Dietary A- and B-type procyanidins

Characterization and biofunctional potential of an abundant and diverse group of phenolics

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Abstract

Procyanidins (PCs) are phenolic compounds that belong to the class of flavonoids and are oligomers of monomeric (epi)catechin units. These monomeric units can be linked to each other by a single C4-C8 or C4-C6 linkage, which is referred to as B-type. Besides these single linkages an additional ether bond can be present, C2-O-C7 or C2-O-C5, which is referred to as A-type. PCs are highly abundant in our diet. Well known PC food sources are cocoa, apple, grape seeds, wine and nuts. After the intake of PC-rich sources health beneficial effects have been detected, which are mainly related to the prevention of cardiovascular diseases such as lowering of blood pressure.

The aims of this thesis were to study the bioavailability, bioconversion and bioactivity of purified PCs. Therefore, we first developed techniques for the efficient purification of both A- and B-type PCs from peanut skins and grape seeds, respectively. Furthermore, tools were set-up to analyze and characterize individual PCs in complex mixtures.

We showed that A-type PC dimers were absorbed from the small intestine of rats and that they were better absorbed than B-type PC dimers. The PC dimers were not conjugated or methylated upon absorption in contrast to their monomeric units (epi)catechin. Furthermore, the presence of A-type PC tetramers enhanced the absorption of B-type PC dimers.

The microbial conversion of B-type PC dimers was studied by exposing them to human microbiota. The main microbial metabolites were 2-(3,4-dihydroxyphenylacetic acid and 5-(3,4-dihydroxyphenyl)-γ-valerolactone. Based on these and other metabolites that were detected, a tentative microbial degradation route was proposed for B-type PC dimers, in which the interflavanic bond does not need to be cleaved upon degradation.

Subsequently, the vasorelaxing potential of purified PCs and their microbial metabolites was analyzed by measuring their effect on the NO production of endothelial cells. Both A- and B-type PCs showed a tendency (insignificant) to increase NO production with increasing degree of polymerization and several of their human microbial metabolites that were tested were inactive. Besides enhancing NO production, several other mechanisms could be targets of PCs and were also discussed.

This thesis increased our knowledge on the absorption, biotransformation and bioactivity of A- and B-type PCs. A possible interaction between oligomers with a high and low degree of polymerization, influencing absorption processes has been discussed, which suggests that until now the biofunctional potential of PAs has been underestimated.

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Chapter 1

General introduction

Proanthocyanidins in relation to cardiovascular health

The modern sedentary life together with a high-calorie diet has a large impact on health. As a consequence, lifestyle-related obesity is rapidly growing (*1-3*). Obesity is an important characteristic feature of the metabolic syndrome, which reflects the increase in risk factors for type 2 diabetes and cardiovascular diseases (CVD) (*4, 5*).

Mortality due to CVD, as one of the life-style related diseases, accounts currently for more than 30% of the deaths in the Netherlands (*6*). The number of hospitalizations for people with CVD was over 300.000 in 2004 (*7*), in a population of 16 million people. Smoking, hypertension and hyperlipidaemia are the prevalent risk factors related to the development of CVD, while obesity and diabetes mellitus have become more important (*5, 8*).

Efforts are made to unravel relations between our diet and the occurrence or, more important, the prevention of CVD. As an example, French people exhibited a lower incidence of cardiovascular mortality than expected based on their relatively high intake of saturated fat ("the French paradox"). The consumption of red wine was considered to be an important factor and an inverse relation between red wine consumption and mortality from CVD was found (*9*). Besides, evidence has been collected on beneficial effects of cocoa (chocolate) on CVD. Several studies reported that both after frequent intake and after a single dose of cocoa blood pressure was reduced (*10-13*).

One group of phenolic compounds, the proanthocyanidins, highly abundant in both product groups, gained considerable attention. This introduction will give background information on the occurrence of proanthocyanidins in dietary sources and the current knowledge on their bioavailability and bioactive properties.

Nature and occurrence of proanthocyanidins

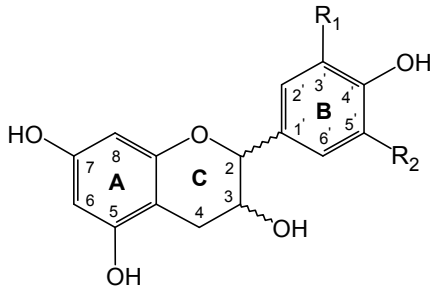
Classification of proanthocyanidins

Proanthocyanidins belong to the class of flavonoids, which have a characteristic C6-C3-C6 diphenylpropane skeleton. Biosynthesis of proanthocyanidins follows the phenylpropanoid pathway and as a final step condensation of flavan-3-ols and flavan-3,4-diols into oligomers, the proanthocyanidins, occurs (*14, 15*). The actual mechanism of condensation and the proportion flavan-3-ols and flavan-3,4-diols involved is not known (*16*). In the older literature, often the name leucoanthocyanidins or condensed tannins were used for proanthocyanidins (*17*). Later this was redefined and leucoanthocyanidins represented the class of monomeric flavan-3,4-diols, while proanthocyanidins were defined as oligomers of

monomeric flavan-3-ols. Both leucoanthocyanidins and proanthocyanidins bear the name of anthocyanidin. Leucoanthocyanidins refers to their biosynthetic conversion into anthocyanidins by the enzyme anthocyanidin synthase (leucoanthocyanidin dioxygenase) (*18*). The translation of leuco is white/colorless and a leuco compound is defined as a colorless compound, which can be converted to a dye by oxidation. The name of proanthocyanidins does not relate to their biosynthetic origin, but rather to their chemical behavior. Proanthocyanidins are converted into anthocyanidins when they are incubated in hot mineral acid. Leucoanthocyanidins also possess this feature. Hence the difficulties to distinguish the presence of both groups using a colorimetric method based on the formation anthocyanidins.

