contained an average of 15%, while the others had only 9–10%. With regard to the phloretin glucosides and the quercetin glycosides, 'Fiesta' contained significantly less of these, at 8% and 23%, compared with 12–14% and 28–41% for the other cultivars, respectively.

Kschonsek et al. (2018) also reported high levels of chlorogenic acid in various apple cultivars. For this purpose, they analyzed the old cultivars 'Ontario' and 'Dülmener Rosenapfel' and the comparatively newer cultivars 'Braeburn' and 'Granny Smith' and found significant differences between the old and the new cultivars. Regarding the profile of phenolic compounds, chlorogenic acid was the main polyphenol in the old apple cultivars with a percentage of around 63%. The new apple cultivars 'Braeburn' and 'Granny Smith', on the other hand, contained a significantly lower proportion of chlorogenic acid, amounting for 15.4% [2].

In the present study, correlation analyses were performed for the main individual phenolic compounds and Mal d 1 content (Table 3). Across all samples, without considering cultivar or biofortification, the correlation coefficient between Mal d 1 content and the individual phenolic compounds was highest for the fraction of procyanidin trimers, followed by the caffeoylglycosides. For the more complex procyanidins, there was a positive correlation, with a high significance; samples with a higher content of procyanidin trimers also had a higher content of Mal d 1. In contrast, there was an inverse correlation for caffeoylglycosides and Mal d 1.

A separate analysis of the correlation between the individual phenolic compounds and the allergenic potential for the controls of each cultivar showed different relationships, depending on the cultivar. For chlorogenic acid, a negative correlation was found for 'Fiesta', 'Golden Delicious', and 'Jonagold' (Figure 3A). High levels of epicatechin were observed in association with high Mal d 1 levels for 'Fiesta' and 'Jonagold', whereas there was a negative correlation for 'Golden Delicious' (Figure 3B). Regarding the fraction of procyanidin trimers, a positive correlation was observed for 'Fiesta' and 'Jonagold' and a negative correlation for 'Jonica' and 'Golden Delicious' (Figure 3C). The correlation coefficients of caffeoylglucosides and Mal d 1 were low ($-0.35 \geq R^2 \leq 0.06$), except for 'Fiesta'. Therefore, the content of this phenolic compound probably plays only a minor role with regard to the allergenic potential. The sum of phloretin glucosides correlated positively with the Mal d 1 content in 'Jonica' and 'Jonagold'. Furthermore, a negative correlation was observed between the sum of quercetin glycosides and the Mal d 1 content in all cultivars, except 'Jonagold'.

The correlation between individual phenolic compounds and the Mal d 1 content of numerous cultivars has already been determined and described in the literature [16,17,20,27,35]. Kiewning et al. (2013) analyzed the abovementioned parameters for the cultivars 'Elstar', 'Diwa', and 'Boskoop' and found a low to moderate correlation between catechin, as well as epicatechin, and Mal d 1 content. In contrast to 'Elstar' and 'Boskoop', the correlation between Mal d 1 and catechin, as well as epicatechin, of the cultivar 'Diwa' was negative [27]. Moreover, in the present study, low to moderate correlation coefficients were found with regard to epicatechin, as well as different dependencies on cultivar.

Bernert et al. (2012) performed an analysis of the correlation between the content of phenolic compounds and the apple allergy tolerance for different cultivars, including 'Golden Delicious'. They identified chlorogenic acid as the main polyphenol in all apple cultivars tested. A statistical evaluation showed a negative correlation between the chlorogenic acid content and the tolerance claims. When apples contained high levels of chlorogenic acid, they were better tolerated by allergy sufferers [16]. The present study confirmed this relationship to a large extent, since in most varieties a high chlorogenic acid content was correlated with a low content of Mal d 1. Due to this, a better tolerance is assumed.

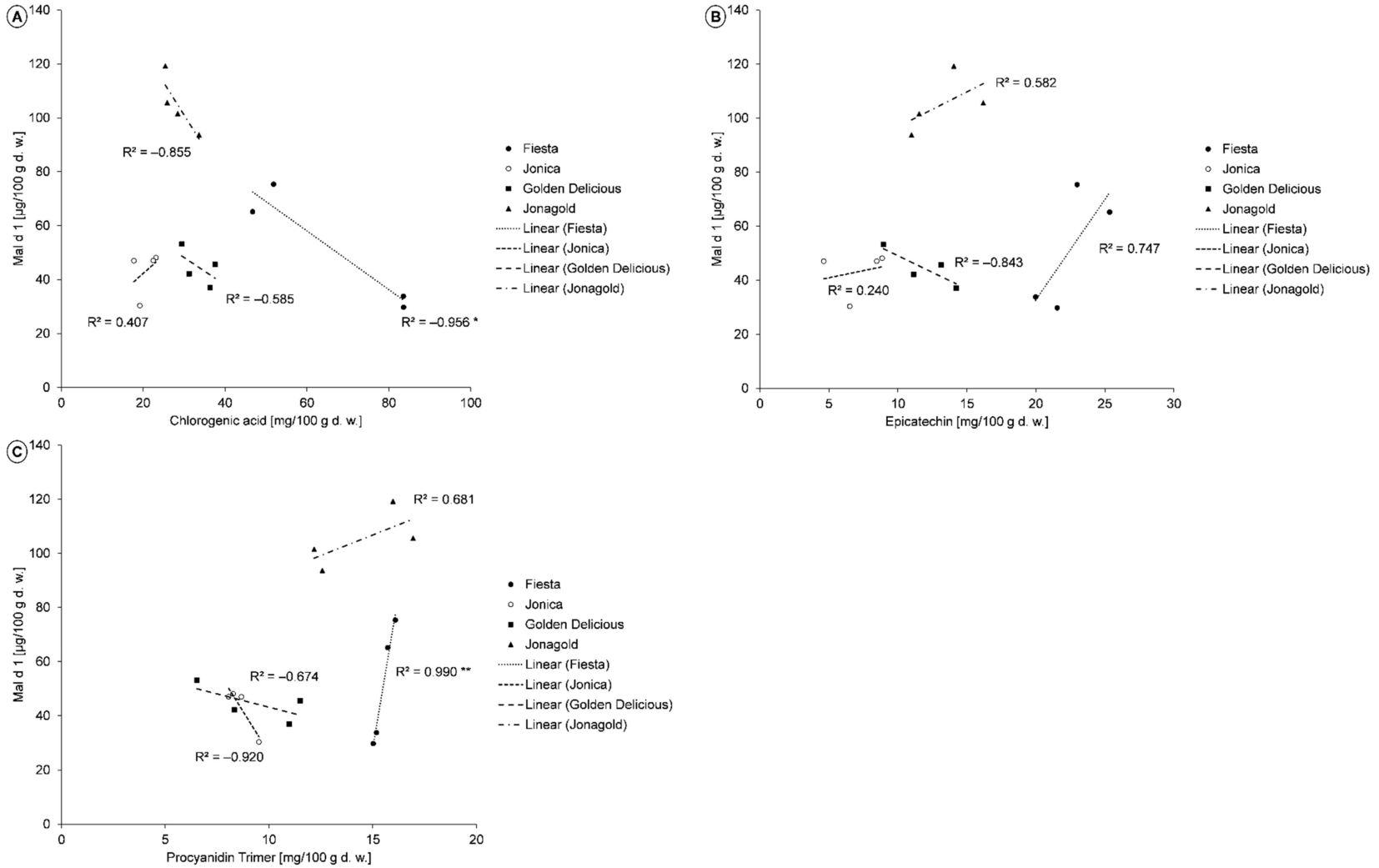


Figure 3. A–C. Correlation between the individual phenolic compounds and the Mal d 1 content in apple samples of the cultivars 'Fiesta', 'Jonica', 'Golden Delicious', and 'Jonagold', $n = 4$ for each variety. (**A**) Chlorogenic acid; (**B**) epicatechin; (**C**) procyanidin trimer. * $p \leq 0.05$; ** $p \leq 0.01$.

Kschonsek et al. (2019b) conducted experiments on the influence of enzymatic browning with regard to in vitro allergenicity in two old and two new apple cultivars and drew conclusions on the relationship between phenolic compounds and allergenic potential. A more intense enzymatic browning occurred in the cultivar 'Ontario' compared to 'Dülmener Rosenapfel'. At the same time, a 25% higher decrease in TPC was observed for 'Ontario'. This may have been due to the higher content of total flavanols (50%) and total hydroxycinnamic acids (15%), as the phenolic compound classes are very good substrates for PPO [20,27,62]. The higher degree of browning was associated with a lower in vitro allergenicity. Correlation analyses showed that high levels of chlorogenic acid, caffeic acid, and epicatechin were associated with the lower in vitro allergenicity of the apples [20]. The present study could only partially confirm these results. Thus, a negative correlation between chlorogenic acid and Mal d 1, which is directly related to allergenicity, was also found in three of the four varieties analyzed. Caffeic acid was not identified in the apple samples. In comparison to the study by Kschonsek et al., high levels of epicatechin associated with low Mal d 1 contents were observed only in Golden Delicious [20]. In contrast, the varieties Fiesta, Jonica, and Jonagold showed a positive correlation of these two parameters. These differences can be explained by the different cultivars.

In a recent study by Romer et al. (2020), the correlation between the phenolic profile and Mal d 1 content was investigated in 16 different apple cultivars. No correlation with the allergen content was found with regard to the levels of flavonols, anthocyanins, and phenolic acids. The flavan-3-ols catechin and epicatechin, as well as the procyanidins B1, B3, and a non-specified procyanidin, showed a high positive correlation with the allergen content [35]. As already explained, the present study was able to confirm the positive correlation between the content of epicatechin and Mal d 1. Variety-specific differences were present with regard to procyanidins. A positive correlation was also observed for 'Fiesta' and 'Jonagold', whereas a low procyanidin content was correlated with a low Mal d 1 content in 'Golden Delicious' and 'Jonagold'.

The allergenicity of apples seems to be mostly influenced by the procyanidins, as well as their monomer epicatechin, and chlorogenic acid. However, there are differences between the varieties. Here, a low procyanidin and epicatechin content, as well as a high content of chlorogenic acid, had an enhanced effect on Mal d 1 content, since low levels of the allergen were present here. With regard to cultivars being generally low in allergens, cultivars with a low procyanidin and epicatechin content and a high chlorogenic acid content seem to, therefore, be advantageous. As only very low correlation coefficients were measured between the other phenolic compounds and Mal d 1, the content of these substances probably had no influence on the overall allergenic potential of the apples.

In most cases, biofortification resulted in lower procyanidin and epicatechin contents and higher levels of chlorogenic acid associated with a lower Mal d 1 content. Therefore, this agronomic practice seems to be suitable for the reduction of allergenic potential. Polyphenols, and especially their oxidation products, quinones, are among the most reactive ingredients in apples. The reaction of phenolic compounds, as phenoxy radicals, quinones, or semiquinone radicals, results in irreversible interactions with proteins [38,39]. The oxidative degradation of phenolic compounds catalyzed by PPO leads to the formation of *o*-quinones (Figure 4).

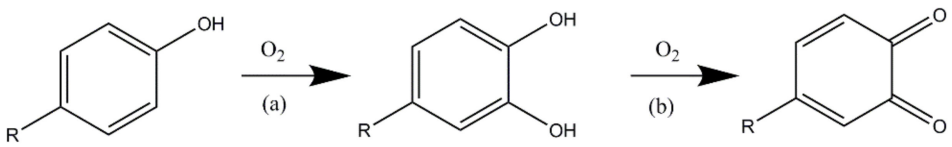


Figure 4. Reactions of PPO as (**a**) monophenolase: in the presence of oxygen, the hydroxylation of phenol derivatives to catechols is catalyzed. (**b**) *o*-Diphenolase activity: the catechols are oxidized to *o*-quinones by the activity of PPO.

The *o*-quinones are very reactive, they can subsequently form dimers/oligomers/ polymers with other phenolic compounds (brown colored melanins), as well as adducts with proteins. The oligomers, in turn, can be re-oxidized and covalently crosslink proteins [38,39].

The potential anti-allergenic properties of the phenolic compounds are based on different molecular mechanisms: On the one hand, the tertiary structure of the proteins can be altered to produce a lack of antibody recognition. This can be caused either by the polyphenols themselves, their oxidized forms (*o*-quinones), or even more directly by PPO. First of all, polyphenols can act as ligands for the hydrophobic cavity [35,63,64]. Due to structural similarity, PPO can use the phenolic amino acid tyrosine as a substrate, in addition to other phenolic compounds. When tyrosine is in the protein structure of the allergens oxidized, there can be a formation of covalent crosslinks within the protein(s) and, consequently, a conformational change and a loss of antibody recognition [20,35,65]. Another mechanism concerns the influence of phenolic compounds on mast cells and the prevention of histamine secretion [27,35,42,66]. Thus, polyphenols are able to influence the binding between IgE antibodies and the FCεRI receptor on the mast cell surfaces [23–67], resulting in a lower amount of released histamine and, thus, in a lower allergic recruitment [35].

Furthermore, interactions between the phenolic compounds and the allergenic proteins are possible, which can influence digestion in the gastrointestinal tract and, thus, inactivating the allergenic effect. Thus, protein–phenol adducts can be formed, which are enzymatically less digestible [38,39]. As already described above, irreversible bonds between phenolic compounds and proteins can be formed, whereby phenolic compounds are oxidized into quinones, which in turn can react with nucleophilic groups of the protein molecule. These interactions can affect the structure, functionality, and quality of the proteins, while bioavailability can also be affected by reduced digestibility in the gastrointestinal tract [68,69].

2.5. Relationship between AOA and Mal d 1

AOA is a measure for the reactivity of phenolic compounds. In the present study, it was determined using the two well-known assays TEAC and ORAC, which are based on different reaction mechanisms and, thus, allow a broader measurement of the AOA and reactivity, respectively. With regard to the determination of AOA in phenol-rich samples such as apples, the TEAC approach is well established. The stable ABTS-$^{+}$ radical used here reacts rapidly with antioxidants and many phenolic compounds with low redox potential. When using the ORAC assay, AOA can be measured over a longer period via the antioxidant inhibition being induced by exogenous peroxyl radicals, and representing a biologically relevant mechanism. The potential effects of secondary antioxidant compounds can also be measured and underestimation can be prevented [70].

Correlation analyses between the AOA measured with the TEAC assay and the Mal d 1 content showed no correlation for all samples, and for the selenium-biofortified samples, in particular (Table 2). However, the controls showed an inverse correlation, with the AOA being higher at low Mal d 1 levels. The analysis of the individual cultivars showed a positive correlation for 'Golden Delicious' from the year 2018 and 'Elstar'. For all other cultivars, an inverse correlation of varying degree was observed (Table 2). Furthermore, correlation analyses were performed between the ORAC value and the Mal d 1 content. However, the correlation between AOA and Mal d 1 content was weakly negative in all samples, as well as in all controls and in all selenium-biofortified samples (Table 2). 'Golden Delicious' and 'Jonagold' showed a positive correlation between ORAC values and Mal d 1 contents in both years of cultivation (Figure 5A), whereas a negative relation was present for 'Fiesta', 'Jonica', and 'Elstar' (Figure 5B). Only in the case of 'Jonagold' rom 2017 was the correlation of statistical significance.

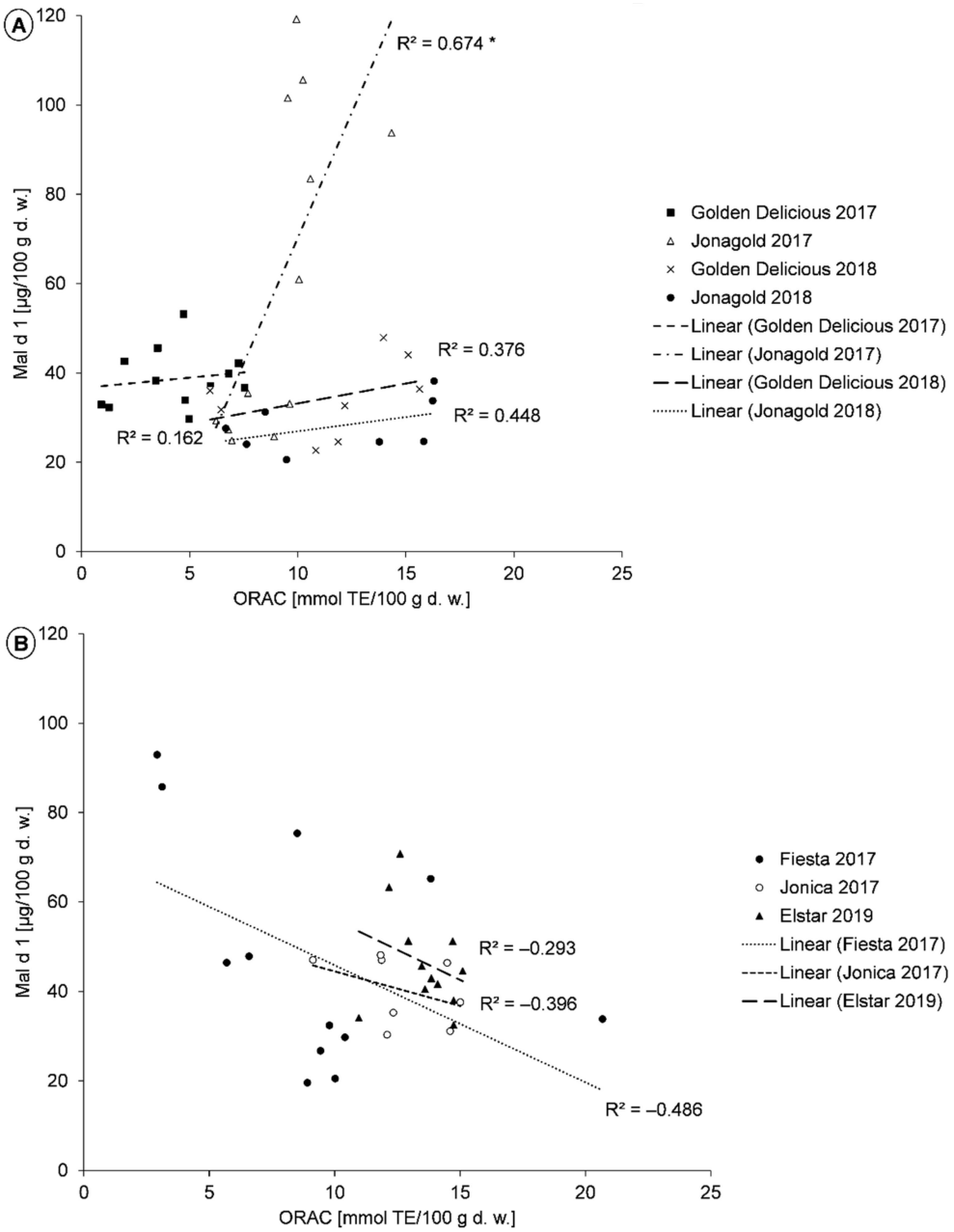


Figure 5. A,**B**. Correlation between ORAC-values and Mal d 1 contents in the apple fruits of the cultivars (**A**) 'Golden Delicious' and 'Jonagold', harvested in 2017 (n = 12 for each variety) and 2018 (n = 8 for each variety) n Osnabrück; (**B**) 'Fiesta' (n = 12), and 'Jonica' (n = 8), harvested 2017 in Osnabrück and 'Elstar' (n = 12), harvested 2019 in Jork. * $p \leq 0.05$.

In addition to the TPC and the PPO activity, AOA can be assigned to a certain role in apple allergenicity [22,27,31,32,40,71,72]. Garcia et al. (2007) and Schmitz-Eiberger et al. (2011) investigated in their studies the relationship between AOA and allergenicity in 'Golden Delicious' apples. They found that AOA and allergenicity were positively correlated [22,32]. This correlation was only partially observed in the present study. Based on the data available, it is clear that a positive correlation is only valid for individual cultivars. This applies for example to 'Golden Delicious' and 'Jonagold'.

Garcia et al. (2007) treated apples of the cultivar 'Golden Delicious' with the synthetic antioxidant dietyldithiocarbamic acid (DIECA). DIECA was added to the samples in sodium phosphate buffer or in succinate-lactate buffer and incubated for 5 to 24 h. A significant inhibition of the IgE-binding of Mal d 1 was found to result from an inhibition of the complex reaction between oxidized phenolic compounds and Mal d 1. The Mal d

1 content in the samples treated with DIECA was higher than the controls. Compared to the controls, the IgE-binding of Mal d 1 in the DIECA-treated samples did not decrease as much, due to a parallel inhibition of further endogenous enzymes [32].

Schmitz-Eiberger et al. (2011) determined the antioxidant capacity of the three apple cultivars 'Braeburn', 'Topaz', and 'Golden Delicious'. Unfortunately, they did not specify the method or specific values for AOA in their publication. Regarding the relationship between the AOA, the PPO activity, and the Mal d 1 content, it was found that the Mal d 1 content and the AOA were lowest and the PPO activity was highest in 'Braeburn'. For 'Golden Delicious', the three parameters were in a medium range. For 'Topaz', a high TPC, a high catechin content, a relatively low PPO activity, and a high AOA were measured. Schmitz-Eiberger et al. (2011) assumed that the IgE-binding of Mal d 1 was reduced by the low progression of oxidative processes (low PPO activity) or by the inhibition of these processes resulting from a high AOA. The authors found that a higher PPO activity and TPC resulted in a diminished extraction of the protein Mal d 1, whereas higher AOA inhibited the interactions between oxidized phenolic compounds and Mal d 1. This results in a higher allergenicity and a "normal" extractability of Mal d 1 [22,72].

With regard to AOA measured by TEAC, different changes were found between the respective controls and biofortified samples. Thus, a consistent trend by biofortification with selenium was excluded. The evaluation of the correlations between the ORAC value and the Mal d 1 content, depending on the selenium biofortification, showed a trend across several cultivars. A positive correlation was found for the biofortified samples of 'Jonica', 'Golden Delicious', and 'Jonagold' from the year 2017, and for 'Boskoop', 'Jonica', and 'Jonagold' from the year 2018. However, the correlation was negative in the corresponding controls.

3. Materials and Methods

3.1. Chemicals

Disodium hydrogen phosphate dodecahydrate was purchased from Bernd Kraft GmbH (Duisburg, Germany). Sodium dihydrogen phosphate monohydrate and 3,3′,5,5′-tertamethylbenzidine were from AppliChem GmbH (Darmstadt, Germany). Catechol was from ThermoFisher GmbH (Kandel, Germany). Aceton and ethanol were purchased from VWR International LLC (Fontenay-sous-Bois, France). Bovine serum albumin (BSA), citric acid monohydrate, hydrochloric acid (25%), hydrogen peroxide (30%), sodium chloride, and Tween® 20 were purchased from Carl Roth GmbH & Co. KG (Karlsruhe, Germany). Potassium dihydrogen phosphate, sodium carbonate, and sulphuric acid were purchased from Grüssing GmbH (Filsum, Germany) and potassium peroxodisulphate was from Fisher Scientific UK Ltd. (Loughborough, UK). Folin-Ciocalteu's phenol reagent, nitric acid (65%), polyvinylpyrrolidone, potassium dihydrogen phosphate, and sodium diethyldithiocarbamat were from Merck KgaA (Darmstadt, Germany). Galllic acid and 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH) were from Fisher Scientific GmbH (Schwerte, Germany). 2,2′-Azino-bis-(3-ethylbenzthiazoline−6-sulfonic acid) diammonium salt (ABTS), trolox, and fluorescein were purchased from Sigma-Aldrich Chemie GmbH (Deisenhofen, Germany). All of the chemicals were of analytical grade. Water was purified using a Milli-Q water system (PURELAB®, Elga LabWater, Veolia Water Technologies GmbH, Celle, Germany) and used for buffers, extraction solvents, and for the dilution of sample extracts.

3.2. Sample Material

For the analysis of the relationship between antioxidant properties, phenolic compounds, and the allergenic protein Mal d 1, six different apple cultivars, grown in three subsequent years in two different locations, were characterized. Apples of the cultivars 'Fiesta', 'Golden Delicious', 'Jonagold', and 'Jonica' were cultivated in 2017 at the Horticultural Research Station of the Osnabrück University of Applied Sciences, Germany (52°31′06.5″N 8°02′84.4″E; 69 m a.s.l.). In the following year, the cultivars 'Boskoop',

'Golden Delicious', Jonagold', and 'Jonica" were cultivated in Osnabrück as well. In 2019, apples of the cultivar 'Elstar' were cultivated in an orchard of a commercial fruit farm in the "Alte Land" region, Jork, Germany (53°30′37.4"N 9°44′44.6"E; 4 m a.s.l.). The location conditions in Osnabrück and Jork and the design of the field experiments have been already described [34,48]. The apple trees were biofortified with a total of 0.075–0.450 kg selenium per hectare and at a meter canopy height (Se/ha x m CH) by applying foliar sprays. Apples of the cultivar 'Fiesta' were sprayed once every two weeks before the harvest in 2017. All other cultivars were treated repeatedly (2–7 times) between mid-June and the end of September. The last application always took place at least two weeks before harvest.

The detailed composition of the selenium-containing fertilizers used and the equipment for application have already been described by Groth et al. [34]. The selenium content was determined in air-dried, ground material of fresh apple samples, while the activity of the polyphenoloxidase was measured in frozen and thawed samples. All other parameters were determined in lyophilized apples. Freeze-drying was performed after homogenization, as described in Groth et al. [48]. For the determination of the selenium content, a sample set of ten randomly chosen apples per treatment and repetition was analyzed. For the determination of the other parameters, a sample set of four randomly chosen apples per treatment and repetition was analyzed.

3.3. Determination of the Polyphenol Oxidase (PPO) Activity

PPO activity was determined as described by Groth et al. [48]. About 10 g of the frozen sample was weighed, crushed in a mortar, and mixed with 25 mL of a phosphate buffer (0.05 M, pH 7.0). The subsequent incubation time was 120 min at 4 °C, in the dark. The supernatant obtained after centrifugation (15 min, 4 °C, 3225 g) was used for photometric measurement in a 96-well microtiter plate. First, 30 µL of the sample extract was pipetted into a well and either 270 µL of a phosphate buffer (0.2 M, pH 5.5) as blank sample or 270 µL of a catechol solution as appositive control (0.1 M in 0.2 M phosphate buffer, pH 5.5) was added. Measurement over a period of 10 min was performed at a wavelength of λ = 420 nm at 25 °C with a microplate reader (BioTek Synergy HT, BioTek Instruments Inc., Winooski, VT, USA). The change in absorbance was recorded every 60 s. The enzyme activity of the samples was expressed as activity units per 100 g of fresh weight (f.w.), where one unit was defined as the change of 0.01 in the absorbance value per minute [48].

3.4. Method for Extracting Phenolic Compounds

The phenolic compounds were extracted from the apple samples using the method according to Groth et al. [48]. For this, 60 mg of the lyophilized sample was mixed with 1 mL of extraction solvent (50% aqueous acetone and 0.1% HCl (*v/v*)) and treated in an ultrasonic bath (5 min, 30 °C). Four glass beads (i. d. 4 ± 0.3 mm) were added and the sample was ground and mixed in a ball mill (5 min, 25 Hz) (RETSCH® MM 400, Retsch GmbH, Haan, Germany) and then centrifuged (5 min, 20,817 g). Three treatments were carried out with the ball mill. The supernatants were combined and filled up to a volume of 4 mL [48].

3.5. Determination of the Total Phenolic Content (TPC) according to Folin-Ciocalteu

The TPC was evaluated using a modified Folin-Ciocalteu method [48]. Twenty microliters of the sample extract was mixed with 100 µL Folin-Ciocalteu phenol reagent (1:10; *v/v*) and 80 µL of an aqueous 7.5% (*w/v*) sodium carbonate solution in a 96-well microtiter plate and incubated in the dark for 2 h. The photometric determination of TPC was performed at a wavelength of λ = 765 nm with a microplate reader (BioTek Synergy HT). TPC values are given in gallic acid equivalents per 100 g of dry weight (mg GAE/100 g d.w.) [48].

3.6. Identification and Quantification of Single Phenolic Compounds Using High Performance Liquid Chromatography Mass Spectrometry (HPLC-MS)

Extraction of phenolic compounds from the lyophilized apple samples was carried out with 60% aqueous methanol in a triple extraction, according to Groth et al. (2020a) and Neugart et al. (2017) [48,73]. Phenolic compound identification and quantification were determined using an 1100 series HPLC system (Agilent Technologies GmbH, Waldbronn, Germany) equipped with an Ascentis® Express F5 column (150 mm × 4.6 mm, 5 µm, Sigma-Aldrich Chemical Co., St. Louis, MO, USA) and a photodiode array detector. Eluent A was 0.5% acetic acid, and eluent B was 100% acetonitrile, used in a gradient modus. The wavelengths of 280 nm, 320 nm, and 370 nm were used for the determination of phloretin glycosides and flavan−3-ols, hydroxycinnamic acid derivatives, and non-acylated flavonol glycosides, respectively. The hydroxycinnamic acid derivatives and flavonoid glycosides (chlorogenic acid, catechin, epicatechin, phloretin-2-*O*-glucoside, and quercetin-3-*O*-glucoside) were identified as deprotonated molecular ions and characteristic mass fragment ions, according to Schmidt et al. (2010) [74], by HPLC-DAD-ESI-MS^n with an Agilent ion trap mass spectrometer (Agilent Technologies Deutschland GmbH, Waldbronn, Germany) in negative ionization mode. The results are presented as mg/100 g dry weight [48].

3.7. Analysis of the Antioxidant Activity (AOA) Using the Trolox Equivalent Antioxidant Capacity Assay (TEAC) and the Oxygen Radical Absorbance Capacity Assays (ORAC)

AOA was determined by TEAC and ORAC assays. As both are based on different reaction mechanisms, more information about the AOA and reactivity of the phenolic compounds can be made than when using only one assay. The measurements were performed as described by Groth et al. [48]. For determining TEAC, a solution was first prepared with the $ABTS^+$ radical and diluted with a phosphate buffer (75 mM, pH 7.4) for reaching an absorbance of E_{730} = 0.700 ± 0.050 (ABTS working solution II). Twenty microliters of various dilutions of the samples, trolox for calibration, or water (blank value) was applied in a 96-well microtiter plate, and then 200 µL of ABTS working solution II was added. The absorption was measured after 6 min incubation at 30 °C and a wavelength of λ = 730 nm with the BioTek Syngergy HT microplate reader. AOA was calculated as trolox equivalent per 100 g dry weight (mmol TE/100 g d.w.) [48].

For the determination of the ORAC, 10 µL of each sample, trolox, or water was applied in a 96-well microtiter plate. Thirty-five microliters of a fluorescein solution (1.2 µM) was added. Subsequently, 100 µL phosphate buffer, or 250 µL in the case of the negative control, was added. After a 10 min-incubation period at 37 °C in the BioTek Synergy HT microplate reader, 150 µL of an AAPH solution (c = 129 mM) was added to the blank value, standards, and samples. The measurement, which is based on fluorescence quenching, was performed at 37 °C, an excitation wavelength of λ = 485 nm, and an emission wavelength of λ = 528 nm. The course of the reaction was recorded for 120 min, with one measurement every two minutes. AOA was also calculated as trolox equivalent per 100 g dry weight (mmol TE/100 g d.w.) [48].

3.8. Extraction of Proteins

For the determination of the Mal d 1 content, proteins were first extracted from the lyophilized apple samples using the method described by Groth et al. [34]. For this purpose, 1.0 g was weighed into grinding bowls and 15 mL of a Björksten extraction buffer with some modifications was added [40]. The addition of sodium azide was omitted, due to its inhibitory effect on the polyclonal, HRP-labeled goat anti-mouse antibody used for the measurement of the Mal d 1 content by direct ELISA (MERCK KGAA, 2019). Extraction was performed with a ball mill for 10 min at 25 Hz (RETSCH® MM 400). Then, samples were transferred into 15 mL-tubes and subsequent centrifugation was performed (10 min, 20,817× *g*). The supernatant was transferred to another 15 mL-tube and extraction was repeated twice more using the ball mill. All supernatants were combined, concentrated to

a volume of 3–4 mL by a gaseous stream of nitrogen, and filled up to 5 mL in a volumetric flask with the phosphate buffer solution [34].

3.9. Determination of the Mal d 1 Content Using ELISA

The use of a direct ELISA for the determination of the Mal d 1 content has already been described by Groth et al. [34]. First, a 1:10 dilution of the sample extracts was prepared and 10 µL thereof was pipetted into a 96-well microtiter plate. These were further diluted by adding 190 µL of a Björksten extraction buffer and incubated for 22 h at 4 °C. For calibration, 200 µL of a recombinant, commercially available Mal d 1 solution (2 µg/mL, Biomay AG, Vienna, Austria) was added to each well. A calibration series from 0.1 to 2.0 µL/mL was prepared. After incubation, washing was repeated five times with 300 µL PBS-T buffer each time (PBS buffer: sodium chloride 0.034 mmol/L, potassium hydrogen phosphate 0.016 mmol/L; pH 7.0; + 0.5% Tween 20). A 1% BSA solution was added as blocking reagent and incubated for 2 h at room temperature. Washing was then done five times with 300 µL PBS-T solution each. Then, 200 µL of a HRP-labelled goat anti-mouse antibody (goat anti-mouse IgG antibody, peroxidase conjugated, H+L, Merck KGaA, Darmstadt, Germany) [75] was added and incubated for 18 h at 4 °C. For the preparation of the reaction solution, 10 mL of a citric acid buffer (6.327 g/L citric acid monohydrate in bidest. water, pH 4.1) was mixed with 0.5 mL of a TMB reagent (2.410 g/L 3,3′,5,5′-tertamethylbenzidine, 0.5 mL hydrogen peroxide (30%), 100 mL acetone, and 900 mL ethanol) [76]. Two-hundred microliters were pipetted into every well and incubated for 90 min at room temperature in the dark. As stop solution, 50 µL sulphuric acid (2 M), was added and the photometric measurement was performed at λ = 450 nm at 30 °C in a microplate reader (BioTek Synergy HT). The Mal d 1 content was given in mg/100 g f.w. [34].

