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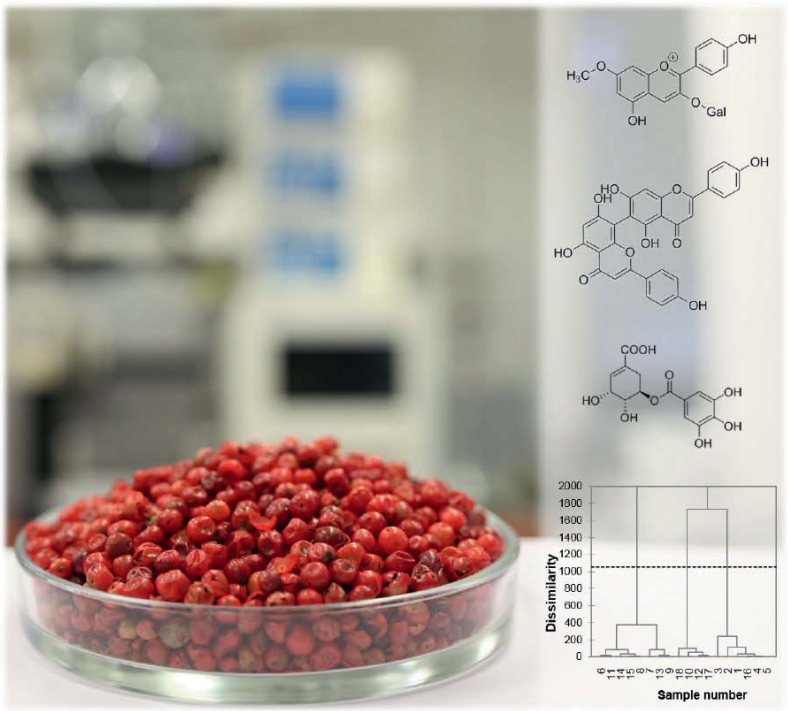
Michelle Feuereisen

Characterization of Polyphenols from *Schinus* sp. Fruits:

Implications for Chemotaxonomy and Authentication



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Characterization of Polyphenols from *Schinus* sp. Fruits: Implications for Chemotaxonomy and Authentication

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To the special people in my life.

The important thing is not to stop questioning. Curiosity has its own reason for existence. One cannot help but be in awe when he contemplates the mysteries of eternity, of life, of the marvelous structure of reality. It is enough if one tries merely to comprehend a little of this mystery each day.

Albert Einstein (1879-1955)

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Preliminary remarks

List of abbreviations

Ac	anthocyanin
AfE	amentoflavone equivalent
ANOVA	analysis of variance
ASE	accelerated solvent extraction
Bf	biflavonoid
CA	cluster analysis
CAE	caffeic acid equivalents
CCC	countercurrent chromatography
DA	discriminant analysis
Da	unified atomic mass unit dalton
DAD	diode array detector
F1	first factor of a principal component analysis
F2	second factor of a principal component analysis
HMBC	heteronuclear multiple bond correlation
HPLC	high-performance liquid chromatography
LC	liquid chromatography
LDA	linear discriminant analysis
MAE	microwave-assisted extraction

MS	mass spectrometry
MvE	malvidin equivalent
m/z	mass-to-charge ratio
nd	not detected
NgE	naringenin equivalent
PCA	principal component analysis
PDA	photo diode array
PLE	pressurized liquid extraction
RSM	response surface methodology
RT	retention time
SFE	supercritical fluid extraction
SPE	solid phase extraction
TPC	total phenolic content
UAE	ultrasound-assisted extraction
UHPLC	ultra high-performance liquid chromatography
UPLC	ultra performance liquid chromatography
UV	ultraviolet

List of publications

Feuereisen, M. M.; Zimmermann, B. F.; Schulze-Kaysers, N.; Schieber, A. Differentiation of Brazilian peppertree (*Schinus terebinthifolius* Raddi) and Peruvian peppertree (*Schinus molle* L.) fruits by UHPLC−UV−MS analysis of their anthocyanin and biflavonoid profiles. *Journal of Agricultural and Food Chemistry*, **2017**, 65, 5330–5338.

Feuereisen, M. M.; Gamero Barraza, M.; Zimmermann, B. F.; Schieber, A.; Schulze-Kaysers, N. Pressurized liquid extraction of anthocyanins and biflavonoids from *Schinus terebinthifolius* Raddi: A multivariate optimization. *Food Chemistry*, **2017**, 214, 564–571.

Schulze-Kaysers, N.; Feuereisen, M. M.; Schieber, A. Phenolic compounds in edible species of the Anacardiaceae family – a review. *RSC Advances*, **2015**, 5, 73301–73314.

Feuereisen, M. M.; Schulze-Kaysers, N.; Schieber, A. Brazilianischer Pfeffer - Quelle bioaktiver Polyphenole. *Deutsche Lebensmittel-Rundschau* **2015**, 111, 514–515.

Feuereisen, M. M.; Hoppe, J.; Zimmermann, B. F.; Weber, F.; Schulze-Kaysers, N.; Schieber, A. Characterization of phenolic compounds in Brazilian pepper (*Schinus terebinthifolius* Raddi) exocarp. *Journal of Agricultural and Food Chemistry* **2014**, 62, 6219–6226.

Conferences

Vandrovcová, M.; Keppler, J. K.; Feuereisen, M. M.; Ricquier, P.; Schieber, A; Skirtach, A. G.; Douglas, T. E. L. Gallotannin enhancement of biomimetic enzymatic hydrogel mineralization. 4th European Symposium and Exhibition on Biomaterials and Related Areas in Weimar, Germany, 9–10 May, **2017**. [Oral poster presentation by Timothy Douglas]

Feuereisen, M. M.; Zimmermann, B. F.; Schulze-Kaysers, N.; Schieber, A.: From Chemotaxonomy to Bioactivity – A Case Study on Brazilian Pepper (*Schinus terebinthifolius* Raddi). 9th World Congress on Polyphenol Applications in Malta, St. Julian's, Republic of Malta, 3–5 June, **2015**, *Book of Abstracts*, 34. [Oral presentation]

Feuereisen, M. M.; Zimmermann, B. F.; Weber, F.; Schulze-Kaysers, N.; Schieber, A. Charakterisierung des Polyphenolprofils von Früchten des Brasilianischen Pfefferbaumes (*Schinus terebinthifolius* Raddi). 43th Deutscher Lebensmittelchemikertag in Gießen, Germany, 22–24 September, 2014, *Lebensmittelchemie* **2015**, 69, 34–35. [Poster]

Feuereisen, M. M.; Zimmermann, B. F.; Schulze-Kaysers, N.; Schieber, A. Characterization and Antimicrobial Activity of Polyphenols from Brazilian Pepper Fruits (*Schinus terebinthifolius* Raddi). 7th World Congress on Polyphenol Applications in Bonn, Germany, 6–7 June, **2013**, *Book of Abstracts*, 30. [Oral presentation]

Declaration of contribution as co-author

The contribution of the co-authors to the papers presented in Chapter 2, Chapter 3, and Chapter 4 are as follows:

Prof. Dr. Andreas Schieber was the supervisor of this thesis. He proofread all manuscripts and contributed to the publication.

Dr. Nadine Schulze-Kaysers advised on experimental work, supported interpretation and publication of the results, and proofread all manuscripts. As the corresponding author she was responsible for all formal aspects of the publications.

Dr. Benno Zimmermann supported interpretation and publication of the LC-MS results and proofread manuscripts.

Dr. Fabian Weber advised on experimental work for the isolation of polyphenols and supported interpretation and publication of the NMR results.

Julia Hoppe conducted the polyphenol extraction and purification and contributed to the subsequent isolation of selected polyphenols.

Mariana Gamero Barraza conducted the PLE experiments for the optimization of the extraction and contributed to the subsequent HPLC analysis.

Chapter 1

General introduction

1 Need for new and natural bio-functional and techno-functional compounds

Human nutrition has always been a subject of constant change influenced by principal factors such as the way of life, current trends, academic research, and increasing global availability of eatables. In recent years, a major trend which partly stands in contrast to the convenience food trend has lead toward an increasingly health conscious and environment conscious lifestyle, most notably regarding the diet. This includes the recollection to more natural, less processed and sustainable groceries often under the label “all natural”. As a consequence, there is an increasing demand for natural products and natural food additives which, in contrast to synthetic additives like azo dyes or sodium nitrite, are valued as beneficial for health and fulfill the consumer expectation. For the industry, the so called “green processing” and “clean label” also implicate the challenge of ensuring the same food quality, shelf life and convenience while avoiding the use of synthetic additives and a reduction of food processing. (Daglia, 2012; Maqsood et al., 2013)

Besides the nutritional and the health value of groceries, food safety is an issue concerning consumers, regulatory agencies, and food industries. Contamination of food with bacteria and fungi causing spoilage and food intoxications are issues which have not yet been overcome. To ensure safe products, effective preservatives and detergents are some of the basic requirements. Unfortunately, there is an increase in microbial resistance against common preservatives and disinfectants. This can interfere with product shelf life and safety, elevating the need for novel antimicrobial agents. Progress in the development of novel antimicrobials is moreover essential

due to the increasing prevalence of antibiotic-resistant bacteria and at the same time a decrease in the development of new antibiotics. Research is focusing on nature as a source of new agents, not only because of consumers' preferences and possible health benefits but also since nature has revealed distinguished bioactive and techno-functional compounds. (Brul & Coote, 1999; Chapman, 2003; Daglia, 2012; Negi, 2012) To enable pharmaceutical or food use, identification and characterization of promising natural sources as well as their compounds is a prerequisite. This is a challenging task, not only because of the overwhelming number of possible raw materials but also since chemical, bioactivity, and industrial aspects like synergetic effects, interactions, sensory properties, toxicity, and applicability of bioactive compounds should be taken into consideration. Therefore, interdisciplinary approaches, as conducted in this thesis, are recommended or even required for a selection and investigation of botanical sources and the establishment of a crucial basis for further research regarding their bioactivity.

2 Nature as a source of bioactive compounds

Ever since natural products have been a rich source of antimicrobial agents. For example, the first discovered and approved antibiotics like penicillin in 1940, tetracycline in 1948, and glycopeptides in 1955 are natural compounds. More precisely, they were yielded from fungi and bacteria. Spices have been used for the preservation for food since ancient days, and various plants have been employed in traditional medicine all the while. Although nowadays up to 25–50% of pharmaceutical products are of plant origin, humans have relied on these antibiotics of microbial origin. Due to the aforesaid reasons of consumers´ preferences and the increasing resistance of microorganisms against classic antimicrobials, there is a rising demand for alternative agents, also of natural origin. Plants are of increasing interest for pharmacology and food safety purposes because they have successfully been utilized in traditional medicine, including the treatment of infections, and since they represent a nearly inexhaustible source of novel molecules. At present, they are scanned for new antimicrobial or otherwise bioactive compounds. Secondary plant metabolites, especially phenolics, are a rather young research domain compared to other food related constitutes such as e.g. macronutrients. Thus, this class of compounds has not been evaluated entirely and further research is essential for the recovery and approval of effective novel and natural agents. Nevertheless, this field

has already revealed promising results of which a few have been verified by *in vivo* studies. Encouraging examples are the neutralization of *Staphylococcus aureus* α-toxin by polymerized catechin, the disruption of *Helicobacter pylori* pathogenesis by the flavonol quercetin in guinea pigs, and the clinical efficacy of the chalcone derivative sofalcone on human *Helicobacter pylori* infection in a multidrug treatment (Cushnie & Lamb, 2011). In addition, techno-functional and bio-functional properties in respect of disease prevention, health benefits, and food application are of major interest. (Bourgaud et al., 2001; Cowan, 1999; Cushnie & Lamb, 2005; Sugathan et al., 2017)

2.1 Secondary plant metabolites

Secondary plant metabolites, also referred to as phytochemicals, occur ubiquitously in plants. They are products of the secondary metabolism, meaning that they have no anabolic, katabolic, or energetic function, and are, therefore, not part of the primary metabolism. Nevertheless, they possess a major role for the reproduction, proper growth, and survival of plants as well as their interaction with the environment and adaptation to environmental influences. Plant secondary compounds provide defense against abiotic and biotic stress caused by herbivores (anti-feeding properties), phytopathogens (phytoalexins), competing plants (allelopathy), UV radiation, and environmental pollution. Furthermore, they are attractants for pollinators or seed dispersing animals. As they are commonly classified according to their biosynthetic pathways, three major groups are considered: phenolic metabolites, terpenoids, and alkaloids including other nitrogen-containing metabolites. Since phytochemicals are part of the daily dietary intake, and thus likely also influence ingesting humans and animals, they have been focused by research in the last decades. Besides some toxic or anti-nutritive effects, various health benefits including prevention and treatment of diseases have been associated with the application or consumption of these plant constituents. (Bourgaud et al., 2001; Dillard & German, 2000; Molyneux et al., 2007) Due to the manifold of compounds, the further delineation in the present work is focused on the group of polyphenols, more precisely on some of their relevant subclasses which will be discussed in the following.

2.2 Polyphenols

With several thousands of identified structures, polyphenols including simple phenolics are the most abundant phytochemicals and widely distributed in higher plants. Basically, the polyphenol molecule consists of at least one aromatic phenyl ring linked to one or more hydroxyl groups, reaching from simple structures such as phenolic acids to highly polymerized tannins. Diversity and ubiquity of these natural plant compounds have led to different classification regarding their chemical structure, biological function, or source of origin. Categorization based on the chemical and structural characteristics usually results in the following subclasses: phenolic acids, flavonoids, stilbenes, and lignans. Frequently, further subgroups such as tannins or phenolic lipids are included. Polyphenols contribute to the organoleptic and nutritive properties of plant based food and several hundred structures can be found in edible plants. Besides fruits, vegetables, nuts, and whole grains, also processed products such as chocolate, wine, coffee, and tea contain high amounts that contribute to the daily intake. (Dai & Mumper, 2010; Dillard & German, 2000; Manach et al., 2004; Tsao, 2010)

The basic flavonoid structure C6-C3-C6 is composed of two aromatic rings (A, B) connected by a heterocyclic pyran ring (C). According to the different substitution degrees of the central C ring, flavonoids are further divided into six subgroups: flavones, flavonols, flavanols, flavanones, isoflavones, and anthocyanidins. The number and complexity of corresponding compounds is increased by the substitution patterns of rings A and B, for example, by hydroxylation, methoxylation, alkylation, or glycosylation. (Dai & Mumper, 2010; Manach et al., 2004; Tsao, 2010)

In nature, the less soluble free aglycones anthocyanidins rather occur glycosylated to one or more sugar moieties that are then called anthocyanins. Anthocyanins are water-soluble pigments imparting many flowers, leaves, fruits, vegetables, and some grains their red, pink, violet, purple, or blue coloration. Color, structure, and consequently stability are influenced, among other factors, by the pH value and metal ion complexation. From the basic structure of a 3,5,7,4’-tetrahydroxyflavylium cation more than 500 known anthocyanins can be derived by different methoxylation and hydroxylation patterns of ring B and glycosidic substitution. The predominantly occurring aglycones in nature are delphinidin, cyanidin, petunidin, peonidin, malvidin, and pelargonidin. (Dillard & German, 2000; Manach et al., 2004; Tsao, 2010)

Biflavonoids are dimeric flavonoids arising by oxidative coupling of two monomers. The heterocyclic ring (C) always has a single functional group, namely a carbonyl function leading to biflavones, flavanon-flavones, or biflavanones. Most naturally occurring dimers have a biphenyl linkage, but almost all possible positions of the interflavonoid bond exist. The variety is expanded by methoxylation, hydroxylation, and glycosylation as well as different oxidation states of the C ring. Nevertheless, there are only approximately 90 known natural biflavonoids, of which amentoflavone occurs most frequently. Further, biflavonoids are only found in a limited number of plants, for example, St. John's wort (*Hypericum perforatum* L.) or ginkgo (*Ginkgo biloba* L.), which display pharmacological activities. (Barnes et al., 2001; Hemingway, 1989; Hyun et al., 2005)

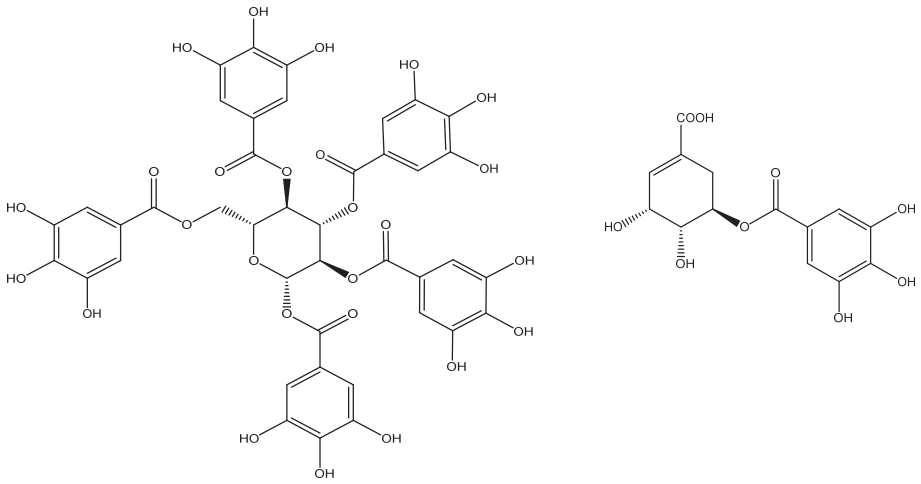

Figure 1: Chemical structure of penta-*O*-galloylglucose and of a mono-*O*-galloyl shikimic acid.

Tannins are commonly subdivided into condensed and hydrolyzable tannins and contribute to the overall sensory impression of many foods like vegetables, fruits, chocolate, and wine due to their astringent and bitter character. Condensed tannins are flavan-3-ol dimers to polymers also called proanthocyanidins. Hydrolyzable tannins, as exemplarily shown in Figure 1, consist of a central molecule (glucose or other polyol, e.g., shikimic acid) esterified with gallic acid (gallotannins) or hexahydroxydiphenic acid (ellagitannins) in different quantities and positions. (Dai & Mumper, 2010; Manach et al., 2004) An additional group also assigned to tannins are the oligomers of phloroglucinol, named phlorotannins. They are less common and have so far been reported only in brown algae (Okuda & Ito, 2011).

2.2.1 Bioactivity of polyphenols

Consumption of polyphenol rich food is associated with a decreased risk for acute and chronic diseases like cancer, cardiovascular indispositions (e.g. coronary heart disease, stroke), and neurodegenerative disorders (e.g., Parkinson's, Alzheimer's disease). For a long time, this was solely attributed to the antioxidative properties of polyphenols, but recent findings have shown that secondary compounds also seem to interact with cellular signaling pathways as well as cell receptors and thereby influence cell function. In addition, polyphenols have revealed further biochemical and pharmacological properties such as antiradical activity, chelation of metal ions, modulation of enzyme activity, anti-inflammatory, anti-proliferative, anti-diabetic, anti-allergic, spasmolytic, hepatoprotective, antiviral, antifungal, and antibacterial activity. (Daglia, 2012; Pietta et al., 2003; Vauzour et al., 2010) Due to its relevance for this work, the latter activity will further be discussed in the following.

Many previous studies dealing with plant extracts hamper insights into structure-activity relationships, modes of action, and the determination of active compounds. This is mainly because they lack an identification of polyphenols and since they apply crude extracts rather than purified fractions or isolated phenolics. Nevertheless, polyphenol rich plant extracts and some pure polyphenols have revealed convincing evidence for their potential as antimicrobial agents. Due to their higher antimicrobial activity and their wide activity spectrum in comparison to other classes of polyphenols, mainly flavonoids and tannins were in the focus of attention. Structure-activity relationships have been examined for several flavonoids and tannins. However, the results are partly ambiguous and to a certain extent contradictory. Therefore, more research is necessary since individual compounds likely have multiple cellular targets rather than one specific site of action and structural features might simply serve the uptake into bacterial cells. Reported modes of action are the inhibition of nucleic acid synthesis as well as cell wall and cell membrane synthesis, influence on cytoplasmic membrane function, inhibition of energy metabolism, and complexation of essential nutrients. Furthermore, polyphenols can suppress microbial virulence factors such as toxins or biofilm formation by influencing the crucial factors like enzymes, attachment to host ligands, or quorum-sensing and thereby attenuate the bacterial pathogenicity. (Cushnie & Lamb, 2005; Cushnie & Lamb, 2011; Daglia, 2012; Engels et al., 2011)

Various pathogenic and non-pathogenic bacterial strains have been included in a multitude of assays and some polyphenols have shown promising antimicrobial effects even against drug-resistant bacteria such as multi-antibiotic resistant *Staphylococcus aureus*. Thereby, some compounds showed the highest activity alone or in synergy with other phenolics or other antibacterial agents like antibiotics. Chemical modification of naturally occurring polyphenols can also enhance their antibacterial effects. Since polyphenols from edible plants have also revealed potential as antimicrobial agents and, among others, have an impact on food-borne bacteria, they could possibly be utilized as new plant based preservatives and prevent food spoilage as well as food intoxications. (Cushnie & Lamb, 2005; Cushnie & Lamb, 2011; Daglia, 2012; Taguri et al., 2004)

2.2.2 Polyphenols as chemotaxonomic and authenticity markers

Chemotaxonomy is a classification system for organisms based on the convergence and deviation of their chemical composition. Plant chemotaxonomy utilizes a broad spectrum of macromolecules (proteins, nucleic acids) and micromolecules (secondary plant products) for the elucidation of plant affiliations and can provide insights into plant systematics and evolution. Thus, the classification of plants has been constantly revised, considering the recent chemotaxonomic findings. (Crawford & Giannasi, 1982; Crockett & Robson, 2011; Feuereisen et al., 2017; 1989; Okuda et al., 1992)

Food fraud has a long and disreputable history reaching back to ancient times. Although statutory regulation und effective verification methods have reduced food adulteration, there still is a temptation to increase the rather low profit margin of the food sector in this way. Thus, the advancement of reliable methods for food authentication and species differentiation is relevant for participants of the supply chain as well as for food control authorities (Feuereisen et al., 2017; Schieber, 2008).

As the boundaries between chemotaxonomy and authentication are indistinct, species differentiation and phenolic profile determination, respectively, can be of use for both areas of application (see Chapter 3 and 5). Phenolic compounds and particularly the subgroup of flavonoids have been shown to be useful and suitable chemotaxonomic markers but also authentication markers. Their use is especially helpful when species differentiation based on morphological characteristics is

challenging or when plant based food products are concerned. These compounds are suitable due to their ubiquity in higher plants, vast structural variety, and their comparatively straightforward extraction and analysis. (Crawford & Giannasi, 1982; Okuda et al., 1992; Schieber, 2008) Relevant techniques and methods of sample preparation, isolation and determination are discussed in Section 5.4.

2.3 Phenolic plant extracts as food ingredients

As mentioned, consumers prefer natural food and consequently additives of natural origin due to positive associations such as health benefits. The development of new preservatives is also necessary to overcome resistances (see section 1). Due to the great variety of bioactivities and structures, the application of suitable plant extracts or polyphenols as natural food additives is an encouraging approach. In this regard, phenolics may be, and in some cases have already been, utilized successfully as antioxidants, colorants, texturizers, and preservatives for food. Among other resources, agro- and food industry by-products are promising sources for the recovery of natural and sustainable food additives because they often contain high amounts of polyphenols and are accumulated as side streams in high quantities. As some polyphenols have been shown to prevent lipid oxidation and microbial growth, both principal causes for food spoilage, their application is conceivable and has partly been approved, even for perishable food like fish or meat. (Ayala-Zavala et al., 2011; Daglia, 2012; Maqsood et al., 2013; Negi, 2012; Pokorný, 1991; Schieber, 2017)

Although polyphenols from edible plants are regularly consumed by humans and, therefore, are considered safe, the toxicity of plant extracts, especially pure phenolics, and their reactions with food ingredients needs to be assessed. For example, essential oils from herbs and spices revealed distinguished antimicrobial activity but also strongly alter the sensory quality of products. Therefore, their application in foods is limited. This example demonstrates that the influence of the utilized phenolic source on the overall sensory impression of food should also be evaluated. (Negi, 2012; Pokorný, 1991)

3 Plant family Anacardiaceae

The Anacardiaceae, more commonly referred to as “cashew family” or “sumac family”, belongs to the Sapindales order and the immediate phylogenetic relatives are the Burseraceae (Kubitzki et al., 2011). For a long time, the classification of the Anacardiaceae into the five tribes Dobineeae, Mangifereae, Rhoideae, Semecarpeae, and Spondieae was common (Engler, 1892; Hegenauer, 1989). More recent publications prefer the denomination as Anacardieae, Rhoeae, Spondiadeae, Semecarpeae, and Dobineeae as characterized by Mitchell and Mori 1987 and shown in Figure 2 (Mitchell & Mori, 1987; Kubitzki et al., 2011; Pell, 2004). The tribes encompass approximately 72 genera with several hundreds of species (Hegenauer, 1989; Simpson, 2006; Kubitzki et al., 2011; Pell, 2004). It should be mentioned that the number of taxa and its order is conflicting as it can be based on different characteristics such as morphological or phylogenetic properties and is constantly revised (Barkley, 1957; Pell, 2004; Schulze-Kaysers et al., 2015).

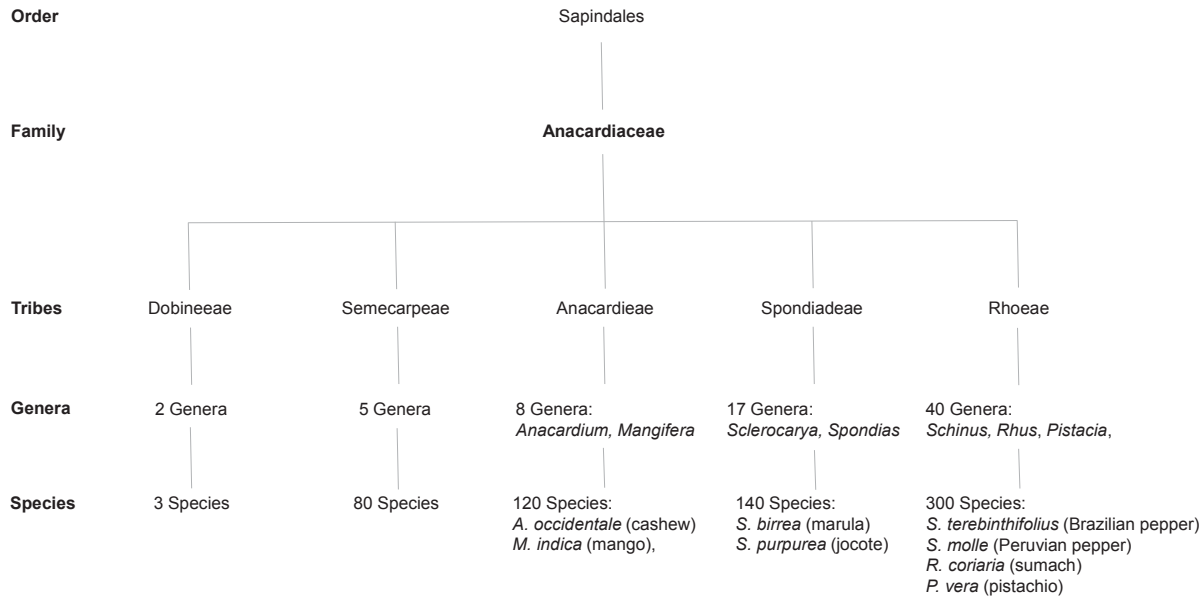

Figure 2: Taxonomic relationships of selected Anacardiaceae species (Tianlu & Barfod).

This plant family is of regional, general economic, and scientific interest due to a broad range of properties. On the one hand, some of the species of the Rhoeae tribe such as Eastern poison ivy (*Toxicodendron radicans* (L.) Kuntze), Western poison ivy (*Toxicodendron rydbergii* (Small ex Rydb.) Greene), Atlantic poison oak (*Toxicodendron pubescens* P. Mill.), Pacific poison oak (*Toxicodendron diversilobum* (Torr. & A. Gray) Greene), and poison sumac (*Toxicodendron vernix* (L. Kuntze)) are

known to produce urushiol, which can cause contact dermatitis if oxidized. (Gladman, 2006; Senchina, 2006) On the other hand, many other species are used in traditional medicine or are of technological value. For example, *Spondias*, *Rhus* and *Schinus* representatives are exploited for diverse medicinal applications reaching from wound treatment over gastrointestinal diseases to various infections. *Schinopsis* and *Rhus* species are applied in the leather and textile industries for tanning and coloring purposes. (Kubitzki et al., 2011; Schulze-Kaysers et al., 2015; Shabbir, 2012; Venter et al., 2012) Furthermore, several edible fruits, some of which are of economic importance, can be found in this plant family. The most important food crops are cashew (*Anacardium occidentale* (L.)), mango (*Mangifera indica* (L.)), pistachio (*Pistacia vera* (L.)), but also pink pepper (*Schinus terebinthifolius* (Raddi), *Schinus molle* (L.)), and recently marula (*Sclerocarya birrea* (A. Rich.)) have gained attention. Many other species such as jocote (*Spondias purpurea* (L.)), sumac (*Rhus coriaria* (L.)), cajú (*Anacardium giganteum* (W. Hancock ex Engl.)) or blood plum (*Haematostaphis barteri* (Hook. F)) are of regional importance (Kubitzki et al., 2011; Schulze-Kaysers et al., 2015; Pell, 2004).