Subgroups of proanthocyanidins

As mentioned before, proanthocyanidins derive their name from the fact that they can be converted into the colored anthocyanidins when incubated in hot mineral acid (*19*). This phenomenon is still used as a rapid method to determine their presence in extracts (*20*). Proanthocyanidins can be subdivided into at least fifteen subgroups based on their hydroxylation patterns (*21*) on the A and the B-ring of the monomeric flavan-3-ol. The subgroups procyanidins, prodelphinidins and propelargonidins **(Figure 1)** are of prime importance in terms of human intake. The other 12 subgroups have been detected mainly in non-food sources (*21*).



PA subgroup	monomeric unit	R_1	R_2	food source
procyanidin	(epi)catechin	OH	H	apple, grape seeds, sorghum
prodelphinidin	(epi)gallocatechin	OH	OH	nuts, grapes, black currants
propelargonidin	(epi)afzelechin	H	H	berries, beans, cinnamon

Figure 1. Common proanthocyanidin subgroups and selected food sources in which they are present (*22*).

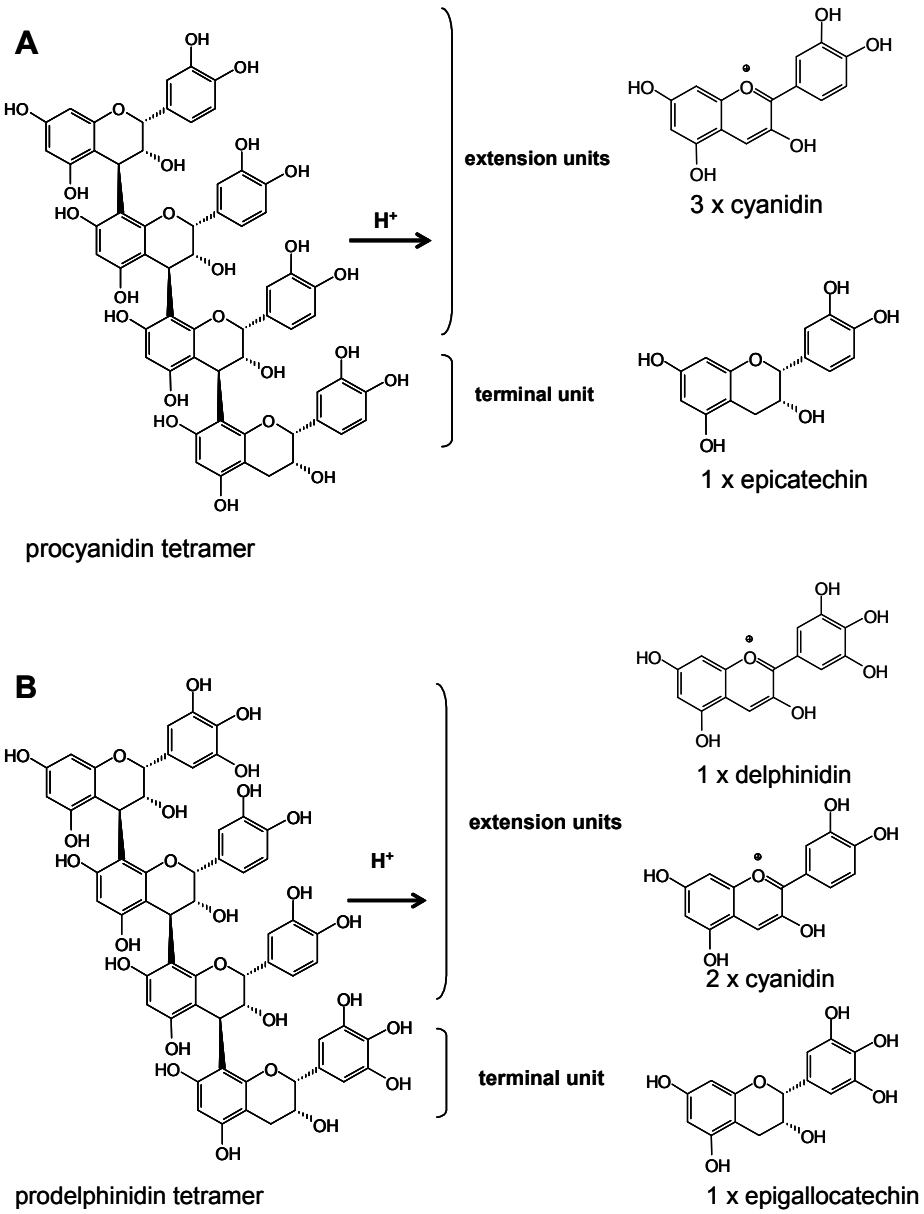


Figure 2. Depolymerization of a procyanidin (4 x epicatechin) (**A**) and an arbitrarily chosen prodelphinidin (2 x epicatechin and 2 x epigallocatechin) (**B**) tetramer when incubated with mineral acid.