3.10. Statistical Analysis

The number of analyses per application with selenium fertilizer or control was $n = 2$. All of the analyses were repeated twice. The data in the Supplementary Materials, Tables 1 and 2, are given in mean ± standard deviation and were further evaluated using Microsoft Excel (Microsoft Office Professional Plus 2016, Redmond, WA, USA). To test the correlation between the individual parameters, correlation analyses were performed, also using Microsoft Excel, and the coefficient of determination R^2 was determined.

4. Conclusions

Several influencing factors have been identified regarding the allergenicity of apples with Mal d 1 content as a measure. In particular, PPO activity and the content of individual phenolic compounds, such as chlorogenic acid, epicatechin, and the fraction of procyanidin trimers, were related to the Mal d 1 content. Biofortification of apples with selenium seems to be promising as an agronomic practice for reducing the allergenic potential of apples. The molecular mechanisms are mainly based in the phenol–protein interactions, where the *o*-quinones resulting from oxidation by PPO lead to an irreversible conformational change of the allergens. As a result, the conformal epitopes of the allergen are affected and allergenicity is reduced. Consequently, it seems to be valuable to take apples into account that already have a low content of allergenic proteins, to biofortify them with selenium, and stimulate TPC formation in this way.

Supplementary Materials: The following are available online, Table S1: Results of the determination of the selenium content, Mal d 1 content, polyphenol oxidase activity, total phenolic content, and antioxidant activity in all apple samples. Table S2: Results of the determination of phenolic compounds using HPLC-MS^n.

Author Contributions: Methodology, S.G.; software, S.G.; validation, S.G.; formal analysis, S.G., C.B., T.W., S.N., S.B., M.H., B.C.S.; resources, D.D., S.R.; data curation, C.B., S.G.; writing—original draft preparation, S.G.; writing—review and editing, C.B., D.D., S.N., S.R.; visualization S.G.; supervision,

D.D., S.R.; project administration, D.D., S.R.; funding acquisition, D.D., S.R. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Federal Ministry of Education and Research (BMBF), grant number FKZ: 031B0299A, B, and C.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Acknowledgments: The authors gratefully acknowledge Hartmut Schlüter and his team (Department of Clinical Chemistry, Mass Spectrometric Proteomics, University Medical Center Hamburg-Eppendorf, Hamburg, Germany) for the nanoLC-MS/MS analyses.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study, in the collection, analyses, or interpretation of data, in the writing of the manuscript, or in the decision to publish the results.

Sample Availability: Samples of the compounds are available from the authors.

References

1. Hyson, D.A. A comprehensive review of apples and apple components and their relationship to human health. *Adv. Nutr.* **2011**, *2*, 408–420. [CrossRef]
2. Kschonsek, J.; Wolfram, T.; Stöckl, A.; Böhm, V. Polyphenolic compounds analysis of old and new apple cultivars and contribution pf polyphenolic profile to the in vitro antioxidant capacity. *Antioxidants* **2018**, *7*, 20. [CrossRef]
3. Souci, S.W.; Fachmann, W.; Kraut, H. *Lebensmitteltabelle für die Praxis*, 5th ed.; Wissenschaftliche Verlagsgesellschaft: Stuttgart, Germany, 2011; pp. 873–876.
4. Tsao, R.; Yang, R.; Xie, S.; Sockovie, E.; Khanizadeh, S. Which polyphenolic compounds contribute to the total antioxidant activities of apple? *J. Agric. Food Chem.* **2005**, *53*, 4989–4995. [CrossRef]
5. Knekt, P.; Jarvinen, R.; Reunanen, A.; Maatela, J. Flavonoid intake and coronary mortality in Finland: A cohort study. *Br. Med. J.* **1996**, *312*, 478–481. [CrossRef]
6. Le Marchand, L.; Murphy, S.P.; Hankin, J.H.; Wilkens, L.R.; Kolonel, L.N. Intake of flavonoids and lung cancer. *J. Natl. Cancer Inst.* **2000**, *92*, 154–160. [CrossRef]
7. Gerhauser, C. Cancer chemopreventive potential of apples, apple juice, and apple components. *Planta Med.* **2008**, *74*, 1608–1624. [CrossRef]
8. Knekt, P.; Isotupa, S.; Rissanen, H.; Heliovaara, M.; Jarvinen, R.; Hakkinen, S.; Aromaa, A.; Reunanen, A. Quercetin intake and the incidence of cerebrovascular disease. *Eur. J. Clin. Nutr.* **2000**, *54*, 415–417. [CrossRef]
9. Botton, A.; Lezzer, P.; Dorigoni, A.; Barcaccia, G.; Ruperti, B.; Ramina, A. Genetic and envirnonmental factors affecting allergen-related gene expression in apple fruit (*Malus domestica* L. Borkh). *J. Agric. Food Chem.* **2008**, *56*, 6707–6716. [CrossRef]
10. Grafe, C. Apfelallergie: Aktueller Wissensstand und Ausblick. *Obstbau* **2009**, *34*, 618–620.
11. Burney, P.G.J.; Potts, J.; Kummeling, I.; Mills, E.N.C.; Clausen, M.; Dubakiene, R.; Barreales, L.; Fernandez-Perez, C.; Fernandez-Rivas, M.; Le, T.-M.; et al. The prevalence and distribution of food sensitization in European adults. *Allergy* **2014**, *69*, 365–371. [CrossRef]
12. Breiteneder, H.; Ebner, C. Molecular and biochemical classification of plant-derived food allergens. *J. Allergy Clin. Immunol.* **2000**, *106*, 27–36. [CrossRef] [PubMed]
13. Bergmann, K.; Zuberbier, J.; Zuberbier, T.; Zapp, J.; Hennebrüder, W. Apfelallergie—Toleranzentwicklung durch regelmäßigen Konsum allergenarmer Äpfel. Eine Beobachtungsstudie. *Erwerbs-Obstbau* **2020**, *62*, 264–273. [CrossRef]
14. Gilissen, L.J.W.J.; Bolhaar, S.T.H.P.; Matos, C.I.; Rouwendal, G.J.A.; Boone, M.J.; Krens, F.A.; Zuidmeer, L.; van Leeuwen, A.; Akkerdaas, J.; Hoffmann-Sommergruber, K.; et al. Silencing the major apple allergen Mal d 1 by using the RNA interference approach. *J. Allergy Clin. Immunol.* **2005**, *115*, 364–369. [CrossRef]
15. Gell, P.; Coombs, R. The classification of allergic reactions underlying disease. In *Clinical Aspects of Immunology (Chapter 13)*; Blackwell Scientific Publications: Oxford, UK, 1963.
16. Bernert, T.; Münstermann, J.; Kothe, A.; Zapp, J. *Polyphenolgehalt in Alten und Neuen Apfelsorten im Bezug auf Allergene Wirkungen*; Hochschule Ostwestfalen-Lippe: Lemgo, Germany, 2012.
17. Kschonsek, J.; Wiegand, C.; Hipler, U.C.; Böhm, V. Influence of polyphenolic content on the in vitro allergenicity of old and new apple cultivars: A pilot study. *Nutrition* **2019**, *58*, 30–35. [CrossRef]
18. Marzban, G.; Puehringer, H.; Dey, R.; Brynda, S.; Ma, Y.; Martinelli, A.; Zaccarini, M.; van der Weg, E.; Housley, Z.; Kolarich, D.; et al. Localisation and distribution of the major allergens in apple fruits. *Plant Sci.* **2005**, *169*, 387–394. [CrossRef]
19. Carnés, J.; Ferrer, A.M.; Fernández-Caldas, E. Allergenicity of 10 different apple varieties. *Ann. Allergy Asthma Immunol.* **2006**, *96*, 564–570. [CrossRef]
20. Kschonsek, J.; Dietz, A.; Wiegand, C.; Hipler, U.C.; Böhm, V. Allergenicity of apple allergen Mal d 1 as effected by polyphenols and polyphenol oxidase due to enzymatic browning. *LWT* **2019**, *113*, 108289. [CrossRef]

21. Matthes, A.; Schmitz-Eiberger, M. Apple (*Malus domestica* L. Borkh.) Allergen Mal d 1: Effect of Cultivar, Cultivation System, and Storage Conditions. *J. Agric. Food Chem.* **2009**, *57*, 10548–10553. [CrossRef] [PubMed]
22. Schmitz-Eiberger, M.; Matthes, A. Effect of harvest maturity, duration of storage and shelf life of apples on the allergen Mal d 1, polyphenoloxidase activity and polyphenol content. *Food Chem.* **2011**, *127*, 1459–1464. [CrossRef]
23. Son, D.; Lee, S. Comparison of the characteristics of the major allergen Mal d 1 according to apple varieties. *Food Sci. Biotechnol.* **2001**, *10*, 132–136.
24. Zuidmeer, L.; van Leeuwen, W.A.; Kleine Budde, I.; Breiteneder, H.; Ma, Y.; Mills, C.; Sancho, A.I.; Meulenbroek, E.J.; van de Weg, E.; Gilissen, L.; et al. Allergenicity Assessment of Apple Cultivars: Hurdles in Quantifying Labile Fruit Allergens. *Int. Arch. Allergy Immunol.* **2006**, *141*, 230–240. [CrossRef]
25. Sancho, A.I.; Foxall, R.; Browne, T.; Dey, R.; Zuidmeer, L.; Marzban, G.; Waldron, K.W.; van Ree, R.; Hoffmann-Sommergruber, K.; Laimer, K.; et al. Effect of postharvest storage on the expression of the apple allergen Mal d 1. *J. Agric. Food Chem.* **2006**, *54*, 5917–5923. [CrossRef]
26. Bolhaar, S.T.; van de Weg, W.E.; van Ree, R.; Gonzalez-Mancebo, E.; Zuidmeer, L.; Bruijnzeel-Koomen, C.A.; Fernandez-Rivas, M.; Jansen, J.; Hoffmann-Sommergruber, K.; Knulst, A.C. In vivo assessment with prick-to-prick testing and double-blind, placebo-controlled food challenge of allergenicity 930 of apple cultivars. *J. Allergy Clin. Immunol.* **2005**, *116*, 1080–1086. [CrossRef] [PubMed]
27. Kiewning, D.; Baab, G.; Schmitz-Eiberger, M. Effect of 1-MCP treatment on the apple (*Malus domestica* L. Borkh.) allergen Mal d 1 during long-term storage. *LWT* **2013**, *53*, 198–203. [CrossRef]
28. Kiewning, D.; Schmitz-Eiberger, M. Effects of long-term storage on Mal d 1 content of four apple cultivars with initial low Mal d 1 content. *J. Sci. Food Agric.* **2014**, *94*, 798–802. [CrossRef] [PubMed]
29. Klockenbring, T.; Bez, C.; Klowat, B.; Liappis, N.; Köpke, U.; Noga, G.; Zielen, S.; Goerlich, R. Allergenic potential of apple cultivars from organic and integrated fruit production. *Allergy Suppl.* **2001**, *68*, 889.
30. Rudeschko, O.; Fahlbusch, B.; Henzgen, M.; Schlenvoigt, G.; Herrmann, D.; Vieths, S. Investigation of the stability of apple allergen extracts. *Allergy* **1995**, *50*, 575–580. [CrossRef] [PubMed]
31. Rudeschko, O.; Fahlbush, B.; Henzgen, M.; Schlenvoigt, G.; Herrmann, D.; Jager, L. Optimization of apple allergen preparation for in vitro and in vivo diagnostics. *Allergy* **1995**, *50*, 262–268. [CrossRef]
32. Garcia, A.; Wichers, J.H.; Wichers, H.J. Decrease of the IgE-binding by Mal d 1, the major apple allergen, by means of polyphenol oxidase and peroxidase treatments. *Food Chem.* **2007**, *103*, 94–100. [CrossRef]
33. Nothegger, B.; Reider, N.; Covaciu, C.E.; Cova, V.; Ahammer, L.; Eidelpes, R.; Unterhauser, J.; Platzgummer, S.; Tollinger, M.; Letschka, T.; et al. Allergen-specific immunotherapy with apples: Selected cultivars could be a promising tool for bich pollen allergy. *J. Eur. Acad. Dermatol. Venerol.* **2020**. [CrossRef]
34. Groth, S.; Budke, C.; Weber, T.; Oest, M.; Brockmann, S.; Holz, M.; Daum, D.; Rohn, S. Selenium biofortification of different varieties of apples (*Malus domestica*)—Influence on protein content and the allergenic proteins Mal d 1 and Mal d 3. 2021; submitted.
35. Romer, E.; Chebib, S.; Bergmann, K.C.; Plate, K.; Becker, S.; Ludwig, C.; Meng, C.; Fischer, T.; Dierend, W.; Schwab, W. Tiered approach for the identification of Mal d 1 reduced, well tolerated apple genotypes. *Sci. Rep.* **2020**, *10*, 9144. [CrossRef] [PubMed]
36. Fernández-Rivas, M.; Bolhaar, M.; González-Mancebo, E.; Asero, R.; van Leeuwen, A.; Bohle, B.; Ma, Y.; Ebner, C.; Rigby, N.; Sancho, A.I. Apple allergy across Europe: How allergen sensitization profiles determine the clinical expression of allergies to plant foods. *J. Allergy Clin. Immunol.* **2006**, *118*, 481–488. [CrossRef] [PubMed]
37. Wang, J.; Vanga, S.K.; Raghavan, V. Effect of pre-harvest and post-harvest conditions on the fruit allergenicity: A review. *Crit. Rev. Food Sci. Nutr.* **2017**. [CrossRef] [PubMed]
38. Rawel, H.M.; Kroll, J.; Rohn, S. Reactions of phenolic substances with lysozyme—Physicochemical characterisation and proteolytic digestion of the derivatives. *Food Chem.* **2001**, *72*, 59–71. [CrossRef]
39. Rohn, S. Possibilities and limitations in the analysis of covalent interactions between phenolic compounds and proteins. *Food Res. Int.* **2014**, *65*, 13–19. [CrossRef]
40. Björksten, F.; Halmepuro, L.; Hannuksela, M.; Lahti, A. Extraction and Properties of Apple Allergens. *Allergy* **1980**, *35*, 671–677. [CrossRef]
41. Chung, S.Y.; Champagne, E.T. Reducing the allergenic capacity of peanut extracts and liquid peanut butter by phenolic compounds. *Food Chem.* **2009**, *115*, 1345–1349. [CrossRef]
42. Singh, A.; Holvoet, S.; Mercenier, A. Dietary polyphenols in the prevention and treatment of allergic diseases. *Clin. Exp. Allergy* **2011**, *41*, 1346–1359. [CrossRef]
43. Rana, S.; Bhushan, S. Apple phenolics as nutraceuticals: Assessment, analysis and application. *J. Food Sci. Technol.* **2016**, *53*, 1727–1738. [CrossRef]
44. Dhyani, P.; Bahukhandi, A.; Rawat, S.; Bhatt, I.D.; Rawal, R.S. Diversity of bioactive compounds and antioxidant activity in Delicious group in Western Himalaya. *J. Food Sci. Technol.* **2018**, *55*, 2587–2599. [CrossRef]
45. Zardo, D.M.; Silva, K.M.; Guyot, S.; Nogueira, A. Phenolic profile and antioxidant capacity of the principal apples produced in Brazil. *Int. J. Food Sci. Nutr.* **2013**, *64*, 611–620. [CrossRef]
46. Wojdylo, A.; Oszmianski, J.; Laskowski, P. Polyphenolic compounds and antioxidant activity of new and old apple varieties. *J. Agric. Food Chem.* **2008**, *56*, 6520–6530. [CrossRef]

47. Mussaci, S.; Serra, S. Apple fruit quality: Overview in pre-harvest factors. *Sci. Hortic.* **2018**, *234*, 409–430. [CrossRef]
48. Groth, S.; Budke, C.; Neugart, S.; Ackermann, S.; Kappenstein, F.-S.; Daum, D.; Rohn, S. Influence of a selenium biofortification on antioxidant properties and phenolic compounds of apples (*Malus domestica*). *Antioxidants* **2020**, *9*, 187. [CrossRef] [PubMed]
49. Bachiega, P.; Salgado, J.M.; de Carvalho, J.E.; Ruiz, A.L.; Schwarz, K.; Tezotto, T.; Morzelle, M.C. Antioxidant and antiproliferative activities in different maturation stages of broccoli (*Brassica oleracea* var. *italica*) biofortified with selenium. *Food Chem.* **2016**, *190*, 771–776. [CrossRef] [PubMed]
50. D'Amato, R.; Proietti, P.; Onofri, A.; Regni, L.; Esposto, S.; Servili, M.; Businelli, D.; Selvaggini, R. Biofortification (Se): Does it increase the content of phenolic compounds in virgin olive oil (VOO)? *PLoS ONE* **2017**, *12*. [CrossRef] [PubMed]
51. D'Amato, R.; Fontanella, M.C.; Falcinelli, B.; Beone, G.M.; Bravi, E.; Marconi, O.; Benincasa, P.; Businelli, D. Selelium biofortification in rice (*Oryza sativa* L.) sprouting: Effects on Se yield and nutritional traits with focus on phenolic acid profile. *J. Agric. Food Chem.* **2018**, *66*, 4082–4090. [CrossRef]
52. Pezzarossa, B.; Remorini, D.; Gentile, M.L.; Massai, R. Effects of foliar and fruit addition of sodium selenate on selenium accumulation and fruit quality. *J Sci. Food Agric.* **2012**, *92*, 781–786. [CrossRef] [PubMed]
53. Schiavon, M.; dall'Acqua, S.; Mietto, A.; Pilon-Smits, E.A.H.; Sambo, P.; Masi, A.; Malagoli, M. Selenium fertilization alters the chemical composition and antioxidant constituents of tomato (*Solanum lycopersicon* L.). *J. Agric. Food Chem.* **2013**, *61*, 10542–10554. [CrossRef]
54. Schiavon, M.; Berto, C.; Malagoli, M.; Trentin, A.; Sambo, P.; Dall'Acqua, S.; Pilon-Smits, E.A.H. Selenium biofortification in radish enhances nutritional quality via accumulation of methyl-selenocystein and promotion of transcripts and metabolites related to glucosinolates, phenolics, and amino acids. *Front. Plant Sci.* **2016**, *7*, 1371. [CrossRef]
55. Zhao, Y.; Wu, P.; Wang, Y.; Feng, H. Different approaches for selenium biofortification of pear-jujube (*Zizyphus jujuba* M. cv. Lizao) and associated effects on fruit quality. *J. Food Agric. Environ.* **2013**, *11*, 529–534.
56. Gupta, M.; Gupta, S. An overview of selenium uptake, metabolism, and toxicity in plants. *Front. Plant Sci.* **2017**, *7*, 2074. [CrossRef]
57. Feng, R.; Wei, C.; Tu, S. The roles of selenium in protecting plants against abiotic stresses. *Environ. Experim. Bot.* **2013**, *87*, 58–68. [CrossRef]
58. Hawrylak-Nowak, B.; Hasanuzzaman, M.; Matraszek-Gawron, R. Mechanisms of selenium-induced enhancement of abiotic stress tolerance in plants. In *Plant Nutrients and Abiotic Stress Tolerance*; Springer: Singapore, 2018; pp. 269–295.
59. WETTERKONTOR GMBH, Monats-und Jahreswerte für Deutschland. Available online: https://www.wetterkontor.de/de/wetter/deutschland/monatswerte.asp?y=2018&m=15 (accessed on 8 September 2020).
60. Kroll, J.; Rawel, H.M.; Rohn, S. A review. Reactions of plant phenolics with food proteins and enzymes under special consideration of covalent bonds. *Food Sci. Technol. Res.* **2003**, *9*, 205–218. [CrossRef]
61. Gruber, P.; Vieths, S.; Wangorsch, A.; Nerkamp, J.; Hofmann, T. Maillard reaction and enzymatic browning affect the allergenicity of Pru av 1, the major allergen from cherry (*Prunus avium*). *J. Agric. Food Chem.* **2004**, *52*, 4002–4007. [CrossRef]
62. Rocha, A.M.C.N.; Morais, A.M.M.B. Characterization of polyphenol oxidase (PPO) extracted from 'Jonagored' apple. *Food Control* **2001**, *12*, 85–90. [CrossRef]
63. Ullah, C.; Unsicker, S.B.; Fellenberg, C.; Constabel, C.P.; Schmidt, A.; Gershenzon, J.; Hammerbacher, A. Flavan-3-ols are an effective chemical defense against rust infection. *Plant Physiol.* **2017**, *175*, 1560–1578. [CrossRef] [PubMed]
64. Casanal, A.; Zander, U.; Dupeux, F.; Valpuesta, V.; Marquez, J.A. Purification, crystallization and preliminary X-ray analysis of the strawberry allergens Fra a 1E and Fra a 3 in the presence of catechin. *Acta Cryst. F* **2013**, *69*, 510–514. [CrossRef] [PubMed]
65. Wu, Z.; Lian, J.; Han, Y.; Zhou, N.; Li, X.; Yang, A.; Tong, P.; Chen, H. Crosslinking of peanut allergen Ara h 2 by polyphenol oxidase: Digestibility and potential allergenicity assessment. *J. Sci. Food Agric.* **2016**, *96*, 3567–3574. [CrossRef]
66. Kanda, T.; Akiyama, H.; Yanagida, A.; Tanabe, M.; Goda, Y.; Toyoda, M.; Teshima, R.; Saito, Y. Inhibitory effects of apple polyphenol on induced histamine release from RBL-2H3 cells and rat mast cells. *Biosci. Biotechnol. Biochem.* **1998**, *62*, 1284–1289. [CrossRef] [PubMed]
67. Tokura, T.; Nakano, N.; Ito, T.; Matsuda, H.; Nagasako-Akazome, Y.; Kanda, T.; Ikeda, M.; Okumura, K.; Ogawa, H.; Nishiyama, C. Inhibitory effect of polyphenol-enriched apple extracts on mast cell degranulation in vitro targeting the binding between IgE and FcERI. *Biosci. Biotechnol. Biochem.* **2005**, *69*, 1974–1977. [CrossRef] [PubMed]
68. Jakobek, L. Interactions of polyphenols with carbohydrates, lipids, and proteins. *Food Chem.* **2015**, *175*, 556–567. [CrossRef]
69. Rohn, S.; Rawel, H.M.; Kroll, J. Inhibitory effects of plant phenols on the activity of selected enzymes. *J. Agric. Food Chem.* **2002**, *50*, 3566–3571. [CrossRef] [PubMed]
70. Prior, R.L.; Wu, X.; Schaich, K. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *J. Agric. Food Chem.* **2005**, *53*, 4290–4302. [CrossRef]
71. Vieths, S.; Janek, K.; Aulepp, H.; Petersen, A. Isolation and characterization of the 18-kDa major apple allergen and comparison with the major birch pollen allergen (Bet v I). *Allergy* **1995**, *50*, 421–430. [CrossRef] [PubMed]
72. Schmitz-Eiberger, M.; Weber, V.; Treutter, D.; Baab, G.; Lorenz, J. Bioactive components in fruits from different apple varieties. *J. Appl. Bot.* **2003**, *77*, 167–171.
73. Neugart, S.; Baldermann, S.; Ngwene, B.; Wesonga, J.; Schreiner, M. Indigenous leafy vegetables of Eastern Africa—A source of extraordinary secondary plant metabolites. *Food Res. Int.* **2017**, *100*, 411–422. [CrossRef] [PubMed]

74. Schmidt, S.; Zietz, M.; Schreiner, M.; Rohn, S.; Kroh, L.W.; Krumbein, A. Identification of complex, naturally occurring flavonoid glycosides in kale (*Brassica oleracea* var. *sabellica*) by high-performance liquid chromatography diode-array detection/electrospray ionization multistage mass spectrometry. *Rapid Commun. Mass Spectrom.* **2010**, *24*, 2009–2022. [CrossRef]
75. Merck KGaA. *Package insert for the goat-anti-mouse IgG antibody assay, conjugated with peroxidase (H+L)*; Sigma-Aldrich: Darmstadt, Germany, 2019.
76. Luttmann, W.; Bratke, K.; Küpper, M.; Myrtek, D. *Der Experimentator Immunologie*; Springer: Berlin-Heidelberg, Germany, 2014; pp. 111–118.

7. DISCUSSION

Biofortification of plant foods is well suited as an agronomic measure to increase the content of certain nutrients, especially minerals. With regard to selenium, it has already been shown in different crops such as rice, lentils, sweet basil, lettuce plant, tomato, radish, and pear-jujube that biofortification resulted in significantly higher contents (D'Amato et al., 2018; Ekanayake et al., 2015; Hawrylak-Nowak, 2008; Hawrylak-Nowak, 2013; Ríos et al., 2008; Schiavon et al., 2013; Schiavon et al., 2016; Zhao et al., 2013).

Increasing the mineral content is necessary and sensible to improve the supply of minerals to the population, as increasingly the soils in Germany and large parts of Europe are becoming depleted in the important minerals (Poňavič & Scheib, 2014). Especially vegetarian or vegan diets are therefore at risk of a deficiency of minerals such as selenium (Fallon & Dillon, 2020). Deficiency of selenium is associated with various symptoms such as reduced immune function, degeneration of the cardiovascular system, and cognitive decline (Gupta & Gupta, 2017; Kielliszek, 2019; Rayman, 2012) and can lead to the endemic disease Keshan and Keshin-Beck (Kielliszek, 2019).

Successful biofortification to increase the population's intake of selenium has already been achieved through a decision by the Ministry of Agriculture and Forestry in 1984 in Finland and subsequent implementation nationwide in the form of biofortification of plant foods with sodium selenate (Alfthan et al., 2015).

Secondary plant metabolites have an important role in human nutrition mainly due to their pharmacological effects (Lieberei & Reisdorff, 2012). Phenolic compounds represent the largest group of secondary plant metabolites and have anticarcinogenic, antioxidant, antimicrobial, immunomodulatory, and antithrombotic effects, based on the antioxidant properties of the substances (Belitz et al., 2008; Ebermann & Elmadfa, 2008; Hyson, 2011; Watzl & Leitzmann, 2005). Among others, it has already been observed that procyanidins are associated with a reduced cancer risk and flavonoids can prevent heart attacks due to their anti-inflammatory effects (Boyer & Liu, 2004; Bravo, 2009; Ebermann & Elmadfa, 2008). Both classes of compounds are abundant in apples (Lee et al., 2003; Neveu & Perez-Jiménez, 2010; Podsedek & Wilska-Jeszka, 2000; Valavandis & Vlochogianni, 2009; Vrhovsek et al., 2004; Wojdylo et al., 2008).

A high content of phenolic compounds in apples is thus desirable due to their nutritional effects. In numerous studies, biofortification with selenium - in addition to a significant

increase in selenium content - also resulted in an increase in phenolic compounds. Thus, in olives, higher levels of phenolic compounds and changes in the phenolic profile were observed, with the contents of certain antioxidant phenolic compounds increasing (D'Amato et al., 2017). An increase in antioxidant flavonoids was also observed in broccoli as well as tomatoes (Bachiega et al., 2016; Schiavon et al., 2013).

However, in addition to nutritive ingredients, apples also contain antinutritive substances such as allergenic proteins. In secondary IgE-mediated food allergy, vasoactive inflammatory mediators such as histamine are released during the allergic reaction, leading to different clinical symptoms (Gallin et al., 1992; Grafe, 2009; Kleine-Tebbe et al., 2016; Marzban et al., 2005; Paschke, 2008; Ring, 2007; von Baehr, 2017; Worm et al., 2014). In Northern and Central Europe, allergy to Mal d 1 is particularly common (Ballmer-Weber & Hoffmann-Sommergruber, 2011; Kleine-Tebbe & Jakob, 2015). Thus, in Germany, about 4 million people are affected by sensitization to apples (Bernert et al., 2012; Kschonsek et al., 2019).

So far, many factors could be related to the content of allergenic proteins in apples such as cultivar (Bolhaar et al., 2005; Kschonsek et al., 2019a; Matthes & Schmitz-Eiberger, 2009; Sancho et al., 2006a; Schmitz-Eiberger & Matthes, 2011; Son & Lee, 2001; Zuidmeer et al., 2006), storage duration and storage conditions (Bolhaar et al., 2005; Kiewning et al., 2013; Kiewning & Schmitz-Eiberger, 2014; Matthes & Schmitz-Eiberger, 2009; Schmitz-Eiberger & Matthes, 2011; Sancho et al., 2006a) as well as ecophysiological and cultivation conditions of the growing sites (Schmitz-Eiberger & Matthes, 2011; Wang et al., 2017; Zuidmeer et al., 2006) or organically cultivation (Fernández-Rivas et al., 2006) on allergenic protein levels in apples were identified.

The influence of selenium biofortification of apples on allergenic proteins has not yet been the subject of research. In other crops such as rice, winter jujube, peas, bread-making wheat, durum wheat, and upland rice, a relationship between selenium biofortification and protein biosynthesis has already been established (D'Amato et al., 2018; Jing et al., 2017; Poblaciones et al., 2013; Poblaciones et al., 2014a; Poblaciones et al., 2014b; Reis et al., 2018).

In the present work, the influence of selenium biofortification of apples on important value-giving metabolites of primary and secondary metabolism was analyzed. Due to the nutritional and health relevance, phenolic compounds as well as allergenic proteins are in the focus. Besides the analysis of different parameters of the selenium-biofortified apples and apples of the control groups as well as a subsequent statistical evaluation, the development of a direct

ELISA for the quantitative determination of the content of Mal d 1 was the subject of the present work.

7.1. Analysis of antioxidant properties and phenolic compounds in selenium biofortified apples

Analysis of antioxidant properties was performed using the TEAC and ORAC photometric assays. The total phenolic content according to FOLIN-CIOCALTEU was also measured photometrically (Müller et al., 2010; Singleton & Rossi, 1965). Subsequent qualitative and quantitative determination of phenolic compounds was performed by HPLC-DAD-ESI-MS^n. In addition, a determination of the enzyme polyphenoloxidase, which plays an important catalytic role in the oxidative degradation of phenolic compounds, was performed. In order to establish the relationship between selenium biofortification and the above parameters and to be able to analyze an influence on the level of selenium content in the fruits, the selenium content was also measured. This was done by means of GF-AAS at the Osnabrück University of Applied Sciences by the project partner.

The influence of selenium biofortification was evaluated on apple samples of six different cultivars, grown in three subsequent years in two different locations. Apples of the cultivars 'Fiesta', 'Golden Delicious', 'Jonagold', and 'Jonica' were cultivated in 2017 at the Horticultural Research Station of the Osnabrück University of Applied Sciences, Germany. In the following year, the cultivars 'Boskoop', 'Golden Delicious', 'Jonagold', and 'Jonica' were cultivated in Osnabrück as well. In 2019, apples of the cultivar 'Elstar' were cultivated in an orchard of a commercial fruit farm in the “Alte Land” region, Jork, Germany. Different selenium forms and application heights were used to find the optimal conditions to achieve the set aims. The biofortification was performed as a foliar application implemented in usual calcium fertilization. In 2017, the apples were biofortified with 0.15 kg selenium per hectare and meter canopy height (Se/ha x m CH) in the form of sodium selenite or sodium selenate with a hand-held spray system. Pure water was sprayed on the trees for the control treatments. In 2018, the application rate of the fertilizer was reduced to 0.075 kg Se/ha x m CH due to slight fruit damage occurring in the year 2017. In this year, only sodium selenate was applied using a backpack sprayer, together with the calcium-containing foliar fertilizer WUXAL®Ascofol Calcium (Ca). For the control treatments, pure water and WUXAL®Ascofol Ca were sprayed on

the trees. For the field trials conducted in 2019 a trailed, air-assisted parcel tunnel sprayer was in use.

7.1.1. Evaluation of the methods

Prior to the analytical determination of the above parameters, optimization of the method for extraction of phenolic compounds was first performed. This required the optimization of individual parameters such as the choice and concentration of the extraction agent, extraction procedure, -temperature, and -time. In a first step, methanol, acetone, and acetonitrile mixed with bidestilled (bidest.) water were used as solvent mixtures in different volume concentrations. The extraction was carried out from a multistage combination of ultrasonic bath and ball mill. In the ultrasonic bath, the slightly elevated temperature at 30°C improves the solubility of the phenolic compounds in the extractant. Due to mechanical effects, the ultrasonic waves additionally lead to the destruction of the cells, which improves the solvent penetration into the sample matrix and promotes the diffusion of the phenolic compounds into the liquid phase (Ghafoor et al., 2009). The use of the ball mill results in complete disruption due to the destruction of the cellular material. The extraction was performed using selected samples and then the total phenolic content was determined according to FOLIN-CIOCALTEU.

Using the composition of the extraction solvent acetone/bidest. water (50/50; v/v), the sum of extracted phenolic compounds was highest in comparison. In a subsequent optimization step, the extraction performance could be increased by adding small amounts of hydrochloric acid, so that for the extraction of phenolic compounds from the apple samples the solvent acetone/bidest. water + 0.1% hydrochloric acid (50/50; v/v) was used. A subsequent comparison of different extraction procedures showed that the most optimal results were obtained with a three-stage extraction with a single use of the ultrasonic bath as well as the use of the ball mill three times. In addition to total phenolic content, antioxidant activity was also determined using TEAC and ORAC assays.

The FOLIN-CIOCALTEU analysis of total phenolic content is an easy to perform and rapid method. The disadvantage is that not only phenolic compounds, but also other reducing substances are detected.