The Anacardiaceae exhibit additional unique features, e.g., they are known to extensively accumulate polyphenols (Hegenauer, 1989). This is one of the reasons why they have been increasingly focused by science. As a consequence, there are numerous studies demonstrating that this plant family, particularly the edible Anacardiaceae, harbor a high potential for the recovery of bioactive compounds (Schulze-Kaysers et al., 2015). Referring to mango and its varieties, a multitude of studies on the phenolic profiles of the fruit have revealed a broad spectrum covering alkylresorcinols, anthocyanins, tannins, gallic acid derivatives, flavonols, phenolic acids, and xanthones (Schulze-Kaysers et al., 2015). Furthermore, techno-functional properties as well as medicinally relevant bioactivities like effects on cancer cell lines could be shown (Noratto et al., 2010; Luo et al., 2014). For example, alkylresorcinols and gallotannins from mango peel and kernels exhibited antifungal (Cojocaru et al., 1986; Droby et al., 1987) or antibacterial activity (Engels et al., 2009; Engels et al., 2010; Engels et al., 2011; Engels et al., 2012). Although they have been investigated to a different extent, results regarding the bioactivity of their phenolics could be observed for several other edible species, such as cashew (Kubo et al., 1993a; Kubo et al., 1993b), marula (Street & Prinsloo, 2013), pistachio (Lim, 2012; Bisignano et al., 2013) or sumac (Shabbir, 2012; Rayne & Mazza, 2007). For further

details on these species and particularly their phenolic profiles and chemotaxonomic relations, the reader is referred to a recent review (Schulze-Kaysers et al., 2015). The chemotaxonomy aspect is updated based on the findings of this work in Chapter 5.

Considering chemotaxonomic coherences, the notion of this thesis was that other, so far poorly investigated Anacardiaceae species, might also be promising sources of bioactive and antimicrobial compounds. The species *S. terebinthifolius* was chosen since it particularly exhibits characteristics like invasiveness, while the responsible compounds are yet unclear.

4 Genus *Schinus* L.

Approximately 30 species have been described within the *Schinus* genus. All of them are native to South American countries but some have been introduced or spread to further tropical and subtropical regions. (Barkley, 1944; Murray et al., 2012) Phytochemical studies of different plant parts have revealed fatty acids, phytosterols, esters and secondary plant metabolites like terpenes and phenolic compounds and have mainly focused on the composition of the essential oils. The use in traditional medicine is known for many species and some of the reported effects on pathologies have already been verified. According to numerous studies, the pharmacological and biological properties related to *Schinus* are: analgestic, allelopathic, antibacterial, antifungal, antidepressant, anti-inflammatory, antioxidant, antitumor, antispasmodic, antiprotozoal, antiulcerogenic, hypotensive, insecticidal, and repellant activities, as well as cholinesterase inhibition, and positive effects on wound healing. (Murray et al., 2012) Although many representatives seem to be of great regional and traditional importance, *S. terebinthifolius* and *S. molle* are the only internationally known and economically relevant species. Therefore, they were the focus of this work and will be described more detailed in the following.

4.1 Economically relevant species

Fruits of both *S. terebinthifolius* and *S. molle* are used as spices, for example, in the United States and Europe. According to leading standard works, the species can clearly be distinguished by their vegetative traits (Barkley, 1944; Barkley, 1957). Nevertheless, the species are said to be hard to differentiate based on the

appearance of the fruits as their size and color variations are not always pronounced (Barkley, 1944; Kramer, 1957; Schrutka-Rechtenstamm et al., 1988). This apparently leads to problems when it comes to authenticity questions and the correct declaration of the spices. This issue is further discussed in Chapter 3.

4.1.1 Schinus terebinthifolius Raddi (Brazilian peppertree)

S. terebinthifolius initially originated from Brazil but spread to further countries in South and Central America (Carvalho et al., 2013; Invasive Species Compendium, 2016; Morton, 1978). Due to its ornamental appearance, the species was introduced to parts of Africa, Asia, Europe and the United States. Consequently, it has often naturalized and showed extensive growth, leading to the suppression of native plants. By now this aggressive plant has been classified as an invasive species in numerous countries (see Section 4.2). (Morton, 1978; Invasive Species Compendium, 2016; Donnelly et al., 2008; Morgan & Overholt, 2005; Florida Exotic Pest Plant Council, 2015). Pursuant to its wide habitat and its usage, *S. terebinthifolius* is regionally more commonly known as “Brazilian pepper”, “Aroeira”, “Chichita”, “Christmas berry” or “Florida holly” (Carvalho et al., 2013; Invasive Species Compendium, 2016; Morton, 1978).

The scrubs to 7 m tall trees have a moderate number of branches that are comparatively thick for the genus. They carry 8–17 cm long leaves and, in maturity, red to pink drupes with an average diameter of approximately 5 mm (Barkley, 1944; Barkley, 1957).

The whole Brazilian peppertree is used traditionally and some plant parts also commercially. For instance, in Brazil, tannin rich extracts of the bark are applied to increase the durability of fishing equipment such as nets and lines, and also the wood is utilized regionally. Cultivation for ornamental purposes or to provide shade is reported for several countries (Morton, 1978; Invasive Species Compendium, 2016; Schrutka-Rechtenstamm et al., 1988). The fact that fruit carrying branches serve as a Christmas decoration, e.g., in Florida or Hawaii, explains some of the above mentioned common names (Morton, 1978; Invasive Species Compendium, 2016; Donnelly et al., 2008). Locally produced but nevertheless commercially available is Brazilian peppertree honey (Morton, 1978). Dried fruits are used as a spice that can be purchased at local markets or at gourmet food stores, online shops and supermarkets (see Chapter 3; Schrutka-Rechtenstamm et al., 1988). They are then

also declared as berries and frequently offered as a colorful supplement in pepper mixtures. Preferably, the pure fruits are used in fine dining and modern cuisine, reaching from seafood, meat, and asparagus to sweat dishes and chocolate. In traditional Brazilian medicine, almost all plant parts are used to treat various ailments, ranging from dermal, cardial, gastro-intestinal, urinary, and respiratory disorders to wound healing and rheumatism (Carvalho et al., 2013; Morton, 1978). Toxic effects of Brazilian pepper fruits were reported formerly, but by now the usage as a spice is considered safe (Carvalho et al., 2013; Morton, 1978; Stahl et al., 1983; Skopp et al., 1987).

4.1.2 Schinus molle L. (Peruvian peppertree)

Before *S. molle* was distributed to other countries in South and Central America, it was indigenous to the Andean region namely Peru and its adjoining countries Chile, Colombia and Ecuador. Eventually, it spread over Mexico to California and has been cultivated and naturalized in other tropic and subtropic regions. To the present, it can even be found in Mediterranean, European countries such as Italy or Spain. It should be mentioned that the precise distribution of some *Schinus* species and especially their varieties remains ambiguous since they can easily be confounded and the taxonomic order was constantly revised. (Lim, 2012; Barkley, 1944; Murray et al., 2012; Kramer, 1957; Martínez-Crovetto, 1963; Invasive Species Compendium, 2017; Goldstein & Coleman, 2004) More commonly, *S. mollle* is referred to as “Peruvian pepper”, “Piru´”, “Pirul”, “Molle”, or “California peppertree”. (Lim, 2012; Invasive Species Compendium, 2017; Kramer, 1957)

The species occurs as trees that have many slender branches compared to other *Schinus* representatives. The leaves have the size of 10–30 cm and the fruits are averagely 5–8 mm in diameter with a red, pink to lavender color. (Barkley, 1944; Barkley, 1957)

As the Peruvians utilize all plant parts, the Peruvian peppertree is of major importance, for instance, to provide traditional medicine. Furthermore, the leaves yield a yellow dye and in several countries the tree was induced due to its ornamental properties and to provide shade. In Mexico and especially in the countries of its origin, syrup, vinegar as well as alcoholic and non-alcoholic beverages are produced from the fruits. (Lim, 2012; Invasive Species Compendium, 2017; Kramer, 1957; Goldstein & Coleman, 2004) Dried fruits are used for seasoning

and at times are also commercially available (Feuereisen et al., 2017; Schrutka-Rechtenstamm et al., 1988; Invasive Species Compendium, 2017). This plant, therefore, also serves culinary and ornamental purposes beyond Peru.

4.2 Characteristics of *Schinus*

Referring to scientific and non-scientific literature, it is striking that there are numerous publications dealing with *S. terebinthifolius* as an invasive plant rather than a spice crop. Nevertheless, there is also a great interest regarding its bioactivity, presumably due to its extensive use in traditional medicine and its aggressive expansion. These considerations have been a motif also of this work. There are historic indications that *S. terebinthifolius* was initially introduced to non-domestic regions as an ornamental plant. Such references, for example, are mentioned for the United States, more precisely Florida (Morton, 1978). Presumably, the distribution and naturalization was then promoted by birds and small mammals, as there is evidence that the ingestion of the fruits and the removal of the exocarp increase the germination rates (Panetta & McKee, 1997; Mandon-Dalger et al., 2004). There is also evidence that the suppression of native plants by *S. terebinthifolius* is facilitated by allelopathic effects (Morgan & Overholt, 2005; Donnelly et al., 2008). However, once naturalized, it spreads rapidly and colonizes densely and even monotypically, especially on disturbed areas. The Brazilian peppertree was rated as an invasive species because such plants can compromise the biodiversity and functions of affected ecosystems and therefore are harmful to the native flora and fauna. Refocusing on Florida, it is said to be a major invader causing severe problems in the Everglades, since the removal causes great costs and eradication is challenging (Schmitz et al., 1997; Ferriter, 1997; Morgan & Overholt, 2005; Williams et al., 2007; Iponga et al., 2009). But also for other regions like Asia (Japan), Africa (Mauritius, Mayotte, Namibia, Réunion, South Africa), Oceania (Australia, New Zealand and further islands), North America (California, Hawaii, Bermuda), Central America, Europe (Canary Islands) and the Caribbean (Bahamas, Cuba, Jamaica, Puerto Rico, Virgin Islands) there are reports according to which *S. terebinthifolius* is invasive and has, if it not already is, potential to become a threat due to its aggressive expansion (Morton, 1978; Invasive Species Compendium, 2016; Donnelly et al., 2008; Florida Exotic Pest Plant Council, 2015).

S. molle is also listed among the invasive species although the threat does not seem as severe and widespread as by *S. terebinthifolius*. The Peruvian peppertree was introduced, for example, to South Africa and California in the 1800s. There, it only showed limited invasiveness but especially in South Africa without prevention it has been expected to expand further. Thereby, the dissemination proceeds similar to that of its Brazilian relative and is promoted by birds, other animals as well as water (Howard & Minnich, 1989; Iponga et al., 2009; Invasive Species Council of California, 2010). *S. molle,* unlike *S. terebinthifolius* which re-vegetates disturbed sites, preferably grows below other tree species and on uncultivated land, resulting in competition for light and takeover of areas (Morgan & Overholt, 2005; Iponga et al., 2008; Iponga et al., 2009). By now, the species grows in Asia, Africa, America, Europe as well as Oceania and experts caution not to underestimate the invasiveness and potential risk of the species (Invasive Species Compendium, 2017; Iponga et al., 2008; Iponga et al., 2009)

4.3 *Schinus* phenolics

The first reports on secondary plant metabolites found in *Schinus* species date back to the early 1960s. The findings mainly concern essential oils and more detailed simple phenols and tannic compounds that were found in plant parts such as leaves, bark and wood, but also polyphenols are mentioned for the first time. Besides shikimic acid, myricitin, quercetin, kaempferol as well as leucodelphinidin and leucocyanidin were listed. Concerning the fruits, to that point, the composition of the essential oils of three species were investigated briefly. (Hegenauer, 1989) Also in later times, the volatile constituents of the different plant parts such as leaves and fruit, especially of *S. molle* and *S. terebinthifolius,* were primarily characterized. (Carvalho et al., 2013; Rita Richter, 2008)

Initially, the determination of the phenolic profile of *S. terebinthifolius* fruits was of interest because of reports about their skin irritating and toxic properties, which stood in contrast to the positive medicinal effects observed (Morton, 1978; Stahl et al., 1983; Skopp et al., 1987; Skopp & Schwenker, 1986). The first phenolic compound ascertained in the drupes was the alkylphenol cardanol 15:1 (n-7), which showed skin irritating effects (Stahl et al., 1983). These results were verified by Skopp et al. (1987) a few years later. Beyond this, they revealed the presence of three further

cardanols: 15:0, 15:2 (n-4) and 15:3 (n-1) as well as four analogous cardols: 15:0, 15:1 (n-7), 15:2 (n-4) and 15:3 (n-1). (Skopp et al., 1987) Although the identification was tentative, there was also evidence for four methylcardols with a chain length of 15 C-atoms and side chains of different degrees of saturation. Some of these compounds were isolated and tested for their skin-irritating effects, resulting in the assumption that cardols and cardanols possess an allergic rather than a general inflammatory character (Skopp et al., 1987). Gallic acid and biflavonoids (amentoflavone, dihydroamentoflavone and tetrahydroamentoflavone) were identified from the fruits almost simultaneously and the latter were associated with the positive impact on health of the Brazilian Pepper (Skopp & Schwenker, 1986). It was not until the 21st century that three other biflavonoids, namely agathisflavone, robustaflavone, tetrahydrorobustaflavone and two biphenyl esters were reported in the fruits (Kassem et al., 2004). Compounds responsible for the bright red color of the fruits had not been identified. Results for *S. molle* fruits suggested that the pigments might also be anthocyanins but no data had been reported. It was quite evident that the phenolic profile was far from being completely characterized. In addition, the information available was scattered and conflicting with respect of the biflavonoids. Accordingly, as described in Chapters 2–4, the phenolic composition of different fruit parts was further investigated in this thesis.

Comparatively little was known about the phenolic composition of *S. molle* fruits. First of all, the three anthocyanins cyanidin-3-galactoside, cyanidin-3-rutinoside and peonidin-3-glucoside were characterized by paper chromatography using standards and UV analysis for *Schinus molle* var. *areira* (Rahman et al., 1974). Later on, two triterpenoids and the biflavonoid chamaejasmin were found in *Schinus molle* L. fruits (Yueqin et al., 2003). These findings were extended by two further biflavonoids: agathisflavone and tetrahydroamentoflavone as well as the dihydroflavonol engeletin and the flavonol quercetin-3-rhamnoside (Ono et al., 2008). Hence, the identification and characterization of the phenolic compounds in *S. molle* also lacked completeness. Consequently, the elucidation of the phenolic profile was also part of this thesis and will be further discussed in Chapter 3. The possibility to differentiate the two species via determination and comparison of their phenolic profiles was also examined in detail (see Chapter 3).

In previous times, not much was known about the chemotaxonomic relationships within the Anacardiaceae family based on the polyphenols, except that many species are rich in tannins and that several species contain anacardic acids and/or cardanols and cardols (Hegenauer, 1989). The chemotaxonomic knowledge was then expanded by more recent findings, which are mostly discussed in the current review concerning edible species (Schulze-Kaysers et al., 2015). An update and a classification of *S. molle* and *S. terebinthifolius* including chemotaxonomic consideration are given in Chapter 5.

5 Techniques to obtain and characterize bioactive phenolics

For the extraction and isolation of polyphenols, a number of aspects need to be considered alongside basic requirements. To avoid degradation and derivatization during processing, extraction, and analysis, the stability of the analytes against different pH values, temperature, light and oxygen exposure should be taken into consideration (Dai & Mumper, 2010). Consequently, not only the selection of the right extraction and isolation conditions are of major importance but also the choice of suitable techniques is challenging and the optimization of the process is often necessary. Many studies using crude plant extracts have been performed but often they lack the determination of the phenolic composition. There are different possibilities to characterize and identify plant polyphenols for various objectives. One example is the use of the phenolic profiles for authentication purposes or to clarify chemotaxonomic relationships (see Section 2.2.2). Further, the obtained and characterized extracts as well as isolated phenolics can be applied knowledge-based to investigate their bio-functional and techno-functional potential, e.g., their antimicrobial properties. In the following, these aspects are discussed regarding their methodological implementation. For this reason, a brief overview of suitable techniques is given and the methods of choice for the aims of this work are outlined.

5.1 Extraction of phenolics from plant material

The first step to yield plant polyphenols is the extraction, which usually starts with the preparation of the plant material. Fresh, frozen, dried, or otherwise processed products may be used, but only gently treated samples are recommended to preserve the original phenolic composition. Since lyophilization is rather mild

compared to air drying, it is a preferred drying method. As the particle size influences the extraction, the plant material is usually comminuted by grinding, milling and/or homogenization in a solvent. (Bucić-Kojić et al., 2007; Dai & Mumper, 2010)

Solid-liquid extraction is well-established and aside from the above mentioned factors, the solvent composition, temperature, pH value, extraction time and sample-to-solvent ratio influence the extraction yield. The chemical nature of the phenolics and the composition of the sample are also relevant as the solubility varies from simple to highly polymerized substances and whether these compounds interact with other plant components such as carbohydrates or proteins. (Dai & Mumper, 2010; Naczk & Shahidi, 2006) Since conventional extraction techniques such as non-instrumental or Soxhlet extraction are often inefficient, various novel techniques have been developed in the last years. Modern extraction techniques range from rather low-cost and simple variants such as microwave-assisted extraction (MAE) or ultrasound-assisted extraction (UAE) to more sophisticated techniques like supercritical fluid extraction (SFE) or pressurized liquid extraction (PLE). These extraction variants have advantages like reduction of extraction time and temperature as well as lower solvent consumption and toxicity. They can also help to prevent undesired reactions such as degradation and derivatization of polyphenols and are often less labor intense and easier to standardize (Alonso-Salces et al., 2001; Dai & Mumper, 2010; Hossain et al., 2011a; Nayak & Rastogi, 2013). Since UAE is suitable for high throughput analysis of a broad spectrum of sample amounts, this technique was selected when many samples available in small quantities were investigated (Chapter 3). In contrast, PLE was chosen for the extraction of *S. terebinthifolius* fruits when a high sample throughput and/or a maximization of the yield was necessary (see Chapter 2 and 4). PLE is a technique that extracts under high pressure and temperatures above the usual boiling points of the solvents used. This technique shows the above mentioned advantages and further increases solvent diffusion, mass transfer and solubility of target compounds. It is suitable for less stable compounds such as polyphenols because high temperatures, if necessary, are usually applied only for a short time. Additionally, the exposure to oxygen and light is much lower than with conventional extraction methods, as the samples are filled in extraction cells, extracted automatically and are therefore retained in a rather oxygen- and light- free environment. Since it is an automated extraction technique, it is also preferable for an optimization of the extraction

conditions and recovery of larger extract amounts. (Alonso-Salces et al., 2001; Dai & Mumper, 2010; Hossain et al., 2011a)

An optimization of the yielding is particularly reasonable if the extraction and isolation procedure or the plant material is expensive or otherwise limited. The required resources can be saved throughout the entire process and a standardization of the extraction procedure is enabled. To achieve an optimization of the extraction as described in Chapter 4, several steps are required. First, the factors that influence the extraction yield and that can be modified should be determined. The number and the type of factors depend on the selected extraction technique, sample material, and target compounds. Depending on the number of identified factors, a design of experiments can be useful. For this purpose, factorial design and response surface methodology (RSM) is widely used, as it allows the investigation of several influence factors. At the same time, the number of necessary experiments can be reduced to a manageable amount. Thus, identification of parameters significantly influencing the extraction and interactions between these parameters is possible in one step. Also the proposed optimized method can be verified easily. RSM was introduced by Box and Wilson and by now is well-established for the optimization of the secondary plant metabolites extraction from food. (Barton, 2013; Box & Wilson, 1992; Hossain et al., 2011a; Nayak & Rastogi, 2013) The obtained plant extracts usually consist of a complex composition of various phenolic and non-phenolic compounds. Thus, after extraction, purification and isolation steps are often required for the recovery of fractions or individual compounds. (Dai & Mumper, 2010; Naczk & Shahidi, 2006)

5.2 Fractionation and isolation of phenolic compounds

Purified fractions and individual compounds may be essential for different purposes, e.g., for identification of polyphenols, use as authentic references otherwise not available, or bioassays (see Section 5.3 and 5.5). Usually, a combination of several concentration and purification steps is necessary to obtain phenolic fractions or single polyphenols with a high purity from a complex crude extract. For a first purification, methods that are based on the polarity of the polyphenols like liquid-liquid partition, column chromatography or solid-phase extraction (SPE) are suitable. Liquid-liquid extraction uses immiscible solvents in which the polyphenols migrate depending on their polarity, while SPE purification is based on interaction of

compounds with the solid stationary and liquid mobile phase. SPE can be applied to eliminate polar, non-phenolic compounds such as organic acids and sugars from extracts. Liquid-liquid partition of extracts with non-polar solvents like hexane or dichloromethane can remove undesired lipoid fractions (Dai & Mumper, 2010; Feuereisen et al., 2014; Naczk & Shahidi, 2006). As mentioned in Chapter 2, these methods are convenient in handling and can also be used for purification and separation of polar and less polar polyphenols when using different solvent systems.

Paper chromatography, thin-layer chromatography, countercurrent chromatography (CCC) as well as preparative or semi-preparative HPLC are suitable techniques for the separation of phenolics. Among these, the latter are preparative-scale techniques and therefore allow the application of higher sample amounts. Depending on the complexity of the extract and effects such as co-elution, yielding of single compounds of high purity may be challenging. (Dai & Mumper, 2010; Naczk & Shahidi, 2006) Analogous to the other mentioned techniques, preparative-scale HPLC is based on the partition and adsorption of analytes to a stationary phase and a mobile phase. Compared to CCC, it has a higher peak resolution and the results are very reproducible. Furthermore, single compounds can be monitored and collected separately, which enables isolation with very high purity. However, compared to CCC, with semi-preparative HPLC usually rather small amounts of the desired compounds are obtained at a time. Nevertheless, it was the method of choice for this work, because for structure elucidation via nuclear magnetic resonance (NMR) spectroscopy, a very high purity of isolated analytes is required (see Chapter 2). This is also preferable for subsequent bioactivity assays. (Dai & Mumper, 2010; Naczk & Shahidi, 2006; Sanbongi et al., 1998)

5.3 Characterization and identification of polyphenols

As mentioned before, the characterization and identification of phenolics and thus also the selection of suitable and reliable methods is crucial for valid research. There are various techniques to quantify and characterize the total phenolic content, a specific class of polyphenols, or even individual phenolic compounds in plant extracts. Liquid chromatography (LC) is most common for the qualitative and quantitative analysis of polyphenols and represents a very reliable method, especially when combined with diode array and mass spectrometry (MS) detection.

LC, usually referred to as high-performance liquid chromatography (HPLC), enables a separation of analytes and is based on the same chromatographic principles as described in Section 5.2. A distinction is made between normal-phase HPLC (polar stationary phase, non-polar eluents) and reversed-phase HPLC (non-polar stationary phase, polar eluents) that can both be combined with different elution methods (isocratic or gradient) and a number of detectors. As a result, both polar and non-polar compounds can be analyzed and various applications are possible. For the analysis of polyphenols, reversed-phase LC is generally combined with a diode array detector (DAD). Corresponding to its conjugated double and aromatic bonds, each phenolic compound absorbs in a lower or higher ultraviolet or visible region of light and therefore exhibits a characteristic absorption spectrum. Coupling to a mass spectrometer enables the detection of molecular masses by ionization of the molecules. Also, an additional fragmentation of the molecules is possible, which allows further inferences regarding the structure of the analyzed phenolic compound. Using HPLC-DAD-MS techniques, a complex mixture of polyphenols present in crude plant extracts can be analyzed. (see Chapter 2–4). However, for reliable and conclusive identification, the comparison of UV/Vis-spectra, retention time, molecular mass and fragmentation patterns with the data of authentic reference standard compounds is highly recommended. Since the availability of standards is limited and novel compounds are detected frequently, characterization and identification can be challenging and further structure elucidation is necessary. (Dai & Mumper, 2010; Schieber, 2008)

NMR spectroscopy is a well-suited technique for structure elucidation of prior purified polyphenols, especially for unknown compounds as described in Chapter 2. This method is based on the magnetic resonance of atoms in a molecule. By applying an external magnetic field, the resonance frequencies of the nuclei deliver information of the molecule structure (e.g. double bonds, position of functional groups, and certain atoms) since the intramolecular magnetic field influences the frequencies (Dai & Mumper, 2010; Schieber, 2008).

5.4 Differentiation of plant species based on polyphenols

The differentiation of plant species can be of interest for authentication purposes as high-cost or scarce foodstuff may be falsified by dilution with less expensive goods

(see Section 2.2.2). Modern analytical techniques for detecting food fraud encompass chromatographic and spectroscopic techniques as well as enzyme-based and stable isotope analysis. Polyphenol analysis is a possible and accepted leverage point for the authentication of plants and foodstuff. Plant authenticity is commonly determined by absolute or relative amounts of secondary metabolites, respectively the presence or absence of marker compounds (Schieber, 2008).

As described in Section 2.2.2, phenolic profiles can be used not only for food authentication but also to clarify chemotaxonomic relationships and similarities. Thereby, the presence or absence of specific secondary plant compounds, so-called chemotaxonomic markers, can enable a differentiation of plants on a higher taxonomic level (i.e. family to species). In other cases, the determination of the relative amounts of phytochemicals can be target-aimed. The knowledge about chemotaxonomic coherences such as relations and similarities is not only of general scientific interest but can also be utilized for the selection of sources containing promising bioactive compounds from the multitude of existing plant species. Thus, chemotaxonomic considerations might be a possibility to gain novel bioactive compounds that are also of economic interest. (Crockett & Robson, 2011; Feuereisen et al., 2017; Hossain et al., 2011b; Schulze-Kaysers et al., 2015).

Frequently, a complex marker profile rather than individual polyphenols are required for reliable authentication of food products or the differentiation of plant species. Consequently, a univariate or bivariate validation is not possible and a statistical evaluation as enabled by a multivariate data analysis becomes necessary (Chapter 3). This application allows the identification of patterns in a large number of analyzed variables. Techniques commonly applied are principal component analysis (PCA), discriminant analysis (DA) or cluster analysis (CA). PCA reduces a large set of attributes (variables/experimental data) to a few factors by the attributes with the largest variations, conserving as much information as possible. Thereby, data groups, their patterns, and the importance of each variable become obvious. CA classifies data depending on the similarity of the attribute values. It is therefore a useful tool for finding groups in a data set (Backhaus, 2008; Hossain et al., 2011b).

Collection of authentic samples is essential for both authentication and chemotaxonomy and a challenging task, particularly when concerning rather exotic plants. As this work has shown, the declaration of food may be deficient or even

wrong. Therefore, besides unambiguously declarated commercially available samples also authentic samples should be involved. Authentic plant material can usually be obtained from botanical gardens via plant databases or when the samples are collected and identified by trained botanists (see Chapter 4).

5.5 Evaluation of bioactivities associated with plant phenolics

There is convincing evidence that plant extracts possess numerous bioactivities. However, there is still a lack of knowledge regarding structure-activity relationships, modes of action, and synergetic effects of polyphenols. The missing of corresponding studies is reasoned by the fact that often the phenolic profile of the plant extracts was not determined and only crude extracts rather than single phenolics were applied. Therefore, it is not possible to assign the observed activities to certain compounds and a need to close this knowledge gap arises.

With the aforementioned techniques, extracts, purified fractions and single polyphenols can be gained from plant material in amounts and purities suitable for bioactivity assays. This is of particular importance if compounds are not commercially available or costly. Determination of the phenolic composition of plant extracts, isolation of phenolics and reliable structure characterization are also crucial for the investigation of their bioactivity. Only then, a suitable and standardized application of plant extracts and single polyphenols in assays as well as determination of the active compounds, structure-activity relationships and modes of action is possible. The knowledge of the phenolic composition is also important because the polyphenols are employed in a variety of assays which require, among others, the consideration of molecule structure-associated factors like stability, solubility, precipitation, and/or diffusion ability of the compounds used.

Knowledge about the phenolic profile of plant extracts is also essential for the potential use as natural food additives, since the assessment and standardization of associated bioactivity, as well as legal approval, and an accurate labeling is necessary. Additionally, the examination of further aspects relevant for food applications like toxicity, influence on organoleptic properties and the transferability of the bioactivity observed *in vitro* to the food matrix is facilitated.