The specific anthocyanidins that are formed upon heating in mineral acid (*23*) determine the name of the subgroup. Procyanidin is the most abundant subgroup present in foods and is a homogeneous group exclusively consisting of (epi)catechin units (**Figure 1**). When procyanidins are incubated in hot mineral acid, the extension units will be converted into cyanidins and the terminal unit will be released as the flavan-3-ol (epi)catechin. An example of the conversion of a procyanidin tetramer is given in **Figure 2A**. A second subgroup is prodelphinidin, a heterogeneous group, consisting of at least one (epi)gallocatechin unit besides (epi)catechin units. An example of acid-induced depolymerization of a prodelphinidin tetramer is given in **Figure 2B**. This tetramer is composed of two epigallocatechin units and two epicatechin units. The terminal unit will be released as flavan-3-ol, in this particular case epigallocatechin. The extension units, one epigallocatechin and two epicatechins, will be released as delphinidin and cyanidins, respectively. The third subgroup present in foods is propelargonidin.

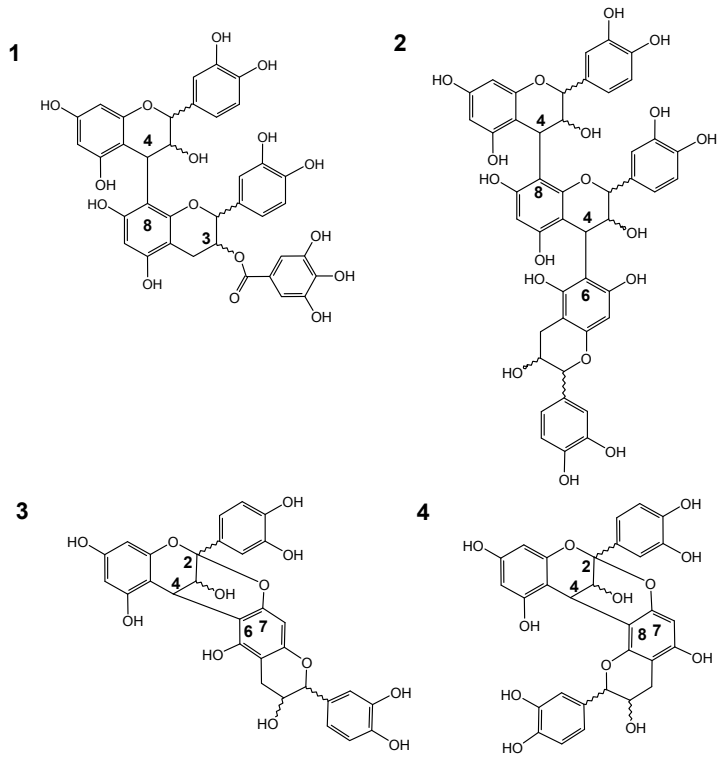


Figure 3. Representative structures of proanthocyanidins. The monomeric units can be linked through a single carbon-carbon bond (C4-C8 (**1**) or C4-C6 (**2**)) or with an additional ether bond (C4-C6, C2-O-C7 (**3**) or C4-C8, C2-O-C7 (**4**)). Galloyl substituents can also be present (**1**).

Similarly as prodelphinidins, it is a heterogeneous group, consisting of at least one (epi)afzelechin unit besides (epi)catechin units. Therefore, depending on the position of the monomeric units, acid depolymerization can result in the anthocyanidins pelargonidin and/or cyanidin (from the extension units) and the flavan-3-ols (epi)afzelechin or (epi)catechin (from the terminal unit).

The monomeric units can be linked through a single carbon-carbon bond: a C4-C8 or a C4-C6 linkage (**Figure 3**, structures 1 and 2, respectively). Proanthocyanidins that exclusively contain one of these C-C bonds are referred to as B-type proanthocyanidins. An additional bond, an ether bond, can be present between the monomeric units, usually a C2-O-C7 (**Figure 3**, structures 3 and 4) or a C2-O-C5 ether bond. Proanthocyanidins that contain both a C-C and an ether bond (C-O-C) between the monomeric units are called A-type proanthocyanidins. Furthermore, galloyl substituents at the C3 position (**Figure 3**, structure 1) have been detected, which are highly abundant in grape seed (*24, 25*).

The size of proanthocyanidins is indicated by their degree of polymerization (DP), which implies the number of monomeric units that are attached to each other. In certain cider apple varieties, the degree of polymerization ranged from 2 up to 190 (*26*). The DP can vary between different sources (**Figure 4**). Sources like barley, peanuts and raspberry mainly contain low DP oligomers, while the opposite applies to cranberry and pecan nuts (*22*).