The determination of PPO activity was performed because these enzymes play an important catalytic role in the oxidative degradation of phenolic compounds to quinones. These react widely to form brown colored melanins. Rapid browning of freshly cut apples is undesirable by the consumer (Nicolas et al., 1994). Furthermore, the substrates of PPO, phenolic compounds, have a number of health-promoting properties (Bravo, 1998; Kroon & Williamson, 2005; Del Rio et al., 2010; Tomás-Barberán & Andrés-Lacueva, 2012). Thus, high PPO activity is undesirable.

Antioxidant activity was determined using two established assays that differ in their reaction mechanism and therefore allow different assessments of AOA. The hydrogen transfer based ORAC assay measures the antioxidant inhibition being induced by peroxyl radicals. Thus, this assay represents a biologically relevant mechanism. The antioxidant activity is measured over a period of time so that the potential effects of secondary antioxidant compounds can also be measured, and an underestimation can be prevented. The advantage of the often-used TEAC, on the other hand, is that there are many comparative values in the literature and the measured values can therefore be easily classified and compared with existing data. In the assay based on electron transfer reactions, both hydrophilic and lipophilic antioxidants are determined. The TEAC is well suited for the determination of antioxidant activity in phenolic rich samples, such as apples, as the ABTS• radical used here reacts quickly with antioxidants and many phenolic compounds of low redox potential (Prior et al., 2005).

7.1.2. Influence of biofortification on the selenium content

Biofortification resulted in significantly higher selenium content in apples compared to controls (Manuscripts I and III). The implementation of foliar fertilization showed significantly increased selenium content in apples of different varieties. Table 7 shows the results of the determination of selenium content in the control and biofortified apple samples in µg/100 g f.w.

While the selenium content in apples of the control treatments ranged between 0.1 – 0.7 µg/100 g f.w., selenium-sprayed fruits reached 2.1 – 23.2 µg/100 g f.w. (Table 7). Thus, the selenium content of apples was increased by the aerial application up by a factor between about 10 and 40. The low native selenium content in the apples confirmed findings of soil analyses, carried out at the Horticultural Research Station of the Osnabrück University of Applied Sciences. These showed that only little selenium was present in the root zone of

the apple trees at the two experimental sites (0.25 – 0.52 mg/kg extracted with aqua regia). A total selenium soil content of < 0.60 mg/kg is considered indicative of deficiency for food production (Poňavič & Scheib, 2014).

Table 7: Selenium content in control and biofortified apples in µg/100 g f.w. and the increase factor.

Variety and Year of Cultivation	Application*	Se [µg/100 g f.w.]	Increase factor
'Fiesta' 2017	control (HS)	0.1 ± 0.1	---
	0.1 kg selenite (HS)	3.1 ± 1.5	31.0
	0.1 kg selenate (HS)	3.1 ± 1.4	31.0
'Jonica' 2017	control (HS)	0.7 ± 0.2	---
	0.15 kg selenite (HS)	13.9 ± 1.3	19.9
'Golden Delicious' 2017	control (HS)	0.4 ± 0.2	---
	0.15 kg selenite (HS)	5.6 ± 0.5	14.0
	0.15 kg selenate (HS)	5.6 ± 0.8	14.0
'Jonagold' 2017	control (HS)	0.4 ± 0.2	---
	0.15 kg selenite (HS)	5.6 ± 1.2	14.0
	0.15 kg selenate (HS)	4.5 ± 1.6	11.3
'Golden Delicious' 2018	control (BS)	0.3 ± 0.0	---
	0.075 kg selenate (BS)	3.7 ± 0.4	12.3
'Jonagold' 2018	control (BS)	0.2 ± 0.0	---
	0.075 kg selenate (BS)	2.1 ± 0.7	10.5
'Boskoop' 2018	control (OS)	0.4 ± 0.1	---
	0.075 kg selenate (OS)	5.3 ± 0.3	13.3
'Jonica' 2018	control (OS)	0.3 ± 0.1	---
	0.075 kg selenate (OS)	3.9 ± 0.7	13.0
'Elstar' 2019	control (OS)	0.6 ± 0.0	---
	0.15 kg selenate (OS)	8.7 ± 1.4	14.5
	0.45 kg selenate (OS)	23.2 ± 2.7	38.7

** Foliar spray rate of selenium per hectare and meter canopy height applied with a hand-held sprayer (HS), a backpack sprayer (BS) or a trailed orchard sprayer (OS).*

The results of the present study are in line with published data. A significant increase of the selenium content resulting from biofortification with foliar fertilization has already been observed by other research groups in a variety of plant foods, especially on vegetables (D'Amato et al., 2018; Ekanayake et al., 2015; Hawrylak-Nowak, 2008; Ríos et al., 2008; Schiavon et al., 2013; Schiavon et al., 2016; Bachiega et al., 2016). In those studies, the dosage form and the fertilizer level played a significant role. It was often found that selenate leads to higher selenium accumulations than selenite (D'Amato et al., 2018; Ekanayake et al., 2015; Hawrylak-Nowak, 2008; Ríos et al., 2008) and the selenium content in the plants increased with increasing application level (D'Amato et al., 2018; Hawrylak-Nowak, 2008; Ríos et al.,

2008; Hawrylak-Nowak, 2013; Schiavon et al., 2013). An increase in selenium concentration was also observed in different fruits. Pezzarossa et al. carried out a biofortification with 1.0 mg Se/L in the form of sodium selenate on peach (*Prunus persica* Batch. cv. Flavorcrest) and pear (*Pyrus communis* L. cv. 'Conference') and increased the selenium concentration in the fruits from < 0.1 µg Se/100 g f.w. to 0.9 µg Se/100 g f.w. and 3.6 µg Se/100 g f.w., respectively (Pezzarossa et al., 2012).

A selenium biofortification in the lower range of the magnitude we observed was reported for selenium-sprayed apples of the variety 'Starking Delicious' (3.5 – 4.2 µg/100 g f.w.) (Babalar et al., 2019). In this field experiment, however, the selenium application rate was also much lower (about 0.022 kg/ha). On the other hand, the amount of water applied on the apple trees was more than twice as high as in our trials. This may have promoted the wetting of the fruits with the spray solution. Liu et al. investigated soil fertilization as an approach to biofortify selenium in apples of the variety 'Gala'. For this purpose, a selenium-rich organic fertilizer was applied to the soil around the trees with a rate of 150 – 300 g Se/ha. In this way, the selenium content of the fruits was increased to a maximum of 0.6 – 2.1 µg/100 g (Liu et al., 2019). Thus, the selenium accumulation remained clearly below the level that could be achieved with foliar fertilization tested in this study. This is in line with the results of Jakovljevic et al., who found that soil fertilization required five times the amount of selenium fertilizer to achieve a selenium enrichment similar to foliar fertilization (Jakovljevic et al., 1996). Zhao et al. investigated three different agronomic techniques to enrich pear-jujube with selenium: soil fertilization, trunk injection, and foliar fertilization. Again, the spray treatment proved to be the most effective method (Zhao et al., 2013).

The selenium form (selenite vs. selenate) applied by foliar fertilization did not significantly affect the level of selenium biofortification in apples. In the literature, different findings on the impact of the selenium species were reported. After foliar fertilization of potatoes, Poggi et al. could not detect any difference in the selenium content of the tubers if pure solutions of sodium selenite or sodium selenate were applied. However, if humid acids were added to the spray solution, selenate proved to be superior (Poggi et al., 2000). Likewise, in pear, carrots, and several allium species, selenate sprays led to a higher uptake and translocation of selenium in plants (Deng et al., 2019; Kápolna et al., 2009; Golubkina et al., 2012). In contrast, the selenium content in grains of rice plants was more than twice as high following a foliar

fertilization with selenite than after a corresponding treatment with selenate (Lidon et al., 2019). Selenium-related phytotoxic effects may also depend on the applied selenium form. In apple trees, the doses of selenium tested in this study generally resulted in mild to moderate damage to foliage, which appeared as leaf edge and tip necrosis. At application rates of ≥ 0.15 kg Se/ha x m CH, necrotic spots were also occasionally observed on the fruits, especially around the calyx area. In general, the damage was more pronounced when apples were treated with selenite than with selenate. This is consistent with previous reports indicating that selenate is usually better tolerated by plants than selenite, especially in the higher concentration range (Puccinelli et al., 2017). Therefore, only selenate was selected for the field experiments during the last two trial years.

Different application systems were used for the foliar sprays performed in this study. When using hand-held or backpack sprayers, the highest selenium accumulation of 13.9 µg/100 g f.w. was found for the variety 'Jonica' following an application of 0.15 kg Se/ha x m CH (= 0.45 kg Se/ha). In 2018 and 2019 fertilization trials were also carried out with trailer-mounted orchard sprayers, which are commonly used in commercial fruit growing. Using this application technique, the selenium content of apples increased proportionally with increasing selenium fertilizer quantity (see Figure 27). At the highest fertilization rate of 1.35 kg Se/ha the fruit selenium content rose to 23.2 µg/100 g f.w. in 'Elstar' apples. Similarly, Ren et al. found rising selenium levels in 'Fuji' apples when they were sprayed with increasing doses of selenite (Ren et al., 2020). Surprisingly, the selenium content of these fruits did not exceed 15.6 µg/100 g f.w., although the apple trees received even higher selenium fertilization rates (up to 6.5 kg Se/ha). This discrepancy is probably due to several causes. First of all, in the 'Fuji' apple orchard, only the leaves were sprayed, while the fruits were protected by packing in bags before spraying. In contrast, in the present field experiments the whole tree including the fruits was treated. Furthermore, the spray solution we applied contained a surfactant to improve the wetting of the hydrophobic surfaces of leaves and fruits. In the work of Ren et al., the addition of such spray adjuvants is not mentioned. Another important difference is that the 'Fuji' apples were always peeled during sample preparation, whereas in present study the fruits were usually examined with peel. Analyses on 'Elstar' apples treated with a high dose of selenium revealed that the selenium content in the fruit peel was seven times higher than in the fruit flesh. In total about 43% of the selenium was in the fruit peel, while 57% had already penetrated the flesh. Similarly,

Deng et al. reported that in selenium-sprayed pears the fruit peel accounted for up to 58% of the selenium amount in the whole fruit. Interestingly, selenate was significantly less retained by fruit peel and thus penetrated better into the fruit than selenite (Deng et al., 2019).

Overall, the results of the present field experiments show that it is possible to biofortify apples with selenium to an extent that can significantly improve the dietary selenium intake of humans. In a normal apple orchard with approx. 3.0 m high trees, a foliar fertilization of approx. 0.5 kg/ha split into several treatments during the fruit development is advised. This would increase the selenium content of fruits to about 9 µg/100 g f.w. The consumption of such an apple of medium size (180 g) would cover about a quarter of the daily requirement of adults, estimated at 70 µg for men and 60 µg for women (Kipp et al., 2015). Selenate is recommended for spraying, as it is better tolerated by apple trees than selenite. A combined spraying of selenate with a calcium-containing fertilizer proved to be feasible.

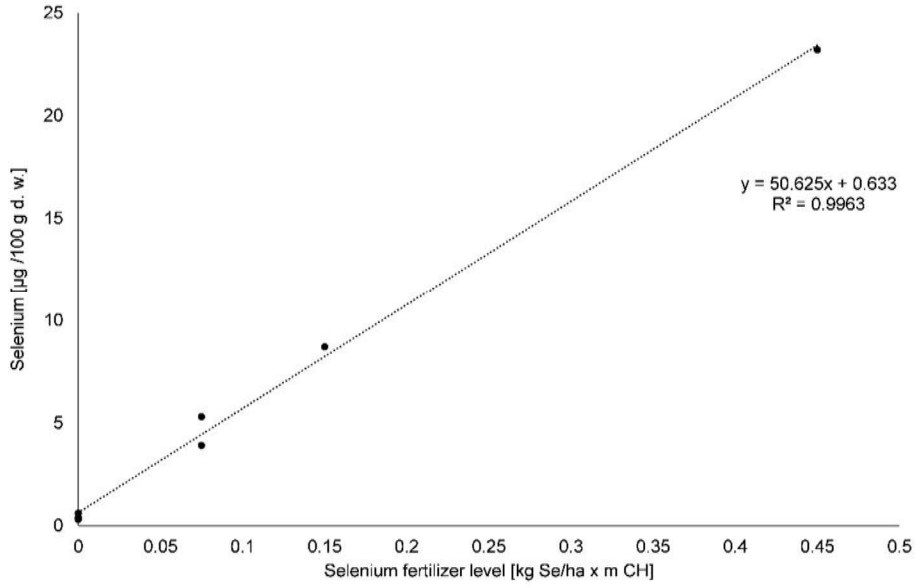


Figure 27: Correlation between selenium fertilizer level and selenium content in the apples using trailer-mounted orchard sprayers for the application of selenium fertilizers in 2018 and 2019.

7.1.3. Influence of biofortification on the PPO activity

Table 8 shows the results of the determination of PPO activity in the different varieties or application forms. The respective factor of change was calculated in each case in relation to the controls. A factor > 1 means that the biofortified apples have a higher PPO activity than the respective controls, a factor < 1 implies a lower PPO activity in the biofortified fruits.

Table 8: PPO activity in control and biofortified apples in Units/100 g f.w. and the change factor.

Variety and Year of Cultivation	Application*	PPO [Units/100 g f.w.]	Change factor
'Fiesta' 2017	control (HS)	10.4 ± 7.6	---
	0.1 kg selenite (HS)	1.9 ± 0.3	0.18
	0.1 kg selenate (HS)	2.5 ± 1.4	0.24
'Jonica' 2017	control (HS)	3.2 ± 1.7	---
	0.15 kg selenite (HS)	5.5 ± 3.0	1.72
'Golden Delicious' 2017	control (HS)	12.0 ± 2.2	---
	0.15 kg selenite (HS)	18.5 ± 2.8	1.54
	0.15 kg selenate (HS)	24.5 ± 9.5	2.04
'Jonagold' 2017	control (HS)	2.4 ± 0.7	---
	0.15 kg selenite (HS)	4.1 ± 1.5	1.71
	0.15 kg selenate (HS)	5.8 ± 5.2	2.42
'Golden Delicious' 2018	control (BS)	142.3 ± 34.9	---
	0.075 kg selenate (BS)	61.1 ± 17.5	0.43
'Jonagold' 2018	control (BS)	33.5 ± 6.9	---
	0.075 kg selenate (BS)	42.4 ± 35.0	1.27
'Boskoop' 2018	control (OS)	41.8 ± 10.5	---
	0.075 kg selenate (OS)	18.8 ± 4.7	0.45
'Jonica' 2018	control (OS)	11.4 ± 9.2	---
	0.075 kg selenate (OS)	3.8 ± 1.5	0.33
'Elstar' 2019	control (OS)	3.8 ± 0.8	---
	0.15 kg selenate (OS)	35.5 ± 8.9	9.34
	0.45 kg selenate (OS)	3.6 ± 1.1	0.95

** Foliar spray rate of selenium per hectare and meter canopy height applied with a hand-held sprayer (HS), a backpack sprayer (BS) or a trailed orchard sprayer (OS).*

It could be shown that the amount of applied selenium has an influence on the PPO activity. Thus, the application of a higher amount of selenium (0.15 kg Se/ha x m CH) resulted in higher enzyme activities in all cultivars, whereas the application of a lower dose (0.075 kg Se/ha x m CH) resulted in mostly lower values compared to the controls (Manuscripts I and III). In most cases, increased PPO activity occurred in the biofortified apples, probably related to TPC and phenolic profile. An increase in TPC was observed. Since there are more substrates for the PPO in the form of phenolic compounds, the enzyme activity is higher.

Biofortification with selenium also resulted in increased PPO activities in other crops. Smolén et al. found that biofortification of potatoes *(Solanum tuberosum* L., cv. 'Vineta') with selenium

(6.3 µM in the form of sodium selenite) and iodine resulted in increased PPO activities in comparison to the untreated controls (Smolén et al., 2016).

In addition to the influence of biofortification on PPO activity, other factors also play a role. Variety-specific differences as well as an influence of ecophysiological conditions were found. The PPO activity of the cultivar 'Golden Delicious' was highest in both growing years, whereas the cultivars 'Jonica' and 'Elstar' were characterized by comparatively low activities. Holderbaum et al., Kolodziejczyk et al., and Kschonsek et al. also found cultivar-specific differences in PPO activity, with 'Golden Delicious' having the highest PPO activity of all the varieties tested. Furthermore, differences were observed between the two growing years due to different ecophysiological conditions. In 2018, a sunshine duration of 756 h, an average rainfall of 88.5 L/m², and an average temperature of 19.8°C were recorded at the cultivation site Osnabrück, Germany. In the previous year, however, the sunshine duration was only 549.4 h with an average rainfall of 224.3 L/m² and an average temperature of 18.2°C (Wetterkontor, 2019). The difference in the amount of rain only plays a marginal role because of the use of artificial irrigation. Consequently, sunshine duration in particular seems to be the dominant influence on the level of the PPO activity.

Kolodziejczyk et al. have already observed differences in PPO activity within one variety in two consecutive years on a number of different apple varieties harvested in 2007 and 2008 (Kolodziejczyk et al., 2010). UV-C treatment is an important postharvest treatment and influences the PPO activity. Thus, Manzocco et al. observed an inactivation of PPO and the prevention of enzymatic browning in 'Golden Delicious' apples by UV-C radiation (Manzocco et al., 2009). Müller et al. also found a reduction of PPO activity in apple juices, when apples have been treated with UV-C light. In contrast, treatment with UV-B radiation did not show any effects (Müller et al., 2014). Additionally, reduced PPO activities after UV-C treatments were observed in other vegetable crops (Cirilli et al., 2017; Lei et al., 2018).

7.1.4. Influence of biofortification on the TPC

The results of the determination of the TPC are shown in Table 9. The differences between the respective controls and the biofortified apples are quickly recognizable on the basis of the change factor, thus a factor > 1 means a higher TPC of the biofortified apples, a factor < 1 means a lower TPC compared to the controls.

Table 9: TPC in control and biofortified apples in mg GAE/100 g d.w. and the change factor.

Variety and Year of Cultivation	Application*	TPC [mg GAE/ 100 g d.w.]	Change factor
'Fiesta' 2017	control (HS)	1,141.3 ± 419.7	---
	0.1 kg selenite (HS)	838.5 ± 273.0	0.73
	0.1 kg selenate (HS)	843.9 ± 54.5	0.74
'Jonica' 2017	control (HS)	735.6 ± 66.6	---
	0.15 kg selenite (HS)	843.0 ± 169.2	1.15
'Golden Delicious' 2017	control (HS)	863.8 ± 123.8	---
	0.15 kg selenite (HS)	797.9 ± 44.7	0.92
	0.15 kg selenate (HS)	851.4 ± 14.9	0.99
'Jonagold' 2017	control (HS)	938.2 ± 78.0	---
	0.15 kg selenite (HS)	956.5 ± 36.7	1.02
	0.15 kg selenate (HS)	893.8 ± 74.0	0.95
'Golden Delicious' 2018	control (BS)	1,212.2 ± 164.3	---
	0.075 kg selenate (BS)	1,206.9 ± 226.4	1.00
'Jonagold' 2018	control (BS)	900.1 ± 136.4	---
	0.075 kg selenate (BS)	815.4 ± 103.5	0.91
'Boskoop' 2018	control (OS)	745.2 ± 66.1	---
	0.075 kg selenate (OS)	743.5 ± 51.3	1.00
'Jonica' 2018	control (OS)	785.1 ± 177.3	---
	0.075 kg selenate (OS)	841.7 ± 106.7	1.07
'Elstar' 2019	control (OS)	828.1 ± 48.0	---
	0.15 kg selenate (OS)	750.7 ± 121.4	0.91
	0.45 kg selenate (OS)	902.7 ± 26.8	1.09

** Foliar spray rate of selenium per hectare and meter canopy height applied with a hand-held sprayer (HS), a backpack sprayer (BS) or a trailed orchard sprayer (OS).*

With the exception of the 'Fiesta' variety, biofortification resulted in only minor changes in TPC. Here, differences were found due to the applied amount of selenium. The application of the higher amount of selenium (0.15 kg Se/ha x m CH) mostly led to stronger changes than the application of only 0.075 kg Se (Manuscripts I and III). The cultivar 'Jonica' showed an increase in TPC in the biofortified samples of 15% in 2017 and 7% in the following year in both crop years. The TPC of the other cultivars was increased or decreased in the range of up to 9%.

Elevated TPC in various selenium biofortified plant foods have been frequently reported in the literature. Bachiega et al. performed an application of 50 µM selenate to broccoli, which led to a significant increase in TPC (Bachiega et al., 2016). In onion *(Allium cepa* L., cv. 'Hercules'), Põldma et al. observed that an application of 50 µg/mL selenate via foliar treatment led to increased TPC when compared to the untreated controls, whereas a higher level with

100 µg/mL resulted in lower TPC (Põldma et al., 2013). In tomatoes, Schiavon et al. found that selenate in low concentrations also led to an increase in TPC, when performing foliar fertilization of up to 20 mg Se/plant (Schiavon et al., 2013). In a follow-up study on radish in 2016, an increase in TPC of 10% in the leaves when compared to the controls was recorded (Schiavon et al., 2016). Hawrylak-Nowak found that the application of a moderate level of selenite (63.3 µM) applied via foliar fertilization led to enhanced TPC with a maximum increase of 43.9% in basil leaves (*Ocimum basilicum* L.) (Hawrylak-Nowak, 2008).

In the present work, cultivar-specific differences in TPC were found. Thus, 'Golden Delicious' is characterized by a comparatively high TPC, whereas 'Jonica' and 'Boskoop' are poorer in phenolic compounds. Kschonsek et al. and Xu et al. also describe different TPC in different apple cultivars (Kschonsek et al., 2018; Kschonsek et al., 2019; Xu et al., 2016). Kschonsek et al. determined the TPC in 15 different apple cultivars and found that the 'Golden Delicious' cultivar had a comparatively low TPC, containing 521.9 mg GAE/100 g peel and 136.5 mg GAE/100 g fruit flesh, respectively (Kschonsek et al., 2018). The measured values are in a comparable size range with the results of the previous work.

In addition to genotypic differences, differences in TPC were also found due to the ecophysiological conditions of the growing years. This comparison can be made using the cultivars 'Jonica', 'Golden Delicious', and 'Jonagold'. The TPC of the three varieties was higher in 2018 than in the previous year. Here, especially the difference in sunshine duration seems to have a dominant influence on the height of the TPC. This has a direct correlation with UV radiation, which in turn is an influencing factor on the biosynthesis of phenolic compounds. Eichholz et al. showed that light intensity and quality are some of the most effective factors on the biosynthesis of phenolic compounds in white asparagus (*Asparagus officinnalis* L., cv. 'Gijnlim') on the basis of UV-B treatments (Eichholz et al., 2012). Scattino et al. could also demonstrate that a postharvest UV-B irradiation induced changes of TPC in peaches (*Prunus persica* L., cv. 'Suncrest') and nectarines (*Prunus persica* var. nucipersica, cv. 'Big Top') (Scattino et al., 2016).

There is an inverse correlation between the TPC and the PPO activity, because the enzyme catalyzes the oxidation reaction of phenolic compounds to quinones, which further react to brown colored polymeric melanins (Bravo, 1998; Kolodziejczyk et al., 2010). This could explain the influence of the selenium biofortification as well as the variety-specific differences, where

lower PPO activities are associated with higher TPC and higher enzyme activities with lower TPC. This correlation has already been described in the literature by Song et al., Kolodziejczyk et al., and Allahveran et al. Song et al. found positive correlations between PPO and TPC based on studies of ten apple varieties (Song et al., 2007). In contrast, Kolodziejczyk et al. found no correlation between these two parameters on the basis of 22 apple varieties (Kolodziejczyk et al., 2010).

In the present work, higher PPO activities and lower TPC were measured at an application level of 0.15 kg Se/ha compared to the untreated controls. In contrast, the reduction to 0.075 kg Se/ha tended to result in lower PPO activities as well as higher TPC. Allahveran et al. performed biofortification with ascorbic acid and citric acid on apples of the variety ‘Red Spur and determined the PPO activity and TPC among other parameters. There, biofortification led to a significant increase in TPC and a decrease in PPO activity (Allahveran et al., 2018).

7.1.5. Influence of biofortification on the AOA

Table 10 shows the AOA of the analyzed apple samples by TEAC and ORAC assay and the respective factors of change.

Biofortification with selenium showed no clear tendencies of an influence on AOA, which was determined by TEAC. Variety specific differences were observed. The AOA of 'Fiesta' decreased in the biofortified apples, whereas the other cultivars mostly showed an increased AOA as a result of biofortification (Manuscripts I and III). 'Jonagold' showed the strongest increase here with an increase in AOA of up to 80%. The other cultivars were only affected to a minor extent. When evaluating the ORAC results, it is noticeable that in some cases different effects occurred compared to the TEAC values. For example, a reduction in AOA of up to 35% was observed in the ORAC of the 'Jonagold' variety, whereas the TEAC values of the biofortified samples were significantly higher. This can be explained by the different reaction mechanism of the two assays as well as by a change in the phenolic profile. On the one hand, the content of individual phenolic compounds may be altered as a result of biofortification, and on the other hand, the substances also show different levels of antioxidant activity.

An increase in AOA due to biofortification with selenium has already been described in the literature. In lettuce plants (*Lactuca sativa* L. cv 'Philipus') increasing doses of selenite and selenate lead to an increase in AOA, as measured by Fluorescence Recovery after

Photobleaching (FRAP) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. Selenate showed higher AOA when compared to selenite (Ríos et al., 2008).

Table 10: AOA in control and biofortified apples measured by TEAC and ORAC assay in mmol TE/100 g d.w. and the change factors.

Variety and Year of Cultivation	Application*	TEAC [mmol TE/ 100 g d.w.]	Change factor	ORAC [mmol TE/ 100 g d.w.]	Change factor
'Fiesta' 2017	control (HS)	15.3 ± 4.9	---	13.4 ± 5.4	---
	0.1 kg selenite (HS)	11.1 ± 2.6	0.73	4.6 ± 1.8	0.34
	0.1 kg selenate (HS)	13.0 ± 0.6	0.85	9.5 ± 0.5	0.71
'Jonica' 2017	control (HS)	5.5 ± 0.5	---	11.2 ± 1.4	---
	0.15 kg selenite (HS)	5.7 ± 0.9	1.04	14.1 ± 1.2	1.26
'Golden Delicious' 2017	control (HS)	6.8 ± 1.1	---	5.4 ± 1.6	---
	0.15 kg selenite (HS)	6.0 ± 0.3	0.88	1.9 ± 1.1	0.35
	0.15 kg selenate (HS)	7.6 ± 1.6	1.12	6.0 ± 1.4	1.11
'Jonagold' 2017	control (HS)	7.4 ± 0.9	---	11.0 ± 2.2	---
	0.15 kg selenite (HS)	11.0 ± 3.9	1.49	7.2 ± 1.2	0.65
	0.15 kg selenate (HS)	13.3 ± 1.3	1.80	9.5 ± 1.3	0.86
'Golden Delicious' 2018	control (HS)	12.1 ± 0.8	---	14.2 ± 1.5	---
	0.075 kg selenate (BS)	10.7 ± 1.1	0.88	8.8 ± 3.0	0.62
'Jonagold' 2018	control (BS)	11.2 ± 2.8	---	8.1 ± 1.2	---
	0.075 kg selenate (BS)	11.4 ± 0.8	1.02	15.5 ± 1.2	1.91
'Boskoop' 2018	control (OS)	15.6 ± 2.4	---	17.0 ± 7.1	---
	0.075 kg selenate (OS)	16.5 3.4	1.06	14.7 ± 0.9	0.86
'Jonica' 2018	control (OS)	12.8 ± 1.8	---	8.9 ± 1.5	---
	0.075 kg selenate (OS)	11.8 ± 1.6	0.92	5.6 ± 0.9	0.63
'Elstar' 2019	control (OS)	7.0 ± 0.3	---	14.1 ± 1.0	---
	0.15 kg selenate (OS)	5.7 ± 0.5	0.81	14.4 ± 0.5	1.02
	0.45 kg selenate (OS)	7.0 ± 0.2	1.00	12.3 ± 1.0	0.87

** Foliar spray rate of selenium per hectare and meter canopy height applied with a hand-held sprayer (HS), a backpack sprayer (BS) or a trailed orchard sprayer (OS).*

Põldma et al. determined an increase in AOA, as measured by TEAC, in onions (*Allium cepa* L. cv. 'Hercules') at a dose of 50 µg/mL Se (Põldma et al., 2013). Bachiega et al. found a significant increase in AOA as a result of a biofortifiaction of broccoli in addition to a significantly higher TPC. There, 50 µM selenate was used as fertilizer. The positive correlation can be explained by the fact that phenolic compounds represent the largest group of antioxidant active substances in broccoli (Bachiega et al., 2016). Additionally, Ekanayake et al. observed an increase of the AOA of lentils (*Lens culinaris* cv. 'Medikus'), due to a biofortification with selenium (Ekanayake et al., 2015).

Variety-specific differences occurred in the present study. For example, the AOA of 'Jonagold' was higher in both years of cultivation than for 'Golden Delicious'. Variety-specific differences in the AOA of apples have already been described in the literature by Kschonsek et al., Wojdylo et al., and Xu et al. In those studies, the higher AOA of the variety 'Jonagold' as compared to 'Golden Delicious' were found (Kschonsek et al., 2018; Wojdylo et al., 2008; Xu et al., 2016).

Kschonsek et al., Wojdylo et al., and Xu et al. were able to show that there are significant positive correlations between TPC (measured according to FOLIN-CIOCALTEU or via HPLC) and AOA (Kschonsek et al., 2018; Wojdylo et al., 2008; Xu et al., 2016). Kschonsek et al. and Wojdylo et al. also determined that the TPC was different, both between the individual substance groups of the polyphenols and between the individual compounds (Kschonsek et al., 2018; Wojdylo et al., 2008). There, Kschonsek et al. measured the highest positive correlation between flavanols and ORAC. Those compounds are the major contributors to AOA. Within the flavanols, epicatechin had the strongest influence on the intensity of the AOA (Kschonsek et al., 2018). Wojdylo et al. found the highest correlations between AOA and procyanidins and hydroxycinnamic acids, while using the TEAC, FRAP, and DPPH assay. The different AOA of the individual varieties are, therefore, due to the different composition of the phenolic compounds, as these show different antioxidant capacities and potentials (Wojdylo et al., 2008). An influence of the weather can also be deduced when comparing the controls from the years 2017 and 2018, similarly to the TPC values.

7.1.6. Influence of biofortification on the phenolic compounds

Tables 11 and 12 show the contents of the various phenolic compounds in the apple samples in mg/100 g d.w.

Table 11: Contents of the various phenolic compounds (chlorogenic acid, epicatechin, procyanidin trimer) in the apple samples in mg/100 g d.w.

Variety and Year of Cultivation	Application*	Chlorogenic acid [mg/100 g d.w.]	Change factor	Epicatechin [mg/100 g d.w.]	Change factor	Procyanidin Trimer [mg/100 g d.w.]	Change factor
'Fiesta' 2017	control (HS)	66.43 ± 19.90	---	22.47 ± 2.28	---	15.51 ± 0.49	---
	0.1 kg selenite (HS)	50.13 ± 15.05	0.75	14.00 ± 4.38	0.62	11.81 ± 5.65	0.76
	0.1 kg selenate (HS)	56.83 ± 2.78	0.86	11.00 ± 0.57	0.49	12.35 ± 1.24	0.80
'Jonica' 2017	control (HS)	20.64 ± 2.58	---	7.12 ± 1.95	---	8.63 ± 0.64	---
	0.15 kg selenite (HS)	20.75 ± 2.85	1.01	7.11 ± 2.53	1.00	10.23 ± 1.12	1.19
'Golden Delicious' 2017	control (HS)	33.65 ± 3.92	---	11.86 ± 2.32	---	9.34 ± 2.33	---
	0.15 kg selenite (HS)	33.48 ± 0.29	0.99	14.98 ± 1.63	1.26	10.27 ± 1.29	1.10
	0.15 kg selenate (HS)	32.59 ± 2.78	0.97	12.89 ± 2.32	1.09	8.46 ± 1.67	0.91
'Jonagold' 2017	control (HS)	28.36 ± 3.77	---	13.19 ± 2.40	---	14.42 ± 2.40	---
	0.15 kg selenite (HS)	30.20 ± 5.48	1.06	14.75 ± 3.46	1.12	11.45 ± 1.24	0.79
	0.15 kg selenate (HS)	31.25 ± 3.09	1.10	13.04 ± 2.37	0.99	11.14 ± 1.89	0.77

** Foliar spray rate of selenium per hectare and meter canopy height applied with a hand-held sprayer (HS), a backpack sprayer (BS) or a trailed orchard sprayer (OS).*

Table 12: Contents of the various phenolic compounds (caffeeoylglucoside, sum of phloretin glucosides, sum of quercetin glycosides) in the apple samples in mg/100 g d.w.