6 Aims of this thesis

This thesis aimed to characterize, recover, and isolate polyphenols from promising edible *Schinus* species to establish a foundation for their application as bio-functional and techno-functional compounds. In this process, the assessment of their suitability as chemotaxonomic and authentication markers was also of major importance. Likewise, the intention was to pave the way for bioactivity and application trials in the search for novel bioactive compounds, such as natural antimicrobials. These should preferably be utilizable as food additives, ideally with an added value for health by avoiding synthetical additives. Chemotaxonomic consideration and specific characteristics of this plant were the reasons to select the so far sparely investigated species *S. terebinthifolius* as a potential source for such polyphenolic compounds. To achieve these objectives, also the investigation of the related *S. molle* was required for differentiation purposes, especially since both species are common, confusable, and used as a spice. An interdisciplinary approach, encompassing various analytical and statistical methods, but also involving botanical, bioactivity, chemotaxonomic, and authentication aspects, was essential to overcome the challenges accompanying the specific aims further stated in the following.

Specific aims of the included studies are (Chapters 2-4):

- Characterization of the phenolic profile of *S. terebinthifolius* fruit parts (Chapter 2)
- Structure elucidation to identify the chemical structure of unknown polyphenols (Chapter 2)
- Phytochemical based differentiation of *S. terebinthifolius* and *S. molle* (Chapter 3)
- Development of a suitable and convenient authentication method for selected *Schinus* species (Chapter 3)
- Optimization of the polyphenol extraction from *S. terebinthifolius* to enhance yield, standardization, and efficiency (Chapter 4)
- Recovery of extracts, fractions, and isolated compounds in quantities and purity suitable for future bioactivity and application studies (Chapters 2 and 4)

For this purpose, the phenolic composition of both *Schinus* species fruits was determined via UHPLC-MS and, where required, further structure elucidation was conducted by 2D NMR analysis after purification of the relevant analytes. The phenolic profiles were compared and further evaluated statistically to gain insights for chemotaxonomy and authentication. These results are compared with previous findings of other Anacardiaceae species to obtain further chemotaxonomic knowledge. In addition, the extraction parameters (ethanol and acid concentration, temperature, and extraction time) of PLE were optimized via RSM and standardized to increase the yield of phenolic rich extracts and consequently desired compounds. This work also involved the development of methods for the purification and isolation of selected compounds, such as anthocyanins and biflavonoids. In this way, a profound basis for subsequent bioactivity testing should be established. This is important in terms of the search for new bio-functional and techno-functional compounds that may, for example, be used as natural food additives. Since findings on the antimicrobial activity of the extracts, fractions, and isolated polyphenols are not included in this thesis, a prospect on the suitability of *S. terebinthifolius* phenolics as natural antimicrobials and food preservatives is given based on literature investigation and the knowledge of the polyphenol profile.

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Chapter 2

Characterization of Phenolic Compounds in Brazilian Pepper (*Schinus terebinthifolius* Raddi) Exocarp

The objective of this study was to characterize the phenolic composition of Brazilian pepper (*Schinus terebinthifolius* Raddi) exocarp extract. Using UHPLC-DAD-MS/MS analysis, four anthocyanins, three biflavonoids, gallic acid, and two types of hydrolyzable tannins (galloyl glucoses, galloyl shikimic acids) were tentatively identified. The structure of the so far unknown 7-*O*-methylpelargonidin 3-*O*-*β*-D-galactopyranoside was elucidated by 2D NMR. Within the group of gallotannins, galloyl shikimic acids with uncommon degrees of galloylation (tetra- to hexagalloyl shikimic acids) were detected. Among the biflavonoids, I3′,II8-biapigenin (amentoflavone), I6,II8-biapigenin (agathisflavone), and II-2,3-dihydro-I3′,II8-biapigenin were identified, which have already been described for Anacardiaceae. From the results of the present study together with previous findings on the phenolic profile of other Anacardiaceae plants, it is concluded that 7-methoxylated flavonoids are a chemotaxonomic trait frequently found in this family.

Keywords: Anacardiaceae, biflavones, chemotaxonomy, galloyl glucose, galloyl shikimic acid, methylated anthocyanidins, NMR, *Schinus terebinthifolius*, tandem mass spectrometry, UHPLC

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1 Introduction

The Brazilian peppertree (*Schinus terebinthifolius* Raddi) belongs to the Anacardiaceae family, which also encompasses economically relevant species such as mango (*Mangifera indica* L.), pistachio (*Pistacia vera* L.), and cashew nut (*Anacardium occidentale* L.) as well as plants of regional importance such as jocote (*Spondias purpurea* L.) and marula (*Sclerocarya birrea* (A. Rich) Hochst.).[1-4] The fruits of *S. terebinthifolius* are commonly used as a spice, either alone or in mixtures with pepper. *S. terebinthifolius*, which is indigenous to Brazil, has meanwhile spread to other countries and is even classified as an invasive species in Florida.[5,6] Its success in foreign habitats and the aggressive expansion of *S. terebinthifolius* suggest that this plant harbors phytochemicals which protect it from biotic stress. There is ample evidence for the presence of allelopathic antimicrobial compounds in various plant parts. Among these, phenolics presumably contribute to the biological activity observed.[6-8]

Although some information is available on the chemical composition of different parts of *S. terebinthifolius*,[9] detailed studies on the nature of phenolic compounds are scarce. Our previous investigations into the phytochemical profile of mango and jocote have revealed the presence of a broad spectrum of various types of phenolic compounds, such as phenolic acids, hydrolyzable tannins, and anthocyanins.[10-12] In mango peels, a novel anthocyanin, namely, 7-*O*-methylcyanidin 3-*O*-*β*-D-galactopyranoside, has been found.[11] Because of the chemotaxonomic relationship between *Mangifera indica, Spondias purpurea*, and *S. terebinthifolius*, we hypothesized that Brazilian pepper contains phenolic compounds of similar composition. Therefore, the objective of this work was to characterize the phenolic composition of the exocarp from *S. terebinthifolius* by UHPLC-DAD-MS/MS. The structure of a so far unknown anthocyanin was elucidated by NMR spectroscopy.

2 Materials and methods

Chemicals. All solvents and reagents were of analytical grade and were purchased from Roth (Karlsruhe, Germany), Sigma-Aldrich (Steinheim, Germany), and VWR (Darmstadt, Germany). Technical grade ethanol was obtained from Hofmann (Düsseldorf, Germany). Unless noted otherwise, deionized water was used throughout. All solvents used as UHPLC-MS eluents were of LC-MS grade and

obtained from the following suppliers: Optigrade water was from Promochem (Wesel, Germany), acetonitrile was from Mallinckrodt Baker (Deventer, The Netherlands), and formic acid was from Fluka/Sigma-Aldrich. Amentoflavone (I3',II8-biapigenin) was purchased from Extrasynthèse (Lyon, France); pelargonidin 3-*O*-glucoside and cyanidin 3-*O*-glucoside were from PhytoLab (Vestenbergsgreuth, Germany).

Sample Preparation and Extraction of Phenolic Compounds. Air-dried fruits from *S. terebinthifolius* Raddi were obtained from Fuchs (Dissen, Germany). Most of the phenolic compounds are located in the outer layer (exocarp), especially the red pigments, which have been characterized here for the first time. Thus, the extraction of phenolic compounds is more efficient and selective when the exocarp is extracted separately. Therefore, the exocarp was manually separated from the rest of the fruit and ground using a model S100 ball mill (Retsch, Haan, Germany). Accelerated solvent extraction (ASE) was performed with a Dionex ASE 350 (Thermo Scientific, Idstein, Germany) system. The powdered exocarp (1.5 g) was mixed with diatomaceous earth (Thermo Scientific) and filled into 10 mL Dionex (ASE 350) stainless steel cells. For extraction, the cell was filled with extraction solvent (EtOH/water/acetic acid, 70:25:5, v/v/v), pressurized (1500 psi), and heated (to 160 °C). Then, the cell was rinsed with fresh extraction solvent (150% of the cell volume) and purged with a flow of nitrogen (100 psi, 60 s).

Purification of Anthocyanin Ac4. Because MS analysis was not sufficient for structure elucidation of anthocyanin Ac4, purification by SPE and preparative HPLC followed by structure elucidation by NMR spectroscopy was necessary. For this purpose, the extract obtained from ASE was evaporated in vacuo at 30 °C in a rotary evaporator (Büchi, Essen, Germany). After dilution with water (1:3, v/v), liquid-liquid extraction with ethyl acetate (1:1, v/v) was performed, followed by rotary evaporation of the aqueous phase. Subsequently, this fraction was purified[13] using a C18 (ec) SPE column (Macherey-Nagel, Düren, Germany). The solvents were removed from the eluate by evaporation and freeze-drying prior to storage in a vacuum desiccator.

For preparative HPLC, the lyophilisate was reconstituted (10 mg/mL) in acetonitrile/water/formic acid (18:80:2, v/v/v) and filtered through 0.2 µm Chromafil RC-20/15 MS filters (Macherey-Nagel).

The anthocyanins were separated on a semi-preparative HPLC from Knauer (Berlin, Germany) consisting of a pump 1050, an autosampler 3950, a manager 5050, and a

UV detector 2550. Using a reversed phase C18 column (Vertex, 250 x 16 mm Eurosphere II 100-5 C18, Knauer) with a C18 guard column (Vertex, 30x16 mm Eurospher 100-5 C18, Knauer), the compounds were eluted with 2.9% (v/v) formic acid in water (solvent A) and 2.9% (v/v) formic acid in acetonitrile (solvent B) at a constant flow rate of 6.0 mL/min. An isocratic separation with 18% of solvent B was performed for 20 min followed by conditioning and equilibration (0 min, 18% B; 4 min, 100% B; 8 min, 100% B; 10 min, 18% B; 20 min, 18% B). The injection volume was 500 µL. The peak recognition mode (500 nm, start/stop 70/100 AU) was used for collection of the anthocyanin fractions with a Foxy R1 fraction collector (Teledyne ISCO, Lincoln, NE, USA). During removal of the organic solvent and the following lyophilization, the fraction containing peak Ac4 was protected from light and temperatures above 30 °C. The lyophilisate was dissolved in deuterated methanol acidified with d1-TFA (1%) for analysis by NMR spectroscopy. All samples were stored at -80 °C until further use.

UHPLC-DAD-MS/MS A**nalysis of Phenolic Compounds.** For the analysis of phenolic compounds, an Acquity UPLC system (Waters, Milford, MA, USA) consisting of a binary pump (BSM), an autosampler (SM) cooled at 10 °C, a column oven (CM) set at 40 °C, a diode array detector (PDA) scanning from 190 to 500 nm, and an Acquity TQD triple-quadrupole mass spectrometer with an electrospray interface was used.

Anthocyanins were separated using an Acquity HSS-T3 RP18 column (150 mm × 2.1 mm; 1.8 µm particle size) from Waters with a guard column (5 mm × 2.1 mm). Eluent A was water/1% formic acid, and eluent B was acetonitrile/0.1% formic acid. The gradient program was as follows: 0 min, 0% B; 0.2 min, 5% B; 12 min, 16.9% B; 13 min, 98% B; 15 min, 98% B; 16 min, 5% B; 18 min, 5% B. The flow rate was 0.4 mL/min. The mass spectrometer was tuned using a solution of cyanidin 3-*O*-glucoside. The following parameters were used: capillary voltage, 1.6 kV; cone voltage, 32 V; extractor voltage, 3.0 V; RF voltage, 1.3 V; source temperature, 150 °C; desolvation temperature, 450 °C; cone gas (nitrogen) flow, 50 L/h; desolvation gas (nitrogen) flow, 800 L/h. The collision gas (argon) flow used in MS/MS experiments was 0.3 mL/min.

For the separation of nonanthocyanin phenolics, an Acquity BEH Shield RP18 column (150 mm × 2.1 mm, particle size = 1.7 µm) from Waters with a guard column

(5 mm × 2.1 mm) was used. Eluent A was water/0.1% formic acid, and eluent B was acetonitrile/0.1% formic acid. The gradient program at a flow rate of 0.4 mL/min was as follows: 0 min, 2% B; 20 min, 60% B; 20.3 min, 100% B; 21.3 min, 100% B; 22 min, 2% B; 23.5 min, 2% B. The mass spectrometer was tuned using a solution of I3',II8-biapigenin, resulting in the following parameters: capillary voltage, −1.0 kV; cone voltage, 25 V; extractor voltage, 2.0 V; RF voltage, 2.0 V; source temperature, 150 °C; desolvation temperature, 450 °C; cone gas (nitrogen) flow, 50 L/h; desolvation gas (nitrogen) flow, 800 L/h. Argon was used as the collision gas in tandem mass spectrometry experiments at a flow rate of 0.25 mL/min. The MS system was controlled by MassLynx 4.1 software.

The compounds were first detected using single MS scanning followed by fragment ion scans of the peaks showing major signals in the MS or UV−vis chromatogram. The UV−vis and mass spectra were used as a basis for structural assignments. To obtain more structural information about the anthocyanins, the cone voltage was set at 50 V for enhanced in-source fragmentation. The aglycones generated were then subjected to fragment ion scans. For the identification of gallic acid derivatives, fixed product ion scan (*m/z* 169) and neutral loss scans (152 u) were conducted. Furthermore, the cone voltage was set at 100 V, resulting in in-source fragmentation. The resulting fragment at *m/z* 169 was then used for fragment ion scan.

The exact masses of the glycosides and the corresponding aglycones were determined by HPLC-QToF-MS using a 6530 Accurate-Mass QToF mass spectrometer (Agilent, Waldbronn, Germany) connected to an Agilent 1260 series HPLC consisting of a degasser, binary pump, autosampler, and DAD. The mass spectrometer was used in positive ionization mode with the following settings: capillary voltage, 3000 V; fragmentor voltage, 200 V; and skimmer, 65 V. The separation was performed on a Kinetex C18 column (150 mm × 2.1 mm, 2.6 µm particle size) from Phenomenex (Aschaffenburg, Germany). The gradient program was as follows: 0 min, 0% B; 7 min, 5% B; 15 min, 15% B; 24 min, 50% B; 25 min, 100% B; 27 min, 100% B; 28 min, 0% B. Eluent A was 93% water, 5% acetonitrile, and 2% formic acid (v/v/ v), and eluent B was 5% water, 93% acetonitrile, and 2% formic acid (v/v/v). The flow rate was 0.4 mL/min, and the column temperature was set to 40 °C.

NMR Spectroscopy. NMR experiments were carried out on a Bruker Avance DPX-300 spectrometer (Rheinstetten, Germany). The sample was dissolved in d4-methanol (99.8% D, Deutero, Kastellaun, Germany) acidified with d1-TFA (99.5% D, Sigma-Aldrich).

^{1}H NMR (300 MHz, CD_3OD wih CF_3COOD) δ 2.71 (d, H3'', 1H, J = 3.4 Hz), 3.83 (m, H6', 1H), 3.86 (m, H5'', 1H), 3.99 (d, H4'', 1H, J = 3.4 Hz), 4.04 (m, H2'', 1H), 4.08 (s, H7a, 3H OCH_3), 5.34 (d, H1'', 1H, J = 7.5 Hz), 6.72 (s, H6, 1H), 7.09 (d, H3' and H5', 2H, J = 8.7 Hz), 7.25 (s, H8, 1H), 8.71 (d, H2' and H6', 2H, J = 8.7 Hz), 9.11 (s, H4, 1H); ^{13}C NMR (75 MHz CD_3OD wih CF_3COOD) δ 57.5 (C7a (OCH_3)), 62.4 (C6''), 70.1 (C4''), 72.4 (C2''), 74.1 (C3''), 77.8 (C5''), 92.9 (C8), 103.1 (C6), 104.3 (C1''), 114.4 (C4a), 117.9 (C3' and C5'), 120.2 (C1'), 136.0 (C4), 136.2 (C2' and C6'), 146.4 (C3), 157.2 (C8a), 158.4 (C5), 165.5 (C2), 167.1 (C4'), 170.7 (C7).

3 Results and discussion

Anthocyanins. The separation of phenolic compounds in exocarp extracts from *S. terebinthifolius* detectable at 500 nm is shown in Figure 1. Four peaks are designated Ac1, Ac2, Ac3, and Ac4, respectively.

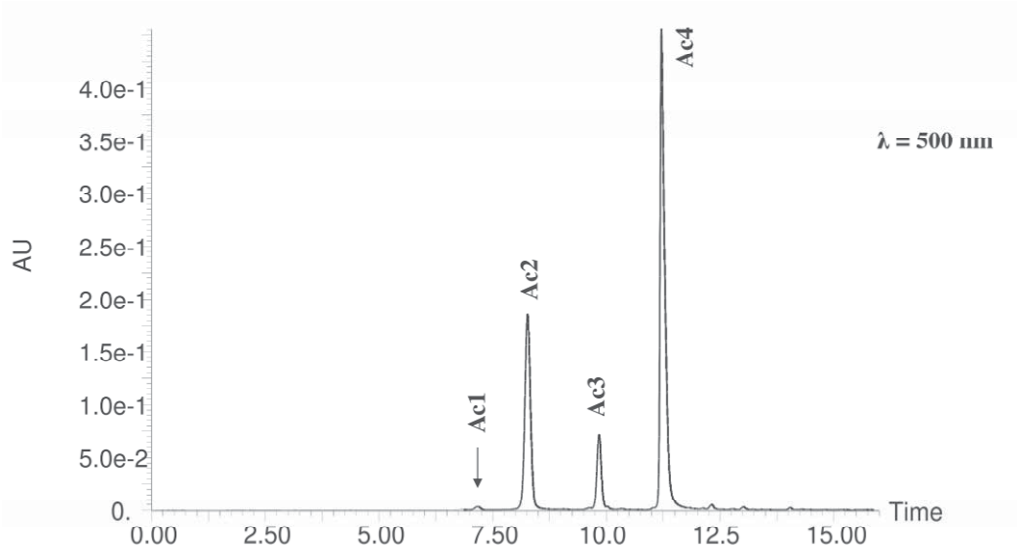
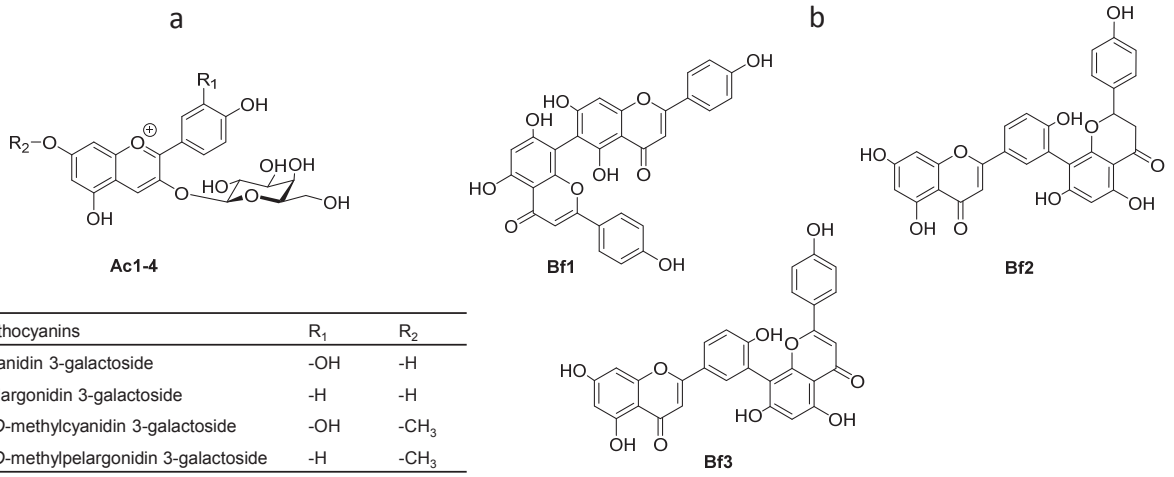


Figure 1: Separation of anthocyanins from Brazilian pepper exocarp extracts by UHPLC (detection wavelength = 500 nm). Ac1, cyaniding 3-*O*-galactoside; Ac2, pelargonidin 3-*O*-galactoside; Ac3, 7-*O*-methyl-cyanidin 3-*O*-galactoside; Ac4, 7-*O*-methylpelargonidin 3-*O*-galactoside.

Compound Ac1. Peak Ac1 showed a molecular ion at *m/z* 449 and a dominant fragment *m/z* 287 (M^+ – 162) in the MS^2 experiment, corresponding to a hexose attached to cyanidin. The standard of cyanidin 3-*O*-glucoside eluted later than Ac1. It is well-known that in reversed-phase chromatography, galactosides of a given aglycone elute prior to the glucosides.[14]

The product ion scan of the in-source fragment with *m/z* 287 of peak Ac1 gave rise to product ions at *m/z* 241, 213, 137, 121, and 109. Identical fragmentation was observed for the cyanidin reference (originating from cyanidin 3-*O*-glucoside). Because an authentic reference compound was not available, the identity of peak Ac1 was proven indirectly by analyzing extracts of mango (*Mangifera indica* L.) peels, chokeberry (*Aronia melanocarpa* [Michx.] Elliot) juice, and Cornelian Cherry (*Cornus mas* L.) fruit extracts. These plants have previously been demonstrated to contain cyanidin 3-*O*-galactoside.[15-18] By comparison of the retention time and the UV−vis and mass spectrometric data, compound Ac1 was identified as cyanidin 3-*O*-galactoside (Figure 2a).



Anthocyanins	R_1	R_2
Cyanidin 3-galactoside	-OH	-H
Pelargonidin 3-galactoside	-H	-H
7-*O*-methylcyanidin 3-galactoside	-OH	$-CH_3$
7-*O*-methylpelargonidin 3-galactoside	-H	$-CH_3$

Figure 2: Structures of the anthocyanins (Ac1, cyanidin 3-*O*-galactoside; Ac2, pelargonidin 3-*O*-galactoside; Ac3, 7-*O*-methylcyanidin 3-*O*-galactoside; Ac4, 7-*O*-methylpelargonidin 3-*O*-galactoside) and biflavones (Bf1, I6,II8-biapigenin; Bf2, II-2,3-dihydro-I3′,II8-biapigenin; Bf3, I3′,II8-biapigenin) identified in *Schinus terebinthifolius* Raddi exocarp.

Compound Ac2. Peak Ac2 showed a molecular ion at *m/z* 433 (M^+) and a dominant fragment at *m/z* 271 (M+ − 162) in the MS^2 experiment, corresponding to a hexose attached to the pelargonidin aglycone. The standard of pelargonidin 3-*O*-glucoside eluted later than Ac2. As mentioned above, in reversed-phase chromatography, galactosides of a given aglycone elute prior to the glucosides.[14]

The product ion scan of the in-source fragment with *m/z* 271 of peak Ac2 gave rise to product ions at *m/z* 197, 169, 141, and 121, which matched the fragmentation of the pelargonidin 3-*O*-glucoside reference. Corresponding data have previously been reported in multistage mass spectrometric studies on black carrot anthocyanins[15]. A peak with the same retention time, UV−vis spectrum, and fragmentation pattern has been found in the above-mentioned extract from Cornelian cherry. Seeram and co-workers[16,17] had identified pelargonidin 3-*O*-galactoside in Cornelian cherry by NMR spectroscopy. On the basis of these findings, compound Ac2 was identified as pelargonidin 3-*O*-galactoside (Figure 2a).

Table 1: UV−Vis and Mass Spectrometric Characteristics (Observed and Theoretical Fragments) of Anthocyanidins Found in Exocarp Extracts from *Schinus terebinthifolius* Raddi[a] (after in-source fragmentation of the glycosides).

Compound	Peonidin	Ac3	Ac4	
	3'-*O*-Methyl-cyanidin	7-*O*-Methyl-cyanidin	7-*O*-Methyl-pelargonidin	4'-*O*-Methyl-pelargonidin (hypothetic)
Vis max (nm)	526	513	499	
$[M]^+$	**301** (20)	**301** (40)	**285** (40)	285
$[M - CH_3]^+$	**286 (**100)	**286** (55)	**270** (55)	270
$[M - CH_3CO]^+$	**258** (70)	**258** (100)	**242** (100)	242
$1,3B^+ - CO$	137	**123** (10)	**107** (25)	121
$0,2B^+$	**151** (5)	**137** (15)	**121** (20)	135
$0,2B^+ - CO$	123	109	93	107
$0,3A^+$	121	135	135	121
$0,2A^+$	**151** (5)	165	165	151

[a]Nomenclature of the fragment ions according to Olivera et al.[47] Data of peonidin and the hypothetical 4′-*O*-methyl-pelargonidin are included for comparison.[b] *m/z* of observed product ions in bold face; relative abundance in parentheses.

Compound Ac3. The retention time of peak Ac3 matched that of the peonidin 3-*O*-glucoside standard. For both Ac3 and peonidin 3-*O*-glucoside, a molecular ion at *m/z* 463 (M^+) and a loss of 162 in MS/MS were obtained. In both cases, the product ion scan of the in-source fragment at *m/z* 301 showed three predominant product ions at *m/z* 301, 286, and 258, but several minor fragments were different (Table 1).

In mango peel extract, a compound with the same retention time, UV−vis spectrum, and fragmentation pattern as Ac3 has been described as 7-*O*-methylcyanidin 3-*O*-galactoside.[11] The fragmentation patterns of the in-source fragment with *m/z* 301 of Ac3 and the major anthocyanidin in a mango peel extract used in our study for

comparison matched well, even regarding the minor fragments and the abundance of each fragment. On the basis of these data, compound Ac3 was identified as 7-*O*-methylcyanidin 3-*O*-galactoside (Figure 2a). Until recently, 7-*O*-methylcyanidin had been detected only in mango peels as its galactoside and in Madagascar rosy periwinkle (*Catharanthus roseus* (L.) G. Don) as its rhamnogalactoside.[18] However, a number of unusual anthocyanins including 7-*O*-methylcyanidin 3-*O*-galactoside have recently been identified in the fruits of Staghorn sumac (*Rhus typhina* L.).

Compound Ac4. Prior to NMR analysis, compound Ac4 was isolated using SPE and semipreparative HPLC. These fractionation and purification processes were necessary because of the complex composition of the crude extract. The anthocyanins not only had to be concentrated, but some of the phenolic compounds, namely, hydrolyzable tannins, coeluted with the anthocyanins and therefore needed to be removed. The chromatographical purity of Ac4 was determined at 280 nm and was ≥96%.

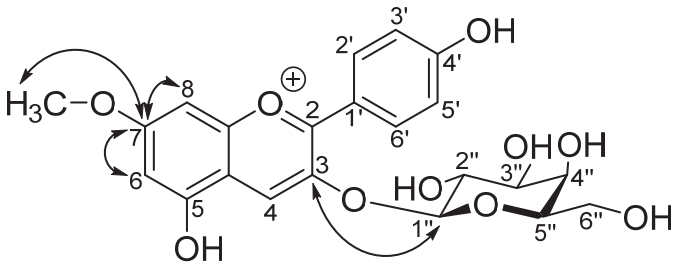


Figure 3: Structure of 7-*O*-methylpelargonidin 3-*O*-*β*-D-galactopyranoside identified in *Schinus terebinthifolius* Raddi exocarp. Arrows show important HMBC correlations.

The proton and carbon signals of Ac4 were attributed according to previously published data.[11,17,19] The ^{1}H NMR displays the typical AA′XX′ signal pattern with two doublets at δ 8.71, J = 8.6 Hz, and δ 7.06, J = 8.6 Hz, which is characteristic of a symmetric monosubstituted B-ring. The anomeric proton of the hexose moiety displays a doublet with a coupling constant of 7.5 Hz, confirming a *β*-glycosidic bond. The two A-ring protons exhibit two relatively broad singlets (δ 6.72 and 7.25), revealing a *meta* substitution. The H4 proton shows a typical high-field shifted singlet at δ 9.11. Besides the signals of the glycosyl moiety between δ 3.8 and 4.1, the spectrum includes a large singlet of a methyl group (δ 4.08). The identity of the galactosyl moiety may be deduced from the small coupling constant (J = 3.6 Hz) of

the proton H4" (δ 3.99), which is characteristic of an equatorial proton. The corresponding proton in a glucose moiety would exhibit a larger coupling constant due to the larger dihedral angle to the adjacent protons.[21] Two-dimensional NMR experiments were conducted to determine the positions of the galactose moiety and the methyl group. HMBC correlations of both the methyl protons and the A ring protons H8 and H6 with the carbon at position C7 revealed that the methoxy group is located at C7. Furthermore, the 3-glycosidic bond was deduced from the correlation of the anomeric proton H1" and the C4 carbon (Figure 3). The signal pattern of Ac4 is in agreement with the data observed by Berardini et al.,[11] who isolated 7-*O*-methylcyanidin 3-*O*-*β*-D-galactopyranoside from mango peel. To our knowledge, 7-*O*-methylpelargonidin 3-*O*-*β*-D-galactopyranoside has not been described before.

Mass Spectrometric Considerations. Although the structure was elucidated by NMR spectroscopy, it is appropriate to discuss the mass spectrometric data of Ac4 together with those of Ac3 to confirm the proposed identity of Ac3 as 7-*O*-methylcyanidin 3-*O*-galactoside (Figure 2a). Compound Ac4 showed a molecular ion at *m/z* 447 (M^+) with a loss of 162 in the MS^2 experiment, resulting in a fragment with *m/z* 285 (M+− 162). The fragments of the aglycones match the theoretically possible fragments of 7-*O*-methylpelargonidin and also those of 4′-*O*-methylpelargonidin for Ac4 and 7-*O*-methylcyanidin or 4′-or 3′-*O*-methylcyanidin (peonidin) for Ac3 (Table 1).