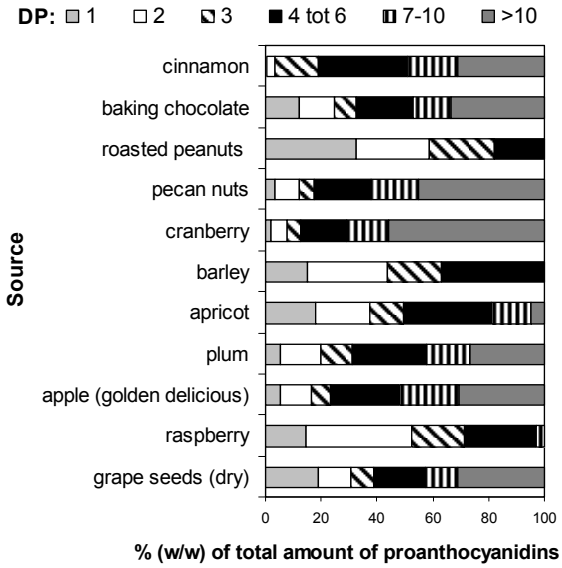


Figure 4. Abundance of proanthocyanidins of various degrees of polymerizations in different dietary sources expressed as proportion (% w/w) of the total amount of proanthocyanidins present (*22*).

Variations in the number and position of the bonds between the monomeric units, the structure of the monomeric units, additional substituents, and the DP, results in an enormous structural variety of naturally occurring proanthocyanidins.

Nomenclature

Initially, proanthocyanidins were named on the basis of an alphanumeric system (*27*). Dimers with an A-type interflavanic linkage were named A with an additional number as new structures were discovered, for example A1 and A2 (**Figure 5**) present in peanut skins (*28*). Dimers containing a single linkage (B-type) were annotated as B followed by numbers, for example B1 and B2 (**Figure 5**), which are often detected as the main dimers in major proanthocyanidin sources like apple (*29*), cocoa (*30*) and wine (*31*). This was continued with the notation C for trimers containing single linkages. However, this nomenclature was not informative about the monomeric composition. Furthermore, the interflavanic linkages were only indicated for dimers (A= double and B= single linkage) and not for higher oligomers, as C represents trimers without indicating if it contains single or double linkages. Nowadays, most authors use the nomenclature of proanthocyanidins that was first suggested by Hemmingway and coworkers (*32*), which has similarities with polysaccharide nomenclature. Translating this nomenclature to proanthocyanidins, the monomeric units are defined and the configuration of the interflavanic linkage (α or β) is given between brackets. Besides the hydroxylation pattern, the nomenclature of the monomeric units depends on the configuration at C2 and C3. This configuration determines whether the prefixes epi (epimer) or *ent* (enantiomer) should be used. For example the configuration 2R:3S represent catechin ((+)-catechin) and 2R:3R represents epicatechin ((-)-epicatechin). The configuration at C2 is almost always R, but when this is S the prefix *ent* should be used. For example 2S:3R represents *ent*-catechin ((-)-catechin) and 2S:3S represents *ent*-epicatechin ((+)-epicatechin).

Using this nomenclature, B1 becomes epicatechin-(4β-8)-catechin or abbreviated as EC-(4β-8)-C and A1 becomes epicatechin-(2β-O-7, 4β-8)-catechin. The disadvantage of this notation is that it becomes quite extensive for higher oligomers. Often both nomenclatures are used together.

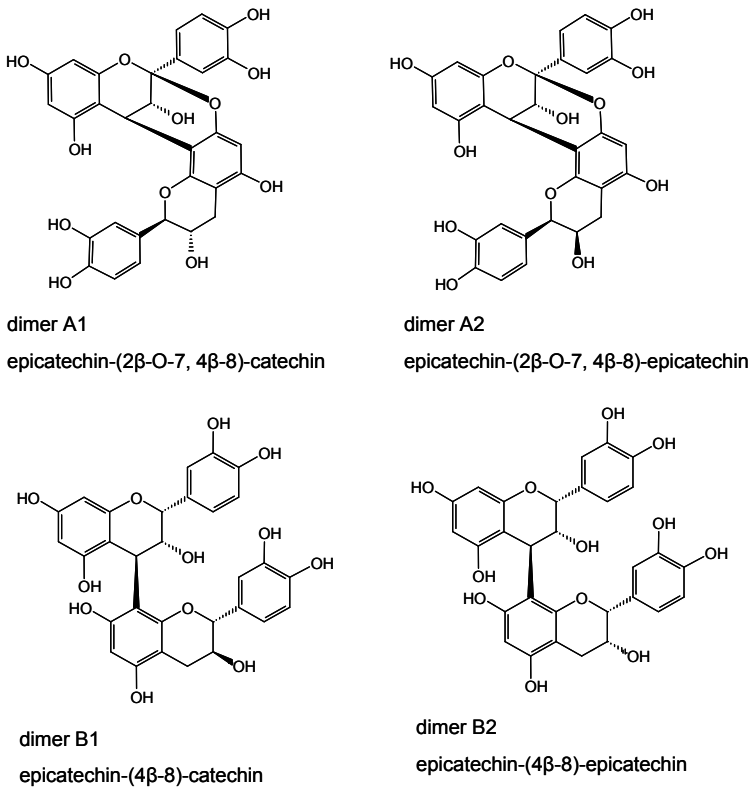


Figure 5. Structures and nomenclature of procyanidin dimers A1, A2, B1 and B2.