Variety and Year of Cultivation	Application*	Caffeeoylglucoside [mg/100 g d.w.]	Change factor	Σ Phloretin glucoside [mg/100 g d.w.]	Change factor	Σ Quercetin glycoside [mg/100 g d.w.]	Change factor
'Fiesta' 2017	control (HS)	5.16 ± 0.26	---	13.01 ± 1.66	---	42.18 ± 31.01	---
	0.1 kg selenite (HS)	5.09 ± 0.11	0.99	9.28 ± 1.14	0.71	26.98 ± 1.77	0.64
	0.1 kg selenate (HS)	5.06 ± 0.28	0.98	12.24 ± 1.87	0.94	40.72 ± 7.41	0.97
'Jonica' 2017	control (HS)	5.23 ± 0.25	---	10.92 ± 0.61	---	23.81 ± 1.63	---
	0.15 kg selenite (HS)	5.24 ± 0.20	1.00	14.47 ± 5.13	1.33	37.72 ± 13.76	1.58
'Golden Delicious' 2017	control (HS)	14.81 ± 0.41	---	17.00 ± 5.37	---	35.09 ± 10.44	---
	0.15 kg selenite (HS)	13.88 ± 1.03	0.94	16.86 ± 2.92	0.99	53.33 ± 18.66	1.52
	0.15 kg selenate (HS)	13.42 ± 1.64	0.91	16.04 ± 1.65	0.94	42.57 ± 9.68	1.21
'Jonagold' 2017	control (HS)	4.99 ± 0.12	---	16.74 ± 3.34	---	58.69 ± 16.61	---
	0.15 kg selenite (HS)	8.28 ± 3.79	1.66	19.63 ± 6.25	1.17	46.20 ± 12.20	0.79
	0.15 kg selenate (HS)	8.90 ± 4.57	1.78	18.96 ± 0.29	1.13	49.37 ± 6.07	0.84

** Foliar spray rate of selenium per hectare and meter canopy height applied with a hand-held sprayer (HS), a backpack sprayer (BS) or a trailed orchard sprayer (OS).*

By HPLC-MS^n analysis, the following phenolic compounds were identified and their contents quantified in apples from the 2017 crop year: the dihydrochalcones phloretin-2-xylosyl-glucoside and phloretin-2-glucoside, the flavan-3-ol epicatechin, and a procyanidin dimer, and a procyanidin trimer, the hydrocinnamic acid derivatives caffeoyl glucoside and chlorogenic acid, as well as the flavonols quercetin-3-O-galactoside, quercetin-3-O-xyloside and quercetin-3-O-glucoside (Manuscripts I and III).

In their review, Rana and Bushan evaluated a large amount of data on the analysis of phenolic compounds in apples and found that flavonols, dihydrochalcones, flavan-3-ols, and phenolic acids have already been identified in apples of various varieties. The main components here are epicatechin, procyanidin B2, chlorogenic acid, phloridzin, caffeic acid, and quercetin derivatives (Rana & Bushan, 2016).

The apples studied in the present work contain mainly chlorogenic acid, up to 41.5%, as well as epicatechin and high amounts of various quercetin glycosides and phloretin glucosides. Based on tentative structure elucidation in the present study and literature descriptions, this trimer is suggested to be procyanidin C1 (Masuda et al., 2018). In some cases, significant differences were found between the individual cultivars. Figure 28 shows the average proportions of the individual phenolic compounds in a comparison of the cultivars 'Fiesta', 'Jonica', 'Golden Delicious', and 'Jonagold'. The respective controls are shown.

The 'Fiesta' variety is characterized above all by a high content of chlorogenic acid (40%), whereas 'Jonica' and the other varieties contain significantly less of this substance, at 21-27%. 'Fiesta' is also richer in epicatechin than the other varieties. These in turn contain significantly more phloretin glucosides and quercetin glycosides than 'Fiesta' (8% and 23%, respectively), with percentages of 12-14% and 28-41%, respectively.

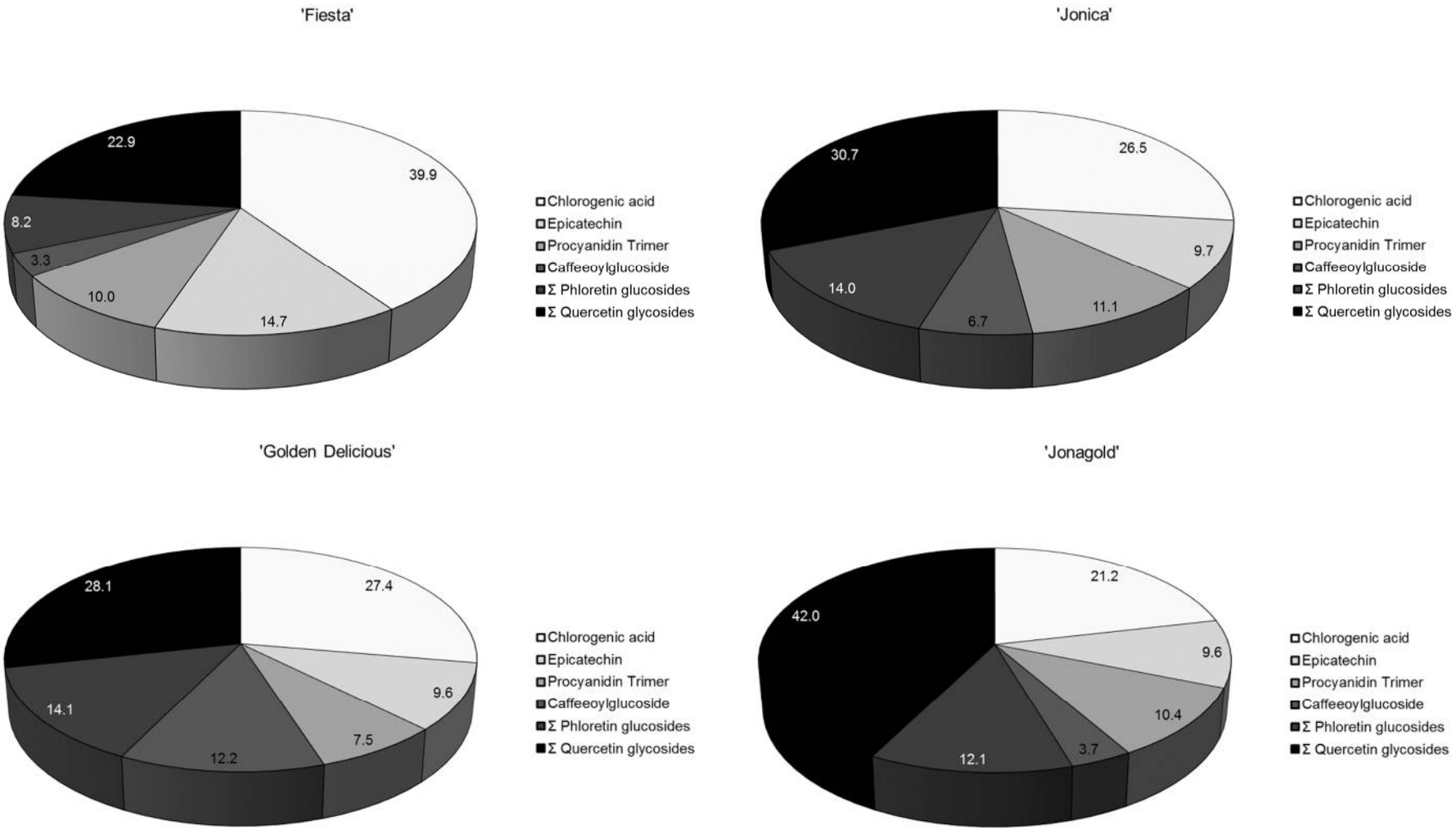


Figure 28: Average proportions of the individual phenolic compounds of control samples of the cultivars 'Fiesta', 'Jonica', 'Golden Delicious', and 'Jonagold'.

Variety specific differences in the content of individual phenolic compounds in apples have also been described in the literature. For example, Kschonsek et al. also reported that chlorogenic acid is the main component in different apple cultivars. They analyzed, among others, the old varieties 'Ontario' and 'Dülmener Rosenapfel' and the comparatively new varieties 'Braeburn' and 'Granny Smith'. They found significant differences in the content of the main component. While the two old cultivars were very rich in chlorogenic acid with a content of about 63%, the new cultivars contained only about 15% (Kschonsek et al., 2018). Dhyani et al. and Zardo et al. identified chlorogenic acid and epicatechin as major components in 'Golden Delicious' (Dhyani et al., 2018; Zardo et al., 2013). In 2005 and 2006, Wojdylo et al. determined the phenolic compounds in 69 apple cultivars, including 'Golden Delicious' and 'Jonagold'. In both varieties, most of all oligomeric procyanidins, and chlorogenic acid were found, whereas 'Jonagold' contained more epicatechin and chlorogenic acid in comparison (Wojdylo et al., 2008).

Biofortification with selenium showed different effects for the cultivars 'Fiesta', 'Jonica', 'Golden Delicious', and 'Jonagold' with regard to the content and proportion of the individual phenolic compounds. The four main components chlorogenic acid, epicatechin, procyanidin trimer, and caffeeoylglucoside and the sum of phloretin glucoside and quercetin glycoside were influenced by biofortification. Further phenolic compounds were not significantly affected.

The content of individual phenolic compounds was strongly influenced by biofortification, especially in the varieties 'Fiesta' and 'Jonagold', whereas 'Jonica' and 'Golden Delicious' were relatively insensitive to the fertilization method. The comparison of the applied selenium forms showed the application of selenite to have stronger effects on the phenolic profile than selenate. In the cultivar 'Fiesta', biofortification resulted in a reduction in the content of all six phenolic compounds by up to 51%. Whether this is a purely variety-specific effect or whether the reduced application rate of 0.1 kg Se/ha x m CH also plays a role cannot be conclusively assessed due to the insufficient data available for this. 'Jonica' showed a 58% increase in quercetin glycoside content. This as well as an increase in epicatechin content was also observed in 'Golden Delicious'. They were 21% and 52% for quercetin and 9% and 26% for epicatechin. In the cultivar 'Jonagold', biofortification led to an increase in the content of

caffeeoyl glucosides (66%, 78%) and the phloretin glucosides (13%, 17%) and to a reduction in procyanidin trimer (21%, 23%) and the quercetin glycosides (16%, 21%).

A change in the phenolic profile resulting from biofortification with selenium has already been described in the literature. D'Amato et al. found that in the biofortification of olives, an increase in the content of oleacein, ligustroside aglycone, and oleocanthal between 32% and 57% was observed in the oil produced from them compared to the untreated controls (D'Amato et al., 2017). In a subsequent study of rice by the research group, an increase in ferulic acid and salicylic acid and a decrease in gallic acid concentration were observed (D'Amato et al., 2018). Schiavon et al. analyzed the influence of biofortification in radish and performed separate analyses of leaves and roots. In roots, the antioxidant flavonoids naringenin chalcone and kaempferol showed enhanced concentrations and a decrease of cinnamic acid derivatives was observed. In leaves, the hydroxycinnamic acids, especially kaempferol derivatives, were increased. Other identified phenolic compounds did not show any variation in concentration or decreased (Schiavon et al., 2013). Pezzarossa et al. performed an application of 1 mg Se/L (as sodium selenate) in tomatoes (*Solanum lycopersicum* cv. 'Red Bunch'), in which a significant increase of quercetin was observed in addition to a decrease of β-carotene and lycopene. Rutin was not influenced (Pezzarossa et al., 2013).

Relationships between AOA and phenolic compounds can be inferred. The determination of AOA by TEAC assay showed a significantly higher AOA with the application of selenate and selenite in 'Jonagold' compared to the control. Therefore, due to the increased amount of caffeeoyl glucoside, it is concluded that this phenolic compound has a high AOA and is mainly responsible for the AOA in 'Jonagold'. Furthermore, high concentrations of caffeoyl glucoside were associated with high AOA by TEAC and low AOA by ORAC and a high content of the procyanidin trimer were measured in association with a low AOA with TEAC and high AOA with ORAC. These results further suggest that these two phenolic compounds have different AOA and - due to the different reaction mechanisms of both antioxidant assays - the AOA of different phenolic compounds were determined, and secondary antioxidant products were additionally measured when using the ORAC assay (Csepregi et al., 2016). A verification of these hypotheses can be performed in further work using HPLConline-TEAC.

7.2. Influence of selenium biofortification of apples on the protein content and the allergenic proteins Mal d 1 and Mal d 3

First, the extraction of proteins from the apple matrix was carried out using an extraction buffer according to BJÖRKSTEN with some modifications (Björksten et al., 1980). Here, a triple extraction was performed in the ball mill. The subsequent determination of the total protein content was performed by the photometric method according to BRADFORD (BRADFORD, 1976). In addition, to separate the allergenic proteins in the apple samples from each other, a gel electrophoretic separation of the protein extracts was performed using disc-SDS-PAGE. Here, triplicate protein extraction was performed using phosphate buffered saline (PBS) buffer in the ball mill. Based on the different band patterns, conclusions can be made about the influence of biofortification on the content of Mal d 1, Mal d 2, Mal d 3, and Mal d 4. For the identification of the apple allergens, LC-MS/MS was subsequently performed. For this purpose, single protein bands from SDS-PAGE were used, with which in-gel digestion was performed. A direct ELISA was also developed and applied to determine the Mal d 1 content in the apple samples. The extracts used for this purpose were obtained from the work-up for the determination of protein content.

The influence of selenium biofortification on the allergenic proteins was carried out on the same apple samples with which an analysis of antioxidant properties and phenolic compounds had already been carried out (see Chapter 7.1.).

7.2.1. Evaluation of the methods

In a first step, different devices for the extraction of proteins from the apple matrix were used and compared on a selection of apple samples. The Ultra-Turrax, the ball mill, and a thermal shaker were used in various combinations. The extraction variant with thermal shaker only showed significantly lower protein contents than with the other methods. One reason for this is the lack of cell disruption by mechanical force, as achieved by ball mill or Ultra-Turrax, so that the proteins could only be insufficiently extracted from the individual cells. No significantly different results were obtained for extraction with ball mill and with or without shaker afterwards. Similarly, no significantly different value was found for extraction using Ultra-Turrax. In order to minimize the time required for sample processing, the apple samples analyzed in this study were finally processed using the ball mill variant without shaker.

Compared to the variant using Ultra-Turrax, this variant offers the advantage that several samples can be processed in parallel and a smaller sample volume can be used. However, a comparison with the literature shows that the proteins could not be quantitatively extracted with any of the variants. Thus, with a maximum result of 0.214 g protein in 100 g dried apple, only about 15% of the proteins could be extracted compared to the literature value of 1.4 g/100 g dry matter (Souci et al., 2011). By Matthes and Schmitz-Eiberger, a maximum of 338.6 µg of protein was extracted from one gram of fresh mass using the same extraction buffer and a similar method. This corresponds to a yield of 11% compared to the literature value of 0.3 g/100 g fresh mass and is thus in the same range (Matthes & Schmitz-Eiberger, 2009; Souci et al., 2011).

Therefore, in the course of further method optimization, the following factors were changed: Extraction vessel and volume, repeats, concentration as well as sample weight. Here, it was found that increasing the extraction volume and sample weight, which were associated with a change of the extraction vessel, as well as a triplicate extraction and the use of the sample concentrator led to the most optimal results. This extraction method was therefore used in the following for the apple samples to be analyzed.

The buffer solution used for extraction was prepared and modified according to Björksten et al. and Matthes and Schmitz-Eiberger. It contains potassium, polyvinylpolypyrrolidone (PVPP), diethyldithiocarbamate (DIECA), and ethylenediaminetetraacetic acid (EDTA) and was adjusted to a pH of 7.0. Here, the buffer components PVPP, EDTA, and DIECA have the task of preventing reactions between polyphenols or quinones and proteins that come into contact with each other after cell disruption (Björksten et al., 1980). The addition of sodium azide as in the method of BJÖRKSTEN, which serves to inhibit the growth of microorganisms, was omitted because, according to the manufacturer's information, there is considerable inhibition of the polyclonal HRP-labeled goat anti-mouse antibody and thus a determination of the Mal d 1 content would not be possible (Merck, 2019).

As part of further work, protein extraction should be further optimized. By comparing the results of the quantitative protein determination according to BRADFORD, a maximum protein content of 241.53 mg/100g d.w. (biofortified sample: 'Jonagold', 2017) was obtained. This corresponds to a percentage of 17.25% of the total protein content of 1,400 mg/100 g d.w. of an average dried apple (Souci et al., 2011). It follows that the extraction is not quantitative.

Matthes et al. were able to obtain a maximum protein content of 33.86 mg/100 g d.w. (Matthes & Schmitz-Eiberger, 2009). In comparison with the literature value of 300 mg/100 g d.w., 11.29 % of the total protein was thus extracted. With the optimized extraction according to BJÖRKSTEN, about 1.5 times the amount of protein could be extracted. The deviation from the total protein content can presumably be attributed to the fact that a large proportion of the proteins are not soluble in the aqueous milieu because they are in part firmly bound to the rest of the apple matrix. To obtain higher extraction yields, different extractants or even acidic or basic digestions of the samples would be an option. However, it must be noted that the extracted proteins must be preserved native in order to be able to subsequently quantify the Mal d 1 by ELISA. Since this protein is heat and acid labile, this further complicates the feasibility with other extraction agents.

Similarly, the use of grinding media for extraction is neither optimal in terms of time nor quantity. This was used due to the high sample volume. Since they are not usable for the subsequent centrifugation step, the extracts often had to be quantitatively transferred back and forth between the grinding media and the extraction tubes, which can result in losses. The grinding media also only allow two samples to be processed in parallel, so the time required is correspondingly high.

The method named according to BRADFORD for the determination of the total protein content can be performed quickly and offers good reproducibility of the results (Bradford, 1976; Lottspeich & Engels, 2006), which could also be confirmed in the present work.

For gel electrophoretic separation, the above-mentioned protein extracts were first used. With the aid of the buffer prepared according to BJÖRKSTEN, no meaningful gels could be obtained, as no bands were detectable. This is attributed to a reaction of the buffer components with the gel. Therefore, for subsequent gel electrophoreses, a PBS buffer consisting of potassium dihydrogen phosphate and sodium chloride was used for protein extraction. To optimize the gel electrophoresis - obtaining higher band sharpness - different options such as different gel compositions, different extraction approaches with PBS buffer as well as mixing ratios of the reduction buffer with the sample were tested. Here, the most optimal results were obtained with an 18% separation gel, which was therefore used for the analysis of the apple samples using disc-SDS-PAGE. In a further optimization of the protein extraction for gel electrophoretic separation, a dialysis following the extraction represents an

alternative. By replacing the buffer by dialysis, the strength of the extraction buffer according to BJÖRKSTEN can be exploited and disc-SDS-PAGE can be performed with the minimal background signals of the PBS buffer.

Several different variants of the ELISA technique have been developed for the quantitative determination of Mal d 1 content in apple samples, as they differ in both specificity and sensitivity. For the analysis of Mal d 1 content, the competitive ELISA is suitable, as it achieves high extinctions especially for small antigene molecules. Therefore, a direct competitive ELISA should be developed based on the method of the indirect competitive ELISA according to Sancho et al. Therefore, a calibration curve was constructed for calibration and estimation of the linear range. This showed a negative slope in the range of 0.01-1.0 mg/l Mal d 1, so that measurements should be made in this range. However, a quantitative determination of the Mal d 1 content in the apple samples could not be performed due to the amounts of recombinant Mal d 1 required for this purpose.

Due to its high specificity, the sandwich variant of the ELISA is also well suited for the determination of the Mal d 1 content from a protein extract. In method development, the method of Matthes et al. served as a template. Since only little unlabeled Mal d 1 was available, a dilution of the antibodies higher by a factor of 10 had to be used. As a result, sufficient staining for detection could only be observed after an incubation period of about 72 h. However, due to the long incubation time, evaporation of the reagents in the wells of the microtiter plates was observed, whereby it is assumed that reproducible measurements are not possible.

The variant of direct ELISA also offers the possibility of allergen determination in apples. The HRP-conjugated goat anti-mouse antibody used for detection is specific enough to distinguish between the homologous proteins Mal d 1 and Bet v 1.

7.2.2. Influence of selenium biofortification on the protein content

Table 13 shows the protein content in the analyzed apple samples in mg/100 g d.w. The change factor can be used to access the influence of selenium biofortification.

Table 13: Protein content in mg/100 g d.w. in the apple samples and the change factors.

Variety and Year of Cultivation	Application*	Protein [mg/100 g d.w.]	Change factor
'Fiesta' 2017	control (HS)	190.03 ± 10.66	---
	0.1 kg selenite (HS)	176.95 ± 43.66	0.93
	0.1 kg selenate (HS)	218.45 ± 22.63	1.15
'Jonica' 2017	control (HS)	141.50 ± 26.83	---
	0.15 kg selenite (HS)	181.07 ± 33.93	1.28
'Golden Delicious' 2017	control (HS)	161.06 ± 5.31	---
	0.15 kg selenite (HS)	177.52 ± 19.22	1.10
	0.15 kg selenate (HS)	171.50 ± 30.61	1.06
'Jonagold' 2017	control (HS)	167.14 ± 8.05	---
	0.15 kg selenite (HS)	201.26 ± 4.59	1.20
	0.15 kg selenate (HS)	241.53 ± 5.13	1.45
'Golden Delicious' 2018	control (BS)	197.38 ± 1.77	---
	0.075 kg selenate (BS)	156.50 ± 31.35	0.79
'Jonagold' 2018	control (BS)	147.37 ± 6.79	---
	0.075 kg selenate (BS)	180.02 ± 31.64	1.22
'Boskoop' 2018	control (OS)	233.01 ± 32.88	---
	0.075 kg selenate (OS)	178.83 ± 5.19	0.77
'Jonica' 2018	control (OS)	188.83 ± 36.68	---
	0.075 kg selenate (OS)	159.38 ± 46.41	0.84
'Elstar' 2019	control (OS)	218.40 ± 6.38	---
	0.15 kg selenate (OS)	214.89 ± 14.94	0.98
	0.45 kg selenate (OS)	215.70 ± 10.53	0.99
'Elstar' – peel 2019	control (OS)	359.92 ± 26.52	--
	0.45 kg selenate (OS)	313.79 ± 11.01	0.87
'Elstar' – pulp 2019	control (OS)	163.89 ± 6.83	--
	0.45 kg selenate (OS)	122.23 ± 10.36	0.75

** Foliar spray rate of selenium per hectare and meter canopy height applied with a hand-held sprayer (HS), a backpack sprayer (BS) or a trailed orchard sprayer (OS).*

Different factors influencing the level of protein content were identified. Besides biofortification technique (form and level of selenium application), the apple variety, and the climatic conditions in the year of cultivation also play a role.

The application of selenate resulted in higher protein contents in most varieties when being compared to the selenite application. For 'Jonagold', the biofortification led to significantly higher protein contents compared to the control. This was especially observed for the application of selenate, independent of level of selenium and cultivation year. For the year 2017, the protein content of the control was 169.5 mg/100 g d.w., while the application of selenate resulted in a content of 241.5 mg/100 g d.w., which represents an increase of almost

45%. In the following year, the difference in protein content between the control and the selenate application was 22% (147.4 mg/100 g d.w. and 180.0 mg/100 g d.w.). Selenite fertilization also resulted tended only to slightly higher protein contents.

The influence of a biofortification with selenium on proteins has already been studied by different research groups on different crops, especially cereals and legumes. However, different effects have been observed. Poblaciones et al. carried out selenium fertilization trials with different cereal crops in two growing periods and determined, among other things, the protein content in grains. Selenite and selenate were applied by foliar sprays at different application levels (Poblaciones et al., 2014a; Poblaciones et al., 2014b). The biofortification of wheat (*Triticum aestivum* L., cv. 'Roxo') did not lead to a significant difference in grain protein content between the application of selenite and selenate. However, in a crop year which was characterized by low precipitation, selenate led to a significantly lower protein content than selenite. Furthermore, the intense drought of this year had a negative influence on the grain protein content (Poblaciones et al., 2014a). Similar results were observed with durum wheat (*Triticum durum* L., cv. 'Marialva'). However, in this cereal species the selenate application always led to higher protein contents than treatments with selenite, regardless of the year (Poblaciones et al., 2014b). Differences in protein content depending on the form of application were also observed in a study on peas (*Pisum sativum* L., cv. 'Lincoln'). Foliar applications of selenite resulted in lower crude protein in grain than the sprays with selenate (Poblaciones et al., 2013). However, this effect does not seem to be valid for all crops, as in a study with barley (*Hordeum vulgare* L. ssp. *distichum*), a difference between the selenium forms on grain protein was also not observed (Rodrigo et al., 2013).

Reis et al. biofortified rice (*Oryza sativa* L., cv. 'Ana 5015') with selenate and an additional treatment with different levels of nitrogen fertilization. Selenium application resulted in lower protein contents compared to the untreated controls, except for the highest nitrogen application. There, a higher protein content was measured (Reis et al., 2018). D'Amato et al. also investigated the influence of a biofortification on rice (*Oryza sativa* L., cv. 'Selenio'). The protein content was slightly affected by selenite. With increasing concentrations of selenate the protein content decreased (D'Amato et al., 2018). Biofortification of pear-jujubes (*Zizyphus jujuba* M. cv. 'Lizao') with selenite in a dose of 300 g/ha and foliar fertilization led to an increase in soluble proteins of 48-52% as described by Zhao et al. (Zhao et al., 2013).

However, this effect was not observed by Jing et al. when studying the variety 'Zhanhua'. There, different concentrations of selenite were applied up to a level of 200 mg/L and no significant differences were found compared to the untreated control (Jing et al., 2017).

In the present study, 'Fiesta' and 'Jonica' showed already initially significant differences in protein content (in 2017). In the following year, significant differences were found between 'Boskoop', the variety with the highest protein content, and 'Jonagold', which had the lowest content. Significant differences were also found between 'Golden Delicious' and 'Jonagold'. A maximum protein content of 233.0 mg/100 g d.w. ('Boskoop') and a minimum of 147.4 mg/100 g d.w. ('Jonagold') was measured.

Matthes et al. also found a difference in the protein content of different apple varieties, with 'Golden Delicious' showing also a significantly higher protein content than 'Jonagold' apples. The protein contents measured in our present study are in comparable ranges to the data of Matthes & Schmitz-Eiberger (Matthes & Schmitz-Eiberger, 2009). Marzban et al. also determined the protein content of different apple varieties and observed significant differences, with 'Jonagold' showing a slightly higher protein content than 'Golden Delicious'. Those values were significantly lower than the protein contents obtained in the present study (Marzban et al., 2005). Differences in the protein content can be explained by different extraction and determination methods. In the present study, the proteins in the apple samples were extracted by a triple treatment in the ball mill. However, Marzban et al. only carried out a simple extraction by stirring the samples.

With regard to the influence of the growth season on the protein content, the controls of the varieties 'Jonica', 'Jonagold', and 'Golden Delicious' from 2017 were compared with those from 2018. The varieties were influenced differently. While the protein content of 'Jonica' and 'Golden Delicious' was higher in 2018, the opposite was found for 'Jonagold'. The differences can be explained by an influence of the different ecophysiological conditions in the years of cultivation such as precipitation, sunshine duration, and the corresponding UV radiation. For the year 2018, a significantly higher sunshine duration (+37%) and significantly lower precipitation (-61%) were recorded (Wetterkontor, 2019). However, the influence of the amount of rain was of lower importance due to the use of irrigation.

The observations made here are only consistent with previously published data on 'Jonagold': significantly lower protein content at high sunshine duration combined with drought. Matthes

and Schmitz-Eiberger (2009) observed significantly different protein contents in the varieties 'Jonagold' and 'Golden Delicious' at two German cultivation sites, which can be differentiated with regard to the environmental conditions such as precipitation and sunshine duration. At the location with the significantly higher sunshine duration (+44%), significantly lower protein contents (-33% and -53%) were found (Matthes & Schmitz-Eiberger, 2009). An influence on the formation of proteins as a function of environmental conditions such as temperature and sunshine duration has also already been described for cereals, where the protein content is an essential quality criterion (Poblaciones et al., 2014a; Poblaciones et al., 2014b; Vollmer et al., 2018). It was found that in dry years, the protein contents of wheat, durum wheat, and barley were lower (Poblaciones et al., 2014a; Poblaciones et al., 2014b; Rodrigo et al., 2013).

The separate analysis of fruit flesh and peel of the variety 'Elstar' from 2019 showed that the proteins are mainly located in the peel. This was independently of the biofortification. The average protein content in the peel was 68.7% in the control and 72.0% in the biofortified samples. Only 31.3% and 28.0% of the proteins are present in the fruit flesh, respectively. The protein content of the fruit flesh was significantly reduced by the biofortification compared to the control, while the total protein content was not affected.

Shah et al. carried out a measurement of the protein content in peel and fruit flesh, with the apple variety not being indicated. They conclude that especially the fruit flesh with a content of 2.0 mg/100 g f.w. (corresponds to a proportion of 71.4%) was particularly richer in protein. The peel, on the other hand, which is low in protein, contained only 0.8 mg/100 g f.w. (28.6%) (Shah et al., 2012). Muhktar et al. analyzed the protein content of various apple varieties, including 'Golden Delicious'. The average protein content was 2.2% in the fruit flesh; values for the peel were not given (Mukhtar et al., 2010).

7.2.3. Influence of selenium biofortification on the pattern of allergenic proteins

The duplicate determinations of the individual apple samples resulted in comparable protein patterns with very similar bands, indicating a good reproducibility of this method inclusive the protein extraction. In general, bands in the range of 18 kDa can be detected, where the well-known apple allergen Mal d 1 (M_w = 17.65 kDa) can potentially be found. Further bands were detected in the range of approx. 9 kDa, indicating the potential presence of the Mal d 3 (molecular weight (M_w) = 11.41 kDa). Slightly above the band close to 25 kDa of the molecular weight marker, broader bands were additionally visible in many samples. These can be

attributed to the apple allergen Mal d 2, which has a molecular weight of 25.68 kDa. Mal d 4, which has a molecular weight of 13.96 kDa was not detected.

Other research groups have also already been able to detect the various allergenic proteins in apples using SDS-PAGE. Matthes and Schmitz-Eiberger (2009) were able to qualitatively detect Mal d 1 by separating protein extracts of the apple variety 'Greenstar' using SDS-PAGE. Because of a worse migration front, no Mal d 3 was identified in that study. Further bands attributable to the other potential apple allergens were also not detected (Matthes & Schmitz-Eiberger, 2009). Marzban et al. were able to detect Mal d 1 and Mal d 2 by means of a gel electrophoretic separation of the protein extracts in various apple cultivars, including 'Golden Delicious' (Marzban et al., 2014). Sancho et al. performed a SDS-PAGE analysis with apple extracts of two different varieties, identifying Mal d 3 (Sancho et al., 2006b).

The different analyzed apple samples showed a similar protein pattern with regard to Mal d 1, Mal d 3, and Mal d 2. Intensity differences between the varieties, the years of cultivation, and due to the biofortification were detected (Manuscript II).

Determination of allergenic protein levels by SDS-PAGE is only semi-quantitative, but allows rapid evaluation and provides indications of differences in levels due to different intensities of band staining. Due to different intensities of band staining, it is assumed that there is an influence of biofortification on the allergenic proteins. Compared to the controls and the application of selenite, fertilization with selenate probably leads to lower Mal d 1 concentrations in most cultivars. Furthermore, a stronger intensity of the Mal d 3 band was observed in the biofortified samples. Therefore, biofortification probably leads to an increased synthesis of this protein. Variety-specific differences appear here: the biofortified apples of the varieties 'Fiesta' and 'Jonagold' seem to synthesize comparatively much Mal d 3, whereas 'Jonagold', 'Golden Delicious', and 'Jonica' contain only few Mal d 3. The differences in 'Elstar' were only marginal. 'Jonagold', on the other hand, seems to contain particularly high levels of Mal d 1.

In the literature, various apple allergens have already been identified by different laboratory analytical methods and differences between individual cultivars have been described. Using immuno-tissue-print-assay (ITP), Marzban et al. found variety-specific differences in the content of Mal d 1, Mal d 2, and Mal d 3 in 'Golden Delicious' (Marzban et al, 2005). In a subsequent study, it was found that the content of Mal d 1 was higher in 'Golden Delicious',

'Granny Smith', 'Fuji', and 'Pink Lady' than in 'Topaz' and 'Braeburn'. Protein extracts here were separated by gel electrophoresis (Marzban et al., 2014). Sancho et al. performed SDS-PAGE with apple extracts from different cultivars, including 'Cox orchard 3' and 'Jonagored', and an indirect competitive ELISA to determine the content of Mal d 3. SDS-PAGE showed an identical protein pattern for the two cultivars, but differences were measured by ELISA: 'Cox orchard 3' had the highest content with 70.2 µg Mal d 3/g peel, while 'Jonagored' and 'Gala' contained only 31 µg/g (Sancho et al., 2006b).

In the present work, it was found that the selenium biofortified apples had a higher content of Mal d 3. This seems to be probably related to the properties of the allergenic protein belonging to the nsLTP. The expression of nsLTPs is affected by different abiotic stress factors. However, in this context, differences have been identified in a bunch of selected crops such as tomato, pepper, and barley. For example, it was observed that salinity led to an induction of gene expression in the three crops mentioned before, while tomato and pepper responded to drought and cold, and only pepper responded to wounding (Jung et al., 2003; Molina & Garcia-Olmedo, 1993; Torres-Schumann et al., 1992; Treviño & O'Connell, 1998). Sancho et al. could show that the content of Mal d 3 depends on the position of the fruit on the tree (sunny *vs.* shady positions): Mal d 3 levels increased 2-fold in apples harvested from the shady site. Furthermore, differences in the content were dependent on lower and upper part of the tree (Sancho et al, 2006b). With regard to apples, the application of selenium-containing fertilizers also seems to be a stress factor for the plant to a certain extent, as the content of Mal d 3 increased.

Separate analysis of protein extracts from peel and fruit flesh of the 'Elstar' variety can determine in which fruit compartment the various allergenic proteins are localized. It was found that Mal d 3 is mainly found in the peel of the apples. Mal d 1 is found in both compartments and Mal d 2 is mainly localized in the fruit flesh. Various research groups have also determined the localization of allergenic proteins in apples. Marzban et al. performed a Northern Blot for the detection of the allergens Mal d 1 and Mal d 3 and an ITP to show the localization of Mal d 1, Mal d 2, and Mal d 3 within apple tissues in four different apple cultivars. They also found that Mal d 1 is present in peel as well as the fruit flesh, while Mal d 2 is mainly expressed in the fruit flesh and Mal d 3 is only present in the peel. The Northern blot analysis showed that Mal d 1 transcripts were found in the peel and in the fruit flesh, whereas

Mal d 3 expression could only be measured in the peel (Marzban et al., 2014). The localization of Mal d 3 is due to the function of the nsLT protein in the biosynthesis of epicuticular wax or cuticula and in plant defense mechanisms such as antimicrobial activities (Hoffmann-Sommergruber, 2005; Marzban et al., 2014; Salcedo et al., 1999; Salcedoet al., 2004).