Ac4 showed a molecular ion at *m*/*z* 447 (M+) (exact mass of 447.12970) with a loss of 162 in the MS2 experiment, resulting in a fragment with *m*/*z* 285 (M+ − 162). The predominant fragments of the aglycones (formed by in-source fragmentation) at *m*/*z* 270 and 242 for Ac4 and at 286 and 258 for Ac3 correspond to the loss of a methyl group (M − 162 − 15) and of a CH3CO moiety (M − 162 − 43), respectively (Table 1). The minor fragments may be used for the localization of the methyl moiety, but the fragments of the aglycones match the theoretically possible fragments of both conceivable structures 7-*O*-methylpelargonidin or 4′-*O*-methylpelargonidin for Ac4 and 7-*O*-methylcyanidin or 4′-or 3′-*O*-methylcyanidin (=peonidin) for Ac3.

Nevertheless, the 7-*O*-position of the methyl group on Ac4 can be deduced by comparison of the mass spectra of the aglycones of Ac3, Ac4, and the peonidin standard. Ac3 and peonidin are isomers and show partially the same fragments in the MS^2 spectra of the aglycones. The major fragments (*m*/*z* 286 and 258; loss of a

methyl group (M − 162 − 15) and of a CH_3CO moiety (M − 162 − 43), respectively) match, whereas the minor fragments differ. Furthermore, the relative intensities of the major fragments and of the unfragmented aglycone (*m/z* 301) differ. These data indicate that the Ac3 aglycone is not peonidin and the methyl moiety is not attached at the 4'-*O* position.

The product ion scan of the in-source fragment with *m/z* 285 of peak Ac4 reveals a fragment pattern similar to that of Ac3, but Ac4 shows fragments that differ from those of Ac3 by 16 u. The relative intensities of all fragments match well considering the mass shift of 16 u, which is a clear indication of close structural similarity. The mass spectrometric data of Ac3 are consistent with those of 7-*O*-methylcyanidin 3-*O*-galactoside found in mango peel extract. Considering the pronounced similarity of the aglycone fragments of Ac3 and Ac4, it is convincing that also Ac4 has the methyl moiety at position 7-*O*. Additionally, it is observed that under the ionization and fragmentation conditions applied in this study, the positive charge of the aglycone fragments is always located on the B-ring and the 0,2B+ − CO fragment, which would be of diagnostic value, is never detected (Table 1).

Chemotaxonomic Considerations. The occurrence of 7-*O*-methylated flavonoids in Brazilian pepper fruits is particularly remarkable in view of the results of previous investigations into the profile of flavonoids in members of the Anacardiaceae family. In mango peel extracts, 7-*O*-methylcyanidin 3-*O*-galactoside and cyanidin 3-*O*-galactoside have been described previously.[11] Mango peels have also been demonstrated to contain both quercetin 3-*O*-glycosides and 7-*O*-methylquercetin (rhamnetin) glycosides.[22,23] In peels of Costa Rican jocote fruits, several glycosides of quercetin and rhamnetin have also been found.[24] Interestingly, Kirby and colleagues detected 7-*O*-methyl derivatives of delphinidin and cyanidin, along with other unusual anthocyanins, in fruits of Staghorn sumac,[19] which is also a member of the Anacardiaceae family. However, the corresponding pelargonidin derivatives were not detected in the sumac fruits. Taking together these results, it becomes evident that 7-methoxylated flavonoids, including anthocyanins, frequently occur in Anacardiaceae and might even be considered a chemotaxonomic trait of this family.

Gallotannins. Gallotannins often occur in complex mixtures, which renders their separation and characterization difficult. The separation by UHPLC of gallotannins and other phenolic compounds from Brazilian pepper exocarp is shown in Figure 4.

The gallotannins were tentatively identified by their MS^2 spectra. There were a number of peaks showing a signal at the fixed product ion scan of *m/z* 169 and the neutral loss scan of 152 suggesting gallotannins. They gave rise to an in-source fragment at *m/z* 169 with the same product ion spectrum as gallic acid, confirming the galloyl moiety. These peaks were further examined. Two groups of compounds were distinguished: (1) Peaks showed mass differences of 152 between each other, starting at *m*/*z* 331 and ending at *m*/*z* 2155; this group was identified as mono- to tridecagalloyl glucoses (Supporting Information Tables S1 and S2). (2) Peaks showed mass differences of 152 between each other, starting at *m*/*z* 325 and ending at *m*/*z* 1085; this group was later tentatively identified as mono- to hexagalloyl shikimic acids (Supporting Information Tables S3 and S4).

In addition, gallic acid was identified by comparing retention time and mass spectrometric data with those of an authentic reference.

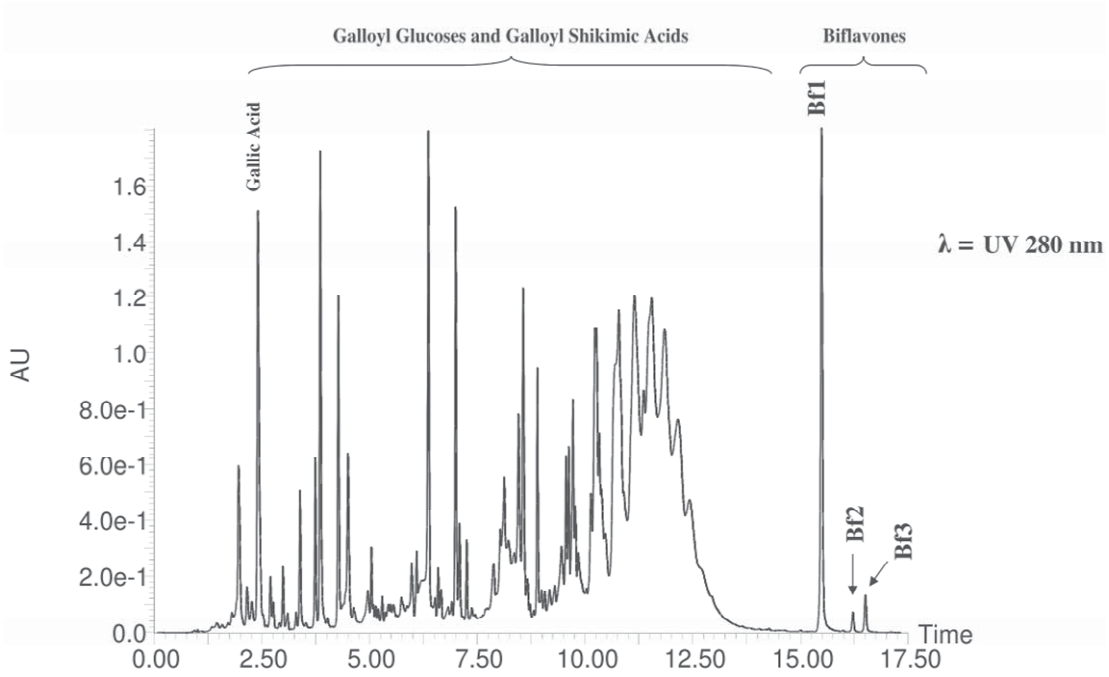


Figure 4: Separation of gallotannins (galloyl glucoses and galloyl shikimic acids) and biflavones by UHPLC (detection wavelength = 280 nm).

Galloyl Glucoses. Three peaks were detected with *m/z* 331 (at 2.7, 2.9, and 3.3 min) showing the same product ions differing in abundance (Supporting Information Tables S1 and S2) as reported by others for monogalloyl glucoses,[25-27] including fragments formed by the loss of water (313), the loss of hexose (169), and cross-ring fragmentation of the glucose (271, 241, 211). The loss of CO_2 was not observed for

monogalloyl glucoses, indicating that the gallic acid moieties are attached to the sugar moiety via an ester linkage rather than an ether linkage.[27]

Five peaks were observed with *m/z* 483 (at 5.1, 5.4, 5.7, 5.8, and 6.1 min) showing the same product ions differing in abundance as reported by others for digalloyl glucoses.[25-27] The peaks with *m/z* 483 had the same product ions as monogalloyl glucose and an additional product ion with *m/z* 331. Furthermore, two of these peaks (eluting after 5.8 and 6.1 min) showed a product ion at *m/z* 439, corresponding to the loss of CO_2 and indicating at least one ether linkage between glucose and gallic acid. This series of peaks with *m/z* of 179 + (n × 152) proceeded consistently until *m/z* 2155, that is, for n = 1−13. Whereas there were three or five peaks with *m/z* 331 or 483, respectively, indicating several isomers of the mono- or digalloyl glucoses, the signals with *m/z* 635 and higher did not split into multiple peaks. It remains to be demonstrated whether this observation may be explained by coelution of isomers or by the absence of different isomers.

Starting from *m/z* 939 (n = 5), the doubly charged ion $[M - 2H]^{2-}$ was more abundant than the singly charged ion, resulting in a series of ions from *m/z* 469 to 1077 with *m*/*z* differences of 76. This behavior has already been described for galloyl glucoses.[28] In most cases, the fragment ions of the $[M - 2H]^{2-}$ precursor ions were interpreted also as doubly charged. Thus, this group was identified as mono- to tridecagalloyl glucoses. 1,2,3,4,6-*O*-Pentagalloylglucose has been reported to occur in *S. terebinthifolius.*[29] Although galloyl glucoses have been intensively investigated,[30] the presence of undeca-, dodeca- and tridecagalloyl glucose has only rarely been reported.[31,32].

Galloyl Shikimic Acids. Three peaks were detected with *m/z* 325 (at 3.4, 3.75, and 3.9 min) showing the same profile of product ions with dominant ions at *m/z* 169, 125 and 173 (Supporting Information Tables S3 and S4), as reported by others for monogalloyl shikimic acid.[33,34] The four peaks with *m/z* 477 (eluting after 6.7, 7.0, 7.6, and 7.75 min) showed the same product ions as monogalloyl shikimic acid and in two cases an additional product ion with *m/z* 325 corresponding to digalloyl shikimic acids. This series of peaks with *m/z* 173 + (*n* × 152) proceeded consistently until *m/z* 1085, that is, for *n* = 1−6. The two heaviest compounds of this series with *m/z* 933 and 1085 showed equally abundant doubly charged ions [M − 2H]− with *m/z* 467 and 543, respectively. Thus, this group was tentatively identified as mono- to hexagalloyl shikimic acids. Monogalloyl,[35-37] digalloyl,[38,39] and trigalloyl[33,38] shikimic

acids have been described before, whereas to our knowledge there is no literature precedent for the presence of tetra-, penta-, and hexagalloyl shikimic acids.

Biflavones. Three major peaks named Bf1, Bf2, and Bf3 were visible in the chromatogram recorded at 280 nm (Figure 4) in the time range from 15.0 to 17.5 min with UV absorption maxima close to 335 nm. Their UV and mass spectrometric data are shown in Table 2.

Table 2: UV Maxima and Positive Product Ions (*m/z*, Relative Abundances in Parentheses; Collision Energy = 40 V) with Hypothetical Structures of the Biflavones Bf1, Bf2, and Bf3[a].

Compound	Bf1	Bf2	Bf3
RT (min)	15.6	16.3	16.6
Assigned Identity	I6,II8-biapigenin	II-2.3-dihydro-I3',II6-biapigenin	I3',II8-biapigenin
UV max (nm)	272, 333	282, 328	269, 335
$[M + H]^+$	539 (6)	541 (2)	539 (55)
$[M + H - H_2O]^+$	521 (5)		521 (3)
$[M + H - C_2H_2O]^+$	497 (2)		497 (9)
$^{1,4}IB^+$		415 (9)	
$^{1,3}IIA^+$	421 (2) [a]		421 (4)
$^{0,2}IIA^+$	419 (49) [a]		419 (3)
$^{1,3}IIA^+ - H_2O$	403 (100) [a]		403 (100)
$^{0,2}IIA^+ - H_2O$	401 (29) [a]		401 (3)
$^{1,3}IB^+$		389 (86)	387 (19)
$^{0,4}IIA^+$	377 (82) [a]		377 (82)
$^{1,3}IIA^+ - H_2O - CO$	375 (45) [a]		375 (48)
$^{1,3}IB^+ - H_2O$		371 (2)	
$^{1,3}IIA^+ - H_2O - 2CO$	347 (50) [a]	347 (1)	347 (20)
$^{0,4}IIA^+ - C_2H_2O$	335 (16) [a]		335 (30)
$^{1,3}IIA^+ - C_2H_2O - C_3O_2$	311 (3) [a]		311 (15)
$^{0,4}IIA^+ - C_2H_2O - CO$	307 (16) [a]		307 (14)
$^{1,4}IB^+ - {}^{1,3}IIB$		297 (4)	
$^{1,3}IIA^+ - C_2H_2O - C_3O_2 - CO$	283 (15) [a]		283 (26)
$^{1,3}IB^+ - {}^{1,3}IIB$		271 (5)	
[Flavone I or II]$^+$	270 (4)	270 (8)	270 (10)
$^{1,3}IB^+ - {}^{1,3}IIB$ (or $^{1,3}IIA^+ - {}^{1,3}IA$)			269 (4)
$^{0,4}IA^+ - {}^{0,2}IIB^+ + H^+$	257 (12)		
$^{0,4}IIB^+$	163 (7) [a]		163 (8)
$^{1,3}IA^+$		153 (100)	153 (32)
$^{0,4}IIB^+ - H_2O$	145 (3) [a]		145 (3)
$^{0,2}IIB^+$	121 (88) [a]	121 (10)	121 (23)
$^{1,3}IIB^+$	119 [a]		119
$^{0,2}IIB^+ - CO$	93 (5) [a]	93 (4)	93 (1)
unknown	229 (4), 361 (15)	111 (6)	453 (3)

[a]Nomenclature of the fragment ions according to Zhang et al.[45] [b]In the case of Bf1 this ring fission in part I leads to the same fragment.

Compound Bf1. Compound Bf1 eluting at 15.6 min showed a UV spectrum similar to that of the I3',II8-biapigenin standard, with the two maxima at 272 and 334 nm (I3',II8-biapigenin, 269 and 335 nm). Identical fragments were found for Bf1 and the reference compound in the negative product ion scans. However, in the positive mode some differences are evident. In the mass spectrum of Bf1, *m/z* 387 and 153 are lacking and *m/z* 419 and 257 are present in significant intensity. The fragment at *m/z* 121 is about 4-fold more abundant in Bf1 than in I3',II8-biapigenin. This fragmentation behavior can be explained by assuming the structure I6,II8-biapigenin.

In I6,II8-biapigenin, the C−C bond between the apigenin moieties links the two A-rings. Therefore, fissions of the C-ring leading to B^+ fragments form relatively light ions, for example, *m/z* 121 ($^{0,2}IB^+$ and $^{0,2}IIB^+$) and 163 ($^{0,4}IB^+$ and $^{0,4}IIB^+$). These ions are also formed from I3',II8-biapigenin, but only when cleavage of part II occurs ($^{0,2}IIB^+$ and $^{0,4}IIB^+$). $^{1,3}IB^+$ fragmentation of I3',II8-biapigenin yields a rather heavy ion (*m/z* 387) because part II is attached to the B-ring of part I. A fragment ion at *m/z* 387 is not observed in Bf1. The A^+ fragments of I6,II8-biapigenin always have the same *m/z* regardless of whether fission occurs in part I or II: *m/z* 421 ($^{1,3}IA^+$ and $^{1,3}IIA^+$) and *m/z* 377 ($^{0,4}IA^+$ and $^{0,4}IIA^+$). These ions are also formed from I3',II8-biapigenin, but only when cleavage of part II occurs ($^{1,3}IIA^+$ and $^{0,4}IIA^+$). After $^{1,3}IA^+$ fragmentation of I3',II8-biapigenin, an ion with *m/z* 153 is formed, which is not observed in Bf1. The fragment with *m/z* 257 in the mass spectrum of Bf1 can be explained by the two ring fissions $^{0,4}IA^+$ and $^{0,2}IIA^+$, if Bf1 is considered as I6,II8-biapigenin (Figure 2b).

Although the mass spectrometric data do not allow the distinction between 6,8-, 6,6- and 8,8-linkages, I6,II8-biapigenin (agathisflavone) confirms the structure, which has been described in *S. terebinthifolius*[9,40] as well as in other *Anacardiaceae* species (*Rhus* ssp.[41,42] and *Anacardium occidentale* (cashew)[43]).

Compound Bf2. Peak Bf2 eluting at 16.3 min with [M − H]− 539 shows UV maxima at 282 and 330 nm, which indicates the presence of a naringenin part. The MS^2 fragments in the positive ionization mode are in agreement with those of a II-2,3-dihydro-I3',II8a biapigenin from *S. terebinthifolius*[44] and a 2,3-dihydro biapigenin from *Selaginella tamariscina*[45]. However, the mass spectrometric data do not allow the distinction between 3′,6- and 3′,8-linkages. We consider our findings as a confirmation of the II-2,3-dihydro-I3′,II8a biapigenin found by Skopp and

Schwenker.[44] Therefore, peak Bf2 was tentatively identified as a II-2,3-dihydro-I3',II8-biapigenin (I3',II8-naringeninyl-apigenin), Figure 2b).

Compound Bf3. Peak Bf3 eluting at 16.6 min with [M −H]− 537 shows the same retention time, UV spectrum, and MS fragments as the I3',II8-biapigenin (amentoflavone) standard. The UV spectrum as well as MS2 spectra in negative and positive mode are in agreement with published data of I3',II8-biapigenin,[45,46] which has been described in *S. terebinthifolius*[44] as well as in other Anacardiaceae species (*Rhus* ssp.[41,42]). Therefore, peak Bf3 was identified as I3',II8-biapigenin (Figure 2b).

In conclusion, this study contributes to the characterization of the phenolic compounds found in *S. terebinthifolius* fruits. The red pigments in the exocarp of *S. terebinthifolius* were characterized for the first time. Besides three anthocyanins, which are known from other Anacardiaceae, the novel anthocyanin 7-*O*-methylpelargonidin 3-*O*-*β*-D-galactopyranoside was identified. Furthermore, two groups of hydrolyzable tannins, namely, galloyl glucoses and galloyl shikimic acids, with partially uncommon degrees of galloylation and three biflavonoids were found.

The presence of 7-*O*-methylated anthocyanins confirms the assumed chemotaxonomic relationship to other Anacardiaceae because 7-*O*-methylcyanidin 3-*O*-*ß*-D-galactopyranoside was also found in mango and Staghorn sumac. These findings expand the knowledge about the phenolic profile of Anacardiaceae and enhance the chemotaxonomic understanding. Antimicrobial effects of extracts from leaves and bark of *S. terebinthifolius* have already been shown. Therefore, microbiological testing with exocarp extracts from *S. terebinthifolius* should be the focus of further investigations. The successful characterization of the phenolic profile facilitates the isolation and identification of promising antimicrobials and may thus enable the assignment of bioactivity to particular compounds. Thus, the present study provides a crucial basis for future research.

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Supporting information

Tables S1−S4. This material is available free of charge via the Internet at http://pubs.acs.org.

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Chapter 3

Differentiation of Brazilian Peppertree (*Schinus terebinthifolius* Raddi) and Peruvian Peppertree (*Schinus molle* L.) Fruits by UHPLC–UV–MS Analysis of Their Anthocyanin and Biflavonoid Profiles

The objectives of this work were to determine the phenolic profile of *Schinus terebinthifolius* and *Schinus molle* fruits and to develop a reliable method for the differentiation of these two similar spices both known as pink pepper. Anthocyanins, biflavonoids and gallotannins, some of which are reported for the first time in these species, were identified by UHPLC−UV/vis−MS/MS. Consideration of the relative and absolute amounts of phenolics as well as indicator compounds from 18 samples revealed that the relative amounts of anthocyanins and biflavonoids are the most trustworthy parameters. Principal component analysis and cluster analysis (CA) allowed a grouping of the samples according to their species, showing that the anthocyanins are most important for the identification of species. As a result, authentication of the two *Schinus* species can be accomplished by UHPLC analysis of the relative amounts of anthocyanins combined with CA.

Keywords: *Schinus terebinthifolius* Raddi, *Schinus molle* L., anthocyanins, biflavonoids, authentication

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1 Introduction

Schinus terebinthifolius Raddi and *Schinus molle* L. are two of approximately 30 *Schinus* species described in the literature and belong to the Anacardiaceae family. Whereas *S. terebinthifolius* (Brazilian peppertree) is indigenous to Brazil, *S. molle* (Peruvian peppertree) initially comes from the Andean region, mainly Peru. Both species have spread to other countries in Southern and Central America and to other tropic and subtropic regions such as parts of the United States, Asia, Africa, Australia, and Mediterranean Europe.[1-8]

Fruits from both species are used as a spice and vary in color and size. Brazilian pepper fruits are described as pink or red with an average diameter of approximately 5 mm. With a size of 7−8 mm in diameter and the same or lavender color, the fruits of the Peruvian pepper are sometimes thought to be hard to distinguish from those of *S. terebinthifolius*. It is therefore difficult to assign the spices to their species only on the basis of the appearance of the fruits.[1,9] Consideration of other distinguishing vegetative traits, for example, phyllotaxis, allows a clear differentiation of the *Schinus* species.[1] Our previous investigations of the phenolic composition of the *S. terebinthifolius* fruit revealed the presence of anthocyanins, biflavonoids, gallic acid, and two groups of hydrolyzable tannins.[10,11] Anthocyanins were detected also in the fruits of *S. molle* var. *areira,*[12] and biflavonoids were reported in the fruits of *S. molle.*[13,14] Some of the biflavonoids match those we identified in *S. terebinthifolius*.[10] Because information about the phenolic profiles of *S. terebinthifolius* and *S. molle* is conflicting and scattered,[15] a differentiation of the species by their profiles of phenolic compounds in the fruits is not yet possible based on the data so far published. Because often the spices on the market lack information about the species and the latter sometimes seem to be unknown even to the suppliers, it would be useful for authentication purposes to identify the species by their phenolic compounds. We found that *S. molle* is commonly sold at higher prices, especially when marketed as “Violetta”. In particular in view of existing price differences, the availability of a reliable method for species differentiation would be of interest both for participants of the supply chain and for food control authorities. Plant authenticity is commonly determined by absolute or relative amounts of secondary plant metabolites or the presence or absence of indicator compounds.[16]

Therefore, the aim of this work was to characterize the phenolic profile of *S. molle* and to develop a method for the phytochemical differentiation of *S. terebinthifolius* and *S. molle* fruits based on their phenolic composition. For this purpose, samples from botanical gardens, local markets, and the food industry were obtained. Phenolic compounds were characterized and quantified using UHPLC−UV/vis−MS/MS, and the results were statistically verified by principal component and hierarchic cluster analyses

2 Material and methods

Chemicals. Solvents and reagents were of analytical grade and were purchased from ChemSolute (Renningen, Germany) or Julius Hoesch GmbH& Co. KG (Düren, Germany). All solvents used as UHPLC-MS eluents were of LC-MS grade and obtained from the following suppliers. Optigrade water and acetonitrile were from ChemSolute, and formic acid was from Fisher Scientific (Geel, Belgium). Amentoflavone (I3',II8-biapigenin) and hinokiflavone (I4'-*O*,II6-biapigenin) were purchased from Extrasynthèse (Lyon, France). Malvidin 3-*O*-glucoside chloride was from Phytoplan (Heidelberg, Germany).

Plant material and extraction of phenolic compounds. Dried and fresh fruits from *S. terebinthifolius* and *S. molle* were obtained from German local and Internet stores, including popular brands, as well as from botanical gardens and local markets in South America. Fruits were also harvested from trees outside botanical gardens in Chile, Peru, and Spain (Table 1). Before analysis, fresh fruits were air-dried or lyophilized. The size of the fruits was determined by measuring 10 fruits per sample with a caliper, and the color was evaluated visually. The exocarp was manually separated from the inner part of the fruit (further referred to as drupe), and 0.35 g of the exocarp was used for extraction. In the case of samples 1−3, only small amounts were available, and therefore, 0.06, 0.17, and 0.13 g respectively of exocarp were used. All samples were extracted three times with 10 mL of a 70% aqueous methanol/ 1% formic acid mixture in an ultrasonic bath for 1 h at room temperature. The drupes were ground with a mortar and pestle and defatted with 6 mL of petroleum ether/g of sample (deviating sample weight; sample 1, 0.25 g; sample 2, 0.43 g; sample 3, 0.53 g) prior to extraction. To remove the remaining organic solvent, the defatted drupes were dried under a nitrogen flow and then extracted

using the same conditions that were described for the exocarp. Subsequently, the volume of the samples was reduced under a nitrogen flow to concentrate the extracts for identification of the analytes. For the quantitation of the anthocyanins and biflavonoids, extraction solvent was added to make the volumes of all samples the same. For identification of the individual compounds, samples 6 and 10 (*S. terebinthifolius* and *S. molle*, respectively; exocarp and drupe) were used because these two samples were available in the largest amounts. Larger quantities of these samples were used for extraction to obtain unambiguous MS^2 spectra even from compounds present in small amounts. Thus, these two samples served as references for comparing retention times and mass spectrometric data of the peaks in the other samples and are further termed references 1 and 2, respectively.

Rhus coriaria (tanner's sumac) was obtained as a ground spice from an online spicer (Eder Gewürze, Mattighofen, Austria) and *Rhus typhina* (staghorn sumac) from a botanical garden (Freie Universität Berlin, Berlin, Germany). These sumac samples were used as references to confirm the presence of rare anthocyanins based on literature reports.[18,19] The *R. typhina* fruits were ground with a mortar and pestle. Both ground spices were subsequently extracted as described above. All plant extracts were filtered through 0.2 µm Chromafil RC-20/15 MS filters (Macherey-Nagel, Düren, Germany) before further analysis.

Table 1: *Schinus* Samples and Their Sources of Supply, Species Specifications, Claimed Origins, and Dry Fruit Appearances

Sample	Source of supply	Species specification	Claimed origin/ cultivation	Harvest year	Mean diameter (mm)	Color
1	University of Bayreuth	*Schinus molle* DT 403 [d]	Ecological-Botanical Garden, University Bayreuth, Germany	2012	-	pale pink/beige
2	Freie Universität Berlin	*Schinus molle* var. *Molle* [d]	Botanical Garden, Freie Universität Berlin, Germany	2014	4.9	pink/beige
3	Brisbane Botanic Gardens	*Schinus molle* var. *areira* [d]	Brisbane Botanic Gardens Mount Coot-tha, Brisbane, Australia	2015	4.8	pale pink/beige[f]
4	Jardín Botánico Nacional	*Schinus molle* var. *areira* [d]	Quilpue, Región de Valparaíso, Chile, identified by Jardín Botánico Nacional, Viña del Mar, Chile	2014	6.1	pink
5				2015	5.7	pink
6[a]	Fuchs [b]	*Schinus terebinthifolius* [e]	Brazil	-	4.7	red
7	Spice Islands [b]	*S. terebinthifolius* (Peruvian peppertree) [e]	not specified	-	4.3	red/beige
8	Lebensbaum [b]	Brazilian Peppertree [e]	Brazil	-	4.8	red
9	Sonnentor [b]	*Schinus terebinthifolius* [e]	Brazil	-	4.5	red
10[a]	Spice for life [c]	*Schinus molle* [e]	Peru	-	6.3	pink/violet
11		Brazilian peppertree [e]	Brazil	-	4.6	red
12	Mandavanilla [c]	-	Peru	-	6.1	pink/violet
13	Chalet Petit [c]	*Schinus molle* [e]	not specified	-	4.3	red
14	Local market, Brazil	-	Marketplace, Belo Horizonte, Brasil	-	3.8	red/beige
15	Local market, Argentina	-	Marketplace, Salta, Argentina	-	4.9	red/beige
16	Hand picked	-	Prado del Rey, province Cádiz, Andalusia, Spain	2014	5.1	pink
17	Hand picked	-	Arica, North of Chile	2015	6.2	pink/violet
18	Hand picked	-	Arequipa, South of Peru	2015	5.3	dark violet

[a] Reference samples. [b] Local shops. [c] Internet shops. [d] As reported by botanical garden. [e] As reported by supplier. [f] Infested with mold.

UHPLC−DAD−MS/MS analysis of phenolic compounds. Two different LC−MS systems were used for the analysis of phenolic compounds. The MS of the first system is more sensitive and therefore particularly suitable for the detection of minor compounds, whereas the UV detector of the second, more acid resistant system has a higher sensitivity and is therefore preferable for the purposes of UV/vis quantitation. For the identification of phenolic compounds, an Acquity UPLC system (Waters, Milford, MA) was used as described previously. Also, the methods were the same with the exception of the eluents and the gradient used for anthocyanin analysis. For enhanced separation, 5 and 3% formic acid in water and acetonitrile, respectively, were used. The gradient for anthocyanin analysis was slightly modified as follows: 0.0 min, 0% B; 0.2 min, 5% B; 12 min, 16.9% B; 16 min, 25% B; 17 min, 98% B; 19 min, 98% B; 20 min, 1% B; 22 min, 0% B. Furthermore, the gradient used for biflavonoid analysis was extended for more efficient detection of hinokiflavone: 0 min, 2% B; 24 min, 71.6% B; 24.3 min, 100% B; 25.3 min, 100% B; 26 min, 2% B; 27.5 min, 2% B.