Dietary sources of proanthocyanidins

Proanthocyanidins are present in a wide variety of plant derived foods and beverages, like chocolate, apple, grapes, legumes, berries, peanuts, wine and several spices (*22, 33*). They are the second most abundant phenolic compound in our diet after lignin (*22*). In the US, the estimated intake of proanthocyanidins over the period 1981-1991 was 53.6 mg/day (*22*). More recently, Mink and coworkers (*34*) assessed the intake of proanthocyanidins in the Iowa Women's Health Study, a cohort of 35.000 postmenopausal women, to determine the relation to CVD mortality. Subjects were divided in five different groups on the basis of their intake. The group with the lowest and highest intake had a median intake of 62 (range of 0-90) and 524 (range of 343-3226) mg/day, respectively. Averaging the median intake over the five different groups that were used in this cohort, an average intake of 228 mg/day

can be estimated. The intake of 53.6 mg/day reported by Gu and coworkers (*22*) is similar to group with the lowest intake reported by Mink and coworkers (*34*). Both used the rather extended USDA database to determine the intake. It seems that 53.6 mg/day would underestimate the intake for a large part of the population, and 228 mg/day might be a more reliable estimate of the US intake.

For the Dutch population the proanthocyanidin intake has not been reported. The intake of monomeric flavan-3-ols in the Dutch population was estimated to be 50 mg/day (*35*) explained by the intake of tea (± 50%) and sources like chocolate, apple and wine. The concentrations (% w/w) of proanthocyanidins in chocolate apple and wine are higher compared to those of monomeric flavan-3-ols (**Figure 4**). For example, apple contains ~10-20 times, and chocolate ~8 times more proanthocyanidins than monomeric flavan-3-ols. (*22, 36*). Therefore, the intake of proanthocyanidins in the Dutch population might be comparable with the US data of Mink and coworkers (*34*).

Absorption of proanthocyanidins

Bioavailability of proanthocyanidins

Proanthocyanidins are known to be present in our diet, but their fate after ingestion is not fully understood. First, they should survive the acidic environment of the stomach. Procyanidin dimers that were incubated in simulated gastric juice (pH 2.0) for 3.5 h (*37*) were not stable and their concentration decreased with 30%. Simultaneously, epicatechin concentrations increased. Nothing was reported on the release of anthocyanidins. In the same study, higher oligomers were completely depolymerized, releasing dimers and epicatechin. In contrast, an *in vivo* study with six human volunteers showed that after ingestion of a cocoa beverage, containing proanthocyanidins, depolymerization did not occur during 1 h (*38*). After ingestion of the cocoa beverage the pH increased from 2.0 to 5.4, which is beneficial for proanthocyanidin stability. Furthermore, the food matrix might have contained protective substances that prevented proanthocyanidin degradation, but this was not reported. A study performed with ileostomy patients who ingested apple juice, confirmed that procyanidins will pass the stomach and reach the colon intact (*39*).

Several *in vivo* studies have been performed, which showed that procyanidins can be absorbed and subsequently appear in plasma (**Table 1**). These studies focused on the absorption of products/extracts containing B-type procyanidins like cocoa (*40*), apple (*41*) and grape seeds (*42-45*), or purified B-type procyanidins (*42, 46*). The majority of these *in vivo* studies suggested that only procyanidin dimers can be absorbed, but trimers have also been detected in urine and plasma of rats (*41, 44, 45*).

Table 1. Overview on the *in vivo* absorption of proanthocyanidins reported in literature.

PC source	species	dose [1]	metabolites [2,3,4,5]	reference
cocoa beverage	human (n=5)	375 mg/kg (~3.6 mg dimers/kg)	B2 (41 ± 4 nM, t= 2h)	(*40*)
grape seed extract	human (n=4)	2 g (18 mg dimers)	B1 (10.6 nM, t= 2h)	(*43*)
apple extract	rat (n=6)	1000 mg/kg (1412 mg dimers/kg)	B1 (170 nM, t=2h)	(*41*)
			B2 (400 nM, t=2h)	
			C1 (140 nM, t=2h)	
B2	rat (n=5)	50 mg/kg	B2 (500 nM, t= 0.5h)	(*46*)
			EC (210 nM, t= 0.5h)	
			3-OMeEC (110 nM, t=0.5h)	
B3	rat (n=15)	2% (w/w) in diet for 2 weeks	< 20 nM	(*42*)
grape seed extract	rat (n=15)	20-40% (w/w) in diet for 2 weeks	< 20 nM	
grape seed extract	rat (n=3)	1 g/kg (28.4 mg/kg dimers)	n.d.	(*44*)
			urine: B1-B4 (2.2% (w/w) of intake)	
			C2 (4.3% (w/w) of intake)	
grape seed extract	rat (n=8)	300 mg/kg, twice a day for 3 days	dimers and trimers, not quantified	(*45*)
			urine: dimers and trimers, not quantified	

[1] the dose was expressed as mg/kg body weight if possible. If applicable, the exposure time is indicated.
[2] plasma metabolites are given unless another matrix is given.
[3] EC= epicatechin, 3-OMeEC = 3'-O-methyl-epicatechin, B1= EC-(4β-8)-C, B2= EC-(4β-8)-EC, B3= C-(4β-8)-C, B4= C-(4β-8)-EC, C1= EC-(4β-8)-EC-(4β-8)-EC.
[4] EC and 3-OMeEC are not given when the source contained EC
[5] n.d. = not detected.