7.2.4. Influence of selenium biofortification on the Mal d 1 content

The levels of the allergenic protein Mal d 1 measured in the apple samples are shown in Table 14. Factors of change were calculated to describe the change in the biofortified apples compared to the controls.

Table 14: Levels of the allergenic protein Mal d 1 in the selenium biofortified and control apples in mg/100 g d.w. and the change factors.

Variety and Year of Cultivation	Application*	Mal d 1 [mg/100 g d.w.]	Change factor
'Fiesta' 2017	control (HS)	51.0 ± 19.2	---
	0.1 kg selenite (HS)	68.8 ± 18.7	1.35
	0.1 kg selenate (HS)	24.8 ± 4.8	0.49
'Jonica' 2017	control (HS)	43.1 ± 4.5	---
	0.15 kg selenite (HS)	37.3 ± 8.9	0.87
'Golden Delicious' 2017	control (HS)	44.5 ± 3.2	---
	0.15 kg selenite (HS)	36.5 ± 3.9	0.82
	0.15 kg selenate (HS)	35.0 ± 3.2	0.79
'Jonagold' 2017	control (HS)	107.5 ± 4.9	---
	0.15 kg selenite (HS)	24.1 ± 4.2	0.22
	0.15 kg selenate (HS)	53.3 ± 19.0	0.50
'Golden Delicious' 2018	control (BS)	40.3 ± 5.7	---
	0.075 kg selenate (BS)	29.9 ± 4.0	0.74
'Jonagold' 2018	control (BS)	25.9 ± 3.5	---
	0.075 kg selenate (BS)	39.0 ± 5.7	1.51
'Boskoop' 2018	control (OS)	43.1 ± 6.5	---
	0.075 kg selenate (OS)	30.9 ± 0.1	0.72
'Jonica' 2018	control (OS)	38.0 ± 7.7	---
	0.075 kg selenate (OS)	32.6 ± 5.2	0.86
'Elstar' 2019	control (OS)	46.9 ± 1.0	---
	0.15 kg selenate (OS)	39.0 ± 3.3	0.83
	0.45 kg selenate (OS)	53.4 ± 13.6	1.14
'Elstar' – peel 2019	control (OS)	78.9 ± 3.0	--
	0.45 kg selenate (OS)	58.9 ± 1.3	0.75
'Elstar' – pulp 2019	control (OS)	29.9 ± 9.9	--
	0.45 kg selenate (OS)	17.1 ± 2.7	0.57

** Foliar spray rate of selenium per hectare and meter canopy height applied with a hand-held sprayer (HS), a backpack sprayer (BS) or a trailed orchard sprayer (OS).*

The different parameters of biofortification have an influence on the content of Mal d 1 in the apples. In general, a reduction of the Mal d 1 content was observed in the selenium biofortified samples. In addition, apple variety and ecophysiological conditions were identified as further factors influencing allergenicity. Furthermore, differences were found in the separately analyzed peel and fruit flesh samples (Manuscript II).

The application of selenite, which was carried out in crop year 2017, resulted in lower Mal d 1 contents for the cultivars 'Jonica' and 'Golden Delicious' and significantly lower Mal d 1 contents for 'Jonagold' compared to the controls. With the exception of 'Jonagold' from 2018, fertilization with selenate also resulted in reduced Mal d 1 contents. For 'Fiesta' and 'Jonagold' from 2017, and 'Golden Delicious' from 2017 and 2018 the differences were even statistically significant. Tendentially, the application of selenate led to lower contents than the application of selenite. However, the differences were not statistically significant.

The results can only be classified to a limited extent due to the lack of comparative studies. The analysis of the influence of biofortification on allergenic proteins has not been the subject of research to date. With regard to the content of Mal d 1 in apples, many influencing factors have been identified so far. For example, it has been shown that organically grown apples have a higher Mal d 1 content (Schmitz-Eiberger & Matthes, 2011) and patients with Mal d 1 allergy show a higher sensitivity when consuming these apples (Klockenbring et al., 2001).

The synthesis of Mal d 1 is influenced by selected ecophysiological stress factors of biotic and abiotic nature. Mal d 1 is a pathogenesis-related protein and mainly synthesized by the fruits as a defense agent against pathogens (Beuning et al., 2004; Botton et al., 2009; Breiteneder et al., 2000; Grafe, 2009; Matthes & Schmitz-Eiberger, 2009; Puehringer et al., 2000). Fruits that are grown organically are thus more subject to the influence of external stress factors like fungal, viral, and bacterial attack which are associated with an increased biosynthesis rate of Mal d 1 (Fernández-Rivas et al., 2006).

With regard to selenium biofortification, it is therefore assumed that the application of selenium and the associated significantly increased selenium accumulation in the apples leads to a better protection of the fruits against certain stress factors, whereby less Mal d 1 is synthesized. The induction of other plant protective substances like phenolic compounds has already been established in the present work (Manuscripts I und III). Furthermore, selenium, which is also a plant-protective agent, plays a role. It has already been shown that this trace

element can boost plants against a plenty of abiotic stresses such as cold, drought, radiation, salinity, and heavy metals (Feng et al., 2013; Gupta & Gupta, 2017). An additionally increased synthesis of the plant-protective Mal d 1 is no longer necessary and the gene expression is therefore downregulated.

With the exception of the variety 'Jonagold', a similar content of Mal d 1 in the range of 38.0 to 51.0 mg/100 g d.w. was measured in the controls of the different apple cultivars. The almost identical Mal d 1 contents of the varieties 'Jonica', 'Golden Delicious', 'Boskoop', and 'Elstar' are assumed to be the result of genetic relations (Jackson, 2003). Various observations have been made in literature on the allergen content of different apple varieties. Thus, Zuidmeer et al. (2006), Matthes and Schmitz-Eiberger (2009), and Kschonsek et al. (2019) found no significant differences between the varieties, whereas Marzban et al. (2014) found significant differences between 'Jonagold' and 'Golden Delicious' with higher values for 'Golden Delicious'. Kiewning and Schmitz-Eiberger (2014) observed higher contents at 'Elstar' compared to 'Boskoop'. Matthes and Schmitz-Eiberger (2009) measured concentrations of Mal d 1 of 'Jonagold' and 'Golden Delicious' in the range of 1.3 µg/g f. w. and 8.7 µg/g f. w. Converted with an approximated dry matter content of 15%, this results in values from 0.9 mg/100 g d. w. to 5.8 mg/100 g d. w. Romer et al. (2020) have also measured values for 'Golden Delicious' in this range, converted to dry matter these are from 1.9 mg/100 g d. w. to 2.3 mg/100 g d. w. These values are higher compared to the present study. Possible causes may lie in the different cultivation locations or the use of different extraction buffer followed by dialysis by the researchers.

An influence of ecophysiological conditions on the content of Mal d 1 could also be detected. The control samples of the three cultivars showed a lower Mal d 1 content in the cultivation year 2018, which was characterized with a high sunshine duration and low precipitation. The influence of environmental conditions such as precipitation, temperature, and solar radiation on the Mal d 1 content has already been described in the literature (Zuidmeer et al., 2006; Matthes & Schmitz-Eiberger, 2009; Botton et al., 2009). In contrast to the present work, Matthes & Schmitz-Eiberger measured significant higher Mal d 1 contents of 12 different apple cultivars at the cultivation site with significantly lower precipitation (-44%) and higher sunshine duration (Matthes & Schmitz-Eiberger, 2009). However, since the apples were

cultivated at two different locations, the influence of other environmental factors such as soil conditions and insects or parasites cannot be excluded.

Based on the separate analysis of peel and fruit flesh of 'Elstar' apples from 2019, it was determined that the allergenic protein Mal d 1 is mainly present in the peel. This is independent of biofortification, as no changes in the proportions in the compartiments were detected there. The Mal d 1 content, on the other hand, is subject to the influence of biofortification, since the Mal d 1 content in the individual plant parts was influenced differently. In the biofortified samples, the Mal d 1 content in the fruit flesh was reduced by 42.8% compared to the control. Whereas, the content of Mal d 1 in the peel was influenced by the biofortification only to a lesser extent – the Mal d 1 content was only 25.4% lower.

7.3. Analysis of the relationship between phenolic compounds and the allergenic protein Mal d 1 in selenium-biofortified apples

The analysis of the relationship between phenolic compounds and their properties with the allergenic protein Mal d 1 was performed using PEARSON'S correlation analyses (Köhler et al., 2007)). Here, the strength and direction of the correlation were investigated and the correlation coefficient R^2 was calculated. Furthermore, the influence of biofortification with selenium was analyzed. For the analysis of the correlation, the contents of the following parameters of selenium biofortified apples and control apples, already partially published in Manuscripts I and II, were used: Selenium content, PPO activity, total phenolic content, contents of the individual phenolic compounds, antioxidant activity by TEAC and ORAC assay, and Mal d 1 content.

7.3.1. Evaluation of the methods

Correlation analyses can be used to determine the strength of the relationship between individual metric variables. The measure of the degree of correlation is the correlation coefficient. This varies from -1.0 to +1.0, with values near +1 indicating a close positive relationship between the variables. Values near 0 indicate no relationship and values near -1 indicate a close negative relationship. This indicates the direction as well as the strength of the relationship. To avoid misinterpretation, the results of the correlation analysis should always be considered in the respective context, including plausibility relationships. To perform a PEARSON statistical analysis, the following requirements must be fulfilled: both variables must

be ordinal or interval scaled, the relationship between the two variables must be linear, and the variables should have a normal distribution (Köhler et al., 2007).

Some studies in the literature have already described a relationship between phenolic compounds and their properties with the allergenic protein Mal d 1 in apples. Since the analyzed parameters are metric data, on the basis of which a correlation is to be verified, and the requirements for PEARSON correlation analysis are fulfilled, the performance of a correlation analysis is suitable.

7.3.2. Correlation Analysis between Selenium Content and Mal d 1 Content

With respect to selenium content, it was found that biofortification resulted in a significant increase in the fruits by a factor of 10 to 40 compared to the respective controls. Furthermore, the Mal d 1 content in the biofortified apples was reduced in most cases (Manuscripts I and II). Manuscript III shows the results of the correlation analyses between the above two parameters. No correlation was found between selenium and Mal d 1 content across all samples. However, when each variety is considered separately, a negative correlation can be observed in most cases, and a high selenium content was therefore associated with a low Mal d 1 content. Variety-specific differences in strength and direction of the correlation were found. Here, the correlation coefficients varied between -0.7673 ('Jonagold' 2017) and 0.0244 ('Fiesta' 2017). Due to the significant negative correlation between selenium and Mal d 1 content, especially the cultivars 'Golden Delicious' and 'Boskoop' seem to be suitable for a future targeted reduction of Mal d 1 content by the applied agronomic approach. In 'Jonagold', differences in correlation were present between the two growing years. The Mal d 1 content of the cultivars 'Jonica' and 'Elstar' was only reduced or increased to a small extent by the biofortification and no association was found for 'Fiesta'.

Due to the lack of comparative studies, a comparison of the results can only be made to a limited extent. The influence of biofortification on allergens has not yet been described. However, a number of other factors influencing the allergenic protein content in apples have already been identified. In connection with biofortification, the cultivation system should be mentioned above all. Schmitz-Eiberger and Matthes (2011) indicated that apples from organic cultivation showed significantly higher Mal d 1 contents (Schmitz-Eiberger & Matthes, 2011). Furthermore, allergic persons revealed a higher sensitivity when consuming such apples (Klockenbring et al., 2001). Since organic cultivation does not involve the use of plant-

protective substances, the apple trees are more exposed to environmental stress factors such as fungal, bacterial, and viral attack. This results in a higher biosynthesis rate of Mal d 1, as this protein is a pathogenesis-related protein, which is synthesized by fruits mainly for defense against such pathogens and occasionally as an response against certain environmental stress conditions (Breiteneder & Ebner, 2000; Fernández-Rivas et al., 2006; Grafe, 2009; Matthes & Schmitz-Eiberger, 2009).

The application of selenium-containing fertilizers seems to lead to a better protection of the fruits against different stress factors, so that a lower synthesis of the also plant-protective protein Mal d 1 is possible. This may result directly from the selenium or indirectly from an increased synthesis of other plant-protective secondary plant metabolites. On the one hand, it has already been shown in selenium-biofortified apples that further plant-protective substances such as phenolic compounds are increasingly synthesized (Manuscripts I and III). This has also been observed previously in other crops (Bachiega et al., 2016; D'Amato et al., 2017; D'Amato et al., 2018; Pezzarossa et al., 2012; Schiavon et al., 2013; Schiavon et al., 2016; Zhao et al., 2013). Furthermore, it has already been shown that selenium can protect plants from a range of abiotic stresses such as cold, drought, radiation, salinity, and heavy metals (Feng et al., 2013; Gupta & Gupta, 2017).

7.3.3. Relationship between PPO Activity and Mal d 1 content

The analysis of correlation between PPO activity and Mal d 1 content showed correlations of different strength and direction in individual varieties. Differences in the correlation were found for one variety, including 'Jonica', from two different growing years. In the cultivation year 2017, there was a significant positive correlation here, whereas a negative correlation was recorded in the following year. In general, a trend towards a negative correlation was observed, with a low Mal d 1 content, a high PPO activity was observed. Therefore, the correlation between PPO activity and Mal d 1 content is probably subject to both cultivar influence and the influence of ecophysiological conditions.

A negative correlation in different apple cultivars is frequently reported in the literature (Garcia et al., 2007; Kiewning et al., 2013; Kschonsek et al., 2019; Schmitz-Eiberger & Matthes, 2011). Garcia et al. reported a reduced allergenicity in the form of a lower IgE-binding capacity of Mal d 1 for 'Golden Delicious' and 'Jonagold', if an excess of exogenous PPO was added to the apple samples (Garcia et al., 2007). Schmitz-Eiberger and Matthes also found higher PPO

activities in connection with lower extractability of Mal d 1 in different apple varieties (Schmitz-Eiberger & Matthes, 2011). The strength of the correlation varies here. Kiewning et al. also performed correlation analyses between Mal d 1 content and PPO activity of different cultivars. 'Elstar' and 'Diwa' showed a high correlation, while the correlation for fruits of 'Boskoop' was only moderate (Kiewning et al., 2013).

The relationship between high PPO activity and low levels of the allergenic protein Mal d 1 can be explained by the reaction of *o*-quinones, derived from the oxidation of phenolic compounds, with proteins. The enzyme PPO catalyzes this reaction, consequently high enzyme activities lead to high levels of *o*-quinones. These in turn enter into reactions with proteins and lead to an irreversible change in the tertiary structure of the allergen by modifying the nucleophilic amino acid side chains of the proteins, with the possibility of follow-up polymerizations (Kroll et al., 2003). Due to these cross-linkages, conformational epitopes of the allergen get lost, which reduces or even eliminates allergenicity (Chung & Champagne 2009; Garcia et al., 2007; Gruber et al., 2004).

With regard to the influence of selenium biofortification on the correlation between PPO activity and Mal d 1 content, partial changes in the strength and direction of the correlation were observed.

7.3.4. Analysis of the Relation between TPC and Mal d 1 Content

The analysis of the relation between TPC and Mal d 1 content in the individual varieties showed only occasional weak correlations. Since no correlation was of statistical significance, no trend was identified. It was therefore assumed that TPC alone does not, or only to a small extent, influence the content of allergenic proteins.

Kiewning et al. and Kschonsek et al. also found that the TPC plays only a minor role with respect to the Mal d 1 content. Rather, the activity of PPO seems to be more significant for the reduction of Mal d 1. It was found that when PPO activity is high, Mal d 1 content is reduced even when TPC is low (Bolhaar et al., 2005; Kiewning et al., 2013; Kschonsek et al., 2019; Matthes & Schmitz-Eiberger, 2009; Sancho et al., 2006; Son & Lee, 2001; Zuidmeer et al., 2006). Other research groups observed an inverse relationship between TPC and Mal d 1 content (Bernert et al., 2012; Kschonsek et al., 2019; Schmitz-Eiberger & Matthes, 2011). Apples with high phenolic compound content were better tolerated by apple allergic

individuals or showed lower in vitro allergenicity. Schmitz-Eiberger and Matthes described the relationship between Mal d 1 content, PPO activity, TPC, and antioxidant capacity in different apple cultivars. Their results showed that higher PPO activity and TPC lead to a diminished extractability of the allergenic protein Mal d 1 (Schmitz-Eiberger & Matthes, 2011). It is suggested that oxidative reactions between phenolic compounds and allergenic proteins in apples are responsible for this relationship (Rudeschko et al., 1995a; Rudeschko et al., 1995b). The reduction in allergenicity could be due to the masking of IgE-binding sites on the allergenic protein, through cross-linking of proteins induced by oxidative enzymes (Chung & Champagne, 2009; Rohn, 2014). PPO is the main factor involved in these oxidative reactions in fruit (Garcia et al., 2007).

A trend in the change of the correlation between TPC and Mal d 1 content in strength and direction was not observed due to biofortification. Thus, in addition to changes in the direction of correlation, stronger or weaker correlations occurred in the different varieties.

7.3.5. Influence of individual phenolic compounds of the Mal d 1 content

The correlation between the individual phenolic compounds and the Mal d 1 content also showed differences between the different varieties. However, trends were evident across several cultivars. For chlorogenic acid, a negative correlation was observed for 'Fiesta', 'Golden Delicious', and 'Jonagold'. High levels of epicatechin were observed in association with high Mal d 1 levels for 'Fiesta' and 'Jonagold', whereas there was a negative correlation for 'Golden Delicious'. For procyanidin trimer, a positive correlation was observed for 'Fiesta' and 'Jonagold', whereas there was a negative correlation for 'Jonica' and 'Golden Delicious'. Since the correlation coefficients between caffeeoylglucoside and Mal d 1 are low, this phenolic compound seems to play only a minor role with respect to allergenic potential. The sum of phloretin glucosides correlated positively with the Mal d 1 content in 'Jonica' and 'Jonagold'. Furthermore, a negative correlation was observed between the sum of quercetin glycosides and the Mal d 1 content in all cultivars, except 'Jonagold'.

In the literature, there are already some studies that analyzed the correlation of individual phenolic compounds and the Mal d 1 content in different apple cultivars (Bernert et al., 2012; Kiewning et al., 2013; Kschonsek et al., 2019a; Kschonsek et al., 2019b; Romer et al., 2020). Kiewning et al. found a low to moderate correlation between catechin and epicatechin and Mal d 1 content, with differences in the direction of correlation among 'Elstar', ' Boskoop', and

'Diwa' cultivars (Kiewning et al., 2013). Also in the present work, low to moderate correlation coefficients were observed for epicatechin and different dependencies due to cultivar. Bernert et al. found that the content of the main polyphenol chlorogenic acid was negatively correlated with tolerance claims in 'Golden Delicious'. When apples contained high levels of chlorogenic acid, they were better tolerated by allergy sufferers (Bernert et al., 2012). The present study confirmed this relationship to a large extent, since in most varieties a high chlorogenic acid content was correlated with a low content of Mal d 1. Due to this, a better tolerance is assumed. Romer et al. conducted a comprehensive study on 16 different apple cultivars to determine the correlation between the phenolic profile and Mal d 1 content. No correlation was found for the levels of flavonols, anthocyanins, and phenolic acids. The flavan-3-ols catechin and epicatechin, as well as the procyanidins B1, B3, and a non-specified procyanidin, showed a high positive correlation with the allergen content (Romer et al., 2020). The correlation between epicatechin content and Mal d 1 was confirmed in the present study. With regard to the procyanidins, variety-specific differences were found here. A positive correlation was also observed for 'Fiesta' and 'Jonagold', whereas a low procyanidin content was correlated with a low Mal d 1 content in 'Golden Delicious' and 'Jonagold'. The allergenicity of apples therefore seems to be mainly influenced by the procyanidins, epicatechin, and chlorogenic acid. Here, a low content of procyanidin and epicatechin and a high content of chlorogenic acid have a positive effect on the Mal d 1 content, since low levels of the allergen are present here. With regard to cultivars being generally low in allergens, cultivars with a low procyanidin and epicatechin content and a high chlorogenic acid content seem to be advantageous, therefore. As only very low correlation coefficients were measured between the other phenolic compounds and Mal d 1, the content of these substances probably had no influence on the overall allergenic potential of the apples.

Biofortification seems to be well suited for the reduction of the allergenic potential, since in most cases the biofortified apple samples had a lower content of procyanidin and epicatechin and a higher content of chlorogenic acid in combination with envious Mal d 1 levels.

The potential anti-allergenic properties of phenolic compounds are based on different molecular mechanisms: on the one hand, the tertiary structure of the proteins can be altered by the polyphenols themselves, by their oxidized forms (*o*-quinones), or directly by PPO, so that recognition of the antibody is no longer possible (Casanal et al., 2013; Romer et al., 2020;

Ullah et al., 2017). Due to the structural similarity between the amino acid tyrosine and phenolic compounds, PPO can also use tyrosine as a substrate. If tyrosine is now present in the protein structure of the allergen, it is oxidized and there is a formation of covalent crosslinks within the protein(s) and, consequently, a conformational change and a loss of antibody recognition (Kschonsek et al., 2019; Romer et al., 2020; Wu et al., 2016). Another mechanism is based on the influence of phenolic compounds on mast cells and the prevention of histamine release (Kanda et al., 1998; Romer et al., 2020; Singh et al., 2011; Wang et al., 2017). Thus, polyphenols are able to influence the binding between IgE antibodies and the FCεRI receptor on the mast cell surfaces, resulting in a lower amount of released histamine and, thus, in a lower allergic recruitment (Romer et al., 2020; Son & Lee, 2001; Tokura et al., 2005). Furthermore, interactions between phenolic compounds and the allergenic proteins may influence digestion in the gastrointestinal tract in a way that inactivates allergenic effects. The phenol-protein adducts formed are enzymatically less digestible (Rawel et al., 2001; Rohn, 2014). During the formation of irreversible bonds between phenolic compounds and proteins, the phenolic compounds are oxidized to quinones, which in turn can react with nucleophilic groups of the protein molecule. These interactions can affect the structure, functionality, and quality of the proteins, while bioavailability can also be affected by reduced digestibility in the gastrointestinal tract (Jakobek, 2015; Rohn et al., 2002).

7.3.6. Relationship between AOA und Mal d 1

There was a negative correlation between the AOA and the Mal d 1 content in the majority of the apple varieties studied. At higher antioxidant activities, the Mal d 1 content tended to be lower. 'Golden Delicious', in contrast to the other cultivars, showed a positive correlation to the Mal d 1 content in the TEAC and ORAC assays. In addition to TPC and PPO activity, it has already been shown that AOA also plays a role in relation to apple allergenicity (Björksten et al., 1980; Garcia et al., 2007; Kiewning et al., 2013; Rudeschko et al., 1995b; Schmitz-Eiberger et al., 2003; Schmitz-Eiberger & Matthes, 2011; Vieths et al., 1995).

Garcia et al. and Schmitz-Eiberger and Matthes analyzed the relationship between AOA and allergenicity of the 'Golden Delicious' cultivar and found a positive correlation (Garcia et al., 2007; Schmitz-Eiberger & Matthes, 2011). Therefore, with respect to the 'Golden Delicious' cultivar, the present work confirms the previously published results of other research groups. Garcia et al. conducted further experiments with 'Golden Delicious' in which the synthesized

antioxidant DIECA was added to the samples. Compared to the untreated controls, the Mal d 1 content was higher in the DIECA-treated samples. It is suggested that the complex reactions between phenolic compounds and Mal d 1 were inhibited (Garcia et al., 2007).

Schmitz-Eiberger and Matthes determined the relationship between AOA, PPO activity, and Mal d 1 content in three apple cultivars 'Braeburn', 'Topaz', and 'Golden Delicious'. They found differences in the level of the three parameters. It was found that the Mal d 1 content and the AOA were lowest, and the PPO activity was highest in 'Braeburn'. For 'Golden Delicious', the three parameters were in a medium range. For 'Topaz', a high TPC, a high catechin content, a relatively low PPO activity, and a high AOA were measured. A high PPO activity and a high TPC resulted in a low Mal d 1 content, whereas a high AOA probably inhibits the interactions between oxidized phenolic compounds and Mal d 1. This results in a higher allergenicity and a "normal" extractability of Mal d 1 (Schmitz-Eiberger et al., 2003; Schmitz-Eiberger & Matthes, 2011).

With respect to AOA by TEAC, no consistent trend through biofortification was observed. Different correlations between ORAC value and Mal d 1 content were observed between controls and biofortified apples. For most varieties, a positive correlation was observed in the biofortified samples, whereas the respective controls showed a negative correlation.

8. REFERENCES

1. Aas, G. Biologie und Ökologie einer gefährdeten Baumart. **2013**, 7-13.
2. Ahammer, L.; Grutsch, S.; Kamenik, A.S.; Liedl, K.R.; Tollinger, M. Structure of the major apple allergen Mal d 1. *J. Agric. Food Chem*. **2017**, *65*(8), 1606-1612.
3. Al-Duais, M.; Müller, L.; Böhm, V.; Jetschke, G. Antioxidant capacity and total phenolics of Cyphostemma digitatum before and after processing: Use of different assays. *Europ. Food Res. Technol.* **2009**, *228*(5), 813-821.
4. Alfthan, G.; Eurola, M.; Ekholm, P.; Venäläinen, E.; Root, T.; Korkalainen, K.; Hartikainen, H.; Salminen, P.; Hietaniemi, V.; Aspila, P.; Aro, A. Effects of nationwide addition of selenium to fertilizers on foods, and animal and human health in Finland: From deficiency to optimal selenium status of the population. *J. Trace Elem. Med. Biol.* **2015**, *31*, 142-147.
5. Allahveran, A.; Farokhzad, A.; Asghari, M.; Sarkhosh, A. Foliar application of ascorbic and citric acids enhanced 'Red Spur' apple fruit quality, bioactive compounds and antioxidant activity. *Physiol. Mol. Biol. Plants* **2018**, *24*, 433-440.
6. Alu'datt, M.H.; Rababah, T.; Ereifej, K.; Brewer, S.; Alli, I. Phenolicprotein interactions in oilseed protein isolates. *Food Res. Int.* **2013**, *52*, 178-184.
7. Ambroziak, U.; Hybsier, S.; Shahnazaryan, U.; Krasnodębska-Kiljańska, M.; Rijntjes, E.; Bartoszewicz, Z.; Schomburg, L. Severe selenium deficits in pregnant women irrespective of autoimmune thyroid disease in an area with marginal selenium intake. *J. Trace Elem. Med. Biol.* **2017**, *44*, 186-191.
8. Amiot, M.J.; Tacchini, M.; Aubert, S.; Nicolas, J. Phenolic composition and browning susceptibility of various apple cultivars at maturity. *J. Food Sci.* **1992**, *57*(4), 958-962.
9. Asano, K.; Shinagawa, K.; Hashimoto, N. Characterization of hazeforming proteins of beer and their roles in chill haze formation. *J. Am. Soc. Brew. Chem*. **1982**; *40*, 147-154.
10. Asero, R.; Mistrello, G.; Roncarolo, D.; Amato, S.; Zononi, D.; Barocci, F.; Caldironi, G. Detection of clinical markers of sensitization to profilin in patients allergic to plant-derived foods. *J. Allergy Clin. Immunol*. **2003**, *112*, 427-432.
11. Babalar, M.; Mohebbi, S.; Zamani, Z.; Askari, M.A. Effect of foliar application with sodium selenate on selenium biofortification and fruit quality maintenance of 'Starking Delicious' apple during storage. *J. Sci. Food Agric.* **2019**, *99*, 5149-5156.

12. Bachiega, P.; Salgado, J.M.; Carvalho, J.E. de; Ruiz, A. L.; Schwarz, K.; Tezotto, T.; Morzelle, M.C. Antioxidant and antiproliferative activities in different maturation stages of broccoli (*Brassica oleracea* var. *italica*) biofortified with selenium. *Food Chem.* **2016**, *190*, 771-776.
13. Ballmer-Weber, B.K.; Hoffmann-Sommergruber, K. Molecular diagnosis of fruit and vegetable allergy. *Curr. Opin. Allergy Clin. Immunol*. **2011**, *11*, 229-235.
14. Bally, R.W.; Gribnau, T.C.J. Some aspects of the chromogen 3,3´,5,5´-tetramethylbenzidine as hydrogen donor in a horseradish peroxidase assay. *J. Clin. Chem. Clin. Biochem*. **1989**, *27*, 791-796.
15. Bañuelos, G. S.; Lin, Z.Q.; Broadley, M. Selenium biofortification. In: Pilon-Smits, E.A.; Winkel, L.H.; Lin, Z.-Q. (eds.) Selenium in plants. *Springer International Publishing* **2017**, Cham, Schweiz, 231-255.
16. Belitz, H.-D.; Grosch, W.; Schieberle, P. Lehrbuch der Lebensmittelchemie. 6. Auflage. *Springer-Verlag* **2008,** Berlin Heidelberg.
17. Belle, C. Catecholoxidase and tyrosinase. In: Kretsinger, R.H.; Uversky, V.N.; Permyakov, E.A. (eds) Encyclopedia of metalloproteins. *Springer* **2013,** New York.
18. Berger, M. M. Spurenelemente – was gibt es Neues? *Swiss Med. Forum* **2003**, *3*(31), 720-726.
19. Bergmann, K.; Zuberbier, J.; Zuberbier, T.; Zapp, J.; Hennebrüder, W. Apfelallergie – Toleranzentwicklung durch regelmäßigen Konsum allergenarmer Äpfel. Eine Beobachtungsstudie. *Erwerbs-Obstbau* **2020**, *62*, 264-273.
20. Bernert, T.; Münstermann, J.; Kothe, A.; Zapp, J. Polyphenolgehalt in alten und neuen Apfelsorten im Bezug auf allergene Wirkungen. *Hochschule Ostwestfalen-Lippe* **2012**.
21. Bernhard W., Gerhard; R: Basiswissen aktualisiert: Flavonoide. *Ernährungs-Umschau* **2001**, *48*.
22. Beuning, L., Bowen, J., Persson, H., Barraclough, D., Bulley, S., Macrae, E. Characterization of Mal d 1-related genes in Malus. *Plant Mol. Biol.* **2004**, *55* (3), 369-388.
23. Biesalski, H.-K. Wissenschaftliche Ernährungsinformation. Antioxidantien. *Forum Ernährungsmedizin* **2000**.
24. Bisswanger, H. Enzyme: Struktur, Kinetik und Anwendungen. *John Wiley & Sons* **2015**.

25. Björksten, F.; Halmepuro, L.; Hannuksela, M.; Lahti, A. Extraction and properties of apple allergens. *Allergy* **1980**, *35*, 671–677.
26. BMEL – Bundesministerium für Ernährung und Landwirtschaft. Statistisches Jahrbuch über Ernährung, Landwirtschaft und Forsten 2019. Bundesanstalt für Landwirtschaft und Ernährung, *Druckerei Hachenburg* **2020**, 359.
27. Bolhaar, S. T.; van de Weg, W. E.; van Ree, R.; Gonzalez-Mancebo, E.; Zuidmeer, L.; Bruijnzeel-Koomen, C. A.; Fernandez-Rivas, M.; Jansen, J.; Hoffmann-Sommergruber, K.; Knulst, A. C. In vivo assessment with prick-to-prick testing and double-blind, placebo-controlled food challenge of allergenicity of apple cultivars. *J. Allergy Clin. Immunol*. **2005**, *116*, 1080-1086.
28. Botton, A.; Lezzer, P.; Dorigoni, A.; Barcaccia, G.; Ruperti, B.; Ramina, A. Genetic and environmental factors affecting allergen-related gene expression in apple fruit (*Malus domestica* L. *Borkh*). *J. Agric. Food Chem*. **2008**, *56* (15), 6707-6716.
29. Botton, A.; Lezzer, P.; Dorigoni, A.; Rupertis, B.; Ramina, A. Environmental factors affecting the expression of apples (*Malus x domestica* L. *Borkh*) allergen-encoding genes. *J. Hortic. Sci. Biotechnol.* Isafruit Special Issue **2009**, 182-187.
30. Boyer, J.; Liu, R. H. Apple phytochemicals and their health benefits. *Nutr. J.* **2004**, 5, 1-15.
31. Bradford, M. M. A Rapid and Sensitive Method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem*. **1976**, *72*, 248-254.
32. Bravo, L. Polyphenols: Chemistry, dietary sources, metabolism and nutritional significance. *Nutr. Rev*. **1998**, *56*, 317-333.
33. Breiteneder, H.; Ebner, C. Molecular and biochemical classification of plant-derived food allergens. *J. Allergy Clin. Immunol*. **2000**, *106*, 27-36.
34. Broadley, M. R.; White, P. J.; Bryson, R. J.; Meacham, M. C.; Bowen, H. C.; Johnson, S. E.; Hawkesforf, M. J.; McGrath, S. P.; Zhao F.; Breward, N.; Harriman, M.; Tucker, M. Biofortification of UK food crops with selenium. *Proceedings Nutr. Soc*. **2006**, *65*, 169-181.
35. Brudzynski, K.; Sjaarda, C.; Maldonado-Alvarez, L. A new look on protein-polyphenol complexation during honey storage: Is this a random or organized event with the help of dirigent-like proteins. *PLoS One* **2013**, *30*, 72897.