Quantitation and determination of relative amounts of anthocyanins and biflavonoids were performed with an Acquity I-Class UHPLC system (Waters) equipped with a binary pump (BSM), an autosampler (SM-FL) cooled at 10 °C, a column oven (CM) set at 40 °C, a diode array detector (PDA eλ) scanning from 200 to 700 nm for the anthocyanins and from 200 to 480 nm for the biflavonoids, and an LTQ XL linear ion-trap mass spectrometer (MS) (Thermo Scientific, Waltham, MA) with an electrospray interface. The separation methods and columns of the second UHPLC were the same as those mentioned above.

The mass spectrometer was tuned using a solution of cyanidin 3-*O*-glucoside for the anthocyanins and I3′,II8-biapigenin for the biflavonoids, resulting in the following settings: source voltage, 4.0 and 5.0 kV (positive ion mode); sheath gas flow, 45 and 52 arbitrary units; auxiliary gas flow, 10 and 15 arbitrary units; sweep gas flow, 3 and 11 arbitrary units; capillary voltage, 18 and 10 V; capillary temperature, 300 °C; multipole 00 offset, −3.75 and −2.25 V; multipole 0 offset, −4.75 and −5.75 V; multipole 1 offset, −13 V and −11 V; front lens, −5.25 and −6.00, respectively. The MS scan was performed in full scan mode, and MS^2 and MS^3 spectra were acquired. Xcalibur version 2.2 (Thermo Electron Corp., San Jose, CA) was used to control the UHPLC−MS system and to evaluate the results. The biflavonoids and

anthocyanidins were quantified by a calibration curve using amentoflavone and malvidin 3-*O*-glucoside as external standards.

Statistical analysis. Statistical analysis was conducted with XLSTAT version 2014.4.06 based on the relative amounts of the main anthocyanins and biflavonoids in the *Schinus* exocarp extracts. The set of observations encompassed the 18 samples, whereas the relative amounts of the eight phenolics served as explanatory variables.

Principal component analysis (PCA). PCA was performed for further data evaluation and visualization as it recognizes patterns and can be used for classification. It shows which samples are similar to each other and which of the polyphenols are characteristic for each sample or a group of samples. The analysis was done without rotation and the filter for the factors was set to a minimum of 80%.

Cluster analysis (CA). CA was used to classify the 18 samples into groups depending on their similarity. An agglomerative hierarchic cluster analysis regarding the similarity was performed with the standard settings.

3 Results and discussion

Appearance of *Schinus* fruits. The size, color, and color intensity of the 18 *Schinus* fruit samples varied considerably. This especially concerned the samples labeled by the suppliers as *S. molle* that were 4.8−6.3 mm in diameter and a pale pink to dark violet color. Sample 3 showed mold infestation, and therefore, only healthy fruits and those that were less affected were considered. The differences among the samples labeled by the suppliers as *S. terebinthifolius* were less pronounced, ranging from 4.3 to 4.8 mm in diameter and a beige-red to bright red color. It needs to be mentioned that the fruits of sample 13 looked identical to those samples labeled as *S. terebinthifolius*, although sample 13 was claimed to be from *S. molle*. The identity of spice sample 7 was also conflicting because the species name *S. terebinthifolius* was linked with the name Peruvian peppertree, which is commonly used for *S. molle*. Although the appearance of the reference samples matched the botanical description of the fruits, the great variety of the samples confirms the aforementioned difficulties in assigning spices to their species exclusively by their fruit morphology.

Phenolic profiles of *S. terebinthifolius* and *S. molle*. Characterization and identification of the phenolics were based on the reference extracts obtained from samples 6 (*S. terebinthifolius*) and 10 (*S. molle*) as described previously. The chemical structures of the anthocyanins and biflavonoids characterized in the following paragraphs can be seen in Figure 1.

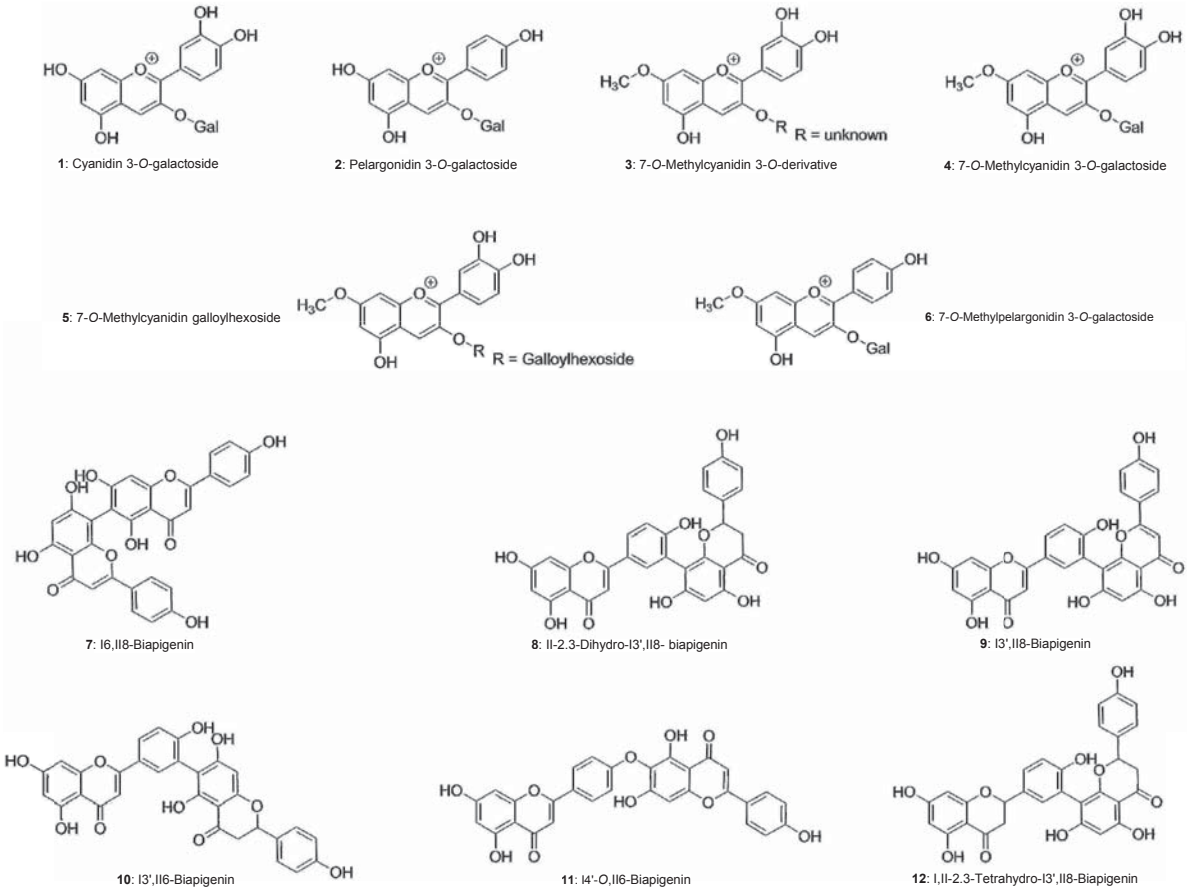

Figure 1: Chemical structures of the found anthocyanins (1–5) and biflavonoids (7–12).

Characterization of anthocyanins. In the extract of *S. molle* exocarp, cyanidin 3-*O*-galactoside, pelargonidin 3-*O*-galactoside, 7-*O*-methylcyanidin 3-*O*-galactoside, and 7-*O*-methylpelargonidin 3-*O*-galactoside were found, i.e., the same anthocyanins as in the exocarp of *S. terebinthifolius* with coinciding retention times, UV spectra, and mass spectra .[10] Additionally, one major (5) and one minor (3) peak visible at 500 nm were characterized as anthocyanins (Figure 2).

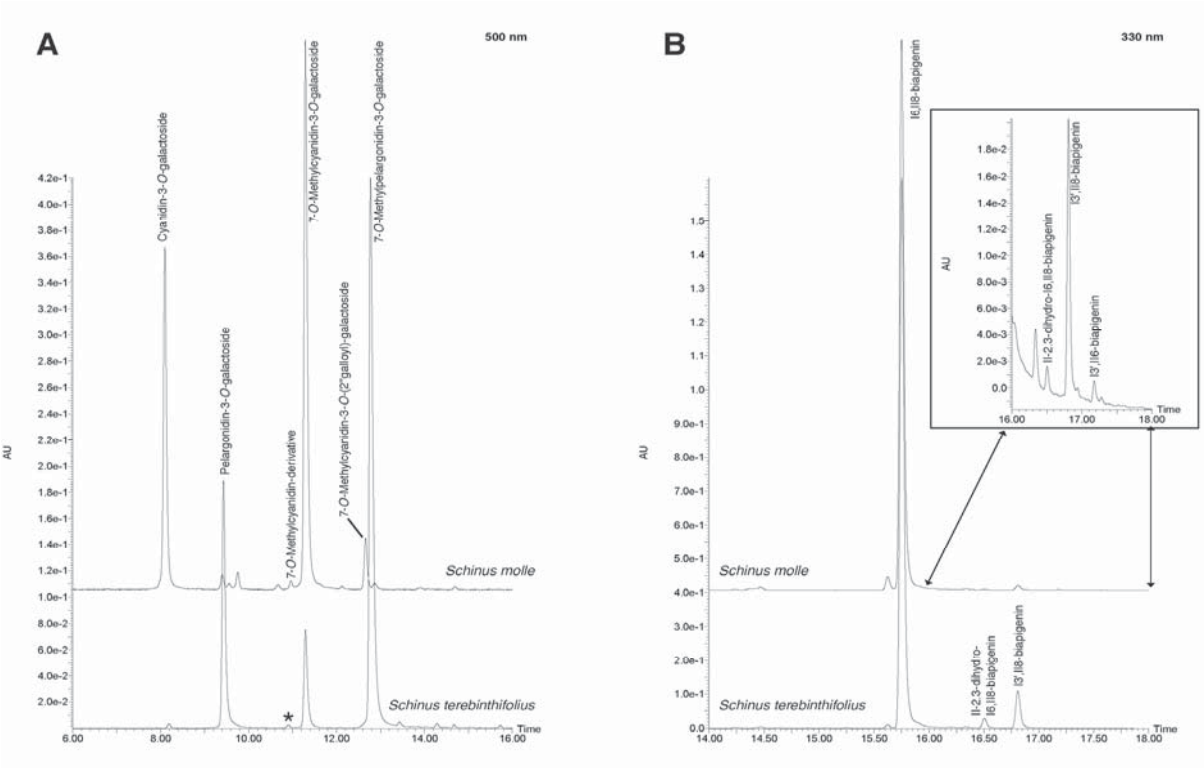

Figure 2: (A) Chromatograms at 500 nm of *S. terebinthifolius* and *S. molle* show the anthocyanins. The peak marked with an asterisk in *S. terebinthifolius* has a retention time shorter than that of the 7-*O*-methylcyanidin derivative in *S. molle*. (B) Chromatograms at 330 nm of *S. terebinthifolius* and *S. molle* show the exocarp biflavonoids. The minor peaks in *S. molle* can be seen in the inset with the enlarged later eluting part of the chromatogram.

*Compound **3**.* **3** eluted shortly before 7-*O*-methylcyanidin 3-*O*-galactoside. The UV/vis spectrum showed maxima at 281 and 517 nm. At a lower collision energy (20 V), the $[M]^+$ ion at *m/z* 595 yielded a fragment at *m/z* 301; i.e., it showed a loss of 294 Da, corresponding to a hexoside pentoside moiety. This possible structure as a diglycoside would usually lead to an elution that was earlier than that of the monoglycosides. Therefore, the identity of the non-anthocyanidin moiety of this molecule is unclear. At a higher collision energy (60 V), the fragments of the aglycone were visible, showing the same spectrum as reported for 7-*O*-methylcyanidin.[10] Thus, anthocyanin **3** in *S. molle* exocarp could be assigned to 7-*O*-methylcyanidin attached to an unknown moiety. Various 7-*O*-methylcyanidin derivatives had been described in different plants,[10,17-21] but none of them matches the observed $[M]^+$ ion at *m/z* 595.

*Compound **5**.* **5** co-eluted with 7-*O*-methylpelargonidin 3-*O*-galactoside at lower (1%) formic acid concentrations in the eluents. The peaks were baseline separated using higher (5%) formic acid concentrations in the eluents; i.e., **5** eluted earlier than 7-*O*-

methylpelargonidin 3-*O*-galactoside. The UV/vis spectrum showed maxima at 281 and 517 nm. At a lower collision energy (20 V), the $[M]^+$ ion at *m/z* 615 yielded a fragment at *m/z* 301, corresponding to a loss of 314 Da. These data can be explained by the loss of a galloylhexoside moiety. This is in agreement with 7-*O*-methylcyanidin 3-*O*-(2''galloyl)-galactoside described in *R. typhina* and *R. coriaria*.[18-19] At a higher collision energy (60 V), the same fragment ion spectrum that was seen for 7-*O*-methylcyanidin was obtained.[10] The analysis of *R. typhina and R. coriaria* extracts showed a major peak with a retention time and spectroscopic behavior in both samples that matched those of peak **5**. Therefore, peak **5** was identified as 7-*O*-methylcyanidin 3-*O*-(2''galloyl)-galactoside. Additionally, a peak with the same spectrometric properties eluted shortly before **5** and occurred in both species. Presumably, this is an isomer of 7-*O*-methylcyanidin 3-*O*-(2''-galloyl)-galactoside with a different substituent position of the galloyl moiety.

To the best of our knowledge, there is only one publication about anthocyanins in *S. molle,*[12] which describes cyanidin 3-*O*-galactoside, cyanidin 3-*O*-rutinoside, and peonidin 3-*O*-glucoside. In our study, only cyanidin 3-*O*-galactoside was confirmed. 7-*O*-Methylpelargonidin 3-*O*-galactoside as described here might have been interpreted by Rahman *et al*.[12] as peonidin 3-*O*-glucoside because these two anthocyanidins show a similar retention behavior. The description of cyanidin 3-*O*-rutinoside prompted us to search for an ion at *m/z* 595, and we found a 7-*O*-methylcyanidin derivative. Additionally, we found 7-*O*-methylcyanidin 3-*O*-galactoside as a major anthocyanin as well as pelargonidin 3-*O*-galactoside and 7-*O*-methylcyanidin 3-*O*-(2''-galloyl)-galactoside as minor anthocyanins in *S. molle*. The latter four compounds are described in *S. molle* for the first time.

Characterization of biflavonoids. In the extract of *S. molle* exocarp, one major and four minor peaks were visible in the later eluting part of the chromatogram recorded at 330 nm (Figure 2). The retention times, UV spectra, and mass spectra of the major peak matched the published data for I6,II8-biapigenin. With regard to the minor peaks, two of them matched the published data of II-2.3-dihydro-I3',II6-biapigenin, and I3',II8-biapigenin in the exocarp of *S. terebinthifolius.*[10] The two other peaks were labeled **10** and **11**. In the drupe extract of *S. molle*, only one major peak, named **12**, was suspected to be a biflavonoid. The retention time, UV spectrum, and mass spectrum matched the published data of I3',II8-binaringenin.[11] The UV and

mass spectrometric data of **10** and **11** are listed in Table 2. For comparison, the spectrometric data of **12** are also reported.

Table 2: UV Maxima and Positive Product Ions (*m/z*, relative abundances in parentheses; collision energy of 40 V) with Hypothetical Structures of Biflavones 10–12.

Compound	**10**	**11**	**12**
Retention time (min)	17.0	19.7	15.7
Biological source	*S. molle* exocarp	*S. terebinthifolius* and *S. molle* exocarp	*S. terebinthifolius* and *S. molle* drupe
Assigned identity	I3',II6-biapigenin	I4'-O,II6-biapigenin	I,II-2.3-tetrahydro-I3',II8-biapigenin
Alternative name	Robustaflavone	Hinokiflavone	I3',II8-binaringenin
UV_{max} (nm)	268, 341	269, 337	290, 330sh
$[M + H]^+$	539 (79)	539 (100)	543 (1)
$[M + H - H_2O]^+$	521 (100)		525 (3)
$^{1,3}IIA^+$	421 (13)	421 (10)	
$^{0,2}IIA^+$	419 (41)	419 (2)	
$^{1,4}IB^+$	413 (12)		
$^{1,3}IIA^+ - H_2O$	403 (67)	403 (2)	405 (1)
$^{0,2}IIA^+ - H_2O$	401 (14)		
$^{1,3}IB^+$	387 (91)	387 (16)	391 (1)
$^{1,3}IIA^+ - C_2H_2O$			381 (1)
$^{0,4}IIA^+$	377 (42)	377 (4)	
$^{1,3}IIA^+ - H_2O - CO$	375 (32)	375 (3)	
$^{1,3}IIA^+ - H_2O - 2CO$	347 (8)	347 (3)	349 (1)
$^{0,4}IIA^+ - C_2H_2O$	335 (5)	335 (3)	
$^{1,3}IIA^+ - C_2H_2O - C_3O_2$	311 (6)		313 (1)
$^{0,4}IIA^+ - C_2H_2O - CO$	307 (20)		
$^{1,4}IB^+ - {}^{1,3}IIB$			297 (14)
$[\text{Flavone II} + OH]^+\cdot$		286 (22)	
$[\text{Flavone II} + O]^+$		285 (10)	
$^{1,3}IIA^+ - C_2H_2O - C_3O_2 - CO$	283 (4)	283 (5)	
$[\text{Flavone I or II}]^+$	270 (21)	270 (26)	
$^{1,3}IB^+ - {}^{1,3}IIB$ (or $^{0,4}IIA^+ - {}^{0,4}IA$)	269 (19)	269 (5)	271 (64)
$^{0,4}IA^+ - {}^{0,2}IIB^+ + H^+$	257 (10)		
$[\text{Flavone II} + O - CO]^+$		257 (27)	
$[\text{Flavone I} - OH]^+\cdot$		254 (12)	
$[\text{Flavone I or II} + H - CO]^+$			245 (3)[a]
$[\text{Flavone I or II} - CO]^+\cdot$		242 (23)	
$^{0,4}IIB^+$	163 (10)	163 (6)	
$^{1,3}IA^+$	153 (35)	153 (8)	153 (100)[a]
$^{0,4}IIB^+ - H_2O$	145 (13)		147 (12)
$^{0,2}IIB^+$	121 (72)	121 (3)	121 (1)
$^{1,3}IIB^+$		119 (3)	119 (1)
$[\text{Flavone I or II} + H^+ - C_2H_2O - C_2H_2O - CO]^+$			159 (47)
unknown	41 (19), 177 (5)	445 (5), 262 (6), 241 (8), 168 (7)	255 (8), 213 (3), 179 (8)[a], 175 (4), 161 (4), 123 (2), 111 (3)

[a]Ions *m/z* 153, 179 and 245 are known naringenin fragments [26, 27] Nomenclature of the fragment ions according to Zhang et al.[22]

*Compound **10**.* Compound **10** eluting at 17.0 min showed a UV spectrum similar to that of the I3',II8-biapigenin standard, with the two maxima at 268 and 341 nm (I3',II8-biapigenin, 269 and 335 nm). In positive ionization mode, an ion at *m/z* 539 was found as the presumable $[M + H]^+$ ion. The product ion scan of **10** is similar to that of I3',II8-biapigenin with marked differences. Ions at *m/z* 539, 521, 387, and 153 have higher abundances. This fragmentation behavior can be explained by assuming the structure I3',II6-biapigenin. According to Zhang *et al.*[22], the high abundance of the fragment ions at *m/z* 387 is typical for I3',II6-linked biapigenins and the fragment ion at *m/z* 413 is characteristic of such biapigenins. The loss of water leading to a *m/z* 521 fragment is preferred in I3',II6-linked rather than in I3',II8- or I6,II8-linked biflavones because of the proximity of two OH functions of unit II to one OH function of unit I.[22]

The UV spectrum and MS^2 spectra were in agreement with published data of I3',II6-biapigenin,[22] which has been described in *Selaginella tamarisca*. I3',II6-Biapigenin has also been described in Anacardiaceae species such as *S. terebinthifolius*[23] and *Rhus succedana,*[24] but not in *S. molle* so far. Therefore, peak **10** was identified as I3',II6-biapigenin (robustaflavone). The occurrence of I3',II6-biapigenin in *S. terebinthifolius* as reported by Kassem *et al.* [23] could not be confirmed.

*Compound **11**.* Peak **11** eluting at 19.7 min with $[M + H]^+$ at *m/z* 539 showed UV maxima at 269 and 337 nm. The retention time, UV spectrum, and MS^2 fragments in positive ionization mode of **11** and the hinokiflavone standard were coincident and in agreement with published data of hinokiflavone from *Se. tamariscina.*[22] Hinokiflavone has further been found in *R. succedana,*[25] but to the best of our knowledge not in *Schinus* species. Peak **11** was present in the exocarp of both *S. terebinthifolius* and *S. molle* and was identified as hinokiflavone.

Characterization of gallic acid derivatives, **including gallotannins.** In the exocarp of *S. molle*, the galloyl glucoses and galloyl shikimic acids were the same as those found in the exocarp of *S. terebinthifolius*, which is described in detail in a previous work.[10] In the drupes of both *Schinus* species, the same galloyl glucoses occurred as in the exocarp but galloyl shikimic acids were lacking.

Table 3: Major and Minor Phenolics in 18 *Schinus* Samples, Detected via LC–MS[a]

	Phenolic compound	References		*S. terebinthifolius* Group 1								*S. molle* Group 2									
		Ref 1 *S. terebinthifolius*	Ref 2 *S. molle*	6	7	8	9	11	13	14	15	1	2	3	4	5	10	12	16	17	18
1	Cyanidin 3-*O*-galactoside	✓	✓	✓	✓*	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
2	Pelargonidin 3-*O*-galactoside	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓*	✓*	✓*	✓*	✓*	✓	✓	✓	✓	✓
3	7-*O*-Methylcyanidin 3-*O*-derivative	n.d.	✓	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	✓*	✓*	✓*	✓	✓	✓	✓	✓	✓*	✓
4	7-*O*-Methylcyanidin 3-*O*-galactoside	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
5	7-*O*-Methylcyanidin galloylhexoside	✓	✓	✓*	✓*	✓*	✓*	✓*	✓*	✓*	✓*	✓*	✓*	✓	✓*	✓*	✓	✓	✓*	✓	✓
6	7-*O*-Methylpelargonidin 3-*O*-galactoside	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓*	✓*	✓*	✓	✓*	✓	✓	✓	✓	✓
7	I6,II8-Biapigenin (agathisflavone)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
8	II-2.3-Dihydro-I3',II8- biapigenin	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
9	I3',II8-Biapigenin (amentoflavone)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
10	I3',II6-Biapigenin (robustaflavone)	n.d.	✓	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	✓	n.d.	n.d.	✓	✓
11	I4'-*O*,II6-Biapigenin (hinokiflavone)	✓	✓	n.d.	✓	n.d.	n.d.	✓	✓	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	✓	n.d.
12	I,II-2.3-Tetrahydro-I3',II8-Biapigenin	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓

[a]Legend: ✓ detected by UV and MS; n.d. not detected; ✓* UV only detectable by MS and not by UV.

Comparison of the phenolic composition. The phenolic composition of the two *Schinus* reference samples differed markedly in the exocarp, while in the drupes, the same polyphenols were detected in both samples. The most evident differences were seen in the anthocyanin profile. The two major peaks at 500 nm in *S. molle* were the two cyanidin derivatives (cyanidin 3-*O*-galactoside and 7-*O*-methylcyanidin 3-*O*-galactoside), whereas in *S. terebinthifolius*, the two pelargonidin derivatives (pelargonidin 3-*O*-galactoside and 7-*O*-methylpelargonidin 3-*O*-galactoside) prevailed (Figure 2). The additional anthocyanin identified as a 7-*O*-methylcyanidin derivative was detected in the exocarp of *S. molle* only. Because the retention times of this compound and of 7-*O*-methylcyanidin 3-*O*-galactoside were similar under the chosen conditions, using the 7-*O*-methylcyanidin derivative as an indicator for *S. molle* is recommended only when MS detection is applied. Furthermore, larger amounts of samples are necessary, because it is a minor compound.

As shown in Figure 2, biflavones require closer examination. The predominant peak in the exocarp of both *Schinus* species was I6,II8-biapigenin. I3',II6-Biapigenin was only present in only *S. molle* and is a possible indicator for this species, although the fact that it is a minor compound has to be considered. II-2.3-Dihydro-I3',II6-biapigenin and I3',II8-biapigenin, both occurring in either *Schinus* species, are also minor constituents relative to I6,II8-biapigenin in *S. molle*.

Differentiation of *S. terebinthifolius* and *S. molle*. Via comparison of the qualitative profile of anthocyanins and biflavonoids in the 18 samples with the two reference samples, two groups representing the two species can be distinguished (Table 3). According to our results, samples 6−9, 11, and 13−15 should be *S. terebinthifolius* fruits (group 1), whereas the rest of the samples belonged to *S. molle* (group 2). This classification reflects the claimed species in Table 1. The only exception is sample 13, which was labeled as *S. molle* but was allocated to *S. terebinthifolius* on the basis of the analytical results, which corresponded to the appearance of this sample. Sample 7 with the conflicting declaration was also allocated to group 1 as it showed the characteristic profile of phenolics in *S. terebinthifolius*. Among the additional compounds found in the reference sample of *S. molle* (**3** and **10**), at least one but not consistently both compounds could be detected in the *S. molle* samples, presumably because they mostly occurred at very low concentrations. Therefore, for a reliable differentiation based on the additional

phenolics, a larger sample amount and time-consuming sample preparation would be necessary. A second problem was that these two indicator compounds tend to co-elute with other anthocyanins or biflavonoids, which may result in erroneous identification of samples. Thus, the two polyphenols found exclusively in *S. molle* seem to be inadequate for authentication purposes.

The quantitation showed that the absolute amounts of anthocyanins and biflavonoids are also not adequate for the differentiation of the two *Schinus* species because the values varied greatly within but not between the groups. Quantitation of the anthocyanins and the biflavonoids revealed that the samples of the *S. terebinthifolius* group contained between 0.009 and 0.018 mg/mL anthocyanins in the extracts. The *S. molle* group had a broader range and contained 0.003−0.073 mg/mL anthocyanins. With 0.062−0.108 mg/mL for group 1 and 0.027−0.167 mg/mL for group 2, the biflavonoid contents of the two species also overlapped. However, for authentication, the relative amounts of the main phenolics (defined as polyphenols obtained in a relative amount of ≥10% in at least one of the samples) were most promising (Table 4).

Considering the anthocyanins, the samples of group 1 showed a high percentage of pelargonidin galactoside (≥23.5%) and methylpelargonidin galactoside (≥59.6%) and small relative amounts of cyanidin derivatives (≤0.7 and ≤14.2%), whereas methylcyanidin galloylhexoside was lacking in all samples (Table 4). In contrast, the samples in group 2 contained larger amounts of cyanidin galactoside (≥6.2%) and methylcyanidin galactoside (≥ 62.3%) and smaller amounts of pelargonidin derivatives (≤1.3 and ≤16.9%). In a considerable fraction of these samples, methylcyanidin galloylhexoside, which seemed to occur in only *S. molle*, was detected in quantifiable amounts. Furthermore, two subgroups (a and b) within the *S. molle* group could be assumed because the peak ratio shifts from cyanidin galactoside to methylcyanidin galactoside for samples 1−5 and 16. Allocation of samples 3 and 18 to the subgroup was not as obvious as for the other fruits.

With regard to the biflavonoids, the differences between the two groups were not that obvious but nevertheless present. Compared to the amount of agathisflavone (**7**), which was predominant in both species, the *S. terebinthifolius* samples contained relevant amounts of **8** and **9**, whereas the percentage of these compounds was much lower in the *S. molle* samples (Table 4).

Table 4: Relative Amounts of the Main Anthocyanins and Biflavonoids Detected at 500 and 330 nm via HPLC−UV/Vis in 18 *Schinus* Exocarp Samples

	Phenolic compound	*S. terebinthifolius* Group 1								*S. molle* Group 2a				*S. molle* Group 2b					
		6	7	8	9	11	13	14	15	10	12	17	18	1	2	3	4	5	16
1	Cyanidin 3-*O*-galactoside	0.4	0.3	0.6	0.7	0.6	0.6	0.7	0.7	29.7	22.7	22.3	18.8	11.8	18.4	6.2	9.4	11.0	6.9
2	Pelargonidin 3-*O*-galactoside	24.2	29.6	27.7	27.6	23.5	28.7	27.3	24.4	1.3	0.6	1.3	0.9	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	0.3
4	7-*O*-Methylcyanidin 3-*O*-galactoside	9.6	6.6	11.2	12.2	11.5	11.0	12.1	14.2	63.4	65.5	67.2	62.3	88.2	81.7	80.0	90.6	89.0	91.6
5	7-*O*-Methylcyanidin galloylhexoside	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	4.8	10.6	8.1	16.9	n.d.[a]	n.d.[a]	13.8	n.d.[a]	n.d.[a]	n.d.[a]
6	7-*O*-Methylpelargonidin 3-*O*-galactoside	65.8	63.5	60.5	59.6	64.4	59.7	60.0	60.8	0.8	0.7	1.0	1.1	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	1.3
7	I6,II8-Biapigenin (agathisflavone)	85.0	70.2	90.0	74.7	80.7	78.9	84.2	88.6	96.0	96.3	93.2	96.7	99.5	98.1	94.9	96.0	95.5	95.8
8	II-2.3-Dihydro-I3',II8- Biapigenin	4.4	15.2	3.5	11.0	6.6	6.8	5.5	3.9	0.3	0.4	0.4	0.2	n.d.[a]	0.1	0.2	0.3	0.2	0.2
9	I3',II8-Biapigenin (amentoflavone)	10.6	14.6	6.5	14.4	12.7	14.2	10.2	7.5	3.8	3.4	6.4	3.1	0.5	1.8	4.9	3.6	4.3	4.0

[a]Not detected.