In vitro, dimer B3 (catechin-(4α-8)-catechin) and trimer C2 (catechin-(4α-8)-catechin-(4α-8)-catechin) were both absorbed by epithelial Caco-2 cells (*47*) in the same order of magnitude as mannitol, a marker for paracellular transport. Higher oligomers (average DP 6) were not absorbed. *In vivo*, trimers did not seem to be absorbed as easy as suggested by the *in vitro* assay (*47*). Nothing has been reported on the bioavailability of A-type procyanidins. This will be explored in this thesis.

A higher absorption of dimers compared to higher DP oligomers, resulted in an increased interest for procyanidin sources rich in low DP oligomers, especially dimers. Research efforts have aimed at developing processing methods to convert the naturally-occurring, more abundant high DP oligomers (**Figure 4**) to monomers and low DP oligomers. For example, the processing of cocoa might be adjusted (unfermented, blanched and without roasting) to obtain cocoa powder with higher amounts of monomers and dimers (*48*). Chocolate products enriched in monomers and low DP oligomers have recently been launched on the market (CocoaVia®, Mars). Another way to obtain low DP oligomers is to depolymerize the proanthocyanidins in mineral acid as indicated in **Figure 2**. The formation of anthocyanidins is prevented by the addition of a nucleophile, like catechin or epicatechin, to create low DP oligomers (*49, 50*). Also cysteine has been used as nucleophile, resulting in the commercially available extract Oligonol (Amino Up Chemical Company, Sapporo, Japan), which is rich in low DP oligomers (*51*).

Methylation and conjugation of proanthocyanidins

Already upon absorption, phenolic compounds can be conjugated by glucuronidation and/or sulfation and additionally, methylation can take place (*52*). The extent of conjugated metabolites can be determined by analyzing samples with and without β-glucuronidase/ sulfatase treatment. Conjugation and methylation are important processes, occurring upon absorption of epicatechin in humans (*48, 53*) and rats (*54-56*). Therefore, it might be hypothesized that conjugation and methylation are also important reactions for procyanidins.

Knowledge on the conjugation of proanthocyanidins is limited. Most studies dealing with absorption of procyanidins (**Table 1**) either analyzed the total amount of procyanidins (*40, 42, 43, 46*) or the unmodified amount of procyanidins (*44*). Only Shoji and coworkers (*41*) determined the difference between total and unmodified PCs, and showed that 10% of the absorbed procyanidins was conjugated.

The enzyme catechol-O-methyl-transferase has been reported to methylate phenolic compounds (*52*). *In vitro*, also procyanidin dimers were shown to be a substrate for this enzyme (*57*). Methylation of procyanidin dimers upon absorption has been reported occasionally. Small amounts of monomethylated procyanidin dimers have been detected twice based on MS data (*41, 57*). Besides monomethylated dimers, recently a tetramethylated procyanidin dimer was tentatively identified after administration of a grape

seed extract to rats (*45*). Also administration of specific wine-derived dimers (*58*), composed of two epicatechin units linked by an ethyl bridge, resulted in tetramethylated dimers, but in contrast to the other studies, in this study they were the only metabolites present in rat plasma (*59*). Standards of methylated and/or glucuronidated procyanidins are still lacking, which limits the development of accurate, rapid, quantitative and sensitive methods to measure them.

Unlike procyanidins, data on the bioavailability and metabolism of B-type dimers/oligomers of the other two subgroups; prodelphinidins and propelargonidins is lacking. Information on A-type proanthocyanidins is completely lacking. In this thesis, absorption and metabolism of A-type and B-type procyanidin dimers will be compared.

Cleavage of the interflavanic linkage of poanthocyanidins upon absorption

No consensus has been reached on the question whether (epi)catechin is a metabolite of procyanidins. This is partly due to the fact that most procyanidin sources that have been used in *in vivo* studies (**Table 1**) also contained (epi)catechin. Therefore, the origin of (epi)catechin and its methylated and/or conjugated metabolites detected in plasma could not be determined. Two studies that have used purified B-type dimers reported (epi)catechin as a procyanidin metabolite. Epicatechin was the major metabolite of B2 (epicatechin-(4β-8)-epicatechin) and B5 (epicatechin-(4β-6)-epicatechin), which was studied *ex vivo* by perfusion of the rat small intestine (*57*) and (methylated)epicatechin was present in rat plasma after the intake of B2 (*46*).

Detection of epicatechin or its metabolites raises the question whether procyanidins are actively converted into epicatechin upon absorption, or whether hydrolysis of procyanidins into epicatechin is caused by the experimental conditions. Therefore, in this thesis, purified procyanidin dimer, trimer, and tetramer fractions, free of monomers, will be used for bioavailability studies.

Microbial metabolism of proanthocyanidins

The majority of proanthocyanidins will reach the colon after passage through the small intestine, where only small amounts are absorbed (*39*). Metabolites formed by the microbiota in the colon might add to the beneficial health effects of procyanidins. It is known that the monomeric units of procyanidins, (epi)catechin, are degraded into phenylvaleric acids, phenylpropionic acids, phenylacetic acids, benzoic acids and hippuric acids (*60-63*). In addition, 5-(3,4-dihydroxyphenyl)-γ-valerolactone and 5-(3-hydroxyphenyl)-γ-valerolactone have been identified in various species, including man (*60, 64-69*).