36. Buitimea-Cantúa, N.E.; Gutiérrez-Uribe, J, A.; Serna-Saldívar, S.O. Phenolic-protein interactions: effects on food properties and health benefits. *J. Med. Food*. **2018**, *21*(2), 188-198.
37. BUND-Ortsgruppe Lemgo, Apfelallergie. Available online: http://www.bund-lemgo.de/apfelallergie.html (accessed on 04 August **2021**).
38. Burney, P.G.J.; Potts, J.; Kummeling, I.; Mills, E.N.C.; Clausen, M.; Dubakiene, R.; Barreales, L.; Fernandez-Perez, C.; Fernandez-Rivas, M.; Le, T.-M.; Knulkst, A. C.; Kowalski, M. L.; Lidholm, J.; Ballmer-Weber, B. K.; Braun-Fahlander, C.; Mustakov, T.; Kralimarkova, T.; Popov, T.; Sakellariou, A.; Papadopoulos, N. G.; Versteeg, S. A.; Zuidmeer, L.; Akkerdaas, J. H.; Hoffmann-Sommergruber, K.; van Ree, R. The prevalence and distribution of food sensitization in European adults. *Allergy* **2014**, *69*, 365–371.
39. Burton, S.G. Biocatalysis with polyphenoloxidase: a review. *Catal. Today* **1994**, *22*(3), 459-487.
40. Cakmak, I. Agronomic approaches in biofortification of food crops with micronutrients. *UC Davis: Department of Plant Sciences*, **2009**.
41. Carbonaro, M.; Mattera, M. Polyphenoloxidase activity and polyphenol levels in organically and conventionally grown peach (*Prunus persica* L., cv. Regina Bianca) and pear (*Pyrus communis* L., cv. Williams). *Food Chem*. **2001**, *72*(4), 419-424.
42. Casanal, A.; Zander, U.; Dupeux, F.; Valpuesta, V.; Marquez, J.A. Purification, crystallization and preliminary X-ray analysis of the strawberry allergens Fra a 1E and Fra a 3 in the presence of catechin. *Acta Cryst. F.* **2013**, *69*, 510-514.
43. Cell Signaling Technology, Überblick über ELISA. Available online: https://www.cellsignal.de/contents/_/overview-of-enzyme-linked-immunosorbent-assay-(elisa)/elisa-educational (accessed on 09 October **2019**).
44. Ceymann, M. Polyphenol content and profile in apples and its potential relevance to human health. **2013**.
45. Chung, S.Y.; Champagne, E.T. Reducing the allergenic capacity of peanut extracts and liquid peanut butter by phenolic compounds. *Food Chem*. **2009**, *115*, 1345-1349.
46. Cirilli, M.; Caruso, G.; Gennai, C.; Urbani, S.; Frioni, E.; Ruzzi, M.; Servili, M.; Gucci, R.; Poerio, E.; Muleo, R. The role of polyphenoloxidase, peroxidase, and ß-glucosidase in phenolics accumulation in *Olea europeaea* L. fruits under different water regimes. *Front. Plant Sci.* **2017**, *8*, 717.

47. Claus H. Laccases: structure, reactions, distribution. *Micron*. **2004**, *35*(1-2), 93-96.
48. Combs Jr, G. F.; Yan, L. Status of dietary selenium in cancer prevention. In: Hatfield, D.L.; Schweizer, U.; Tsuji, P.A.; Gladyshev, V.N. (eds.). Selenium - its molecular biology and role in human health, *Springer International Publishing* **2016**, 321-332.
49. Cornille, A.; Giraud, T.; Smulders, M.J.M.; Gladieux, P.; Rolda, I. The domestication and evolutionary ecology of apples. *Trends Genet.* **2014**, *30*(2), 57-65.
50. Crozier, A.; Jaganath, I.B.; Clifford, M.N. Dietary phenolics: chemistry, bioavailability and effects on health. *Nat. Prod. Rep.* **2009**, *26*(8), 1001-1043.
51. Csepregi, K.; Neugart, S.; Schreiner, M.; Hideg, E. Comparative evaluation of total antioxidant capacities of plant polyphenols. *Molecules* **2016**, *21*, 208.
52. D'Amato, R.; Proietti, P.; Nasini, L.; Del Buono, D.; Tedeschini, E.; Businelli, D. Increase in the selenium content of extra virgin olive oil: quantitative and qualitative implications. *Grasas y Aceites*, **2014**, *65*, e025.
53. D'Amato, R.; Proietti, P.; Onofri, A.; Regni, L.; Esposto, S.; Servili, M.; Businelli, D.; Selvaggini, R. Biofortification (Se): Does it increase the content of phenolic compounds in virgin olive oil (VOO)? *PLoS ONE* **2017**, *12*.
54. D'Amato, R.; Fontanella, M.C.; Falcinelli, B.; Beone, G.M.; Bravi, E.; Marconi, O.; Benincasa, P.; Businelli, D. Selelium biofortification in rice (*Oryza sativa* L.) sprouting: Effects on Se yield and nutritional traits with focus on phenolic acid profile. *J. Agric. Food Chem.* **2018**, *66*, 4082-4090.
55. D'Archivio, M.; Filesi, C. Polyphenols, dietary sources and bioavailability. *Ann. Ist. Super. Sanità* **007**, *43*(4), 348-361.
56. Del Rio, D.; Costa, L.G.; Lean, M.E.J.; Crozier, A. Polyphenols and health: What compounds are involved? *Nutr. Metab. Cardiovasc. Dis.* **2010**, *20*, 1-6.
57. Deng, X. F.; Zhao, Z. Q.; Han, Z. Y.; Huang, L. Q.; Lv, C. H.; Zhang, Z. H.; Zhang, H. Q.; Liu, X. W. Selenium uptake and fruit quality of pear (*Pyrus communis* L.) treated with foliar Se application. *J. Soil Sci. Plant Nutr.* **2019**, *182*(4), 637-646.
58. Ernährungsbericht der Deutschen Gesellschaft für Ernährung e.V. **2004**.
59. Dhyani, P.; Bahukhandi, A.; Rawat, S.; Bhatt, I.D.; Rawal, R.S. Diversity of bioactive compounds and antioxidant activity in delicious group in Western Himalaya. *J. Food Sci. Technol.* **2018**, *55*, 2587–2599.

60. Dixon, R. A.; Paiva, N. L. Stress-induced phenylpropanoid metabolism. *PIlant Cell* **1995**, *7*(7), 1085.
61. Duan, N.; Bai, Y.; Sun, H.; Wang, N.; Ma, Y.; Li, M.; Wang, X.; Jiao, C.; Legall, N.; Mao, L.; Wan, S.; Wang, K.; He, T.; Feng, S.; Zhang, Z.; Mao, Z.; Shen, X.; Chen, X.; Jiang, Y.; Wu, S.; Yin, C.; Ge, S.; Yang, L.; Jiang, S.; Xu, H.; Liu, J.; Wang, D.; Qu, C.; Wang, Y.; Zuo, W.; Xiang, L.; Liu, C.; Zhang, D.; Gao, Y.; Xu, Y.; Xu, K.; Chao, T.; Fazio, G.; Shu, H.; Zhong, G.-Y.; Cheng, L.; Fei, Z.; Chen, X. Genome re-sequencing reveals the history of apple and supports a two-stage model for fruit enlargement. *Nat. Commun.* **2017**, *8*, 249.
62. Dunn, K.L.; Yang, L.; Girard, A.; Bean, S.; Awika, J.M. Interaction of sorghum tannins with wheat proteins and effect on in vitro starch and protein digestibility in a baked product matrix. *J. Agric. Food Chem*. **2015**, 63, 1234–1241.
63. Ebermann, R.; Elmadfa, I. Lehrbuch Lebensmittelchemie und Ernährung. 1. Auflage. *Springer* **2008**.
64. Edreva, A. Pathogenesis-related proteins: Research progress in the last 15 years. *Gen. Appl. Plant Physiol.* **2005** *31*(1-2), 105-124.
65. Eichholz, I.; Rohn, S.; Gamm, A.; Beesk, N.; Herppich, W.B.; Kroh, L.W.; Ulrichs, C.; Huyskens-Keil, S. UVB-mediated flavonoid synthesis in white asparagus *(Asparagus officinalis* L.). *Food Res. Int.* **2012**, *48*, 196-201.
66. Ekanayake, L.J.; Thavarajah, D.; Vial, E.; Schatz, B.; McGee, R.; Thavarajah, P. Selenium fertilization on lentil (*Lens culinaris* Medikus) grain yield, seed delinium concentration, and antioxidant activity. *Field Crop. Res.* **2015**, *177*, 9-14.
67. Elmadfa, I.; Leitzmann, C. Ernährung des Menschen. 5. Auflage. *Ulmer* **2015**.
68. Engelhardt, U.H. Polyphenole in Tee. Wissenschaftlicher Informationsdienst Tee. *Deutsches Tee-Institut* Frühjahr **1998**.
69. European Commission – Food Safety. **2000**. Available online: http://ec.europa.eu/food/fs/sc/scf/out80g_en.pdf
70. Fallon, N. und Dillon, S. A. Low intakes of iodine and selenium amongst vegan and vegetarian women highlight a potential nutritional vulnerability. *Front. Nutr.* **2020**, *7*, 72.
71. Feng, R.; Wei, C.; Tu, S. The roles of selenium in protecting plants against abiotic stresses. *Environ. Experim. Bot*. **2013**, *87*, 58-68.

72. Fernandes, H.; Michalska, K.; Sikorski, M.; Jaskolski, M. Structural and functional aspects of PR-10 proteins. *FEBS J* **2013**, *280*(5), 1169-1199.
73. Fernández-Rivas, M.; Bolhaar, M.; González-Mancebo, E.; Asero, R.; van Leeuwen, A.; Bohle, B.; Ma, Y.; Ebner, C.; Rigby, N.; Sancho, A. I. Apple allergy across Europe: How allergen sensitization profiles determine the clinical expression of allergies to plant foods. *J. Allergy Clinic.Immunol.* **2006**, *118*(2), 481-488.
74. Finaud, J.; Lac, G.; Filaire, E. Oxidative stress: relationship with exercise and training. *Sports medicine* **2006**, *36*(4), 327-358.
75. Frankel, E.N.; Meyer, A.S. The problems of using one-dimensional methods to evaluate multifunctional food and biological antioxidants. *J. Sci. Food Agric*. **2000**, *80*, 1925-1941.
76. Frede, W. Handbuch für Lebensmittelchemiker. 3. Auflage. *Springer* **2010.**
77. Frei, B. Reactive oxygen species and antioxidant vitamins: mechanisms of action. *Americ. J. Med.* **1994**, *97*(3A), 5S–13S; 22S–28S.
78. Gallin, J.I.; Goldstein, I,M.; Snyderman, R. Inflammation: basic principles and clinical correlates. 2. Edition. *Raven Press* **1992**, 701-708.
79. Ganten, D.; Ruckpaul, K. Molekular- und Zellbiologische Grundlagen. 1. Auflage. *Springer* **1997**, 133-167.
80. Gell, P.; Coombs, R. The classification of allergic reactions underlying disease. In: Clinical aspects of immunology (chapter 13). *Oxford: Blackwell Scientific Publications* **1963**.
81. Gerdemann, C.; Eicken, C.; Krebs, B. The crystal structure of catecholoxidase: new insight into the function of type-3 copper proteins. *Acc. Chem. Res*. **2002**, *35*(3), 183-191.
82. Gerhauser, C. Cancer chemopreventive potential of apples, apple juice, and apple components. *Planta Med.* **2008**, *74*, 1608-1624.
83. Gey, M. H. Instrumentelle Analytik und Bioanalytik. 2. Auflage. *Springer* **2008**.
84. Gey, M.H. Instrumentelles und Bioanalytisches Praktikum. *Springer* **2012**.
85. Ghafoor, K.; Hee Choi, Y.; Yeoung Jeon, J.; Hee Jo, I. Optimization of ultrasound-assisted extraction of phenolic compounds, antioxidants, and anthocyanins from grape (*Vitis vinifera*) seeds. *J. Agric. Food Chem*. **2009**, *57*, 4988-4994.
86. Gilissen, L. J. W. J.; Bolhaar, S. T. H. P.; Matos, C. I.; Rouwendal, G. J. A.; Boone, M. J.; Krens, F. A.; Zuidmeer, L.; van Leeuwen, A.; Akkerdaas J.; Hoffmann-Sommergruber, K.; Knulst, A. C.; Bosch, D.; van de Weg W. E.; van Ree, R. Silencing the major apple allergen

Mal d 1 by using the RNA interference approach. *J. Allergy Clin. Immunol*. **2005,** *115,* 364-369.

87. Golubkina, N.A.; Folmanis, G.E.; Tananaev, I.G. Comparative evaluation of selenium accumulation by allium species after foliar application of selenium nanoparticles, sodium selenite and sodium selenate. *Dokl. Biol. Sci.* **2012**, *444*(1), 176-179).
88. Gomez, F.; Aranda, A.; Campo, P.; Diaz-Perales, A.; Blanca-Lopez, N.; Perkins, J. Hohe Prävalenz der Lipidtransferproteinsensibilisierung bei Patienten mit Apfelallergie und systemischen Symptomen. *PLoS ONE* **2014.**
89. Grafe C. Apfelallergie: Aktueller Wissensstand und Ausblick. *Obstbau* **2009**, *618*-620.
90. Gruber, P.; Vieths, S.; Wangorsch, A.; Nerkamp, J.; Hofmann, T. Maillard reaction and enzymatic browning affect the allergenicity of Pru av 1, the major allergen from cherry (*Prunus avium*). *J. Agric. Food Chem.* **2004**, *52*, 4002–4007.
91. Grune, T. Antioxidantien. In: Vitamine, Spurelemente und Mineralstoffe. Prävention und Therapie mit Mikronährstoffen. Biesalski, H. K.; Kohrle, J.; Schumann, K. (Hrsg.). *Georg Thieme* **2002**, 50-56.
92. Güngör, N.; Özyürek, M.; Güclü, K. et al. Comparative evaluation of antioxidant capacities of thiol-based antioxidants measured by different *in vitro* methods. *Talanta* **2011,** *83*, 1650-1658.
93. Günzler, H.; Bahadir, A. M.; Borsdorf, R.; Danzer, K.; Fresenius, W.; Galensa, R.; Huber, W.; Linscheid, M.; Lüderwald, I.; Schwedt, G.; Tölg, G.; Wisser, H. Analytiker Taschenbuch, Band 15. *Springer* **1997**.
94. Gupta, M.; Gupta, S. An overview of selenium uptake, metabolism, and toxicity in plants. *Front. Plant Sci.* **2017**, *7*, 2074.
95. Guyot, S.; Le Bourvellec, C. Procyanidins are the most abundant polyphenols in dessert apples at maturity. *LWT* **2002**, *35,* 289-291.
96. Guyot, S.; Marnet, N. Variability of the polyphenolic composition of cider apple (*Malus domestica*) fruits and juices. *J. Agric. Food Chem.* **2003,** *51*, 7522-7522.
97. Halliwell, B. How to characterize a biological antioxidant. *Free Radical Res.* **1990**, *9*(1), 1-32.
98. Halliwell, B.; Gutteridge, J. M. C. Free radicals in biology and medicine. 15. edition. *Oxford University Press* **2015**.

99. Han, H.M.; Koh, B.K. Effect of phenolic acids on the rheological properties and proteins of hard wheat flour dough and bread. *J. Sci. Food Agric.* **2011**, *91*, 2495-2499.
100. Hanke, M.-V.; Flachowsky, H. Obstzüchtung und wissenschaftliche Grundlagen. *Springer* **2017**.
101. Harborne, J.B. Plant phenolics. Encyclopedia of plant physiology, Volume 8, secondary plant products. *Springer* **1980**, 329–395.
102. Harborne, J.B. Flavonoids in the environment: Structure-activity relationship. *Progress Clin. Biol. Res.* **1988**, *280*, 17-27.
103. Haslam, E. Natural polyphenols (vegetable tannins) as drugs: Possible modes of action. *J. Nat. Prod.* **1996**, *59*, 205-215.
104. Hauser, M.; Egger, M.; Wallner, M.; Wopfner, N.; Schmidt, G.; Ferreira, F. Molecular properties of plant food allergens: A current classification into protein families. *Open Immunol*. J. **2008**, *1*, 1-12.
105. Hawrylak-Nowak, B. Enhanced selenium content in sweet basil (*Ocimum basilicum* L.) by foliar fertilization. *Veg. Crop. Res. Bull.* **2008**, *69*, 63-72.
106. Hawrylak-Nowak, B. Comparative effects of selenite and selenate on growth and selenium accumulation in lettuce plants under hydroponic conditions. *Plant Growth Regul.* **2013**, *70*, 149-157.
107. Hejgaard, J.; Jacobsen, S.; Svendsen, I. Two antifungal thaumatinlike proteins from barley grain. *FEBS Lett* **1991,** *291*(1), 127-131.
108. Heldt, H.; Piechulla, B. Pflanzenbiochemie. *5*. Auflage. *Springer* **2015**.
109. Herrmann, K. Über die Gehalte der hauptsächlichen Pflanzenphenole im Obst. *Flüssiges Obst* **1992**, *59*, 66-70.
110. Hirschi, K.D. Nutrient biofortification of food crops. *Annu. Rev. Nutr.* **2009**, *29*, 401-421.
111. Hoffmann-Sommergruber, K. Plant allergens and pathogenesis-related proteins. What do they have in common? *Int. Arch. Allergy Immunol*. **2000**, *122*, 155-166.
112. Hoffmann-Sommergruber, K. SAFE consortium The SAFEproject: 'plant food allergies: field to table strategies for reducing their incidence in Europe' an EC-funded study. *Allergy* **2005**, *60*, 436-442.
113. Holderbaum, D.F.; Kon, T.; Kudo, T.; Guerra, M.P. Enzymatic browning, polyphenoloxidase activity, and polyphenols on four apple cultivars: Dynamics during fruit development. *Hort. Sci.* **2010**, *45*, 1150-1154.

114. Holtzhauer, M. Methoden in der Proteinanalytik. 1. Auflage. *Springer* **1996**, 405-415.
115. Huang, D.; Ou, B.; Hampsch-Woodill, M.; Flanagan, J.; Prior, R. L. High-throughput assay of oxygen radical absorbance capacity (ORAC) using a multichannel liquid handling system coupled with a microplate fluorescence reader in 96-well format. *J. Agric. Food Chem.* **2002**, *50*(16), 4437–4444.
116. Huang, D.; Boxin, O.U.; Prior, R. L. The chemistry behind antioxidant capacity assays. *J. Agric. Food Chem.* **2005**, *53*(6), 1841-1856.
117. Hughes, D. J.; Duarte-Salles, T.; Hybsier, S.; Trichopoulou, A.; Stepien, M.; Aleksandrova, K.; Fagherazzi, G. Prediagnostic selenium status and hepatobiliary cancer risk in the european prospective investigation into cancer and nutrition cohort. *Am. J. Clin. Nutr.* **2016**, *104*, 406-414.
118. Hughes, D. J.; Fedirko, V.; Jenab, M.; Schomburg, L.; Méplan, C.; Freisling, H.; Hesketh, J. E. Selenium status is associated with colorectal cancer risk in the European prospective investigation of cancer and nutrition cohort. *Int. J. Cancer* **2015**, *136*, 1149-1161.
119. Hyson, D. A. A comprehensive review of apples and apple components and their relationship to human health. *Adv. Nutr*. **2011**, *2*, 408-420.
120. Industrieverband Agrar. Herkunft und Evolutionsgeschichte des Apfels aufgeklärt. Available online: https://www.iva.de/sites/default/files/styles/gallery_popup/public/benutzer/%25uid/magazinbilder/1479341_credit_alexa_m_schmitzyang_bai.jpg?itok=8_9V6ScL (**2018**)
121. Iriti, M.; Faoro, F. Bioactivity of grape chemicals for human health. *Nat. Prod. Commun.* **2009,** *4*(5*),* 611-634.
122. Jackson, J. E. Biology of apples and pears, biology of horticultural crops. 1. edition, *Cambridge University Bridge*, **2003**, 30-31.
123. Jakobek, L. Interactions of polyphenols with carbohydrates, lipids, and proteins. *Food Chem.* **2015**, *175*, 556-567.
124. Jakovljevic, M.; Licina, V.; Antic-Mladenovic, S.; Velickovic, M. The effects of selenium application on replant soil and its content in apple leaves and fruits. *Acta Hort.* **1996**, *477*, 163-166.
125. Janovitz-Klapp, A.; Richard, F.; Nicolas, J. Polyphenoloxidase from apple, particular purification and some properties. *Phytochem*. **1989**, *28* (11), 2903-2907.

126. Jenkins, J.A.; Griffiths-Jones, S.; Shewry, P.R.; Breiteneder, H.; Mills, E.N. Structural relatedness of plant food allergens with specific reference to cross-reactive allergens: an *in silico* analysis. *J. Allergy Clin. Immunol***. 2005**, *115*, 163-170.

127. Jeske, L.; Placzek, S.; Schomburg, I.; Chang, A.; Schomburg, D. BRENDA in 2019: a European ELIXIR core data resource. *Nucl. Acids Res*. **2019**, *47* (D1), D542–D549.

128. Jing, D. W.; Du, Z. Y.; Ma, H. L.; Ma, B. Y.; Liu, F. C.; Song, Y. G.; Li, L. Selenium enrichment, fruit quality and yield of winter jujube as affected by addition of sodium selenite. *Sci. Hortic.* **2017**, *225*, 1-5.

129. Jung, H. W.; Kim, W.; Hwang, B. K. Three pathogen-inducible genes coding lipid transfer protein from pepper are different activated by pathogens, abiotic, and environmental stresses. *Plant Cell Environ.* **2003**, *26*, 915-928.

130. Juniper, B.E.; Watkins, R.; Harris, S.A. The origin of the apple. *Acta Hortic.* **1998**, *484*, 27-34.

131. Jurd, L. Some advances in the chemistry of anthocyanin-type plant pigments. *Adv. Food Res. Suppl*. **1972**, *4*, 123-142.

132. Kadereit, J.W.; Kost, B.; Körner, C.; Sonnewald, U. Lehrbuch der Pflanzenwissenschaften. 37. Auflage. *Springer* **2014**.

133. Kanda, T.; Akiyama, H.; Yanagida, A.; Tanabe, M.; Goda, Y.; Toyoda, M.; Teshima, R.; Saito, Y. Inhibitory effects of apple polyphenol on induced histamine release from RBL-2H3 cells and rat mast cells. *Biosci. Biotechnol. Biochem.* **1998**, *62*, 1284-1289.

134. Kápolna, E.; Hillestrøm, P. R.; Laursen, K. H.; Husted, S.; Larsen, E. H. Effect of foliar application of selenium on its uptake and speciation in carrot. *Food Chem*. **2009**, *115*(4), 1357-1363.

135. Kaufmann, S.H.E. Basiswissen Immunologie. 1. Auflage. *Springer* **2014**, 48-51.

136. Kemeny, D. M. ELISA: Anwendung des Enzyme Linked Immunosorbent Assay im biologisch/medizinischen Labor. *Spektrum Akademischer Verlag* **1994**.

137. Kielliszek, M. Selenium-fascinating microelement, properties and sources in food. *Molecules* **2019**, *24*, 1298.

138. Kielliszek, M.; Błażejak, S. Current knowledge on the importance of selenium in food for living organisms: A review. *Molecules* **2016**, *21*, 609.

139. Kiewning, D.; Baab, G.; Schmitz-Eiberger, M. Effect of 1-MCP treatment on the apple *(Malus domestica* L. Borkh.) allergen Mal d 1 during long-term storage. *LWT* **2013**, *53*, 198-203.

140. Kiewning, D.; Schmitz-Eiberger, M. Effects of long-term storage on Mal d 1 content of four apple cultivars with initial low Mal d 1 content. *J. Sci. Food Agric*. **2014**, *94*, 798-802.

141. Kipp, A.P.; Strohm, D.; Brigelius-Flohé, R.; Schomburg, L.; Bechthold, A.; Leschik-Bonnet, E.; Heseker, H. German Nutrition Society (DGE) Revised reference values for selenium intake. *J. Trace Elem. Med. Biol.* **2015**, *32*, 195-199.

142. Kleine-Tebbe J.; Jakob, T. (Hrsg.): Molekulare Allergiediagnostik. *Springer* **2015.**

143. Kleine-Tebbe, J.; Waßmann-Otto, A.; Mönnikes, H. Nahrungsmittelallergien und andere -unverträglichkeiten. *Bundesgesundheitsbl*. **2016**, *59*, 705-722.

144. Klockenbring, T.; Bez, C.; Klowat, B.; Liappis, N.; Köpke, U.; Noga, G.; Zielen, S.; Goerlich, R. Allergenic potential of apple cultivars from organic and integrated fruit production. *Allergy Suppl.* **2001**, *68*, 889.

145. Knekt, P.; Jarvinen, R.; Reunanen, A.; Maatela, J. Flavonoid intake and coronary mortality in Finland: A cohort study. *Br. Med. J*. **1996**, *312*, 478-481.

146. Knekt, P.; Isotupa, S.; Rissanen, H.; Heliovaara, M.; Jarvinen, R.; Hakkinen, S.; Aromaa, A.; Reunanen, A. Quercetin intake and the incidence of cerebrovascular disease. *Eur. J. Clin. Nutr.* **2000**, *54*, 415–417.

147. Köhler, W., Schachtel, G., Voleske, P. Biostatistik. 4. aktualisierte und erweiterte Auflage. *Springer* **2007**.

148. König, J. Sinn und Unsinn der Angabe der Oxygen Radical Absorbance Capacity (ORAC-Wert) auf Lebensmitteln. *Ernährung/Nutrition* **2011**; *35*, 72-74.

149. Kolodziejczyk, K.; Milala, J.; Sojka, M.; Kosmala, M.; Markowski, J. Polyphenoloxidase activity in selected apple cultivars. *J. Fruit Ornam. Plant Res.* **2010**, *18*, 51–61.

150. Krath, B.N.; Eriksen, F.D.; Pedersen, B.H.; Gilissen, L.J.W.J.; van de Weg, W.E.; Dragsted, L.O. Development of hypo-allergenic apples: silencing of the major allergen Mal d 1 gene in 'Elstar' apple and the effect of grafting. *J. Hortic. Sci. Biotech*. **2009** (*Suppl*), 52-57.

151. Krebitz, M.; Wagner, B.; Ferreira, F.; Peterbauer, C.; Campillo, N. et al. Plant-based heterologous expression of Mal d 2, a thaumatin-like protein and allergen of apple (*Malus domestica*), and its characterization as an antifungal protein. *J. Mol. Biol.* **2003**, *329*, 721-730.

152. Kroll, J.; Rawel, H.M.; Rohn, S. A review. Reactions of plant phenolics with food proteins and enzymes under special consideration of covalent bonds. *Food Sci. Technol. Res.* **2003,** *9*, 205-218.

153. Kroon, P.; Williamson, G. Polyphenols dietary components with established benefits to health? *J. Sci. Food Agric*. **2005**, *85*, 1239-1240.

154. Kschonsek, J.; Wolfram, T.; Stöckl, A.; Böhm, V. Polyphenolic compounds analysis of old and new apple cultivars and contribution pf polyphenolic profile to the *in vitro* antioxidant capacity. *Antioxidants* **2018**, *7*, 20.

155. Kschonsek, J.; Wiegand, C.; Hipler, U.C.; Böhm, V. Influence of polyphenolic content on the in vitro allergenicity of old and new apple cultivars: A pilot study. *Nutrition* **2019a**, *58*, 30-35.

156. Kschonsek, J.; Dietz, A.; Wiegand, C.; Hipler, U.C.; Böhm, V. Allergenicity of apple allergen Mal d 1 as effected by polyphenols and polyphenoloxidase due to enzymatic browning. *LWT* **2019b**, *113*, 108289.

157. Lattanzio V. Bioactive polyphenols: their role in quality and storability of fruit and vegetables. *J. of Appl. Botany* **2003**, *77*, 128-146.

158. Lawson, P.G.; Daum, D.; Czauderna, R.; Vorsatz, C. Factors influencing the efficacy of iodine foliar sprays used for biofortifying butterhead lettuce (*Lactuca sativa*). *J. Plant Nutr. Sci.* **2016**, *179*, 661-669.

159. Le Bourvellec, C.; Renard, C.M.G.C. Interactions between polyphenols and macromolecules: Quantification methods and mechanisms. *Crit. Rev. Food Sci. Nutr.* **2012,** *52*, 213-248.

160. Lee, K.H.; Abdul Karim, M.I. Biochemical studies of cocoa bean polyphenoloxidase. *J. Sci. Food Agric*. **1991**, *55*(2), 251-260.

161. Lee, K.W.; Kim, Y.J.; Kim, D.; Lee, H.J.; Lee, CY. Major phenolics in apple and their contribution to the total antioxidant capacity. *J. Agric. Food. Chem.* **2003**, *51*, 6516-6520.

162. Lei, J.; Li, B.; Zhang, N.; Yan, R.; Guan, W.; Brennan, C.S.; Gao, H.; Peng, B. Effects of UV-C treatment on browning and the expression of polyphenoloxidase (PPO) genes in different tissues of Agaricus bisporus during cold storage. *Postharvest Biol. Technol.* **2018**, *139*, 99-105.

163. Le Marchand, L.; Murphy, S.P.; Hankin, J.H.; Wilkens, L.R.; Kolonel, L.N. Intake of flavonoids and lung cancer. *J. Natl. Cancer Inst.* **2000**, *92*, 154–160.

164. Lewitzki, E. Das Apfelbuch: Alles über Äpfel und Apfelbäume. 1. Auflage. *Books on demand* **2020**, 17-19.
165. Li Li, J. Exercise and oxidative stress: role of the cellular antioxidant systems. *Exerc. Sport Sci. Rev.* **1995**, 23 (1), 135-166.
166. Lidon, F. C.; Oliveira, K.; Galhano, C.; Guerra, M.; Ribeiro, M. M.; Pelica, J.; Pataco, I.; Ramalho, J. C.; Leitão, A. E.; Almeida, A. S.; Campos, P. S.; Ribeiro-Barros, A. I.; Pais, I. P.; Silva, M. M.; Carvalho, M. L.; Santos, J. P.,; Pessoa, M. F.; Reboredo, F. H. Selenium biofortification of rice through foliar application with selenite and selenate. *Experim. Agric*. **2019**, *55*(4), 528-542.
167. Lieberei, R.; Reisdorff, C. Nutzpflanzen. 8. überarbeitete Auflage. **2012.**
168. Liu, L.; Niu, C. Q.; Liu, J. T.; Quan, L.; Wang, Z. M.; Yuan, L. X.; Liu, Z. K. Effects of soil-applied selenium on selenium content of Gala apple fruits. In: Bañuelos, G.; Lin, Z.-Q.; Liang, D.; Yin, X.-B. (eds): Selenium research for environment and human health: perspectives, technologies and advancements: *Proceedings of the 6th International Conference on Selenium in the Environment and Human Health (ICSEHH 2019),* **October 27-30 2019**, Yangling, Xi'an, China, CRC Press, Leiden, The Netherlands, 83-84.
169. Liu, R. H.; Eberhardt, M.; Lee, C. Antioxidant and antiproliferative activities of selected New York apple cultivars. *New York Fruit Quarterly*, **2001**, *9*, 15-17.
170. Liu, Y.; Qiu, S.; Li J. Peroxidase-mediated conjugation of corn fiber gum and bovine serum albumin to improve emulsifying properties. *Carbohydr. Polym*. **2015**, 118, 70-78.
171. Lottspeich, F.; Engels, J. W. Bioanalytik. 2. Auflage. *Elsevier* **2006**.
172. Luttmann, W.; Bratke, K.; Küpper, M.; Myrtek, D. Der Experimentator Immunologie. 4. Auflage. *Springer* **2014**, 111-118.
173. Machholz, R.; Lewerenz, H.J. Lebensmitteltoxikologie. *Springer* **1989**, 226-231.
174. März, L. Das Apfelhandbuch. 1. Auflage. *Diplomica* **2012**.
175. Manach, C.; Scalbert, A.; Morand, C.; Rémésy, C.; Jiménez, L. Polyphenols: food sources and bioavailability. *Am. J. Clin. Nutr.* **2004**, *79*(5), 727-747.
176. Manzocco, L.; Quarta, B.; Dri, A. Polyphenoloxidase inactivation by light exposure in model systems and apple derivatives. *Innov. Food Sci. Emerg. Technol*. **2009**, *10*, 506–511.