Thus, on the basis of the relative amounts of anthocyanins and biflavonoids, the same classification as in Table 3 was achieved, but the differences were even more evident and more reliable because they did not depend on minor compounds that are difficult to separate and identify. These results indicated that anthocyanins and biflavonoids, especially their relative amounts, were suitable indicators of the phytochemical differentiation of *S*. *terebinthifolius* and *S*. *molle*.

Statistical evaluation. Principal component analysis was performed to verify the possibility of differentiating the two *Schinus* species on the basis of the relative amounts of anthocyanins and biflavonoids. This approach allows the reduction of the experimental data consisting of numerous variables (anthocyanins and biflavonoids) to a few factors (F) also called principal components. As this is based on the variance of the variables, as much information as possible is conserved. Thereby, the phenolic patterns of each species and the importance of each phenolic compound for authentication should become more obvious.

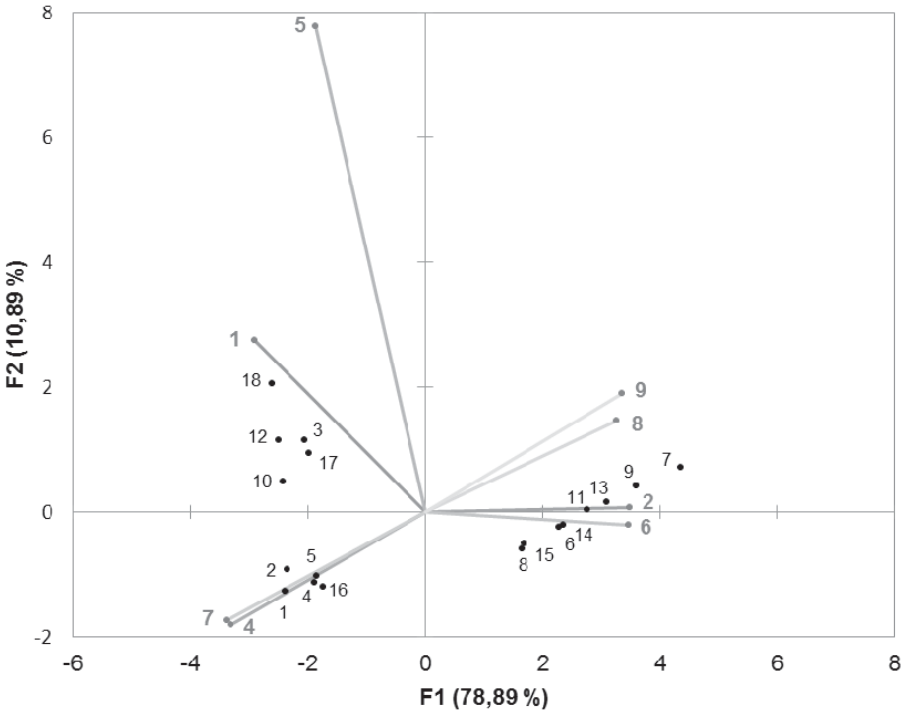

Figure 3: Unrotated principal component loadings (similarity of samples), for loading 1 vs loading 2.

The statistical model shown in Figure 3 describes 89.8% (F1, 78.9%; F2, 10.9%) of the total variance of the polyphenol profile. According to this PCA, the *Schinus*

samples can clearly be distinguished into three groups (A−C). The biplot reveals that group A is positively correlated with F1 and slightly positively or negatively correlated with F2. Group B is positively correlated with F2 and negatively correlated with F1, whereas group C is negatively correlated with both factors. Thus, group A representing *S. terebinthifolius* is defined by a large amount of pelargonidin derivatives and larger amounts of biflavonoids **8** and **9**. The two other groups assigned to *S. molle* have more similarities but are still clearly separated. These two groups are characterized by a large amount of cyanidin derivatives and mainly contain the biflavonoid **7**. Whereas group C displays the largest amounts of anthocyanin **4**, the separation of group B is explained by larger amounts of **1** and **5**. This grouping is in accordance with the species declaration shown in Table 1 except for the already mentioned samples 7 and 13, and sample 3. The reason for this presumably incorrect allocation of the latter might be the mold infestations, which may lead to changes in the phenolic profile. Sample 18 also stands out of group B, which may be explained by the deviating fruit appearance and possible allocation to a different variety of *S. molle.*

These results clearly show that a differentiation of *S. terebinthifolius* from *S. molle* based on the relative amounts of the main compounds is possible. The performance of a PCA exclusively with the anthocyanins exhibited the same grouping. Moreover, the samples in each group move together even closer. We can conclude that for authentication purposes the determination of the relative amounts of anthocyanins is sufficient.

Whereas PCA reduces a set of attributes (variables) to factors by the attributes with the largest variations, CA is a useful tool for finding groups in a data set based on the similarities of the attribute values. Therefore, CA was used to group the 18 samples based on their relative amounts of anthocyanins and biflavonoids and to reveal existing patterns. The dendrogram (Figure 4) of the clustering by Ward's method reveals three groups closely related to those of the PCA. The principal difference is that sample 3 is assigned to group C instead of group B. However, this and all other classifications of the samples are in accordance with the species declaration except for samples 7 and 13, as discussed above. No patterns regarding the sample origin were observed. This is another point supporting the samples being grouped according to their species affiliation rather than other factors. At the same

time, the analysis revealed that the spices in local stores mainly come from *S. terebinthifolius*. Although mentioned differently in the literature, *S. molle* is comparatively rarely sold as a spice and mainly as a specialty named “Violetta”.

A closer examination of groups B and C and their appearance and species declaration leads to the assumption that the two groups may be attributed to different varieties of *S. molle*. This is supported by the fact that samples with similar sizes and colors as well as samples declared to be from the same variety are grouped together. Nevertheless, for a definitive differentiation of varieties, data sets of further *Schinus* fruits would be helpful.

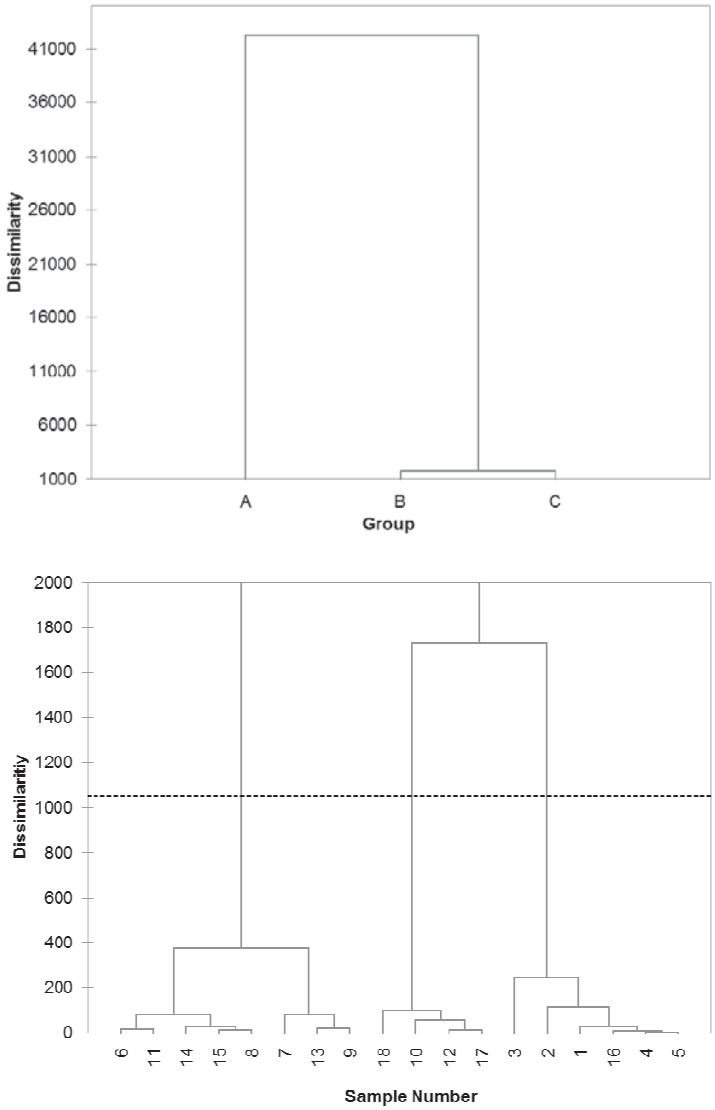

Figure 4: Hierarchic cluster analysis of *Schinus* samples.

CA based on the anthocyanins showed only the same grouping. Thus, differentiation based on CA of the anthocyanins is sufficient for authenticity analysis. Although clustering uses less information than PCA does, the results are in even better accordance with the appearance of the fruits and the species declaration. CA is therefore the method of choice for a quick and easy but yet statistically supported identification of *Schinus* species.

Acknowledgments

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Chapter 4

Pressurized liquid extraction of anthocyanins and biflavonoids from *Schinus terebinthifolius* Raddi: A multivariate optimization

Response surface methodology was employed to investigate the effects of pressurized liquid extraction (PLE) parameters on the recovery of phenolic compounds (anthocyanins, biflavonoids) from Brazilian pepper (*Schinus terebinthifolius* Raddi) fruits. The effects of temperature, static time, and ethanol as well as acid concentration on the polyphenol yield were described well by quadratic models ($p < 0.0001$). A significant influence of the ethanol concentration ($p < 0.0001$) and several interactions ($p < 0.05$) were identified. Identification of the biflavonoid I3′,II8-binaringenin in drupes of *S. terebinthifolius* was achieved by UHPLC-MS^2. Interestingly, at high extraction temperatures (>75 °C), an artifact occurred and was tentatively identified as a diastereomer of I3′,II8-binaringenin. Multivariate optimization led to high yields of phenolic compounds from the exocarp/drupes at 100/75 °C, 10/10 min, 54.5/54.2% ethanol, and 5/0.03% acetic acid. This study demonstrates that PLE is well suited for the extraction of phenolic compounds from *S. terebinthifolius* and can efficiently be optimized by response surface methodology.

Keywords: Anthocyanins, Biflavonoids, Polyphenol extraction, Pressurized liquid extraction, Response surface methodology, *Schinus terebinthifolius*, UHPLC-DAD-MS/MS

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1 Introduction

The Anacardiaceae family encompasses more than 70 genera and over 600 species, including food crops and spices, such as mango (*Mangifera indica* L.), pistachio (*Pistacia vera* L.*),* cashew nut (*Anacardium occidentale* L.), sumac (*Rhus coriaria* L.), and Brazilian pepper (*Schinus terebinthifolius* Raddi). Previous investigations have demonstrated the diversity of phenolic compounds present in this plant family as well as their chemotaxonomic implications (Berardini et al., 2005; Feuereisen et al., 2014; Kirby, Wu, Tsao, & McCallum, 2013; Kosar, Bozan, Temelli, & Baser, 2007; Schulze-Kaysers, Feuereisen, & Schieber, 2015). For example, mango was shown to have an interesting phytochemical profile with a broad spectrum of phenolic compounds, some of which possess antibacterial or anti-inflammatory activity (Berardini, Carle, & Schieber, 2004; Engels, Schieber, & Gänzle, 2011; Knödler et al., 2008).

S. terebinthifolius fruits are commonly used as a spice, whereas the bark, leaves and roots are traditional constituents in folk medicine, due to their anti-inflammatory, antipyretic, and analgesic properties (Carvalho, Melo, Aragão, Raffin, & Moura, 2013). Furthermore, *S. terebinthifolius* presumably contains protective phytochemicals, which support its aggressive expansion. Such invasive growth can for example be seen in Florida with serious effects on native plants. Phenolic compounds are assumed to contribute to the allelopathic effects including the antimicrobial activity (FLEPPC, 2011; Johann, Pizzolatti, Donnici, & de Resende, 2007; Morgan & Overholt, 2005; Rhouma et al., 2009). A recent review summarizes the chemical composition of different parts of *S. terebinthifolius* and its main biological and toxicological properties (Carvalho et al., 2013). To expand the compositional database, we previously characterized the phenolic composition of *S. terebinthifolius* exocarp and detected anthocyanins, biflavonoids and gallotannins. Among the anthocyanins, pelargonidin 3-*O*-galactoside, 7-*O*-methylcyanidin 3-*O*-galactoside, and the novel compound 7-*O*-methylpelargonidin 3-*O*-*β*-D-galactopyranoside were identified as main constituents. Furthermore, the biflavonoids I6,II8-biapigenin (agathisflavone), I3',II8-biapigenin (amentoflavone), and II-2.3-dihydro-I3',II6-biapigenin were found to be characteristic phenolics in Brazilian pepper fruit exocarp (Feuereisen et al., 2014). For further investigations of the biological activities of the identified compounds, their recovery in adequate

amounts is indispensable. As the pressurized liquid extraction (PLE) is suitable for thermolabile compounds (Mustafa & Turner, 2011), it is considered a method of choice for the extraction of Brazilian pepper exocarp since it contains anthocyanins. Furthermore, the use of PLE leads to reduced extraction time and amounts of solvent (Mustafa & Turner, 2011; Papagiannopoulos et al., 2002), which is of particular importance for high sample throughput for bioactivity assays. Therefore, the aim of this work was to develop a process for the recovery of phenolic compounds from Brazilian pepper fruits by PLE. Since it is known that pressurized liquid extraction is affected by several factors, such as temperature, extraction time and solvent composition, depending on the target extractives (Mustafa & Turner, 2011), a comprehensive characterization of the process is crucial. For this purpose, response surface methodology was applied, which allows the identification of parameters significantly influencing the extraction as well as of interactions between these parameters in one step. The resulting empirical models can be used for numerical optimization leading to maximized polyphenol yields. For optimization, the Desirability function method after Derringer and Suich (1980) was applied. The Desirability function approach is one of the most popular and suitable methods for multiple response problems, because it enables the transformation of all response variables into an overall desirability function. Thus, a basis for future applications such as bioactivity assays and isolation of selected phenolics is provided.

2 Material and methods

2.1. Chemicals and plant material

Solvents and reagents were purchased from ChemSolute (Renningen, Germany), Merck (Darmstadt, Germany), Sigma-Aldrich (Steinheim, Germany) or VWR (Darmstadt, Germany) and were of analytical grade. Technical grade ethanol was obtained from Hofmann (Düsseldorf, Germany). Amentoflavone (I3′,II8-biapigenin) was purchased from Extrasynthèse (Lyon, France); malvidin 3-*O*-glucoside chloride was from Phytoplan (Heidelberg, Germany); naringenin and caffeic acid were supplied by Sigma-Aldrich. Deionized water was used throughout all tests.

Dried fruits from *S. terebinthifolius* were obtained from Fuchs (Dissen, Germany). Since pulverization and extraction are more efficient when carried out separately, the exocarp was removed from the remaining parts of the fruit. Thus, the exocarp was

manually separated from the residual pericarp and seed (in the following referred to as drupe) and ground using a model S100 ball mill (Retsch, Haan, Germany). The samples were stored in the dark at room temperature until further use. The drupes contain high amounts of oil and therefore jamming has to be avoided. For this purpose, the drupes were frozen before grinding to grit using a mill (Krups, Offenbach, Germany) and frozen again until further use.

2.2. Pressurized liquid extraction

PLE was performed with a Dionex ASE 350 (Thermo Scientific, Idstein, Germany) system. The powdered exocarp (0.2 g) or drupe grit (0.5 g) were mixed with diatomaceous earth (Thermo Scientific) and filled between two layers of diatomaceous earth (approx. 3.2 g per 0.2 g exocarp and 2.8 g per 0.5 g drupe) in 10 mL Dionex (ASE 350) stainless steel cells, which were fitted with cellulose filters (Macherey-Nagel, Düren, Germany).

The automated extraction cycle included the following steps: the extraction cell containing the sample was filled with extraction solvent, pressurized (1500 psi) and heated until the target temperature was reached. This was followed by the static extraction. Extraction time, temperature, solvent composition, and acid concentration were chosen based on the experimental design given in Table 1. Then, the cell was rinsed with fresh extraction solvent (150% of the cell volume) and purged with a flow of nitrogen (100 psi, 60 s).

Preliminary tests had shown that for an effective extraction, defattening of the drupe grit was necessary. Without delipidation, the phenolic yield was poor, probably because diffusion of the solvent is hindered. Thus, in a first step, the samples were defatted by ASE (Accelerated Solvent Extraction) with petrol ether at 60 °C with a static period of 6 min. In a second step, the extraction of phenolic compounds was then performed through static extraction cycles according to the experimental conditions obtained from the experimental design (Table 1). All extracts were made up to a defined volume of 50 mL and stored at -80 °C until further use.

Table 1: Factor combinations obtained from face-centered central composite design.

Run*	Temperature/°C	Static Time/Min	Ethanol/%	Acetic Acid/%
1	80	5	50	2.5
2	40	10	0	5.0
3	120	0	0	5.0
4	80	5	0	2.5
5	40	10	100	5.0
6	120	0	100	0.0
7	120	0	100	5.0
8	120	0	0	5.0
9	120	10	0	0.0
10	120	10	100	0.0
11	120	5	50	2.5
12	80	5	50	2.5
13	40	0	0	0.0
14	80	10	50	2.5
15	80	5	50	5.0
16	80	5	50	0.0
17	40	0	100	5.0
18	80	0	50	2.5
19	40	5	50	2.5
20	120	10	100	5.0
21	40	0	100	0.0
22	40	10	0	0.0
23	80	5	50	2.5
24	40	10	100	0.0
25	80	5	50	2.5
26	40	0	0	5.0
27	120	0	0	0.0
28	80	5	50	2.5
29	80	5	50	2.5
30	80	5	100	2.5

* in randomized order.

2.3. Folin-Ciocalteu assay

The total phenolic content (TPC) was determined using the Folin-Ciocalteu assay according to Cliffe, Fawer, Maier, Takata, and Ritter (1994). Caffeic acid was dissolved in 50% ethanol and used as a reference. Aliquots of 0.01 mL of extract were mixed with 0.84 mL of water. Folin reagent (0.05 mL) was added and the mixture incubated for 3 min. Finally, 0.1 mL of saturated Na_2CO_3 solution was added under stirring. After 60 min, the absorption was recorded at 720 nm using a Genesys 6 spectrophotometer (Thermo Scientific, Langenselbold, Germany). The results were

expressed as caffeic acid equivalents (CAE) and are further referred to as total phenolic content (TPC). The dry weight of the extract was determined with a moisture analyzer MA 100 (Sartorius, Göttingen, Germany).

2.4. HPLC and UHPLC-DAD-MS/MS analysis

The HPLC system was equipped with a quaternary pump, a degasser, an autosampler, and a column oven (e2965 Separation module, Waters, Milford, CT, USA). UV/Vis spectra were recorded from 210 to 800 nm using a Waters 996 photodiode array detector. A NUCLEODUR 100-5 C18ec analytical column (150 mm x 2 mm i.d.; 3 µm, Macherey-Nagel) with a SecurityGuard Guard Cartridge (AQ C18, 4 mm x 2 mm, Phenomenex, Torrance, CA, USA) was used for the separation of the phenolic compounds. Elution was conducted with 1% (v/v) acetic acid in water (solvent A) and 1% (v/v) acetic acid in acetonitrile (solvent B) at a constant flow rate of 0.3 mL/min. The gradient program was as follows: 0–40% B in 80 min, 40–100% B in 10 min, 100–0% B in 10 min, and 0% B for 10 min for equilibration (based on a method reported by Papagiannopoulos, Wollseifen, Mellenthin, Haber, & Galensa (2004) with modifications). The injection volume was 20 µL for the exocarp samples and 10 µL for the drupe samples. The extracts were filtered through 0.2 µm Chromafil RC-20/15 MS filters (Macherey-Nagel, Düren, Germany) prior to injection. According to their respective absorption maxima the peak integration was done at a wavelength of 500 nm for peaks 1–3, 330 nm for peaks 4–6, and 290 nm for peak 7. For quantification, a calibration was compiled with malvidin for the anthocyanins and with amentoflavone and naringenin for the biflavonoids.

The characterization of the phenolic compounds was performed using an Acquity UHPLC-DAD-MS/MS system (Waters, Milford, MA, USA) as described in our previous work (Feuereisen et al., 2014).

2.5. Statistical analysis

A face-centered central composite design was employed to investigate the effects of four independent variables (extraction temperature, static extraction time, ethanol and acetic acid concentration) on the dependent variables (concentration of the anthocyanins and biflavonoids, TPC). This resulted in separate plans for exocarp and drupes each consisting of 30 experiments including 6 replicates of the center point (Table 1). Design Expert Version 9.0.3.1 (State-Ease, Inc. Minneapolis, MN,

USA) was used for data analysis. Experimental data was fitted with the following second order polynomial equation:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i} \sum_{j=i+1} \beta_{ij} X_i X_j$$

where Y is the predicted response, β_0 , β_i, β_{ii}, β_{ij} represent the model coefficients for intercept, linear, quadratic and interaction terms, respectively, X_i and X_j the independent variables, and k is the number of factors. Transformation was performed for response variables with lack of homoscedasticity and normal distribution as described by Box and Cox (1964). The adequacy of the models was determined by coefficient of regression (R^2) and the F-test value (*F*-value) obtained from the analysis of variance (ANOVA).

Analysis of the phenolic compounds resulted in 9 responses (seven for the exocarp and two for the drupe) displaying their concentrations, and for these separate predictive models have been generated. As the optimum conditions for maxima of the responses can be contradictory, a simultaneous optimization of all responses was necessary to achieve a maximized yield of all phenolics. This was realized by the desirability approach after Derringer and Suich (1980). For this purpose, the models (two for the extraction of the drupe and seven for the extraction of the exocarp) were combined and condensed to overall Desirability functions using the numerical optimization function of Design Expert. For verification of the calculated optimal ASE conditions, further extractions with these parameters were conducted. The resulting concentrations of all phenolic compounds were compared with the predicted values.

3 Results and discussion

3.1. Key phenolic compounds

Six main peaks were detected by HPLC in the exocarp extracts (Fig. 1a and b). Three compounds with retention times between 19.8 and 26.3 min had absorption maxima around 500 nm (peaks 1–3: 499, 513, 497 nm), whereas the other three eluted after 68.0–72.9 min and exhibited absorption maxima around 330 nm (peaks 4–6: 335, 336, 326 nm). By comparison of absorption maxima and retention times with our previous findings (Feuereisen et al., 2014), three anthocyanins (pelargonidin 3-*O*-galactoside, 7-*O*-methylcyanidin 3-*O*-galactoside, and 7-*O*-methylpelargonidin

3-*O*-*β*-D-galactopyranoside) and three biflavonoids (I6,II8-biapigenin (agathisflavone), I3',II8-biapigenin (amentoflavone), and II-2.3-dihydro-I3',II6-biapigenin) were identified. Analysis of the drupe extract revealed one main compound (Fig. 1c) which eluted at 71.1 min and showed an absorption maximum of 290 nm (peak 7).

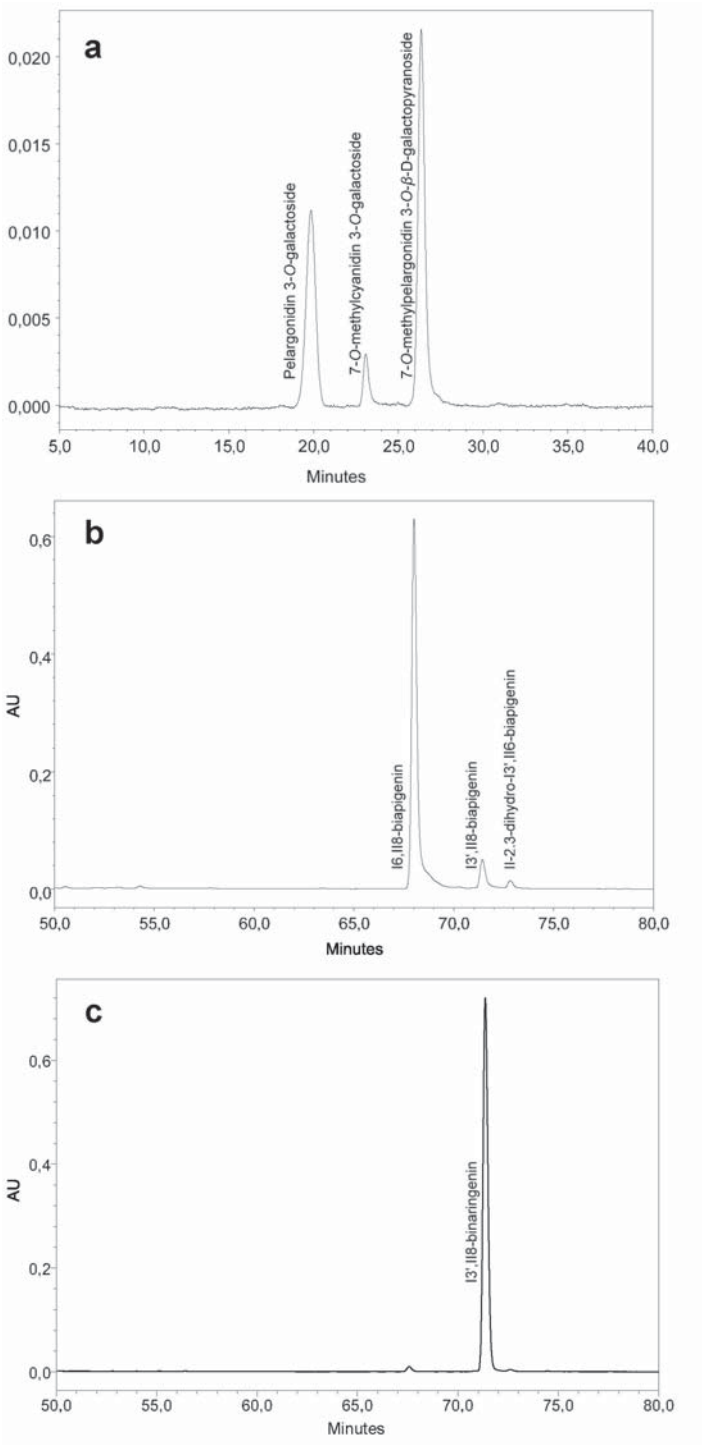

Figure 1: Chromatograms of key phenolics from *Schinus terebinthifolius* exocarp at 500 nm (a), and 330 nm (b) and of the drupe at 290 nm (c).

For identification, peak 7 was further analyzed by UHPLC-MS^2; it eluted at 15.7 min and showed a $[M + H]^+$ ion at *m/z* 543. The molecular mass is in accordance with a tetrahydrobiapigenin (i.e., a binaringenin). The UV maxima at 290 and 330 nm indicate the presence of a naringenin moiety. In the literature, I3',II6-binaringenin (Kassem, El-Desoky, & Sharaf, 2004) and I3',II8-binaringenin (Skopp & Schwenker, 1986) in fruits of *S. terebinthifolius*, and I3',II8-binaringenin (Ono et al., 2008) and I3,II3-binaringenin (Yueqin et al., 2003) in fruits of *Schinus molle* have been reported. The I3,II3 linkage can be excluded for peak 7: the fragment ion at *m/z* 297 (cf. Fig. 2a) cannot be explained assuming the I3,II3 linkage but may be the 1,4IB+ – 1,3IIB[1] ion from I3',II8- or I3',II6-binaringenin. Further, the ion at *m/z* 271 might be formed by two 0,3B-ring fissions of I3,II3-binaringenin, which is rather unlikely. The abundant signal at *m/z* 297 is rather due to a 1,4IB+ – 1,3IIB ion from I3',II8- or I3',II6-binaringenin. Among these two possible structures, I3',II8-binaringenin is more probable because the loss of water (*m/z* 525) is not as prominent as in I3',II6-linked biflavonoids. Such a loss of water is preferred in I3',II6 rather than in I3',II8- or I6,II8-linked biflavonoids because of the proximity of two OH functions of unit II to one OH function of unit I. The naringenin nature of the two subunits is supported by the presence of the ions at *m/z* 153, 179 and 245 that are known naringenin fragments (Fabre, Rustan, de Hoffmann, & Quetin-Leclercq, 2001; Justesen, 2000; Lee, Kim, Liu, Oh, & Lee, 2005; Tong et al., 2012). Therefore, peak 7 present in extracts of *S. terebinthifolius* drupes was identified as I3',II8-binaringenin, which confirms the findings of Skopp and Schwenker (1986) and Ono et al. (2008) in *S. terebinthifolius* and *S. molle*, respectively. Also, I3',II8-binaringenin could be assigned to the drupe rather than to the whole fruit for the first time.