Table 2 summarizes a total of twenty-eight different microbial metabolites that have been reported to originate from procyanidins. Fifteen of these have only been detected once. Phenylpropionic and phenylacetic acids (mainly *meta* hydroxylated) were the main microbial metabolites that have been detected, both *in vitro* and *in vivo*. Additional metabolites like benzoic and hippuric acids have been detected only *in vivo*. From these studies the metabolites derived from procyanidins seem similar to those reported for (epi)catechin, their free monomeric units (see above). However, four out of the six *in vivo* studies (*62, 63, 70-73*) used procyanidin sources that also contained monomers. Therefore, metabolites detected in those studies might also have originated from the monomers.

Three *in vitro* fermentations (*74-76*) with procyanidins have been performed. Two of these studies used a mixture of procyanidin oligomers (DP 2-71, and average DP 7) (*74, 75*) that were fermented with human microbiota. A drawback of using high DP oligomers is their antimicrobial properties (*77, 78*), which might be related to their interaction with proteins (*79, 80*). Only one of these studies used a low DP oligomer, i.e. purified B2 (*81*), but fermentation was performed with microbiota of rats instead of humans.

Fermentations performed with catechin and epicatechin, showed differences in bioconversion between animal and human microbiota (*66, 82*). In this thesis, we study the human microbial conversion of purified B-type dimers. Care was taken to exclude monomers during the isolation of the dimers.

Potential health effects of proanthocyanidins

Bioacitivity of proanthocyanidins (*in vitro* and animal studies)

A range of health related properties are reported for proanthocyanidins: Antiviral activity (*83, 84*), insulin-like activity (*85*), anti-tumor activity (*86, 87*), anti-inflammatory activity (*88, 89*), and antioxidant activity (*90*). Only few studies have used pure proanthocyanidins or proanthocyanidin fractions with a defined degree of polymerization. An increase in antioxidant activity with increasing DP has been reported, which was based on molarities (*91*). In contrast, another study reported that a B-type trimer (C1) showed higher antioxidant activity compared to higher oligomers (*83*). Compared to resveratrol and ascorbic acid, an A-type propelargonidin dimer and trimer had similar or better radical scavenging abilities, tested in different systems on a molar basis (*92*). Thus, both A- and B-type procyanidins have been reported to exert antioxidative capacities.

Only few studies showed the vasorelaxing properties of proanthocyanidins. These studies mostly tested the ability of procyanidins to relax animal aorta rings. Two studies reported vasorelaxing activity of procyanidins from cocoa (*93*) and grape seeds (*94*).

Table 2. Overview of *in vivo* (urine) and *in vitro* microbial metabolites of proanthocyanidins reported in literature.

reference	**74** [A]	**75**	**76**	**62** [C]
procyanidin source/composition	apple extract	willow tree extract	B3	B3, C2 and polymer fraction
		14C-labelled		
	Av DP 2-71	Av DP=7 no DP 1-3		Av DP=7 no DP 1-3
fermentation conditions	*in vitro* human (n=4)	*in vitro* human (n=1)	*in vitro* rat (n= -)	*in vivo* rats (n=5)
concentration/dose	0.2-7.9 mM [B]	5 mM	1.7 mM	1 g/kg diet 5 days
metabolites				
phenylpropionic acid	x	x	x	
3-hydroxy	x	x		x
4-hydroxy		x	x	
3,4-dihydroxy	x			x
phenylacetic acid			x	
3-hydroxy	x	x		x
4-hydroxy		x	x	
3,4-dihydroxy	x			x
3-methoxy-4-hydroxy				
benzoic acid	x			
3-hydroxy				x
4-hydroxy				x
3,4-dihydroxy				
3-methoxy-4-hydroxy				x
4-O-methyl-gallic acid				
hippuric acid				
3-hydroxy				x
4-hydroxy				x
ferulic acid				
m-coumaric acid				x
protocatechuic acid				x
phenylvaleric acid				
3-hydroxy		x		x
phenyl-γ-valerolactone				
3,4-dihydroxy				
3-hydroxy			x	
3-methoxy-4-hydroxy				
ethylcatechol				
3,4-diHPP-2-ol [H]			x	

[A] metabolites that showed a distinctive increase compared to the control
[B] includes flavan-3-ols, procyanidins and chlorogenic acid
[C] metabolites significantly increased ($P < 0.05$) after the B3 diet compared to a control diet
[D] metabolites significantly increased ($P < 0.05$) from 0-48 h
[E] only the dominating metabolites are given, serum metabolites are indicated with x[E]

71 pine bark extract	**63** [D] chocolate	**72** [E] sorghum bran extract	**70** [F] grape seed extract	**73** [G] grape vine extract
DP 2-4	DP 1-10	DP 1-polymers	+ DP 1	+ DP 1
in vivo human (n=1)	*in vivo* human (n=11)	*in vivo* rat (n=5-7)	*in vivo* human (n=69)	*in vivo* rat (n=5) *in vitro* (n= -)
960 mg	80 g	0-400 g/kg diet, 50 days	1000 mg/day 6 weeks	*in vivo* 15-75 mg/kg
	x	x	x	x [G 1-2]
	x	x	x	x [G 1-2]
		x[E]		
	x			
		x		x
				x [G 1-2]
	x			x
		x[E]		
		x[E]		x [G 1]
			x	
				x
				x
	x			
	x			
x				x [G 1-2]
x				
				x [G 1]