177. Marzban, G.; Puehringer, H.; Dey, R.; Brynda, S.; Ma, Y.; Martinelli, A.; Zaccarini, M.; van der Weg, E.; Housley, Z.; Kolarich, D.; Altmann, F.; Laimer, M. Localisation and distribution of the major allergens in apple fruits. *Plant Sci.* **2005**, 169, 387-394.
178. Marzban, G.; Kinaciyan, T.; Maghuly, F.; Brunner, R.; Gruber, C.; Hahn, R.; Jensen-Jarolim, E.; Laimer, M. Impact of sulfur and vitamin C on the allergenicity of Mal d 2 from apple (*Malus domestica*). *J. Agric. Food Chem.* **2014**, *62*, 7622-7630.
179. Masuda, I.; Koike, M.; Nakashima, S.; Mizutani, Y.; Ozawa, Y.; Watanabe, K.; Sawada, Y.; Sugiyama, H.; Sugimoto, A.; Nojiri, H.; Sashihara, K.; Yokote, K.; Shimizu, T. Apple procyanidins promote mitochnondrial biogenesis and proteoglycan biosynthesis in chondrocytes. *Nature* **2018**, *8,* 7729.
180. Matern, U.; Grimmig, B. Polyphenols in plant pathology. In: Scalbert, A. (editor) Polyphenolic phenomena. *INRA Editions* **1993**, 143-147.
181. Matissek, R.; Fischer, M. Lebensmittelanalytik. 5. Auflage. *Springer* **2014**.
182. Matthes, A.; Schmitz-Eiberger, M. Apple (*Malus domestica* L. Borkh.) allergen Mal d 1: effect of cultivar, cultivation system, and storage conditions. *J. Agric. Food Chem.* **2009**, *57*, 10548-10553.
183. Mayer, A.M.; Harel, E. Polyphenoloxidases in plants. *Phytochem*. **1979**, *18*, 193-215.
184. Mayer, A. M. Polyphenoloxidases in plantssrecent progress. *Phytochem*. **1987**, *26*, 11-20.
185. Mayer, A.M. Polyphenoloxidases in plants and fungi: going places? A review. *Phytochem*. **2006**, *67*(21), 2318-2331.
186. Marusek, C.M.; Trobaugh, N.M.; Flurkey, W.H.; Inlow, J.K. Comparative analysis of polyphenoloxidase from plant and fungal species. *J. Inorg. Biochem*. **2006**, *100*, 108-123.
187. Mazza, G.; Miniati, E. Anthocyanins in fruits, vegetables, and grains. *Boca Raton. CRC Press* **1993**, 362.
188. McRae, K.B.; Lidster, P.D. Comparison of the polyphenol profiles of apple fruit cultivars by correspondence analysis. *J. Sci. Food Agric.* **1990**, *50*, 1572-1579.
189. Merck, K.G.a.A. Packungsbeilage zum goat-anti-mouse IgG-Antikörper, Peroxidase konjugiert (H+L). **2019**.
190. Molina, A.; Garcia-Olmedo, F. Development and pathogen induced expression of three barley genes encoding lipid transfer proteins. *Plant J.* **1993**, *4*, 983-991.

191. Müller, A.; Noack, L.; Greiner, R.; Stahl, M.R.; Posten, C. Effect of UV-C and UV-B treatment on polyphenoloxidase acitvity and shelf life of apple and grape juice. *Innov. Food Sci. Emerg. Technol*. **2014**.
192. Müller, L.; Gnoyke, S.; Popken, A.M.; Böhm, V. Antioxidant capacity and related parameters of different fruit formulations. *LWT Food Sci. Technol*. **2010**, 43, 992–999.
193. Mukhtar, A.; Gilani, A. H.; Bhatty, N. Some nutritional and microbiological aspects of apple of common verities available for house hold consumption. *J. Anim. Plant Sci*. **2010** *20*(4), 253-257.
194. Munk, K. Botanik – Taschenlehrbuch Botanik. *Thieme* **2008**.
195. Nakayama, T.; Yonekura-Sakakibara, K.; Sato, T.; Kikuchi, S.; Fukui, Y.; Fukuchi-Mizutani, M.; Ueda, T.; Nakao, M.; Tanaka, Y.; Kusumi, T.; Nishino, T. Aureusidin synthase: A polyphenoloxidase homolog responsible for flower coloration. *Science* **2000**, *290*, 1163-1166.
196. Navarro-Alarcon, M.; Cabrera-Vique, C. Selenium in food and the human body: a review. *Sci. Total Environ.* **2008**, *400*, 115-141.
197. Neumüller, M. Apfelallergie - Was ist das? Available online: https://www.aepfel-fuer-allergiker.de/Apfelallergie-im-Detail (accessed on 04 August **2021**).
198. Neveu, V.; Perez-Jiménez, J. Phenol-Explorer: an online comprehensive database on polyphenol contents in foods 2010, online Database. Version 1.5.2. Available at http://www.phenolexplorer.eu (accessed on 04 August **2021**).
199. Nicolas, J.J.; Richard-Forget, F.C.; Goupy, P.M.; Amoit, M.; Aubert, S.Y. Enzymatic browning reactions in apple and apple products. *Crit. Rev. Food Sci. Nutr.* **1994**, *34*, 109-157.
200. Nies, A. M. et al. Freie Radikale und oxidativer Stress bei körperlicher Belastung und Trainingsanpassung – Eine aktuelle Übersicht. *Deutsche Zeitschrift fur Sportmedizin* **2002**, *53*(12), 345-353.
201. Nimse, S. B.; Pal, D. Free radicals, natural antioxidants, and their reaction mechanisms. *RSC Adv.* **2015**, *5*(35), 27986-28006.
202. O'Connell, J.E.; Fox, P.F. Significance and applications of phenolic compounds in the production and quality of milk and dairy products: A review. *Int. Dairy J.* **2001**, *11*, 103-120.

203. Oh, D.H.; Song, K.J.; Shin, Y.U.; Chung, W.I. Isolation of a cDNA encoding a 31-kDa, pathogenesis-related 5/thaumatin-like (PR5/TL) protein abundantly expressed in apple fruit (*Malus domestica* cv. Fuji). *Biosci. Biotechnol. Biochem*. **2000**, *64*, 355-362.

204. Oster, O.; Prellwitz, W. The daily dietary selenium intake of West German adults. *Biol. Trace Elem. Res.* **1989**, *20*, 1-14.

205. Ou, B.; Hampsch-Woodill, M.; Flanagan, Deemer, E. K.; Prior, R. L.; Huang, D. Novel fluorometric assay for hydroxyl radical prevention capacity using fluorescein as the probe. *J. Agric. Food Chem.* **2002**, *50*, 2772-2777.

206. Ou, B.; Hampsch-Woodill, M.; Prior, R.L. Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. *J. Agric. Food Chem*. **2001**, *49*, 4619-4626.

207. Ozdal, T.; Capanoglu, E.; Altay, F. A review on protein-phenolic interactions and associated changes. *Food Res. Int*. **2013**, *51*, 954-970.

208. Özyürek, M.; Güclü, K.; Apak, R. The main and modified CUPRAC methods of antioxidant measurement. *Trends Anal. Chem.* **2011**, *30*, 652-664.

209. Papas, A.M. Antioxidant status, diet, nutrition, and health. *CRC Press: Boca Raton* **1999**.

210. Paschke, A. Proteine als Lebensmittelallergene. *Nachrichten aus der Chemie* **2008**, 56 (10), 1005-1009.

211. Pezzarossa, B.; Remorini, D.; Gentile, M.L.; Massai, R. Effects of foliar and fruit addition of sodium selenate on selenium accumulation and fruit quality. *J Sci. Food Agric.* **2012**, *92*, 781-786.

212. Pezzarossa, B.; Rosellini, I.; Malorgio, F.; Borghesi, E.; Tonutti, P. Effects of selenium enrichment of tomato plants on ripe fruit metabolism and composition. *Proceedings of the 7th International Postharvest Symposium*, Kuala Lumpur, Malaysia**,** 25-29 June **2013,** 247-251.

213. Pizzocaro, F.; Torreggiani, D.; Gilardi, G. Inhibition of apple polyphenoloxidase (PPO) by ascorbic acid, citric acid and sodium chloride. *J. Food Proc. Preserv*. **1993**, *17*, 21-30.

214. Poblaciones, M. J., Rodrigo, S. M., Santamaría, O. Evaluation of the potential of peas (*Pisum sativum* L.) to be used in selenium biofortification programs under Mediterranean conditions. *Biol. Trace Elem. Res.*, **2013**, *151*, 132-137.

215. Poblaciones, M. J.; Santamaría, O.; García-White, T.; Rodrigo, S. M. Selenium biofortification in bread-making wheat under Mediterranean conditions: influence on grain yield and quality parameters. *Crop Past. Sci.* **2014a**, *65*, 362-369.

216. Poblaciones, M. J.; Rodrigo, S. M.; Santamaría, O.; Chen, Y.; McGrath, S. P. Agronomic selenium biofortification in *Triticum durum* under Mediterranean conditions: From grain to cooked pasta. *Food Chem.* **2014b**, *146*, 378-384.

217. Podsedek, A.; Wilska-Jeszka, J. Compositional characterisation of some apple varieties. *Europ. Food Res. Techn*. **2000**, *210*, 268-272.

218. Poggi, V.; Arcioni, A.; Filippini, P.; Pifferi, P. G. Foliar application of selenite and selenate to potato (*Solanum tuberosum*): Effect of a ligand agent on selenium content of tubers. *J. Agric. Food Chem*. **2000**, *48*(10), 4749-4751.

219. Põldma, P.; Moor, U.; Tõnutare, T.; Herodes, K.; Rebane, R. Selenium treatment under field conditions affects mineral nutrition, yield and antioxidant properties of bulb onion (*Allium cepa* L.). *Acta Sci. Pol. Hort. Cultus* **2013**, *12*, 167-181.

220. Poňavič, M.; Scheib, A. Distribution of selenium in european agricultural and grazing land soil. In: Reimann, C.; Birke, M.; Demetriades, A.; Filzmoser, P.; O'Connor P. (Eds.). Chemistry of Europe's agricultural soils. Geologisches Jahrbuch (Reihe B). *Schweizerbart* **2014**, *103*, 131-144.

221. Prigent, S. Interactions of phenolic compounds with globular proteins and their effects on food-related functional properties. *Wageningen University* **2005.**

222. Prior, R.L.; Wu, X.; Schaich, K. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *J. Agric. Food Chem.* **2005,** *53*, 4290-4302.

223. Prior, R.L.; Wu, X. Anthocyanins: structural characteristics that result in unique metabolic patterns and biological activities. *Free Radic. Res.* **2006**, *40*(10), 1014-1028.

224. Puccinelli, M.; Malorgio, F.; Pezzarossa, B. Selenium enrichment of horticultural crops. *Molecules* **2017**, *22*, 933.

225. Puehringer, H.; Moll, D.; Hoffmann-Sommergruber, K.; Watillon, B.; Katinger, H.; Machado, M. L. D. The promoter of an apple Ypr10 gene, encoding the major allergen Mal d 1, is stress- and pathogen-inducible. *Plant Sci*. **2000**, *152*, 35-50.

226. Rana, S.; Bhushan, S. Apple phenolics as nutraceuticals: Assessment, analysis and application. *J. Food Sci. Technol***. 2016**, *53*, 1727-1738.

227. Rawel, H.M.; Kroll, J.; Rohn, S. Reactions of phenolic substances with lysozyme – Physicochemical characterisation and proteolytic digestion of the derivatives. *Food Chem.* **2001**, *72*, 59-71.

228. Rawel, H.M.; Meidtner, K.; Kroll, J. Binding of selected phenolic compounds to proteins. *J. Agric. Food Chem.* **2005**, *53*(10), 4228-4235.

229. Rawel, H.M.; Frey, S.K.; Meidtner, K.; Kroll, J.; Schweigert, F.J. Determining the binding affinities of phenolic compounds to proteins by quenching of the intrinsic tryptophan fluorescence. *Mol. Nutr. Food Res*. **2006**, *50*(8), 705-713.

230. Rawel, H.; Rohn, S. Nature of hydroxycinnamate-protein interactions. *Phytochem. Rev*. **2010**, *9* (1), 93-109.

231. Rayman, M. Selenium and human health. *Lancet* **2012**, *379*, 1256-1268.

232. Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans, C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Rad. Biol. Med.* **1999**, *26* (9/10), 1231-1237.

233. Rechner, A. Einfluss der Verarbeitungstechnik auf die Polyphenole und antioxidative Kapazität von Apfel- und Beerenobstsäften. *Universität Gießen* **2000.**

234. Reis, H. P. G.; de Queiroz Barcelos, J. P.; Furlani Junior, E.; Santos, E. F.; Silva, V. M.; Moraes, M. F.; Putti, F. F.; dos Reis, A. R. Agronomic biofortification of upland rice with selenium and nitrogen and its relation to grain quality. *J. Cereal Sci.* **2018***, 79*, 508-515.

235. Ren, G.; Ran, X.; Zeng, R.; Chen, J.; Wang, Y.; Mao, C.; Wang, D.; Wang, X.; Feng, Y.; Yang, G. Effects of sodium selenite spray on apple production, quality, and sucrose metabolism-related enzyme activity. *Food Chem.* **2020**, 127883.

236. Renard, C.M.G.C.; Dupont, N. Concentrations and characteristics of procyanidins and other phenolics in apples during fruit growth. *Phytochem*. **2007**, *68*, 1128-1138.

237. Rescigno, A.; Sollai, F.; Pisu, B.; Rinaldi, A.; Sanjust, E. Tyrosinase inhibition: general and applied aspects. *J. Enzym. Inhib. Med. Chem*. **2002**, *17*, 207-218.

238. Rhodes, M.J.C. Physiological roles of phenolic compounds in plants and their interactions with microorganisms and humans. In: Vercauteren, J.; Chèze, C. Triaud J. (Eds.): Polyphenols 96. Bordeaux. *INRA Edition* **1998**, 13-30.

239. Ring, J. Angewandte Allergologie. *3.,* neu bearbeitete Auflage. *Urban und Vogel* **2007.**

240. Ríos, J.J.; Rosales, M.A.; Blasco, B.; Cervilla, L.M.; Romero, L.; Ruiz, J.M. Biofortification of Se and induction of the antioxidant capacity in lettuce plants. *Sci. Hort.* **2008**, *116*, 248-255.

241. Robards, K.; Prenzler, P.D.; Tucker, G.; Swatsitang, P.; Glover, W. Phenolic compounds and their role in oxidative processes in fruits. *Food Chem*. **1999,** *66*(4), 401-443.

242. Rodrigo, S.; Santamaría, O.; López-Bellido, F. J.; Poblaciones, M. J. Agronomic selenium biofortification of two-rowed barley under Mediterranean conditions. *Plant Soil Environ*. **2013**, *59,* 115-120.

243. Rohn, S.; Rawel, H.M.; Kroll, J. Inhibitory effects of plant phenols on the activity of selected enzymes. *J. Agric. Food Chem*. **2002**, *50*, 3566-3571.

244. Rohn, S.; Possibilities and limitations in the analysis of covalent interactions between phenolic compounds and proteins. *Food Res. Int*. **2014**, *65*, 13-19.

245. Rolff, M.; Schottenheim, J.; Decker, H.; Tuczek, F. Copper-O2 reactivity of tyrosinase models towards external monophenolic substrates: molecular mechanism and comparison with the enzyme. *Chem. Soc. Rev.* **2011**, *40*, 4077-4098.

246. Romer, E.; Chebib, S.; Bergmann, K.C.; Plate, K.; Becker, S.; Ludwig, C.; Meng, C.; Fischer, T.; Dierend, W.; Schwab, W. Tiered approach for the identification of Mal d 1 reduced, well tolerated apple genotypes. *Sci. Rep.* **2020**, *10*, 9144.

247. Ros, G. H.; van Rotterdam, A. M. D.; Bussink, D. W.; Bindraban, P. S. Selenium fertilization strategies for biofortification of food: An agro-ecosystem approach. *Plant Soil* **2016**, *404*, 99-112.

248. Rudeschko, O.; Fahlbusch, B.; Henzgen, M.; Schlenvoigt, G.; Herrmann, D.; Vieths, S. Investigation of the stability of apple allergen extracts. *Allergy* **1995a**, *50*, 575-580.

249. Rudeschko, O.; Fahlbush, B.; Henzgen, M.; Schlenvoigt, G.; Herrmann, D.; Jager, L. Optimization of apple allergen preparation for *in vitro* and *in vivo* diagnostics. *Allergy* **1995b**; *50*, 262-268.

250. Salcedo, G.; Diaz-Perales, A.; Sanchez-Monge, R. Fruit allergy: plant defence proteins as novel potential panallergens. *Clinic. Experim. Allergy* **1999**, *29*, 1158-1160.

251. Salcedo, G.; Sanchez-Monge, R.; Diaz-Perales, A.; Garcia-Casado, G.; Barber, D. Plant non-specific lipid transfer proteins as food and pollen allergens. *Clinic. Experim. Allergy*, **2004**, *34*, 1336-1341.

252. Salzbrunn, K.U. Über die Funktion und Struktur der Tyrosinase aus *Streptomyces antibioticus*. *Universität Mainz* **2007.**

253. Sancho, A.I.; Rigby, N.M.; Zuidmeer, L.; Asero, R.; Mistrello, G. Amato, S.; González-Mancebo, E.; Fernández-Rivas M.; van Ree, R.; Mills E.N.C. The effect of thermal processing on the IgE re ctivity of the non-specific lipid transfer protein from apple, Mal d 3. *Allergy* **2005**, *60*, 1262-1268.

254. Sancho, A.I.; Foxall, R.; Browne, T.; Dey, R.; Zuidmeer, L.; Marzban, G. Effect of postharvest storage on the expression of the apple allergen Mal d 1. *J. Agric. Food Chem*. **2006a**, *54*, 5917-5923.

255. Sancho, A. I.; Foxall, R.; Rigby, N. M.; Browne, T.; Zuidmeer, L.; van Ree, R.; Waldron, K. W.; Clare Mills, E. N. Maturity and storage influence on the apple (*Malus domestica*) allergen Mal d 3, a nonspecific lipid transfer protein. *J. Agric. Food Chem*. **2006b**, *54*, 5098-5104.

256. Sanoner, P.; Guyot, S. Polyphenol profiles of French cider apple varieties (*Malus domestica* sp.). *J. Agric. Food Chem*. **1999**, *47*, 4847-4853.

257. Scattino, C.; Castagna, A.; Neugart, S.; Chan, H.M.; Schreiner, M.; Crisosto, C.H.; Tonutti, P.; Ranieri, A. Post-harvest UV-B irradiation induces changes of phenol contents and corresponding biosynthetic gene expression ion peaches and nectarines. *Food Chem*. **2014**, *163*, 51–60.

258. Scheurer, S; Wangorsch, A.; Nerkamp, J.; Skov, P.S.; Ballmer-Weber, B.K.; Wüthrich, B.; Haustein, D.; Vieths S. Crossreactivity within the profilin panallergen family investigated by comparison of recombinant profilins from pear (Pyr c 4), cherry (Pru av 4), and celery (Api g 4) with birch pollen profilin Bet v 2. *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.* **2001**, *756*, 315–325.

259. Schiavon, M.; dall'Acqua, S.; Mietto, A.; Pilon-Smits, E.A.H.; Sambo, P.; Masi, A.; Malagoli, M. Selenium fertilization alters the chemical composition and antioxidant constituents of tomato (*Solanum lycopersicon* L.). *J. Agric. Food Chem*. **2013**, *61*, 10542-10554.

260. Schiavon, M.; Berto, C.; Malagoli, M.; Trentin, A.; Sambo, P.; Dall'Acqua, S.; Pilon-Smits, E.A.H. Selenium biofortification in radish enhances nutritional quality via accumulation of methyl-selenocystein and promotion of transcripts and metabolites related to glucosinolates, phenolics, and amino acids. *Front. Plant Sci*. **2016**, *7*, 1371.

261. Schmid, J.; Amrhein, N. Molecular Organization of the Shikimate Pathway in Higher Plants. *Phytochem*. **1995**, *39*(4), 737-749.

262. Schmitz-Eiberger, M.; Weber, V.; Treutter, D.; Baab, G.; Lorenz, J. Bioactive components in fruits from different apple varieties. *J. Appl. Bot*. **2003**, *77*, 167-171.

263. Schmitz-Eiberger, M.; Matthes, A. Effect of harvest maturity, duration of storage and shelf life of apples on the allergen Mal d 1, polyphenoloxidase activity and polyphenol content. *Food Chem.* **2011**, *127*(4), 1459-1464.

264. Schomburg, L.; Arnér, E. S. Selenium metabolism in herbivores and higher trophic levels including mammals. In: Pilon-Smits, E. A.; Winkel, L. H.; Lin, Z.-Q. (eds.) Selenium in plants, *Springer International Publishing* **2017**, 123-139.

265. Schütt, D. Analytik und Transformation von Flavonoiden durch anaerobe und aerobe Bakterien. *Technische Universität Berlin* **2014.**

266. Selinheimo, E.; Autio, K.; Kruus, K.; Buchert, J. Elucidating the mechanism of laccase and tyrosinase in wheat bread making. *J. Agric. Food Chem.* **2007**, *55*, 6357-6365.

267. Shah, A. M.; Qureshi, M.; Memon, Z. N.; Memon, S. N.; Syed, S. K. Analysis of carbohydrate and protein from pulp and peel of apple (*Malus sylvestis*) and banana (*Musa paradisiacal*). *Sindh Univ. Res. Hour. (Sci. Ser.)* **2012**, *44* (1), 71-74.

268. Shaheen, S. O.; Sterne, J. A.; Thompson, R. L.; Songhurst, C. E.; Margetts, B. M.; Burney, P. G. Antioxidants and Asthma in Adults – Populationbased Case–Control Study. *Am. J. Resp. Crit. Care Med.* **2001**, *164*, 1823-1828.

269. Shahidi, F.; Zhong. Y. Lipid oxidation and improving the oxidative stability. *Chem. Soc. Rev*. **2010**, *39*, 4067-4079.

270. Shahkhalili, Y.; Finot, P.A.; Hurrell, R.; Fern, E. Effects of foods rich in polyphenols on nitrogen excretion in rats. *J. Nutr*. **1990**, *120*(4), 346-352.

271. Simkó, M.; Gazsó, A.; Fiedeler, U.; Nentwich, M. Nanopartikel, freie Radikale und oxidativer Stress. *NanoTrust-Dossiers* **2009**, *012*, 1-4.

272. Singh, A.; Holvoet, S.; Mercenier, A. Dietary polyphenols in the prevention and treatment of allergic diseases. *Clin. Exp. Allergy* **2011**, *41*, 1346-1359.

273. Singleton, V.L.; Rossi, J.A. Jr. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic aid reagents. *Am. J. Enol. Viticult.* **1965**, *16*, 144-158.

274. Smoleń, S.; Kowalska, I.; Czernicka, M.; Halka, M.; Keska, K.; Sady, W. Iodine and selenium biofortification with additional application of salicylic acid affects yield,

selected molecular parameters and chemical composition of lettuce plants (*Lactuca sativa* L. var. *capitata*). *Front. Plant Sci*. **2016**, *7*, 1553.

275. Solomon, E.I.; Sundaram, U.M.; Machonkin, T.E. Multicopper oxidases and oxygenases. *Chem. Rev.* **1996**, 96, 2563-2605.
276. Somogyi, A.; Rosta, K.; Pusztai, P.; Tulassay, Z.; Nagy, G. Antioxidant measurements. *Physiol. Meas*. **2007**, *28*, R41-55.
277. Son, D.; Lee, S. Comparison of the characteristics of the major allergen Mal d 1 according to apple varieties. *Food Sci. Biotechnol*. **2001**, *10*, 132-136.
278. Song, Y.; Yao, Y.; Zhai, H.; Du, Y.; Chen, F.; Wei, S. Polyphenolic compound and the degree of browning in processing apple varieties. *Agric. Sci.* **2007**, 607-612.
279. Souci, S.W.; Fachmann, W.; Kraut, H. Lebensmitteltabelle für die Praxis, 5. Auflage. *Wissenschaftliche Verlagsgesellschaft Stuttgart* **2011**, 873-876.
280. Statista (**2021a**). Pro-Kopf-Verbrauch von Äpfeln in Deutschland in den Jahren 2007/08 bis 2018/19 (in Kilogramm). Available online: https://de.statista.com/statistik/daten/studie/643179/umfrage/pro-kopf-konsum-von-aepfeln-in-deutschland/ (accessed on 04 August **2021**).
281. Statista (**2021b**). Sortenanteile von Apfelsorten in der Europäischen Union im Jahr 2019/2020. Available online: https://de.statista.com/statistik/daten/studie/29114/umfrage/bedeutende-apfelsorten-in-der-eu-nach-sortenanteil/ (accessed on 04 August **2021**).
282. Statista (**2021c**). Erntemengen der wichtigsten Anbauländer von Äpfeln in den Jahren 2018 und 2019 (in 1.000 Tonnen). Available online: https://de.statista.com/statistik/daten/studie/1188595/umfrage/erntemengen-wichtiger-anbaulaender-von-aepfeln-weltweit/ (accessed on 04 August **2021**).
283. Statista (**2021d**). Erntemenge von Äpfeln weltweit von 1980 bis 2019 (in 1.000 Tonnen). Available online: https://de.statista.com/statistik/daten/studie/708202/umfrage/erntemenge-von-aepfeln-weltweit/ (accessed on 04 August **2021**).
284. Statista (**2021e**). Kennzahlen zur Apfelernte in Deutschland in den Jahren 2009 bis 2020. Available online: https://de.statista.com/statistik/daten/studie/166867/umfrage/kennzahlen-zur-apfelernte/ (accessed on 04 August **2021**).

285. Statista (**2021f**). Anteile einzelner Obstsorten an der gesamten Obsternte in Deutschland in den Jahren 2017 bis 2019. Available online: https://de.statista.com/statistik/daten/studie/206331/umfrage/obst-aus-deutschland/ (accessed on 04 August **2021**).
286. Statista (**2021g**). Pro-Kopf-Konsum von Obst in Deutschland in den Wirtschaftsjahren 2004/2005 bis 2018/2019 (in Kilogramm). Available online: https://de.statista.com/statistik/daten/studie/6300/umfrage/pro-kopf-verbrauch-von-obst-in-deutschland/ (accessed on 04 August **2021**).
287. Steinbrenner, H.; Brigelius-Flohé, R. Das essenzielle Spurenelement Selen: Selenbedarf in Gesundheit und Krankheit. *Aktuelle Ernährungsmedizin* **2015**, *40*, 368-378.
288. Stintzi, A.; Heitz, V.; Prasad, S.; Wiedemann-Merdinoglu, S.; Kauffmann, S.; Geofroy, P.; Legrand, M.; Fritig, B. Plant 'pathogenesis-related' proteins and their role in defense against pathogens. *Biochem*. **1993**, *75*, 687-706.
289. Sun, J.; Chu, Y. F.; Wu, X.; Liu, R. H. Antioxidant and antiproliferative activities of common fruits. *J. Agric. Food Chem*. **2002**, *50*(25), 7449-7454.
290. Swieca, M.; Gawlik-Dziki, G.; Dziki, D.; Baraniak, B.; Czyz, J. The influence of protein-flavonoid interactions on protein digest- ibility in vitro and the antioxidant quality of breads enriched with onion skin. *Food Chem.* **2013**, *141*, 451-458.
291. Tepper, A.W.J.W.; Lonadi, E.; Bubacco, L.; Canters, G.W. Structure, spectroscopy and function of tyrosinase; comparison with hemocyanin and catecholoxidase. In: Messerschmidt, A. (eds) Handbook of Metalloproteins. *Wiley/Blackwell Chichester* **2010.**
292. ThermoFisher Scientific, Übersicht über ELISA. Available online: https://www.thermofisher.com/de/de/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/overview-elisa.html (accessed on 04 August **2021**).
293. Tokura, T.; Nakano, N.; Ito, T.; Matsuda, H.; Nagasako-Akazome, Y.; Kanda, T.; Ikeda, M.; Okumura, K.; Ogawa, H.; Nishiyama, C. Inhibitory effect of polyphenol-enriched apple extracts on mast cell degranulation in vitro targeting the binding between IgE and FcERI. *Biosci. Biotechnol. Biochem*. **2005**, *69*, 1974-1977.
294. Tomás-Barberán, F.A.; Clifford, M.N. Flavanones, chalcones and dihydrochalcones – nature, occurrence and dietary burden. *J. Sci. Food Agric*. **2000**, *80,* 1073-1080.

295. Tomás-Barberán, F.A.; Andrés-Lacueva, C. Polyphenols and health: Current state and progress. *J. Agric. Food Chem*. **2012**, *60*, 8773-8775.

296. Torres-Schumann, S.; Godoy, J. A.; Pintor-Toro, J. A. A probable lipid transfer protein gene is induced by NaCl in stems of tomato plants. *Plant Mol. Biol*. **1992**, *18*, 749-757.

297. Treutter, D. Biosynthesis of phenolic compounds and its regulation in apple. *Plant Growth Reg.* **2001**, *34*(1), 71-89.

298. Treviño, M. B.; O'Connell, M. A. Three drought-responsive members of the nonspecific lipid-transfer protein gene family in Lycopersicon pennellii show different development patterns of expression. *Plant Physiol.* **1998**, *116*, 1461-1468.

299. Tron, T.; Laccases. In.; Kretsinger, R.H.; Uversky V.N.; Permyakov E.A. (eds.) Encyclopedia of Metalloproteins. *Springer* **2013.**

300. Tsao, R.; Yang, R.; Young, J. C.; Zhu, H. Polyphenolic profiles in eight apple cultivars using high-Performance liquid chromatography (HPLC). *J. Agric. Food Chem*. **2003**, *51*(21), 6347-6353.

301. Tsao, R.; Yang, R.; Xie, S.; Sockovie, E.; Khanizadeh, S. Which polyphenolic compounds contribute to the total antioxidant activities of apple? *J. Agric. Food Chem*. **2005**, *53*, 4989-4995.

302. Tsao, R. Chemistry and biochemistry of dietary polyphenols. *Nutrients* **2010**, *2*(12), 1231-1246.

303. Ullah, C.; Unsicker, S.B.; Fellenberg, C.; Constabel, C.P.; Schmidt, A.; Gershenzon, J.; Hammerbacher, A. Flavan-3-ols are an effective chemical defense against rust infection. *Plant Physiol*. **2017**, *175*, 1560-1578.

304. Valavanidis, A.; Vlachogianni, T. Polyphenolic profile and antioxidant activity of five apple cultivars grown under organic and conventional agricultural practices. *Int. J. Food Sci. Technol.* **2009**, *44*, 1167-1175.

305. van der Sluis, A.A.; Dekker, M. Activity and concentration of polyphenolicantioxidants in apple: Effect of cultivar, harvest year, and storage conditions. *J. Agric. Food Chem*. **2001,** *49*, 3606-3613.

306. van der Sluis, A. A.; Dekker M. Activity and concentration of polyphenolic antioxidants in apple juice. 1. Effect of existing production methods. *J. Agric. Food Chem*. **2002**, *50*, 7211-7219.

307. van Gelder, C. W. G.; Flurkey, W. H.; Wichers, H. J. Sequence and structural features of plant and fungal tyrosinases. *Phytochem*. **1997**, *45*, 1309-1323.
308. van Ree, R.; Fernandez-Rivas. M.; Cuevas, M.; van Wijngaarden, M.; Aalberse, R.C. Pollen-related allergy to peach and apple: An important role for profilin. *J. Allergy Clin. Immunol*. **1995**, *95*, 726-734.
309. van Ree, R. Analytic aspects of the standardization of allergenic extracts. *Allergy* **1997**, *52*, 795-805.
310. Vermerris, W.; Nicholson, R. Families of phenolic compounds and means of classification. Phenolic compound biochemistry. *Springer* **2006**, 3-25.
311. Vidal, S.; Francis, L. The mouth-feel properties of grape and apple proanthocyanidins in a wine-like medium. *J. Sci. Food Agric*. **2003**, *83*(6), 564-573.
312. Vieths, S.; Janek, K.; Aulepp, H.; Petersen, A. Isolation and characterization of the 18-kDa major apple allergen and comparison with the major birch pollen allergen (Bet v I). *Allergy* **1995**, *50*, 421-430.
313. Vogt, T. Phenylpropanoid biosynthesis. *Mol. Plant* **2010**, *3(*1), 2–20.
314. Vollmer, E.; Michalis, K.; Mußhoff, O. Vorernte-Vorhersage des Proteingehalts im Weizen in Nordost-Deutschland für Marktakteure anhand von Wetterinformationen. *Journal für Kulturpflanzen* **2018**, *70* (2), 45-55.
315. von Baehr, V. Nahrungsmittelallergien vom Typ I bis Typ IV. *Institut für Medizinische Diagnostik Berlin* **2017.**
316. Vrhovsek, U.; Rigo, A.; Tonon, D.; Mattivi, F. Quantitation of polyphenols in different apple varieties. *J. Agric. Food Chem*. **2004**, *52*(21), 6532-6538.
317. Walker, J.R.; Ferrar, P.H. Diphenoloxidases, enzyme-catalysed browning and plant disease resistance. *Biotechnol. Genet. Eng. Rev.* **1998**, *15*, 457-498.
318. Wang, J.; Vanga, S. K.; Raghavan, V. Effect of pre-harvest and post-harvest conditions on the fruit allergenicity: A review. *Crit. Rev. Food Sci. Nutr.* **2017**.
319. Watzl, B.; Leitzmann, C. Bioaktive Substanzen in Lebensmitteln. 3. Auflage. *Hippokrates Verlag* **2005**.
320. Wendelin, S.; Eder, R. Phenolzusammensetzung und antioxidative Kapazität von Trauben und Weinen. *Jahrestagung der Arbeitsgemeinschaft landwirtschaftlicher Versuchsanstalten ALVA* **2002**, 293-296.

321. Wensing, M.; Akkerdaas, J.H.; van Leeuwen, W.A.; Stapel, S.O.; Bruijnzeel-Koomen, C.A.; Aalberse, R.C.; Bast, B.J.; Knulst, A.C.; van Ree, R. IgE to Bet v 1 and profilin: Cross-reactivity patterns and clinical relevance. *J. Allergy Clin. Immunol.* **2002,** *110*, 435-442.

322. Westphal, S.; Kempf, W.; Foetisch, K.; Retzek, M.; Vieths, S.; Scheurer, S. Tomato profilin Lyc e 1: IgE cross-reactivity and allergenic potency. *Allergy* **2004**, *59* (5), 526-553.

323. WetterKontor GmbH. Monats- und Jahreswerte für Deutschland. Available online: https://www.wetterkontor.de/de/wetter/deutschland/monatswerte.asp?y=2018&m=15 (accessed on 18 September 2019).

324. World Health Organisation (WHO). Mortality and burden of disease attributable to selected major risks. Global Health Risks. **2009**.

325. Willers, J.; Heinemann, M.; Bitterlich, N.; Hahn, A. Intake of minerals from food supplements in a German population – A nationwide survey. *Food Nutr. Sci.* **2015**, *6*, 205-215.

326. Winkel, L.H.; Vriens, B.; Jones, G.D.; Schneider, L.S.; Pilon-Smits, E.; Bañuelos, G.S. Selenium cycling across soil-plant-atmosphere interfaces: A critical review. *Nutrients* **2015**, *7*, 4199-4239.