Interestingly, in drupe extracts obtained at higher extraction temperature, a second, less dominant peak eluting at 74.2 min occurred (peak 8). Further investigations showed that the compound appeared only after extraction at temperatures higher than 75 °C and that the amounts increased with elevating temperatures. By re-extraction of the primary extract (obtained at 60 °C) at high temperatures, it was proven that this additional compound is an artifact.

Identical MS^2 spectra were obtained for peaks 7 and 8 (Fig. 2). We therefore hypothesized a stereoisomer of the naturally occurring I3',II8-binaringenin. Naringenin has a chiral carbon atom at position 2. Both possible enantiomers occur

[1] Nomenclature of the fragment ions according to Zhang, Li, Yan & Shi 2011

naturally in various citrus fruits. The epimerisation of naringenin in plants may take place by a non-enzymatic reaction (Caccamese & Chillemi, 2010; Gaffield, Lundin, Gentili, & Horowitz, 1975). The in vitro epimerisation is described at basic pH values by Miles and Main (1988) and has been observed by Krause and Galensa (1991) at high temperatures (70 °C) in neutral methanol/water mixtures, whereas in pure methanol the configuration remained stable. Heat-induced epimerization has been described also for monomeric (Kofink, Papagiannopoulos, & Galensa, 2007) and oligomeric flavanols (Kothe, Zimmermann, & Galensa, 2013). This supports the hypothesis that binaringenin can react in the same way as naringenin under the conditions applied here (ethanol/water mixtures) at temperatures higher than 75 °C. For this reaction, opening of the B-ring is required, which is not impaired by the I3',II8-bonding in either of the two subunits. Considering the two chiral carbon atoms in I3',II8-binaringenin, four stereoisomers are possible, i.e., two pairs of enantiomers, which can also be seen as two pairs of diastereomers. Since the stationary phase used here is not chiral, only the diastereomers can be separated, resulting in two peaks with identical spectral properties as observed here. In conclusion, the artifact found after extraction at high temperatures was tentatively identified as a diastereomer of the naturally occurring I3',II8-binaringenin.

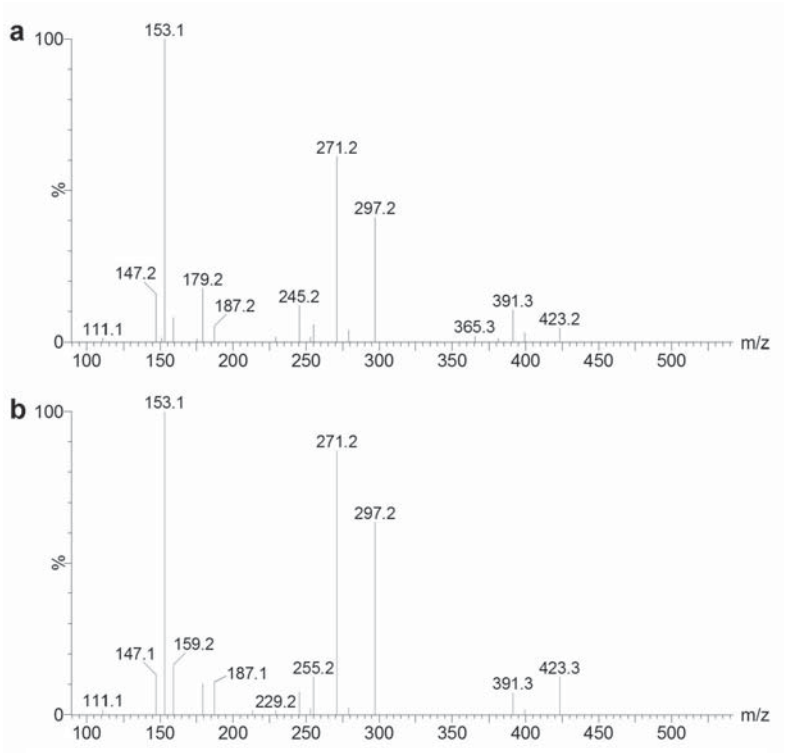

Figure 2: Fragment ion scans of the precursor with *m/z* = 543. Peak 7 (native binaringenin) (a) and peak 8 (artifact after hot extraction) (b).

The compounds representing peaks 1–7 were considered key phenolic substances of *S. terebinthifolius* for the following characterization of the influence of PLE conditions on their extraction yields and are further referred to as anthocyanins 1–3 and biflavonoids 1–4.

3.2. Factors influencing the extraction yields

The regression models for all responses, namely concentrations of anthocyanins 1–3, biflavonoids 1–4 and TPC, as well as their corresponding coefficients, are given in Table 2. All models were highly significant ($p < 0.0001$) and had high regression coefficients (0.82–0.99). Thus, the influence of ASE parameters on extraction yields of anthocyanins 1–3 and biflavonoids 1–4 as well as TPC were well described by the established quadratic models. As seen in Table 2, ethanol (X_3) had a significant, quadratic influence on all response variables ($p < 0.0001$). In fact, the ethanol concentration (X_3) is the most dominant factor that influences the extraction of key compounds from Brazilian pepper fruit. Furthermore, both temperature (X_1) and static time (X_2) had a significant positive linear influence ($p \leq 0.0004$ and $p \leq 0.0409$, respectively) on all responses. Acetic acid (X_4) had an effect only on the anthocyanin yield in interaction with the ethanol concentration ($p \leq 0.0144$). Fig. 3a–c presents response surface plots illustrating the effects of ethanol and acetic acid on the anthocyanin recovery. These results are in agreement with findings of Cacace and Mazza (2003), who also showed a quadratic influence of the ethanol concentration on the anthocyanin yield from black currants.

In addition to the quadratic influence of ethanol on all biflavonoids, slight interactions of ethanol with temperature ($p < 0.0001$) or static time ($p \leq 0.0233$) were observed for the biflavonoids 1 and 2 from the exocarp. Fig. 3d exemplifies the interactions of ethanol and temperature on the concentration of biflavonoid 1. Gaedke (2003) also identified a quadratic influence of ethanol on the extraction of biflavonoids from St. John's wort with an optimum around 60–80% alcohol at moderate temperatures (60–80 °C) and therefore reports similar results. For comparison, Fig. 3e shows the quadratic influence of ethanol ($p <0.0001$) and the influence of the temperature ($p < 0.0001$) on the total phenolic content of the exocarp extract. Furthermore, a slight interaction of X_1 and X_2 ($p = 0.0272$) was found. For TPC of the drupe extract, a quadratic influence of the temperature ($p < 0.0184$) as well as of the ethanol content ($p < 0.0001$) and a slight interaction of X_1 and X_3 ($p = 0.0142$) were observed (Fig. 3f).

Table 2: Second order polynomial equations and regression coefficients of the response variables and optimum ASE conditions.

Fruit part	Response variable*	Second order polynomial equation in coded factors	R^2	Predicted R^2	Optimum ASE conditions (°C / min / % ethanol / % acid)
Exocarp	Anthocyanin 1 (mg MvE/mL)	$\sqrt{(Y + 0.00001891)} = 0.096 + 0.014\,X_1 + 0.008408\,X_2 + 0.003646\,X_3 + 0.017\,X_4 + 0.007297\,X_1X_2 - 0.007076\,X_1X_3 - 0.015\,X_3X_4 - 0.037\,X_3^2$	0.88	0.72	100/10/54.5/5
	Anthocyanin 2 (mg MvE/mL)	$\sqrt{(Y + 0.00000282669)} = 0.040 + 0.005644\,X_1 + 0.002920\,X_2 + 0.004015\,X_3 + 0.004572\,X_4 - 0.005653\,X_3X_4 - 0.015\,X_3^2$	0.83	0.66	
	Anthocyanin 3 (mg MvE/mL)	$(Y + 0.000037872)^{0.75} = 0.053 + 0.009858\,X_1 + 0.004805\,X_2 + 0.008368\,X_3 + 0.005602\,X_4 + 0.004250\,X_1X_2 - 0.004976\,X_3X_4 - 0.024\,X_3^2$	0.88	0.79	
	Biflavonoid 1 (mg AfE/mL)	$\sqrt{Y} = 0.26 + 0.019\,X_1 + 0.009417\,X_2 + 0.096\,X_3 + 0.012\,X_1X_3 + 0.009186X_2X_3 - 0.13\,X_3^2$	0.99	0.99	
	Biflavonoid 2 (mg AfE/mL)	$Y = 0.005432 + 0.0007544\,X_1 + 0.0003745\,X_2 + 0.001763\,X_3 + 0.0005405\,X_1X_3 + 0.0002471\,X_2X_3 - 0.002984\,X_3^2$	0.97	0.95	
	Biflavonoid 3 (mg AfE/mL)	$Y = 0.001769 + 0.0002907\,X_1 + 0.0001271\,X_2 + 0.0006462\,X_3 + 0.00008941\,X_1X_2 - 0.0008355\,X_3^2$	0.96	0.93	
	TPC (mg CAE/100mL)	$Y = 37.75 + 6.16\,X_1 + 3.57\,X_2 + 0.89\,X_3 + 3.03\,X_1X_2 - 15.91\,X_3^2$	0.82	0.68	
Drupe	Biflavonoid 4 (mg NgE/mL)	$Ln(Y + 0.00016484) = -3.24 + 0.94\,X_1 + 0.62\,X_2 + 0.61\,X_3 - 2.70X_3^2$	0.86	0.80	75/10/54.2/0.03
	TPC (mg CAE/100mL)	$\sqrt{Y} = 3.75 + 0.42\,X_1 + 0.33\,X_2 - 0.041\,X_3 - 0.25\,X_1X_3 + 0.51\,X_1^2 - 1.79\,X_3^2$	0.87	0.75	

* expressed as: Malvidin Equivalent (MvE), Amentoflavone Equivalent (AfE), Caffeic Acid Equivalent (CAE), Naringenin Equivalent (NgE)

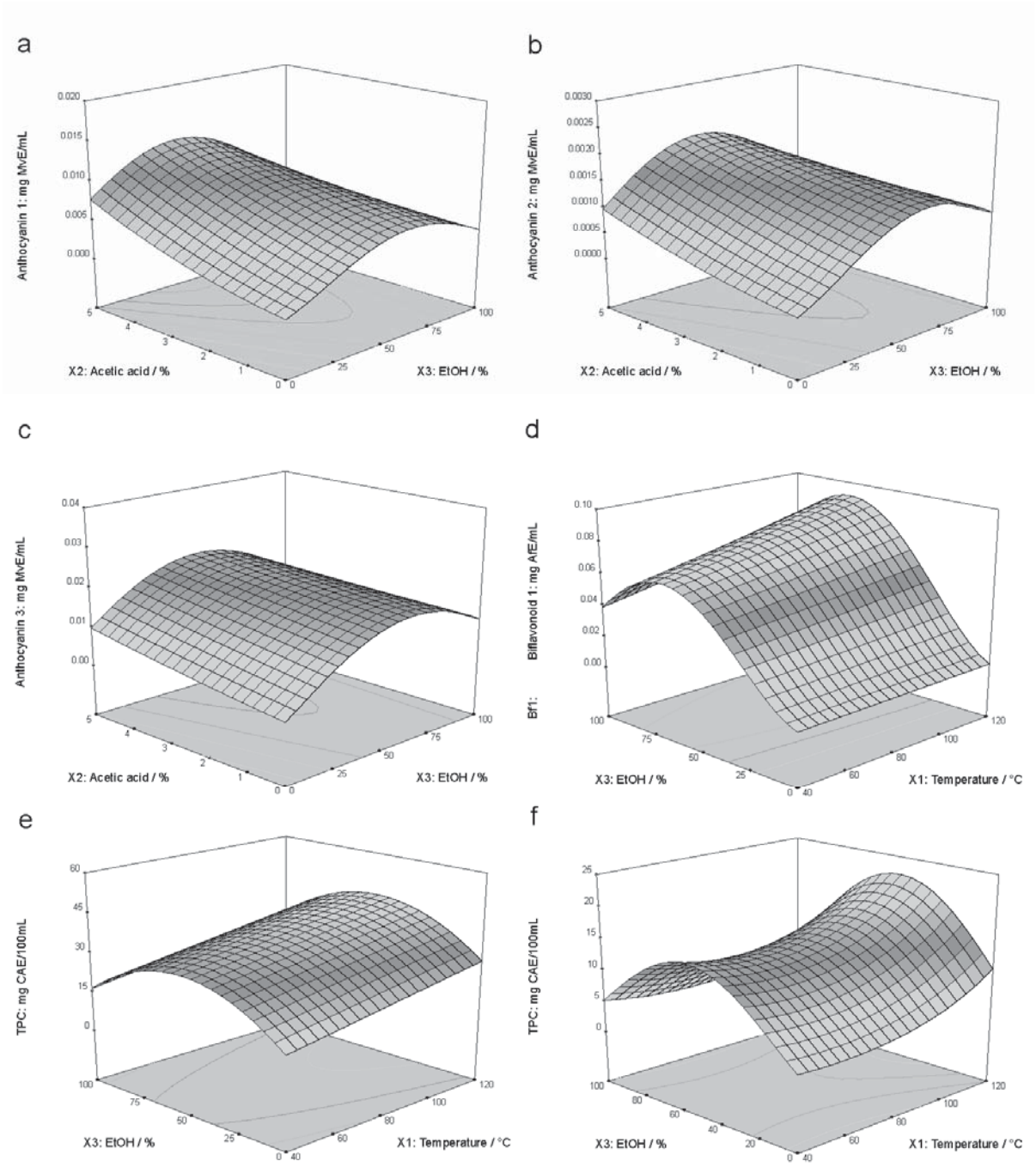

Figure 3: Response surface plots showing the effects of acetic acid and ethanol concentration on the extraction of anthocyanin 1 (a), anthocyanin 2 (b) and anthocyanin 3 (c), of ethanol concentration and temperature on biflavonoid 1 (d), of ethanol concentration and temperature on TPC of the exocarp extract (e), and of ethanol concentration and acetic acid on TPC of the drupe extract (f).

3.3. Optimization and verification of predictive models

Based on the established regression models, a multiple response optimization was conducted using the Desirability function proposed by Derringer and Suich (1980). All responses were considered and set to maximum. Table 3 shows the upper and lower limits of the independent variables.

While time, ethanol concentration, and acetic acid concentration varied in the investigated ranges, temperature was set to a maximum of 100 °C for the exocarp extraction. Thus, degradation of the heat-sensitive anthocyanins or other undesired reactions should be avoided. This temperature was selected because Arapitsas and Turner (2008) and Ju and Howard (2003, 2005) reported that for anthocyanin extraction from red cabbage and dried red grape skin by PLE, temperatures of about 100 °C are most suitable and that higher temperatures may lead to degradation of individual anthocyanins. The results of the numerical optimization are given in Table 2. The ideal ASE conditions for the exocarp were 100 °C, 10 min static time, and the optimum concentrations of ethanol and acetic acid were 54.5% and 5%, respectively (desirability = 0.90). Because static time was ideal at level +1 (10 min) and several short cycles were superior to one extended cycle (data not shown), further tests with up to 3 cycles were conducted. Referred to a total yield obtained after 3 cycles, the first cycle already led to yields of 79.2–83.6% for anthocyanins from the exocarp. However, two cycles of 10 min are considered sufficient for exhaustive extraction of anthocyanins and a time-saving process. Since only 77.1% of biflavonoid 3 was extracted after two cycles, three cycles were required for a complete extraction of biflavonoids.

Table 3: Factor settings for the optimization.

Fruit part	ASE settings	Goal
Exocarp	Temperature	equal to (100°C)
	Static time	in range (0–10 min)
	Ethanol	in range (0–100 %)
	Acetic acid	in range (0–5 %)
Drupe	Temperature	equal to (75°C)
	Static time	in range (0–10 min)
	Ethanol	in range (0–100 %)
	Acetic acid	in range (0–5 %)

When the drupes were extracted at temperatures higher than 75 °C, the tetrahydroamentoflavone isomer mentioned in 3.1 appeared. Since it was shown that it is an artifact and thus undesired for bioactivity testing of naturally present phenolics, the temperature was set at 75 °C for the optimization. The optimal conditions for the drupe extraction were 54.2% ethanol, 0.03% acid and 10 min static time (desirability = 0.59). Here, the desirability was relatively low due to the temperature limit necessary to avoid isomerization as described in 3.1. For the

drupes, up to three extraction cycles were considered appropriate since two cycles resulted in a yield of biflavonoid 4 of 78.6% referred to a total yield obtained after 3 cycles.

The suitability of the established regression models for the prediction of response values was tested using the recommended optimum conditions shown in Table 2. The actual values were found to be in good agreement with the predicted values for TPC and yields of anthocyanin 1–3 and biflavonoid 1–4 (Fig. 4). Thus, the empirical models are suitable for the characterization and optimization of the extraction of key phenolic compounds from *S. terebinthifolius* by PLE. Considering the dry weight of the exocarp extract (0.15%), the optimized extract consisted of more than 34% phenolics and can therefore be considered rich in phenolic compounds. The lower concentration (2.6% phenolics, dry weight 0.55%) of the drupe extract might be attributable to the presence of only one biflavonoid in relevant quantities.

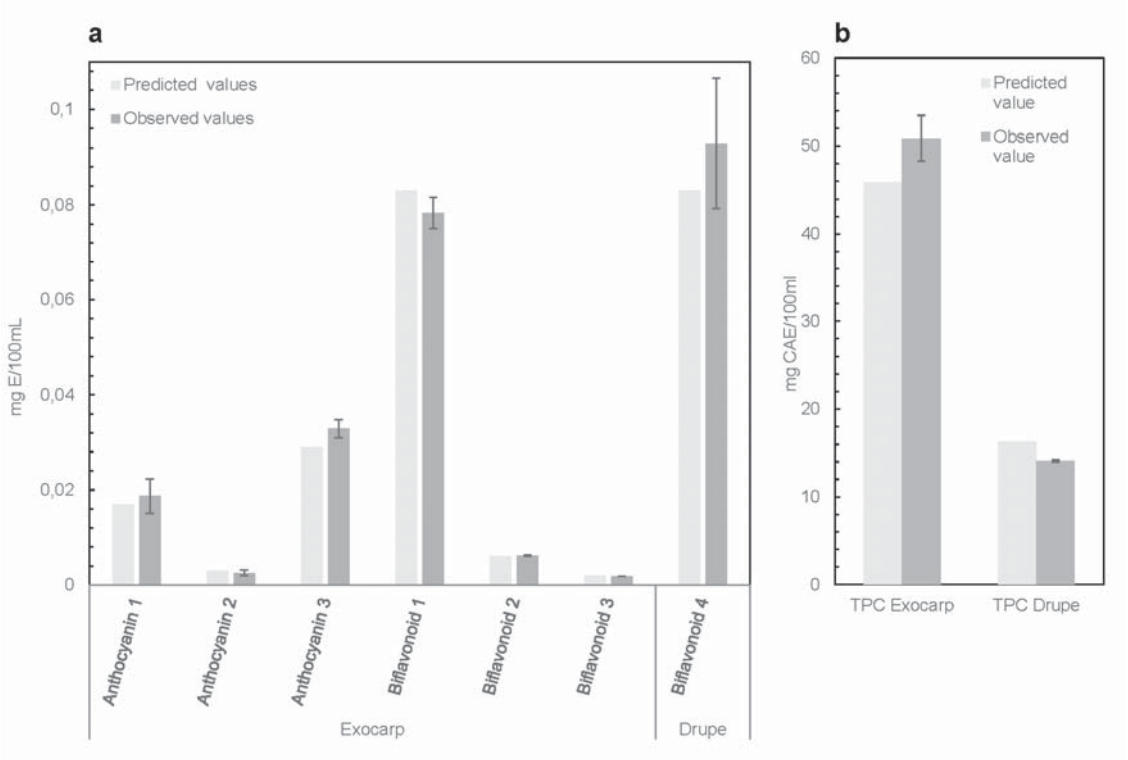

Figure 4: Predicted and actual response values for peak areas of anthocyanin 1–3 and biflavonoids 1–4 (a) and TPC (b).

4 Conclusion

To the best of our knowledge, the extraction of *S. terebinthifolius* fruit exocarp and drupe by means of PLE was investigated for the first time. For this purpose, response surface methodology was found to be a useful tool for the identification of significant extraction parameters as well as their interactions. The highly significant models were suitable not only for a numerical multivariate optimization of the ASE extraction but also for prediction of extraction yields. The presence of I3',II8-binaringenin in *S. terebinthifolius* was confirmed and allocated to the internal part of the fruit (drupe). Furthermore, it was shown that high extraction temperatures result in the formation of an isomer of this compound. These findings facilitate the implementation of PLE for the recovery of anthocyanins and biflavonoids from *S. terebinthifolius* and may serve as a starting point for the extraction of flavonoids also from other sources. Furthermore, the optimized ASE conditions constitute a crucial basis for future research such as bioactivity assays and purification of selected phenolics.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2016. 07.002.

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Chapter 5

Concluding remarks

Trends and negative experiences concerning food engender consumers' desires and anxieties. Therefore, possibilities to react and counteract are needed. On the one hand, this includes methods to prevent food fraud and ensure high quality, safe groceries and on the other hand, to come towards consumers' food preferences of more natural products by using natural food additives. (Negi, 2012, Schieber, 2008) This implies the extension of authentication methods and the search for, characterization, and yielding of novel and natural techno-functional compounds as intended and enabled by this work (see Chapter 2–4).

Edible plants are preferred sources for the recovery of bioactive extracts and compounds because in their original form they are consumed regularly. Therefore, concerns regarding potential toxicity are less pronounced and the approval as a food additive may be facilitated. Among food crops, especially spices have shown encouraging evidence for their bioactivity and potential as food additives with added value for health (Cowan, 1999; Maqsood et al., 2013). Since in addition, several edible Anacardiaceae species have revealed a broad and promising spectrum of bioactive polyphenols, *Schinus* was selected for further research. More precisely, the focus of this thesis was the characterization of the phenolic composition of *S. terebinthifolius* and the implementation of this knowledge also for the estimation of its bioactivity potential. Thus, the most widespread *Schinus* species, which is already used as a spice, was investigated. Its invasiveness suggests that this plant harbors effective phytochemicals. Among these, phenolics presumably contribute to its defense against biotic and abiotic stress and may be valuable for other fields of application. Furthermore, the phenolic profile of *S. molle* was examined because

these fruits are also used as a spice and are difficult to distinguish from those of *S. terebinthifolius*. It was intended to develop a method for the phytochemical differentiation of these species and to gain further insights into the profile of phenolic compounds, also with respect to the chemotaxonomy of both species and the Anacardiaceae family. (Schulze-Kaysers et al., 2015) The findings of this research are not limited to the investigated species since they may be transferred to other plants, especially botanically related species.

1 Polyphenol profiles of *Schinus* species fruits

Although numerous publications on the bioactivity of plant extracts exist, there is a lack of knowledge regarding the active compounds, since the chemical structure of secondary compounds was often not characterized. To overcome this problem, the basis of this thesis was the determination of the phenolic profile of the selected *Schinus* species (see Chapter 2 and 3). In the first instance, the polyphenol spectrum of *S. terebinthifolius* fruits was investigated because of the high importance for this work and as a spice. *S. molle* was included mainly for distinction purposes. Characterization of the polyphenol spectrum enabled their use as a tool for food authentication and chemotaxonomy and also helped to solve challenges concerning analytical methods, maximization of extraction yield, and the recovery of purified phenolics. The knowledge of the phenolic composition allows a more reliable assessment of their health potential and facilitates their use as a source of natural food additives.

Usually, a favored method for the identification of polyphenols is the use of authentic references. Due to the great variety of phenolic compounds, standards are not always available. A useful tool to overcome this problem is the analysis and comparison of results of plant extracts for which the occurrence of the suspected compound has previously clearly been demonstrated. Since even then MS analysis was not sufficient for structure elucidation of all anthocyanins present in *S. terebinthifolius*, the purification of the relevant anthocyanin by SPE and semi-preparative HPLC followed by structure elucidation by NMR spectroscopy was required (see Chapter 2).

Some information on the chemical composition of different parts of *S. terebinthifolius* and *S. molle* had already been published, but these studies mainly relate to the

essential oils. Comprehensive findings on the nature of phenolic compounds, especially those present in the fruits, were not available and the scarce information was scattered and partly conflicting (see Section 4.3). LC-MS and NMR analyses revealed the presence of 30 different polyphenols for *S. terebinthifolius.* The exocarp extract of the Brazilian pepper was complex and contained five anthocyanins and four biflavonoids besides gallic acid, thirteen galloylglucoses, and six galloyl shikimic acids. The drupe extract had a comparatively simple profile of phenolics as it contained the same galloylglucoses but only one, divering biflavonoid. Only five of these compounds had previously been reported for *S. terebinthifolius* fruits, whereas 21 were reported for this species for the first time in the fruit. Additionally, tetra- to hexagalloyl shikimic acids were tentatively identified. To the best of the author's knowledge these have not been reported before. NMR analysis enabled the identification of a so far unknown and uncommonly methylated anthocyanin structure, namely 7-*O*-methylpelargonidin 3-*O*-*β*-D-galactopyranoside. The investigation of *S. molle* revealed 32 different phenolics. More precisely, it shows the same profile as its relative *S. terebinthifolius,* besides one additional anthocyanin and one biflavonoid. Only three of these compounds were known in the Peruvian pepper fruit. All other 29 had so far not been reported. Thus, a comprehensive phenolic profile was ascertained for both species. Interestingly, it was shown that the Peruvian peppertree fruit contains a higher amount and a lager variety of cyanidin derivatives, whereas in the Brazilian species, pelargonidin and its derivatives prevailed (see Chapter 2–3). Previous investigations of the phytochemical profile of other edible Anacardiaceae like mango, jocote, and cashew have revealed the presence of a broad spectrum of various types of phenolic compounds, such as phenolic acids, hydrolyzable tannins, and anthocyanins (Schulze-Kaysers et al., 2015). The hypothesis of this work was that the peppertrees investigated contain a similar composition of phenolics due to their chemotaxonomic relation. This was confirmed by the findings presented in Chapters 2 and 3.

The need for a more efficient and selective extraction was a methodical reason for the separate investigation of the exocarp and the rest of the drupe. However, there are several further aspects of major interest. One was to verify whether the view that fruits contain the highest concentration and variety of phenolic compounds in the outer layer is also valid for the drupe fruits of peppertrees. This was confirmed and might be due to the protective function of the outer layer. The approach to consider

the fruit parts separately supports and complements the findings of several former studies. Panetta & McKee (1997) reported that the germination and seedling rate of whole fruits was multiple times lower than of fruits without exocarp, regardless whether the removal took place manually or by frugivorous digestion (Panetta & McKee, 1997). Other groups showed an inhibition of *S. terebinthifolius* seed germination by its exocarp extract. This effect was much more distinct when seed of other plant species were exposed to *S. terebinthifolius* fruit exocarp or whole fruit extracts (Donnelly et al., 2008; Nilsen & Muller, 1980). This leads to the assumption that the success of Brazilian peppertrees in foreign habitats is based on allelopathic effects and that especially the exocarp seems to contain the highly active compounds that could also derive benefits for other fields of application. Although phenolic acids are suspected to trigger the observed effects, so far no one has verified and confirmed the active compounds. Therefore, especially the Chapters 2 and 3 of this work also provide deeper insights into the phytochemistry of the fruits since it was clearly shown that the phenolics in the exocarp and the rest of the fruit differ noticeably. These variations in the polyphenol composition may be the reasons for the reported deviating functions of the investigated fruit parts.

2 Phytochemical differentiation of *Schinus* species

Because of labeling requirements and price differences, simple and reliable methods for authentication are of interest for all stakeholders of the supply chain and for control authorities. Such methods had not been in place for the differentiation of *S. terebinthifolius* and *S. molle* used as spices. Until now, for an unambiguous differentiation of *S. molle* and *S. terebinthifolius* a detailed examination of various vegetative traits of the whole plants such as phyllotaxis, or a complex combination of cell morphological and chemical investigation of the fruit was necessary (Barkley, 1944; Barkley, 1957; Schrutka-Rechtenstamm et al., 1988). The examination of genetic relationships is also possible but this, as well as the other methods, relies on time consuming and/or costly analyses and the experience of experts. In contrast, the method developed in this work initially enables a quick and easily manageable and yet reliable differentiation of the species by the spices, which is based on the phenolic profiles of the fruit (see Chapter 2–3).

As described in Chapter 3, this method was achieved by considering all commonly performed approaches for authentication by secondary plant metabolites, namely their absolute or relative amounts as well as the presence or absence of indicator compounds. Two minor compounds, one anthocyanin and one biflavonoid, found only in *S. molle* and under specific analytical conditions, may serve as indicator compounds. Nevertheless, it became apparent that the relative amount of anthocyanins is the most reliable approach, especially since the anthocyanin profile of both species differs more pronounced quantitatively rather than qualitatively. Focusing on major rather than minor compounds overcomes analytical difficulties like the co-elution of these substances. Furthermore, the influence of technical differences of the systems used, such as variation of acid tolerance and detection sensitivity, is avoided, enabling a robust method for differentiation. Combined with cluster analysis it was shown that the relative amount of the main anthocyanins is sufficient for the authentication of both *Schinus* species, as the 18 samples, including reference samples, could clearly be allocated to different subgroups of the sample set. To ensure that the implementation of this method is also possible on a low cost basis, rather basic equipment and techniques like UAE and HPLC were chosen intentionally. In this way, a time saving and easily implementable method was developed. Its application does not require much experience since the operation of the necessary equipment and methods is straightforward and the interpretation of results is statistically supported, which makes it attractive also for industrial purposes.

An enlargement of the sample set might have allowed the extension of the method to the differentiation of varieties. Since it is difficult to acquire authentic samples, the selection for this thesis was limited. The less pronounced differences in the anthocyanin profile of the varieties, therefore, can only be assumed but are not distinct enough for a valid statement. Although this aspect is minor and not of great importance for this work, further investigations might be worthwhile.

3 Chemotaxonomic implications and features of the Anacardiaceae

Chemotaxonomic considerations led to *S. terebinthifolius* as a promising source of bioactive compounds. This hypothesis and the assumption were confirmed that this species, as well as *S. molle,* contain phenolic compounds of similar composition, as

frequently reported for edible Anacardiaceae (Schulze-Kaysers et al., 2015). Consideration of these findings from literature research in combination with the results of Chapters 2 and 3 from a chemotaxonomic point of view revealed several interesting and novel aspects and possible chemotaxonomic traits. On the one hand, phenolics such as anthocyanins and phenolic acids rather widespread in fruits can be found in all edible Anacardiaceae so far investigated. Flavonols (mainly quercetin, kaempferol, myricetin and derivatives), tannins (hydrolysable and/or condensed) as well as gallic acid were reported for almost all members of this group. On the other hand, less frequently occurring compounds were determined in several representatives. More precisely, alkylphenols were reported for mango, cashew, pistachio, and *Schinus* sp. and biflavonoids for cashew, *Schinus* sp., and *Rhus* sp.. (Almeida et al., 2011; Emongor & Tautsagae, 2016; Mathew & Parpia, 1970; Schulze-Kaysers et al., 2015) It should be kept in mind that even if these compounds have not been reported for the fruits so far, they might just not have been investigated yet. This is evident as often they have already been found in other parts of the plant like leaves or bark. Moreover, there are some uncommon substitution patterns that were observed for several of the mentioned species. Besides tannins, other galloylated phenolics, mainly flavonol-galloylglycosides or anthocyanidin-galloylglycosides, have so far been reported for six of the eight fruits listed above. Interestingly, there is an increased occurrence of methoxylated phenolic compounds, mainly flavonoids (anthocyanins, flavonols, isoflavones) but also cardols in all mentioned edible species, except the poorly investigated marula. Especially remarkable are the uncommon 7-methoxylated flavonoids found in several members such as mango, jocote, *Rhus* sp. and the *Schinus* species examined here. (Schulze-Kaysers et al., 2015; Silva et al., 2016) The assessment that highly hydroxylated phenolics, such as gallic acid, myricetin, and their derivatives, as well as methoxylated phenolics, occur frequently in the Anacardiaceae family is supported by the previous findings of Umadevi et al. (1988). They reported several methoxylated flavones and flavonols, including 7-methoxylated compounds, but no methoxylated anthocyanins for several of the 20 Anacardiaceae species investigated. (Umadevi et al., 1988) Thus, the rather uncommon polyphenol subclasses and particularly the distinguished substitution patterns seem to constitute a chemotaxonomic trait frequently found within the Anacardiaceae family, specifically the edible species. Although it would be desirable to examine further species for a

conclusive proof whether the presence of these types of polyphenols can be considered a chemotaxonomic trait of the whole plant family, the findings of this work expand the chemotaxonomic understanding in general and especially regarding Anacardiaceae. The characterization of the enzymes (flavonoid-methyltransferases) involved in the biosynthesis would also be of interest. The knowledge gained so far may even facilitate the search for novel antimicrobials in several ways. On the one side, a more frequent occurrence of methylated phenolics was revealed for the investigated species and the whole plant family. This might be of major importance, because it is known that the methylation of phenolics can enhance their bioactivity and antimicrobial impact, respectively (Lam et al., 2007). On the other side, an ongoing chemotaxonomic consideration and knowledge transfer of the findings may simplify the selection of further promising plants as a source of potent bioactive compounds by enabling the estimation of their phytochemical profile.

4 Multivariate optimization of extract yield based on identified polyphenols

For subsequent application studies and the investigations of the biological activities of the identified compounds, their recovery in adequate amounts and thus an optimization of their yield is indispensable, especially since the extract is very complex and numerous phenolics are not available or costly. A multivariate approach conducted by PLE was selected since this meets the aim of an efficient and yet economical optimization of the extraction method (see Chapter 4).

Extraction technique and conditions were selected such that the possibility of a food grade application of the phenolic rich *S. terebinthifolius* extracts is retained. As described in Section 5.1, the separate extraction of the exocarp and the remaining parts of the drupe was of interest for several reasons, but also necessary as otherwise the comminution and extraction were less efficient. Although pressurized liquid extraction is appreciated for the extraction of rather sensitive compounds like polyphenols, this extraction method produced so far unreported artifacts, namely a diastereomer of I3′,II8-binaringenin which occurred when high temperatures were applied. Its occurrence can be prevented by using a lower temperature which, however, was high enough to ensure adequate recovery of the polyphenols.

The multivariate approach based on RSM was favorable since the selected models described the detected data very well, which can be seen from the coefficients of

determination (see Chapter 4). They revealed that the ethanol concentration has a main effect on the yield of key compounds, whereas extraction time as well as temperature have a secondary but still significant effect. Additionally, interactions were determined which, in the case of anthocyanins, included also the acid concentration. The initial optimization of polyphenol extraction by PLE from both *Schinus* fruit parts can be rated as highly suitable, as it enables an effective and resource saving yielding of extracts rich in the original *Schinus* polyphenols. Chapter 4 therefore demonstrates that PLE is well-suited for the extraction of phenolic compounds from *S. terebinthifolius* and can efficiently be optimized by response surface methodology. Thus, this study based on Chapter 2 provides a crucial basis for further research, such as bioactivity assays and purification of desired polyphenols.

5 Recovery of purified and isolated bioactive polyphenols

As mentioned, authentic references are costly and by far not available for all phenolics found in the two *Schinus* species. However, for an evaluation of the bioactivity, purified compounds are essential because only then the observed activities can clearly be allocated to an individual compound. The same holds true for an investigation of structure-activity relationships, modes of action and synergetic effects with other antimicrobials. Such investigations arise for *S. terebinthifolius* polyphenols, in particular as the plant encloses several groups of phenolics in which the substitution patterns differ only at one position (methyl- or hydroxyl group, oxidation status of the C ring) or in the number of gallic acids or the interflavonoid bond. Due to these structural similarities, conclusions about structure-activity relationships can easily be drawn. The bioactivities like antimicrobial properties have been investigated in detail for only some of these polyphenols.

Knowing the identity of the phenolics facilitated their recovery and isolation. The multistage isolation method for anthocyanins as utilized for the yielding of the purified anthocyanin for NMR analysis (see Chapter 2) may also be used for the isolation of anthocyanins for other applications like bioactivity assays. Since the liquid−liquid extraction with ethyl acetate enables a separation of the anthocyanins from the less polar biflavonoids, the latter may also be easily gained in high purity or even as single compounds. For purification of this fraction, only a subsequent liquid-liquid

extraction is necessary to remove the remaining gallotannins. Single biflavonoids may be recovered by a slight modification of the semi-preparative HPLC gradient that was developed for anthocyanins. In this manner, the desired anthocyanins and biflavonoids may be obtained with an excellent chromatographical purity of up to 99.8%, which is sufficient for bioactivity assays.

6 Potential of *Schinus terebinthifolius* as a source of bioactive and antimicrobial agents

So far, little is known about the bioactivity of *S. terebinthifolius* fruits and their phenolics, probably because the knowledge of the phenolic profile has been fragmentary. More information is available for other plant parts, especially the leaves, as they have been investigated more extensively. The following brief overview of associated bioactivities focuses on antimicrobial effects and involves plant parts beside the fruits, since some of the polyphenols are present in several parts of the plant. As far as an estimation is possible, only publications concerning phenolic-rich extracts, fractions or purified phenolic compounds are taken into consideration. It should be kept in mind that essential oils may contain phenolics and, vice versa depending on the extraction solvent and method, polyphenol rich extracts may contain certain amounts of essential oils. Therefore, the activity cannot always be allocated exclusively to one of these groups of secondary plant metabolites or even single compounds. As a consequence, besides the investigation and selection of suitable extracts, the evaluation of purified fractions and isolated compounds should be considered for further research.

Two recent reviews summarize the biological and pharmacological properties of *S. terebinthifolius,* mainly the effects of alcoholic extracts of unverified composition and the essential oils from different plant parts, primarily bark and leaves. The effects directly ascribed to the phenolic compounds detected and in some cases purified, such as antioxidant, antiallergic, antitumor, antibacterial and antifungal activity, are also presented. (Carvalho et al., 2013; Patocka & Almeida, 2017) Several well-elaborated studies regarding the identification of the active fractions or compounds have lately been conducted more frequently, revealing additional findings. Antioxidant, antiproliferative, antiinflammatory, wound-healing, and chemopreventive activities were determined by *in vitro* and/or *in vivo* assays with

phenolic leaf extracts containing flavonoids as well as phenolic acids. In addition, even single phenolics (gallic acid derivatives, polyphenols) were used. (Fedel-Miyasato et al., 2014, Silva et al., 2017) Toxicity has comprehensively been addressed but research has shown conflicting results, especially for plant parts other than the fruits. Further investigation is therefore necessary for a final assessment, even if the consumption of fruits is considered safe. (Carvalho et al., 2013; Patocka & Almeida, 2017)

A vast number of studies tested *S. terebinthifolius* extracts from different plant parts for their antimicrobial activity, mainly using the agar-diffusion and the microdilution methods. The results of these different assays are not always comparable. Furthermore, the comparability of the studies is limited because of differences in the extraction and purification methods. Restrictions have to be made among other reasons due to methodical features, e.g., the agar-diffusion assay depends on the solubility and hence on the diffusion of the applied compounds. To summarize these findings, leaf extracts have most frequently been examined and showed antimicrobial properties such as growth inhibition, microbicidal, and anti-adherent activity against several microorganisms. More precisely, effects were observed against the following facultative pathogenic or obligate pathogenic microorganisms, including mycotoxin producing and plant pathogenic microorganisms: *Aspergillus niger*, *Aspergillus parasiticus*, *Bacillus subtilis*, *Candida albicans*, *Candida glabrata*, *Candida krusei*, *Candida tropicalis*, *Fusarium solano*, *Escherichia coli*, *Paracoccidioides brasiliensis*, *Pseudomonas aeruginosa*, *Pseudomonas savastanoi*, *Rhizoctonia solani*, *Sporothrix schenckii*, *Staphylococcus aureus* and *Streptococcus mutans* (Carvalho et al., 2013; Johann et al., 2008; Patocka & Almeida, 2017; Rhouma et al., 2009; Uliana et al., 2016). Antimicrobial effects of extracts from the combination of stems and leaves were also shown against *Aggregatibacter actinomycetemcomitans*, *Candida albicans, Enterococcus faecalis, Staphyloccocus aureus*, *Staphylococcus epidermidis,* and *Streptococcus mutans* (Cochrane, 1999; Pereira et al., 2011). Mixed extracts obtained from a combination of leaves, stalks, and flowers and an extract from stem bark were also effective against *Candida albicans* (Schmourlo et al., 2005; Alves et al., 2013). Stem or stem bark, however, showed effects against *Candida krusei, Candida glabrata*, *Sporothrix schenckii,* and *Staphylococcus aureus* (de Lima et al., 2006; Johann et al., 2007). To the best of the author's knowledge, only the following four studies have investigated the activity of

the fruit phenolics against microorganisms. A study that tested ethanolic extracts of *S. terebinthifolius* fruits showed antibacterial effects against *Staphylococcus aureus*, *Staphylococcus epidermidis,* and their methicillin-resistant counterparts (Cochrane, 1999). Degáspari et al. (2005) reported growth inhibiting effects of Brazilian pepper fruit extract containing flavone, apigenin, ellagic acid, and syringic acid against *Staphylococcus aureus* and *Bacillus cereus* (Degáspari et al., 2005). A fruit exocarp crude extract, its flavonoid fraction, and the isolated compound apigenin caused inhibition of nitric oxide production. Furthermore it showed antioxidant activity and antimycobacterial activity against *Mycobacterium bovis* but no significant cytotoxic effects (Bernardes et al., 2014). A very recent study demonstrated that *S. terebinthifolius* is a potential source of non-biocide virulence inhibitors, as it reports the inhibitory activity of a flavone rich extract from peppertree fruit on *S. aureus* accessory gene regulator alleles in the absence of growth inhibition. The effective fraction, containing biflavonoids and saponins, was well-tolerated by human cell cultures *in vitro* and mouse skin *in vivo* and might therefore be promising for the treatment of skin infections. (Muhs et al., 2017) Among the bacteria, yeasts, and molds which were partly investigated in multiple studies, clinical isolates, antibiotic-resistant species, but also such causing food borne illnesses were considered. Thus, as far as investigated, the plant shows activity against a broad spectrum of microorganisms and disease-associated effects, e.g., inflammation or oxidation. This corroborates its ethnopharmacological use and confirms that it is a possible and encouraging source of antimicrobial compounds that might be considered for several fields of application (Bernardes et al., 2014; Carvalho et al., 2013; Muhs et al., 2017; Patocka & Almeida, 2017).

Although the chemical composition of the extracts has not always been investigated, there are several studies including the identification, fractionation or even isolation of active compounds (Bernardes et al., 2014; Degáspari et al., 2005; El-Massry et al., 2009; Muhs et al., 2017; Uliana et al., 2016). These studies show conclusive indications that the antimicrobial potential is based on the phenolic compounds rather than only on the essential oils. The same applies to those investigations where aqueous or alcoholic extracts showed a higher activity than the essential oils or undefined extracts gained with less polar solvents such as hexane or dichloromethane. (Bernardes et al., 2014; Uliana et al., 2016, Pereira et al., 2011)

Some of the aforementioned studies investigated further plants, including medicinal plants mainly from Brazil. Also in comparison to those, the Brazilian peppertree shows promising results since it often has the most pronounced antimicrobial activity or at least is ranked among the most effective plants, especially since it has shown activity even against several antibiotic resistant bacteria. (de Lima et al., 2006; Johann et al., 2007; Johann et al., 2008; Patocka & Almeida, 2017; Pereira et al., 2011; Schmourlo et al., 2005) Exceptions arise when other Anacardiaceae are included, since then the predominance of the *S. terebinthifolius* is not always distinct (Pereira et al., 2011; Rhouma et al., 2009; Schmourlo et al., 2005).

Although this overview is focused on Brazilian pepper, it should be mentioned that some studies investigated both *S. molle* and *S. terebinthifolius*. In these cases, both species showed the same effects but the latter mostly revealed stronger antimicrobial activity. (Patocka & Almeida, 2017; Schmourlo et al., 2005; Rhouma et al., 2009) In view of the invasiveness of the Brazilian peppertree, the findings of Cochrane (1999) are very remarkable. Different parts of 37 plant species from the list of invasive species from the Floridas Exotic Pest Plant Council were tested for their antimicrobial activity against 6 pathogenic bacteria, including their antibiotic resistant counterparts and one pathogenic yeast. *S. terebinthifolius* was among the plants with the highest and broadest antibacterial activity, particularly against antibiotic-resistant species. Interestingly, a trend that plants rated with the highest invasiveness also show the most pronounced antimicrobial activity regarding species variety and quantitative effects can be observed. (Cochrane, 1999)

The above presented results, aspects, and conclusions clearly suggest that phenolic compounds especially from invasive plants and/or Anacardiaceae are worth considering when it comes to the search for natural and novel antimicrobials. Additionally, and most important for this thesis, the initial assumptions and the approach of this work, as well as the selection of *S. terebinthifolius* as a potential and promising source of bioactive compounds, have been confirmed. First own investigations have shown that the *S. terebinthifolius* exocarp extract, obtained and characterized with the help of the methods of Chapter 2–4, has an encouraging antimicrobial effect. This extract has revealed bacteriostatic and bactericidal effects on several species, significantly inhibited biofilm formation, and also showed a high antioxidant capacity (data not published so far).

Consideration of the phenolic profile determined in this work may explain the antimicrobial activity already shown and also the corresponding findings of studies where the chemical composition of the extracts has not been investigated. Galloyl glucoses, partly identified also in mango, have been comprehensively investigated regarding their antimicrobial activity, revealing growth inhibition of a broad spectrum of microorganisms. Some mechanisms behind this, as well as a significant reduction of surface contamination with *L. monocytogenes* of green leafy vegetables and decreased attachment of *E.coli*, were also determined. (Engels et al., 2009; Engels et al., 2010; Engels et al., 2011; Engels et al., 2012) In contrast, biflavonoids and particularly anthocyanins have so far been considered little. Nevertheless, there are results for some isolated compounds that show a moderate to high antibacterial potential, suggesting that further consideration of these polyphenol groups might also be worthwhile. (Cisowska et al., 2011; Junqueira-Gonçalves et al., 2015; Kaikabo & Eloff, 2011; Makhafola et al., 2012) In particular, the same applies to a continuing investigation of *S. terebinthifolius* and generally to Anacardiaceae in respect of the recovery of natural and novel antimicrobial compounds. One main reason is that *Schinus* contains methylated polyphenols which lack investigation and that these may possibly possess a stronger antimicrobial activity than their unmethylated derivatives (Lam et al., 2007). This thesis offers a fundamental basis for the proposed investigation.

Further studies are necessary to clarify major aspects like structure-activity relationships, modes of action, synergetic effects, and reactions of the *S. terebinthifolius* polyphenols with non-phenolic compounds. This would also promote the target-aimed application of extracts and individual compounds. Due to the activity against a broad spectrum of microorganisms, several fields of application such as phytopharmaca or food additives are conceivable. However, this work provides the crucial basis for such a knowledge based application of the characterized and identified extracts, purified fractions and single polyphenols to determine bioactivity and techno-functional properties.

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Summary

The need for new and natural techno-functional and bio-functional compounds is caused by the trend to “all natural” food and the increasing prevalence of microorganisms resistant against classical antimicrobial compounds. As a consequence, the search for such compounds is a challenging task concerning both research and industry. The aim of this thesis was the selection of a promising plant and determination of its phenolic composition in course of the search for natural and novel bioactive compounds as well as the evaluation of their suitability as chemotaxonomic and authentication markers. Edible plants are preferred sources for the recovery of such compounds. Representatives of the Anacardiaceae have revealed a broad variety of polyphenols that possess encouraging bioactivity, especially antimicrobial effects, and thus are a promising subject for research. Belonging to the Anacardiaceae, the Brazilian peppertree was assumed to fulfill the described aimes because it is already used as a spice, widely distributed, and shows aggressive invasiveness. Mainly for reasons of differentiation, the phenolic composition of its close relative, the Peruvian peppertree, was also determined.

Structure characterization via LC-MS and 2D NMR revealed 30 polyphenols in *S. terebinthifolius* and 32 in *S. molle* fruits. Most of them were reported in the fruits or the whole species for the first time. The compounds belong to three groups of phenolics, namely phenolic acids, flavonoids (biflavonoids, anthocyanins) as well as hydrolyzable tannins (galloyl glucoses, galloyl shikimic acids) and include some unusual degrees of galloylation. Among the red pigments a so far unknown anthocyanin was characterized via NMR spectroscopy as 7-*O*-methylpelargonidin 3-*O*-*β*-D-galactopyranoside. The separate evaluation of exocarp and drupe provided deeper insights into the phytochemistry of the fruits, as it confirmed that the exocarp encloses a broader spectrum of phenolics and therefore suggests that the reported activities, such as allelopathic effects, are based on these compounds. Furthermore, the assumption that the investigated *Schinus* species possess a phenolic composition similar to those of recently investigated representatives of the Anacardiaceae family was confirmed. Chemotaxonomic considerations of literature data combined with the findings of this work revealed less frequently occurring subgroups of polyphenols and unusual substitution patterns. The occurrence of

galloylated phenolics and particularly of methylated flavonoids, especially the uncommon 7-methoxylated representatives, seem to contribute to chemotaxonomic traits within the Anacardiaceae family. The increase in chemotaxonomic understanding will further facilitate the search for novel and natural compounds. This knowledge may be transferred to other plants, especially botanically related species, to estimate their phenolic composition and thus their bioactivity potential. Likewise, based on the knowledge of the phenolic composition and the analysis of 18 different fruit samples mainly from botanical gardens and commercial suppliers, a method for the differentiation of the two *Schinus* species was developed. This method considers the relative amounts of anthocyanins and uses a statistical tool, the cluster analysis, and hereby achieves a resource saving, facile implementation and reliable distinction of the species for the first time. This enables spice authentication that may easily be utilized by stakeholders of the food industry or control authorities. An extension of the authentic sample set might even facilitate the differentiation of varieties, especially in combination with a reference database.

Additionally, the extraction of the exocarp and drupe was optimized using PLE combined with design of experiments. Thereby, the extraction parameters significantly influencing the amount of identified and desired polyphenols as well as interactions between these parameters were identified simultaneously. The optimization of the PLE settings enables the economical recovery of the extracts in suitable amounts for subsequent purification and bioactivity assays. The purification and isolation of other desired anthocyanins and biflavonoids in high purity is possible based on the separation process of anthocyanins for the NMR analysis. This thesis, therefore, not only provides chemotaxonomic insights and authentication strategies for both *Schinus* species but also constitutes a crucial basis for further investigation of bio-functional and techno-functional properties of their polyphenols. In addition, it was shown that both invasive plants and edible representatives of the Anacardiaceae are promising regarding the search for bioactive and especially antimicrobial polyphenols. On the basis of the findings and insights of this thesis, *S. terebinthifolius* can be rated as a valid source of promising bioactive compounds. The polyphenols may be employed for several fields of application, like the prevention and treatment of diseases or for techno-functional use as natural food additives.

Zusammenfassung

Die Notwendigkeit neuer, natürlicher techno- und biofunktioneller Substanzen wird durch den Trend hin zu natürlichen Lebensmitteln und der Zunahme an Resistenzen von Mikroorganismen gegenüber den klassischen antimikrobiellen Wirkstoffen verursacht. Die Suche nach Alternativen stellt somit eine Herausforderung dar, welche sowohl die Forschung als auch die Industrie betrifft. Das Ziel dieser Arbeit war daher die Auswahl einer vielversprechenden Pflanze und die Bestimmung ihrer phenolischen Zusammensetzung auf der Suche nach natürlichen, neuen und bioaktiven Substanzen. Des Weiteren wurden diese Phenole hinsichtlich ihrer Eignung als chemotaxonomische Marker und zur Authentifizierungskontrolle evaluiert. Für die Gewinnung solcher Verbindungen stellen essbare Pflanzen eine bevorzugte Quelle dar. Diese Vertreter der Anacardiaceae zeigten bisher eine breite Vielfalt an Polyphenolen, die aussichtsreiche Bioaktivitäten, insbesondere antimikrobielle Eigenschaften aufweisen. Diese Spezies sind daher vielversprechende Forschungsobjekte für die Wissenschaft. Es wurde vermutet, dass der Brasilianische Pfefferbaum, welcher ebenfalls zu den Anacardiaceae gehört, die genannten Ziele erfüllt vor allem weil er bereits als Gewürz verwendet wird, weit verbreitet ist und aggressive Invasivität zeigt.

Die Charakterisierung der chemischen Strukturen mittels LC-MS und 2D NMR-Spektroskopie belegte die Anwesenheit von 30 Polyphenolen in den Früchten von *S. terebinthifolius* und von 32 in *S. molle*. Der Großteil von ihnen wurde für die Früchte oder die gesamte Spezies erstmalig berichtet. Die Verbindungen gehören zu drei Gruppen von phenolischen Verbindungen, den phenolischen Säuren, den Flavonoiden (Biflavonoide, Anthocyane) und den hydrolysierbaren Tanninen und beinhalten untypische Galloylierungsgrade. Unter den roten Pigmenten war ein bisher unbekanntes Anthocyan, das mittels NMR-Spektroskopie als 7-*O*-Methylpelargonidin-3-*O*-*β*-D-galactopyranosid charakterisiert wurde. Die separate Untersuchung von Exokarp und dem Rest der Steinfrucht lieferte tiefere Einblicke in die Phytochemie der Früchte. Dadurch konnte belegt werden, dass das Exokarp ein breiteres Spektrum an Polyphenolen aufweist, was vermuten lässt, dass die beobachteten Bioaktivitäten, wie beispielsweise allelopathische Effekte, auf diesen Verbindungen beruhen. Außerdem konnte hierdurch die Annahme bestätigt werden,

dass die untersuchten *Schinus* Spezies eine vergleichbare phenolische Zusammensetzung wie kürzlich betrachtete Vertreter der Anacardiaceae aufweisen. Die chemotaxonomische Betrachtung von Literaturdaten in Kombination mit den Ergebnissen dieser Arbeit zeigte dabei vergleichsweise selten vorkommende Untergruppen von Polyphenolen und ungewöhnliche Substitutionsmuster. Das Auftreten von galloyierten phenolischen Verbindungen und insbesondere methylierten Flavonoiden, darunter seltene 7-methoxylierte Vertreter, scheint ein chemotaxonomisches Merkmal der Anacardiaceae zu sein. Durch den Gewinn an chemotaxonomischem Verständnis wird die Suche nach neuen, natürlichen Verbindungen weiter erleichtern, da dieses Wissen auch auf andere Pflanzen, insbesondere verwandte Spezies, übertragen werden kann. Anhand des so vermuteten polyphenolischen Profils lässt sich ebenfalls ihr Potenzial hinsichtlich der Bioaktivität einschätzen. Auf Basis der Kenntnis der phenolischen Zusammensetzung und der Analyse von 18 verschiedenen Fruchtproben, hauptsächlich aus botanischen Gärten und von kommerziellen Anbietern, konnte des Weiteren eine Methode zur Differenzierung der beiden *Schinus* Spezies entwickelt werden. Diese Methode berücksichtigt die relativen Mengen an Anthocyanen und nutzt die statistische Methode der Clusteranalyse, um dadurch erstmals eine ressourcenschonende, einfache zu implementierende und zuverlässige Unterscheidung der Spezies zu gewährleisten. Dies ermöglicht die Authentifizierung der Gewürze, welche mit geringem Aufwand von Interessenvertretern der Lebensmittelindustrie oder der Lebensmittelüberwachung genutzt werden kann. Durch eine Vergrößerung der Probenanzahl und in Kombination mit einer Referenzdatenbank könnte dadurch sogar eine Unterscheidung von Varietäten erfolgreich durchgeführt werden.

Zusätzlich wurde die Extraktion des Exokarp und der verbleibenden Steinfrucht mit Hilfe der PLE und in Verbindung mit statistischer Versuchsplanung optimiert. Dadurch ließen sich die Extraktionsparameter und ihre Wechselwirkungen bestimmen, welche die Ausbeute der gewünschten Polyphenole signifikant beeinflussen. Durch diese Optimierung der PLE Parameter wird eine ökonomische Gewinnung von Extrakten in adäquaten Mengen für nachfolgende Aufreinigungsschritte und für zukünftige Untersuchungen der Bioaktivität ermöglicht.

Des Weiteren ist mit Hilfe der beschriebenen Methode für die Gewinnung von Anthocyanen für die NMR Analyse auch die Aufreinigung und Isolierung von weiteren gewünschten Anthocyanen und Biflavonoiden mit hoher Reinheit möglich. Diese Dissertation liefert somit nicht nur chemotaxonomische Erkenntnisse und Strategien zur Authentifizierung der beiden *Schinus* Spezies, sondern bereitet auch eine essentielle Basis für weitere Untersuchungen der bio- und technofunktionellen Eigenschaften ihrer Polyphenole. Darüber hinaus konnte gezeigt werden, dass sowohl invasive Pflanzen als auch essbare Vertreter der Anacardiaceae vielversprechende Quellen bei der Suche nach bioaktiven und antimikrobiellen Polyphenolen sind. Basierend auf den Ergebnissen und Erkenntnissen dieser Arbeit kann *S. terebinthifolius* als eine valide Quelle für bioaktive Verbindungen betrachtet werden. Die enthaltenen Polyphenole könnten für verschiedene Anwendungen in unterschiedlichen Bereichen eingesetzt werden, wie beispielweise für die Prävention und Behandlung von Krankheiten oder als natürliche technofunktionelle Lebensmittelzusatzstoffe.

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