327. Wojdyło, A.; Oszmiánski, J.L.; Skowski, P. Polyphenolic compounds and antioxidant activity of new and old apple varieties. *J. Agric. Food. Chem.* **2008**, *56*, 6520-6530.

328. Wolfe, K.; Wu, X.; Liu, R. H. Antioxidant activity of apple peels. *J. Agric. Food Chem.* **2003**, *51*(3), 609-614.

329. Worm, M.; Jappe, U.; Kleine-Tebbe, J.; Schäfer, C.; Reese, I.; Saloga, J.; Treudler, R.; Zuberbier, T.; Wassmann, A.; Fuchs, T.; Dölle, S.; Raithel, M., Ballmer-Weber, B.; Niggemann, B.; Werfel, T.; Food allergies resulting from immunological cross-reactivity with inhalant allergens. *Allergy J. Int*. **2014**, *23*, 1-16.

330. Wortmann, L.; Enneking, U.; Daum, D. German consumers' attitude towards selenium-biofortified apples and acceptance of related nutrition and health claims. *Nutrients* **2018**, *10*, 190.

331. Wu, Z.; Lian, J.; Han, Y.; Zhou, N.; Li, X.; Yang, A.; Tong, P.; Chen, H. Crosslinking of peanut allergen Ara h 2 by polyphenoloxidase: Digestibility and potential allergenicity assessment. *J. Sci. Food Agric*. **2016**, *96*, 3567-3574.

332. Xu, Y.; Fan, M.; Ran, J.; Zhang, T.; Sun, H.; Dong, M.; Zhang, Z.; Zheng, H. Variation in phenolic compounds and antioxidant activity in apple seeds of seven cultivars. *Saudi J. Biol. Sci.* **2016**, *23*, 379-388.

333. Zardo, D.M.; Silva, K.M.; Guyot, S.; Nogueira, A. Phenolic profile and antioxidant capacity of the principal apples produced in Brazil. Int. *J. Food Sci. Nutr.* **2013**, *64*, 611-620.

334. Zhao, Y.; Wu, P.; Wang, Y.; Feng, H. Different approaches for selenium biofortification of pear-jujube (*Zizyphus jujuba* M. cv. Lizao) and associated effects on fruit quality. *J. Food Agric. Environ*. **2013**, *11*, 529-534.

335. Zuidmeer, L.; van Leeuwen, W.A.; Kleine Budde, I. Allergenicity assessment of apple cultivars: hurdles in quantifying labile fruit allergens. *Int. Arch. Allergy Immunol.* **2006**, *141*(3), 230-240.

APPENDIX

A) List of hazardous substances used according to GHS

Table 15 lists the numbering of the GHS symbols and Table 16 lists the chemicals used and their GHS classification and disposal.

Table 15: Numbering of the GHS symbols.

01	02	03	04	05	06	07	08	09
								

Table 16: Listing of chemicals used, including manufacturers, GHS classification, H and P phrases, and disposal.

Chemical	Manufacturer	GHS classification	H phrases	P phrases	Disposal
Acetone	VWR International LLC	02, 07	225, 319, 336	210, 233, 305+351+338	(3)
Acetonitrile	Carl Roth GmbH & Co. KG	02, 07	225, 332, 302, 312, 319	210, 240, 302, 352, 305+351+338	(3)
Acetic acid	Carl Roth GmbH & Co. KG	02, 05	226, 290, 314	210, 280, 301+330+331, 305+351+338, 308+310	(3)
Ammonium hydrogen carbonate	Carl Roth GmbH & Co. KG	07	302	301+312, 330	(1)
Ammonium peroxodisulfate	Carl Roth GmbH & Co. KG	03, 07, 08	272, 302, 315, 317, 319, 334, 335	220, 261, 280, 305+351+338, 342+311	(6)
Ammonium sulfate	AppliChem GmbH	Not a hazardous substance according to GHS			(4)

Chemical	Manufacturer	GHS classification	H phrases	P phrases	Disposal
2,2‘-Azino-bis-(3-Ethylbenz-thiazoline-6-sulfonic acid) diammonium salt (ABTS)	Sigma-Aldrich Chemie GmbH	Not a hazardous substance according to GHS			(6)
2,2’-Azo-bis-(2-Methylamidino-propane) Dihydrochloride (AAPH)	Fisher Scientific GmbH	Not a hazardous substance according to GHS			(6)
Bet v 1 monoclonal antibody	MyBiosource.com	Not a hazardous substance according to GHS			(6)
Bovine serum albumin (BSA)	Carl Roth GmbH & Co. KG	Not a hazardous substance according to GHS			(6)
Bromophenol blue	Carl Roth GmbH & Co. KG	Not a hazardous substance according to GHS			(6)
Catechin	Carl Roth GmbH & Co. KG	07	315, 319	264, 280, 302+352, 332, 313, 362, 364, 305+351+338, 337+313	(6)
Catechol	ThermoFisher GmbH	05, 06, 08	301+311, 332, 351, 318, 341	280, 301+310, 301+351+338, 312	(2)
Citric acid	Carl Roth GmbH & Co. KG	07	319	280, 305+351+338, 337+313	(4)
Citric acid monohydrate	Carl Roth GmbH & Co. KG	07	319	280, 305+351+338, 337+313	(4)
Chlorogenic acid	Carl Roth GmbH & Co. KG	Not a hazardous substance according to GHS			(6)
Coomassie brillant blue G 250	Carl Roth GmbH & Co. KG	Not a hazardous substance according to GHS			(3)
2,2-Diphenyl-1-picrylhydrazyl (DPPH)	Sigma-Aldrich Chemie GmbH	08	317, 334	261, 280, 342+311	(2)

Chemical	Manufacturer	GHS classification	H phrases	P phrases	Disposal
Di-potassium hydrogen phosphate	Merck KGaA	Not a hazardous substance according to GHS			(1)
Disodium hydrogen phosphate dodecahydrate	Bernd Kraft GmbH	Not a hazardous substance according to GHS			(6)
Disodium dihydrogen ethylene-diamine tetraacetate	Carl Roth GmbH & Co. KG	07, 08	332, 373	260	(6)
Epicatechin	Carl Roth GmbH & Co. KG	07	315, 319, 335	261, 305+351+338	(6)
Ethanol	VWR International SLLC	02, 07	225, 319	210, 240, 305+351+338, 403+233	(4)
Fluorescein	Sigma-Aldrich Chemie GmbH	07	319	305+351+338	(6)
Folin-Ciocalteu Reagent	Merck KGaA	05	290, 315, 319	280, 305+351+338, 337+313	(6)
Gallic acid	Fisher Scientific GmbH	07	315, 319, 335	261, 305+351+338	(6)
Glycerol	Merck KGaA	Not a hazardous substance according to GHS			(3)
Glycine	Carl Roth GmbH & Co. KG	Not a hazardous substance according to GHS			(6)
Goat-Anti-mouse-IgG, H+L, HRP-conjugated	Merck KGaA	Not a hazardous substance according to GHS			(6)
Goat-Anti-rabbit IgG (H+L), unconjugated	Novex ®	Not a hazardous substance according to GHS			(6)
Hydrochloric acid (25 %)	Carl Roth GmbH & Co. KG	05, 07	290, 341, 335	260, 280, 303+361+353, 304+340+310, 305+351+338	(4)
Hydrogen peroxide (30 %)	Carl Roth GmbH & Co. KG	02, 07	225, 302+312+ 332, 319	210, 305+351+338, 403+235	(3)

Chemical	Manufacturer	GHS classification	H phrases	P phrases	Disposal
Isopropanol	VWR International S.A.S.	02, 07	225, 319, 336	210, 233, 240, 305+351+338, 403+235	(3)
Mal-d 1-antibody HRP-conjugated	Biobyt. Ltd.	Not a hazardous substance according to GHS			(6)
2-Mercapto-ethanol	Carl Roth GmbH & Co. KG	05, 06, 08, 09	301+331, 310, 315, 317, 318, 373, 410	273, 280, 302+352, 304+340, 305, 351, 338, 308+310	(2)
Methanol	Carl Roth GmbH & Co. KG	02, 06, 08	225, 331, 311, 301, 370	210, 233, 280, 302+352, 304+340, 308+310, 403+235	(2)
n-Hexane	VWR International S.A.S.	02, 07, 08, 09	223, 304, 361f, 373, 315, 336, 411	210, 240, 273, 301+310, 331, 302+352, 403+235	(3)
Nitric acid (65 %)	Merck KGaA	03, 05, 06	272, 290, 314, 331	280, 303+361+353, 304+340, 305+351+338, 310	(5)
Nitrogen	---	04	280	403	---
N,N,N',N'-Tetramethyl-ethylendiamine	Carl Roth GmbH & Co. KG	02, 05, 07	225, 332, 302, 314	210, 280, 305+351+338, 310	(4)
Ortho-phosphoric acid	Grüssing GmbH	05	290, 314	280, 301+330+331, 305+351+338, 308+310	(4)
Phloretin-2-glucoside	Carl Roth GmbH & Co. KG	07	315, 319, 335	261, 305+351+338	(6)
Polyvinyl-polypyrrolidone	Merck KGaA	Not a hazardous substance according to GHS			(6)
Potassium dihydrogen phosphate	Merck KGaA	Not a hazardous substance according to GHS			(4)

Chemical	Manufacturer	GHS classification	H phrases	P phrases	Disposal
Potassium peroxodisulfate	Fisher Scientific UK Ltd.	03, 07, 08	272, 302, 315, 319, 334, 335	220, 261, 280, 305+351+338, 342+311	(2)
Potassium hydroxide	Carl Roth GmbH & Co. KG	05, 07	290, 302, 314	280, 301+330+331, 305+351+338, 308+310	(1)
Quercetin-3-glucoside	Carl Roth GmbH & Co. KG	06	301	301+310+330	(2)
Rabbit anti-Apple Mal d 1, polyclonal antibody	MyBiosource.com	Not a hazardous substance according to GHS			(6)
Recombinant Mal d 1	Biomay AG	Not a hazardous substance according to GHS			(6)
Rotiphorese® Gel 40 (acrylamide, bisacrylamide)	Carl Roth GmbH & Co. KG	06, 08	301, 312, 332, 315, 317, 319, 340, 350, 361f, 372	201, 280, 302+352, 304+340, 305+351+338, 308+310	(5)
Sodium azide	Merck KGaA	06, 08, 09	300+310, 373	273, 280, 301+310+330, 302+352+310, 391, 501	(2)
Sodium carbonate	Grüssing GmbH	07	319	260, 305+351+338	(1)
Sodium chloride	Carl Roth GmbH & Co. KG	Not a hazardous substance according to GHS			(4)
Sodium diethyl dithio-carbamate	Merck KGaA	07, 09	273, 280, 302+352, 304+340, 305, 351, 338, 308+310	301+312+330	(6)
Sodium dihydrogen phosphate monohydrate	AppliChem GmbH	Not a hazardous substance according to GHS			(6)

Chemical	Manufacturer	GHS classification	H phrases	P phrases	Disposal
Sodium dodecyl sulfate	Carl Roth GmbH & Co. KG	02, 05, 07	228, 302+332, 315, 318, 335, 412	210, 261, 280, 301+312+330, 305+351+338 +310, 370+378	(6)
Sodium hydrogen carbonate	Merck KGaA	Not a hazardous substance according to GHS			(6)
Sodium hydroxide	Carl Roth GmbH & Co. KG	05	290, 314	280, 301+330+331, 305+351+338, 308+310	(1)
Sulfuric acid	Grüssing GmbH	05	290, 314	280, 301+330+331, 305+351+338, 308+310	(4)
3,3'5,5'-Tetramethyl-benzidine	AppliChem GmbH	Not a hazardous substance according to GHS			(3)
Trimethyl-sulfonium hydroxide in methanol (0,25 M)	Carl Roth GmbH & Co. KG	02, 06, 08	225, 301+311+ 331, 370	210, 280, 301+310, 303+361+353, 308+311	(2)
Tris-(hydroxyl-methyl)-amino-methane	SERVA Electrophoresis GmbH	Not a hazardous substance according to GHS			(6)
Tris-(hydroxyl-methyl)-amino-methane hydrochloride	Carl Roth GmbH & Co. KG	Not a hazardous substance according to GHS			(6)
Trolox	Sigma-Aldrich Chemie GmbH	07	315, 319, 335	261, 305+351+338	(6)
Tween® 20	Carl Roth GmbH & Co. KG	Not a hazardous substance according to GHS			(3)

Disposal key:

(1) If necessary, dilute with water or dissolve in it, then dispose of in the container for alkalis.

(2) Dispose of in the container for halogenated or toxic organic solvents.

(3) If necessary, dissolve in ethanol and dispose of in the container for halogen-free, organic solvents.

(4) If necessary, dilute with water or dissolve in it, then dispose of in the container for HCl/ H_2SO_4-containing acids.

(5) If necessary, dilute with water or dissolve in it, then dispose of in the container for nitrate-containing acids.

(6) Dispose of in the contaminated equipment collection container.

(7) If necessary, dilute with water or dissolve in it and dispose of in the container for silver waste.

Table 17 lists the CMR substances used in categories 1A and 1B.

Table 17: Listing of the CMR substances used in categories 1A and 1B.

CAS-Number	Chemical	Process
7789-00-6	Acrylamide (40%), K1B, M1B	SDS-PAGE

B) Supplementary material to the publications

Supplementary material to the publications “Selenium Biofortification of Different Varieties of Apples *(Malus domestica)* – Influence on Protein Content and the Allergenic Proteins Mal d 1 and Mal d 3” (*Food Chemistry* 2021) and “Relationship between Phenolic Compounds, Antioxidant Properties, and the Allergenic Protein Mal d 1 in Different Selenium-Biofortified Apple Cultivars *(Malus domestica)”* (*Molecules* 2021) have been published and can be found in the following section. Table S1 supplememts the publication in *Food Chemistry* and tables S2 and S3 belongs the the paper published in *Molecules*.

Table S1: Evaluation of the results of protein separation using SDS-PAGE of all apple samples: bands obtained, intensity of bands and proteins identified. The intensity of the bands was determined in a self-selected three-level scale (weak – medium – strong).

Variety and Year of Cultivation	Application*	Shown in Figure	Bands obtained [M_w]	Intensity of Bands	Proteins identified
'Fiesta' 2017	control (HS)	Fig. 1 (A), line 3	11.41	Weak	Mal d 3
	0.1 kg selenite (HS)	Fig. 1 (A), line 2	11.41	Strong	Mal d 3
			17.65	Strong	Mal d 1
			25.68	Medium	Mal d 2
	0.1 kg selenate (HS)	Fig. 1 (A), line 1	11.41	Strong	Mal d 3
			17.65	Weak	Mal d 1
			25.68	Weak	Mal d 2
'Idared' 2017	0.15 kg selenite (HS)	Fig. 3, line 2	11.41	Medium	Mal d 3
			17.65	Weak	Mal d 1
			25.68	Medium	Mal d 2
	0.15 kg selenate (HS)	Fig. 3, line 1	11.41	Strong	Mal d 3
			17.65	Medium	Mal d 1
			25.68	Medium	Mal d 2
'Jonica' 2017	control (HS)	Fig. 1 (B), line 10	11.41	Medium	Mal d 3
			17.65	Strong	Mal d 1
			25.68	Medium	Mal d 2
	0.15 kg selenite (HS)	Fig. 1 (B), line 11	11.41	Medium	Mal d 3
			17.65	Strong	Mal d 1
			25.68	Medium	Mal d 2

Variety and Year of Cultivation	Application*	Shown in Figure	Bands obtained [M_w]	Intensity of Bands	Proteins identified
'Golden Delicious' 2017	control (HS)	Fig. 1 (A), line 9	17.65	Medium	Mal d 1
	0.15 kg selenite (HS)	Fig. 1 (A), line 8	11.41	Medium	Mal d 3
			17.65	Medium	Mal d 1
			25.68	Weak	Mal d 2
	0.15 kg selenate (HS)	Fig. 1 (A), line 7	11.41	Strong	Mal d 3
			17.65	Weak	Mal d 1
			25.68	Weak	Mal d 2
'Jonagold' 2017	Control (HS)	Fig. 1 (A), line 6	11.41	Weak	Mal d 3
			17.65	Strong	Mal d 1
			25.68	Medium	Mal d 2
	0.15 kg selenite (HS)	Fig. 1 (A), line 5	11.41	Medium	Mal d 3
			17.65	Weak	Mal d 1
			25.68	Weak	Mal d 2
	0.15 kg selenate (HS)	Fig. 1 (A), line 4	11.41	Strong	Mal d 3
			17.65	Medium	Mal d 1
			25.68	Weak	Mal d 2
'Golden Delicious' 2018	Control (BS)	Fig. 2, line 7	11.41	Strong	Mal d 3
			17.65	Weak	Mal d 1
			25.68	Weak	Mal d 2
	0.075 kg selenate (BS)	Fig. 2, line 6	11.41	Strong	Mal d 3
			17.65	Weak	Mal d 1
			25.68	Weak	Mal d 2

Variety and Year of Cultivation	Application*	Shown in Figure	Bands obtained [M_w]	Intensity of Bands	Proteins identified
'Jonagold' 2018	Control (BS)	Fig. 2, line 5	11.41	Strong	Mal d 3
			17.65	Weak	Mal d 1
			25.68	Weak	Mal d 2
	0.075 kg selenate (BS)	Fig. 2, line 4	11.41	Strong	Mal d 3
			17.65	Medium	Mal d 1
			25.68	Weak	Mal d 2
'Boskoop' 2018	Control (OS)	Fig. 2, line 9	11.41	Weak	Mal d 3
			17.65	Medium	Mal d 1
			25.68	Medium	Mal d 2
	0.075 kg selenate (OS)	Fig. 2, line 3	11.41	Strong	Mal d 3
			17.65	Weak	Mal d 1
			25.68	Weak	Mal d 2
'Jonica' 2018	Control (OS)	Fig. 2, line 8	11.41	Strong	Mal d 3
			17.65	Strong	Mal d 1
			25.68	Medium	Mal d 2
	0.075 kg selenate (OS)	Fig. 2, line 2	11.41	Strong	Mal d 3
			17.65	Weak	Mal d 1
			25.68	Medium	Mal d 2
'Elstar' 2019	Control (OS)	Fig. 3, line 5	11.41	Medium	Mal d 3
			17.65	Medium	Mal d 1
			25.68	Medium	Mal d 2
	0.15 kg selenate (OS)	Fig. 3, line 6	11.41	Medium	Mal d 3
			17.65	Medium	Mal d 1
			25.68	Medium	Mal d 2
	0.45 kg selenate (OS)	Fig. 3, line 7	11.41	Medium	Mal d 3
			17.65	Medium	Mal d 1
			25.68	Medium	Mal d 2

Variety and Year of Cultivation	Application*	Shown in Figure	Bands obtained [M_w]	Intensity of Bands	Proteins identified
'Elstar' – peel 2019	Control (OS)	Fig. 3, line 4	11.41	Strong	Mal d 3
			17.65	Medium	Mal d 1
			25.68	Weak	Mal d 2
	0.45 kg selenate (OS)	Fig. 3, line 9	11.41	Strong	Mal d 3
			17.65	Weak	Mal d 1
			25.68	Weak	Mal d 2
'Elstar' – fruit flesh 2019	Control (OS)	Fig. 3, line 3	11.41	Medium	Mal d 3
			17.65	Medium	Mal d 1
			25.68	Strong	Mal d 2
	0.45 kg selenate (OS)	Fig. 3, line 8	11.41	Weak	Mal d 3
			17.65	Medium	Mal d 1
			25.68	Medium	Mal d 2

** Foliar spray rate of selenium per hectare and meter canopy height applied with a hand-held sprayer (HS), a backpack sprayer (BS) or a trailed orchard sprayer (OS).*

Table S2: Results of the determination of the selenium content, Mal d 1 content, polyphenoloxidase activity, total phenolic content, and antioxidant activity in all apple samples. Data are given as mean value ± standard deviation (n = 4; n = 2 for Mal d 1).

Variety and Year of Cultivation	Application*	Se [µg/100 g f.w.]	Mal d 1 [µg/100 g d. w.]	PPO [Units/ 100 g f. w.]	TPC [mg GAE/ 100 g d. w.]	TEAC [mmol TE/ 100 g d. w.]	ORAC [mmol TE/ 100 g d. w.]
'Fiesta' 2017	control (HS)	0.1 ± 0.1	51.0 ± 19.2	10.4 ± 7.6	1,141.3 ± 419.7	15.3 ± 4.9	13.4 ± 5.4
	0.1 kg selenite (HS)	3.1 ± 1.5	68.8 ± 18.7	1.9 ± 0.3	838.5 ± 273.0	11.1 ± 2.6	4.6 ± 1.8
	0.1 kg selenate (HS)	3.1 ± 1.4	24.8 ± 4.8	2.5 ± 1.4	843.9 ± 54.5	13.0 ± 0.6	9.5 ± 0.5
'Jonica' 2017	control (HS)	0.7 ± 0.2	43.1 ± 4.5	3.2 ± 1.7	735.6 ± 66.6	5.5 ± 0.5	11.2 ± 1.4
	0.15 kg selenite (HS)	13.9 ± 1.3	37.3 ± 8.9	5.5 ± 3.0	843.0 ± 169.2	5.7 ± 0.9	14.1 ± 1.2
'Golden Delicious' 2017	control (HS)	0.4 ± 0.2	44.5 ± 3.2	12.0 ± 2.2	863.8 ± 123.8	6.8 ± 1.1	5.4 ± 1.6
	0.15 kg selenite (HS)	5.6 ± 0.5	36.5 ± 3.9	18.5 ± 2.8	797.9 ± 44.7	6.0 ± 0.3	1.9 ± 1.1
	0.15 kg selenate (HS)	5.6 ± 0.8	35.0 ± 3.2	24.5 ± 9.5	851.4 ± 14.9	7.6 ± 1.6	6.0 ± 1.4
'Jonagold' 2017	control (HS)	0.4 ± 0.2	107.5 ± 4.9	2.4 ± 0.7	938.2 ± 78.0	7.4 ± 0.9	11.0 ± 2.2
	0.15 kg selenite (HS)	5.6 ± 1.2	24.1 ± 4.2	4.1 ± 1.5	956.5 ± 36.7	11.0 ± 3.9	7.2 ± 1.2
	0.15 kg selenate (HS)	4.5 ± 1.6	53.3 ± 19.0	5.8 ± 5.2	893.8 ± 74.0	13.3 ± 1.3	9.5 ± 1.3
'G. Del.' 2018	control (BS)	0.3 ± 0.0	40.3 ± 5.7	142.3 ± 34.9	1,212.2 ± 164.3	12.1 ± 0.8	14.2 ± 1.5
	0.075 kg selenate (BS)	3.7 ± 0.4	29.9 ± 4.0	61.1 ± 17.5	1,206.9 ± 226.4	10.7 ± 1.1	8.8 ± 3.0
'Jonagold' 2018	control (BS)	0.2 ± 0.0	25.9 ± 3.5	33.5 ± 6.9	900.1 ± 136.4	11.2 ± 2.8	8.1 ± 1.2
	0.075 kg selenate (BS)	2.1 ± 0.7	39.0 ± 5.7	42.4 ± 35.0	815.4 ± 103.5	11.4 ± 0.8	15.5 ± 1.2
'Boskoop' 2018	control (OS)	0.4 ± 0.1	43.1 ± 6.5	41.8 ± 10.5	745.2 ± 66.1	15.6 ± 2.4	17.0 ± 7.1
	0.075 kg selenate (OS)	5.3 ± 0.3	30.9 ± 0.1	18.8 ± 4.7	743.5 ± 51.3	16.5 3.4	14.7 ± 0.9
'Jonica' 2018	control (OS)	0.3 ± 0.1	38.0 ± 7.7	11.4 ± 9.2	785.1 ± 177.3	12.8 ± 1.8	8.9 ± 1.5
	0.075 kg selenate (OS)	3.9 ± 0.7	32.6 ± 5.2	3.8 ± 1.5	841.7 ± 106.7	11.8 ± 1.6	5.6 ± 0.9

Variety and Year of Cultivation	Application*	Se [µg/100 g f.w.]	Mal d 1 [µg/100 g d. w.]	PPO [Units/ 100 g f. w.]	TPC [mg GAE/ 100 g d. w.]	TEAC [mmol TE/ 100 g d. w.]	ORAC [mmol TE/ 100 g d. w.]
'Elstar' 2019	control (OS)	0.6 ± 0.0	46.9 ± 1.0	3.8 ± 0.8	828.1 ± 48.0	7.0 ± 0.3	14.1 ± 1.0
	0.15 kg selenate (OS)	8.7 ± 1.4	39.0 ± 3.3	35.5 ± 8.9	750.7 ± 121.4	5.7 ± 0.5	14.4 ± 0.5
	0.45 kg selenate (OS)	23.2 ± 2.7	53.4 ± 13.6	3.6 ± 1.1	902.7 ± 26.8	7.0 ± 0.2	12.3 ± 1.0

* *Foliar spray rate of selenium per hectare and meter canopy height applied with a hand-held sprayer (HS), a backpack sprayer (BS) or a trailed orchard sprayer (OS).*

G. Del.: Golden Delicious

Table S3: Results of the determination of phenolic compounds using HPLC-MS^n. Data are in average ± standard deviation. The total phenolic content in mg/100 g d. w. was calculated by the sum of all quantitative determined phenolic compounds. For the four main phenolic compounds the content in mg/100 g d. w. and the percentage share is given (n = 4). Phloretin glycosides and Quercetin glycosides were summed up, respectively.

Variety and Year of Cultivation	Application*	Σ [mg/100 g d.w.]	Chlorogenic acid [mg/100 g d.w.]	%	Epicatechin [mg/100 g d.w.]	%	Procyanidin Trimer [mg/100 g d.w.]	%
'Fiesta' 2017	control (HS)	166.18 ± 48.57	66.43 ± 19.90	39.9	22.47 ± 2.28	14.7	15.51 ± 0.49	10.0
	0.1 kg selenite (HS)	118.72 ± 23.16	50.13 ± 15.05	41.5	14.00 ± 4.38	11.6	11.81 ± 5.65	9.6
	0.1 kg selenate (HS)	139.57 ± 11.16	56.83 ± 2.78	40.8	11.00 ± 0.57	7.9	12.35 ± 1.24	8.9
'Jonica' 2017	control (HS)	77.77 ± 3.37	20.64 ± 2.58	26.5	7.12 ± 1.95	9.1	8.63 ± 0.64	11.1
	0.15 kg selenite (HS)	96.94 ± 24.87	20.75 ± 2.85	21.9	7.11 ± 2.53	7.2	10.23 ± 1.12	10.9
'Golden Delicious' 2017	control (HS)	123.14 ± 16.54	33.65 ± 3.92	27.4	11.86 ± 2.32	9.6	9.34 ± 2.33	7.5
	0.15 kg selenite (HS)	144.19 ± 23.99	33.48 ± 0.29	23.7	14.98 ± 1.63	10.5	10.27 ± 1.29	7.2
	0.15 kg selenate (HS)	127.35 ± 17.63	32.59 ± 2.78	25.8	12.89 ± 2.32	10.1	8.46 ± 1.67	6.6
'Jonagold' 2017	control (HS)	137.76 ± 20.98	28.36 ± 3.77	21.2	13.19 ± 2.40	9.6	14.42 ± 2.40	10.4
	0.15 kg selenite (HS)	131.90 ± 16.66	30.20 ± 5.48	23.4	14.75 ± 3.46	11.2	11.45 ± 1.24	8.7
	0.15 kg selenate (HS)	134.09 ± 6.74	31.25 ± 3.09	23.3	13.04 ± 2.37	9.7	11.14 ± 1.89	8.3

** Foliar spray rate of selenium per hectare and meter canopy height applied with a hand-held sprayer (HS)*

*

Variety and Year of Cultivation	Application*	Σ [mg/100 g d.w.]	Caffeeoyl-glucoside [mg/100 g d.w.]	%	Σ Phloretin glucosides [mg/100 g d.w.]	%	Σ Quercetin glycosides [mg/100 g d.w.]	%
'Fiesta' 2017	control (HS)	166.18 ± 48.57	5.16 ± 0.26	3.3	13.01 ± 1.66	8.2	42.18 ± 31.01	25.4
	0.1 kg selenite (HS)	118.72 ± 23.16	5.09 ± 0.11	4.4	9.28 ± 1.14	8.1	26.98 ± 1.77	22.7
	0.1 kg selenate (HS)	139.57 ± 11.16	5.06 ± 0.28	3.6	12.24 ± 1.87	8.7	40.72 ± 7.41	29.2
'Jonica' 2017	control (HS)	77.77 ± 3.37	5.23 ± 0.25	6.7	10.92 ± 0.61	14.0	23.81 ± 1.63	30.6
	0.15 kg selenite (HS)	96.94 ± 24.87	5.24 ± 0.20	5.7	14.47 ± 5.13	14.7	37.72 ± 13.76	38.9
'Golden Delicious' 2017	control (HS)	123.14 ± 16.54	14.81 ± 0.41	12.2	17.00 ± 5.37	14.1	35.09 ± 10.40	28.5
	0.15 kg selenite (HS)	144.19 ± 23.99	13.88 ± 1.03	9.9	16.86 ± 2.92	11.7	53.33 ± 18.66	37.0
	0.15 kg selenate (HS)	127.35 ± 17.63	13.42 ± 1.64	10.6	16.04 ± 1.65	12.6	42.57 ± 9.68	33.4
'Jonagold' 2017	control (HS)	137.76 ± 20.98	4.99 ± 0.12	3.7	16.74 ± 3.34	12.1	58.69 ± 16.61	42.6
	0.15 kg selenite (HS)	131.90 ± 16.66	8.28 ± 3.79	6.3	19.63 ± 6.25	14.7	46.20 ± 12.20	35.0
	0.15 kg selenate (HS)	134.09 ± 6.74	8.90 ± 4.57	6.7	18.96 ± 0.29	14.2	49.37 ± 6.07	36.8

** Foliar spray rate of selenium per hectare and meter canopy height applied with a hand-held sprayer (HS)*

Acknowledgement

Ich bedanke mich ganz herzlich bei allen, die mich während meiner Promotionszeit und der Anfertigung meiner Publikationen und dieser Dissertation unterstützt haben.

In erster Linie gilt mein Dank Herrn Prof. Dr. Sascha Rohn für die Ermöglichung der Durchführung der Dissertation und die spannenden Fragestellungen, die damit verbunden waren, sowie für die weiteren Projekte, die mir im Laufe meiner Promotionszeit anvertraut wurden. Weiterhin danke ich für die jederzeit wertvolle Unterstützung, die konstruktiven Hinweise sowie die wissenschaftliche Betreuung und die Möglichkeit die Ergebnisse meiner Arbeit auf nationalen Tagungen vorstellen zu können.

Ein besonderer Dank geht an meinen Diplomanden Sofia Ackermann, Fenja-Sarah Kappenstein und Sven Brockmann sowie meine Bachelorandinnen Martina Holz und Bao Chau Sawadski, die während meiner Promotionszeit unter meiner Leitung ihre Abschlussarbeiten angefertigt haben. Ich danke Euch für die gute Zusammenarbeit und Unterstützung, die schöne Zeit und die vielen Erkenntnisse, die wir gemeinsam gewonnen haben und die zum Teil auch in meine Dissertation einflossen.

Den Auszubildenden zur/m Biologisch-Technischen Assistentin/en Caroline Wiemer, Hartwig Kha, Julia Jourdan, Jenny Brester und Linda Weissi von der Berufsschule 06 in Hamburg danke ich für die tatkräftige Unterstützung im Labor, das Messen unzähliger Proben und ihr Interesse an den Tätigkeiten.

Mein Dank gilt des Weiteren dem gesamten Projektteam *BiofortiSe,* insbesondere Herrn Prof. Dr. Diemo Daum und Herrn Christoph Budke von der Hochschule Osnabrück, Abteilung für Pflanzenernährung, für die zur Verfügung gestellten Apfelproben, die gute Zusammenarbeit und die wertvolle Unterstützung bei meinen Publikationen.

Zudem danke ich Frau Prof. Dr. Susanne Neugart (Georg-August-Universität Göttingen, Department für Nutzpflanzenwissenschaften) für die Analytik der phenolischen Verbindungen an einem Teil der Apfelproben, die unter ihrer Leitung am Leibniz-Institut für Gemüse- und Zierpflanzenbau (IGZ) Großbeeren durchgeführt wurde.

Meinen Kollegen des Arbeitskreises Rohn des Instituts für Lebensmittelchemie danke ich für die angenehme und kollegiale Arbeitsatmosphäre und die gute Zusammenarbeit.

Meiner Familie, insbesondere meiner Mutter, danke ich von Herzen für die Unterstützung während meiner gesamten Studien- und Promotionszeit, für das Interesse an meinem Fach und die Rückendeckung, die sie mir stets gegeben haben.

Mein größter Dank gilt meiner Frau Nicole für ihr Verständnis und den liebevollen Zuspruch, und die Kraft und Zuversicht, die sie mir stets gegeben hat.

Affidavit of assurance

Hiermit versichere ich an Eides statt, dass ich die vorliegende Dissertation mit dem Titel

Influence of a Selenium Biofortification of Apples (*Malus domestica* BORKH.) on Nutritionally Important Metabolites of Primary and Secondary Plant Metabolism

selbstständig angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet habe. Die eingereichte schriftliche Fassung entspricht der auf dem elektronischen Speichermedium.

Ich versichere, dass diese Dissertation nicht in einem früheren Promotionsverfahren eingereicht wurde.

---------- ----------

(Ort, Datum) (Sabrina Groth)

Curriculum vitae

Entfällt aus datenschutzrechtlichen Gründen.