[F] metabolites significantly increased ($P < 0.002$)

[G] the main metabolites detected in urine are given. It was indicated when similar metabolites were detected in the faces (x^{G1}) or after *in vitro* fermentation (x^{G2})

[H] 3,4-diHPP-2-ol=1-(3-hydroxyphenyl)-3-(2,4,6-trihydroxyphenyl)propan-2-ol

The activity of the cocoa B-type procyanidins depends on their DP, i.e. only oligomers with a DP of 5 and higher were active (*93*).
Only one study tested the relaxation properties of A-type dimers and trimers (*95*). In that study it was found that trimers were more active than dimers. Several mechanisms might underlie the relaxation of arteries, one of which might be the stimulation of NO production by endothelial cells. Therefore, in this thesis we will test different A- and B-type procyanidin fractions for their NO-stimulating potential.

Bioacitivity of microbial metabolites of proanthocyanidins (*in vitro* and animal studies)

A higher activity for high DP oligomers might not be relevant, because high DP oligomers have a low bioavailability. Besides proanthocyanidins, the activity of microbial metabolites might also be of interest. Data on the bioactivity of such metabolites (phenylpropionic, phenylacetic acids, etc.) are very limited. One of the metabolites of procyanidins (**Table 2**), 5-(3',4'-dihydroxyphenyl)-γ-valerolactone, has been reported to have an antioxidative activity in between that of epicatechin and ascorbic acid (*65*). Furthermore, 3,4-dihydroxyphenylacetic acid showed anti-proliferative activity (*96*). Some of the microbial metabolites enhanced the expression of endothelial nitric oxide synthase (eNOS), the enzyme responsible for the NO production of endothelial cells, but the main metabolites (phenylpropionic and phenylacetic acids) were not included in that study (*97*). Therefore, in this thesis, we performed a study on the potential of several phenolic metabolites to enhance NO production of endothelial cells.

Human intervention studies

Williamson and Manach (*98*) reviewed 21 human intervention studies with proanthocyanidin-rich sources like cocoa (including chocolate), pine bark (Pycnogenol) and grape (including wine, grape juice and grape seed extracts) (**Table 3**). The biomarkers affected included a decreased LDL cholesterol level, decreased platelet aggregation, and increased antioxidant status, which are all related to the functioning of the vascular system.

Intervention studies that reported increased plasma antioxidant status have promoted the development of many commercial proanthocyanidin extracts (Oligonol®, Vitaflavan®, Oligopin®, Pycnogenol®, MegaNatural™ etc.) that have a health claim on this antioxidant activity. Evidence on the health potential of cocoa and grape/wine is focused on blood pressure (*13, 99, 100*), endothelial-dependent relaxation (*101*), and LDL concentration/oxidation (*102, 103*). The improved flow-mediated dilation after a cocoa drink rich in flavan-3-ols coincided with an increase of nitric oxide, a vasorelaxing mediator, in plasma (*104*). Recently, a meta-analysis of randomized placebo controlled trials (*105*) confirmed that chocolate intake increased flow-mediated-dilation and reduced blood pressure.

Table 3. Summary of human intervention studies with procyanidin rich sources in which biomarkers were significantly affected.

biomarkers significantly affected	sources	nr. studies	references
increased plasma anti-oxidant status	cocoa/chocolate, cranberry juice, pomegranate juice	5	(*98*)
decreased lipid peroxide/thiobarbituric acid reactive species	cocoa/chocolate, red wine, grape extract	3	(*98*)
endothelial-dependant dilatation	cocoa	2	(*98*, *101*)
increased flow-mediated dilatation	grape juice, cocoa	3	(*98-100*)
decreased blood pressure	cocoa, Pycnogenol	4	(*13*, *98-100*)
decreased susceptibility of LDL to oxidation	cocoa, grape juice, grape extract, pomegranate juice	7	(*98*,*102, 103*)
decreased platelet aggregation	cocoa, Pycnogenol, grape juice	4	(*98*)
decreased LDL plasma concentration	cocoa, Pycnogenol	3	(*98*, *100, 103*)

A drawback of all intervention studies mentioned is that none of them has been performed with pure procyanidins. Some used procyanidin-rich extracts, but also wine and juices have been used. Besides proanthocyanidins, these products contain a multitude of other compounds. Therefore, it is uncertain whether the potential health effects observed can be ascribed only to proanthocyanidins (*106*).

Thesis Outline

Proanthocyanidins are an important group of phenolic compounds in our diet and evidence on their beneficial health effects is increasing. However, many aspects on their occurrence in the diet, their bioavailability, metabolism, and bioactivity are still unknown, especially with respect to A-type procyanidins. Therefore, the aims of this thesis were: **i)** to determine the bioavailability and metabolism of A-type in comparison to B-type procyanidins, **ii)** to determine the human microbial metabolites of B-type procyanidins, and **iii)** to determine the potential of A- and B-type procyanidins or their microbial metabolites to improve vascular function by influencing NO-release of endothelial cells.

To achieve these aims it is important to isolate A- and B-type dimers and other oligomers, and concomitantly to develop of appropriate analytical tools for their characterization.

Peanut skins are a by-product from the peanut butter industry and a cheap, rich source of A-type procyanidins. Considering the reported health beneficial effects of proanthocyanidins, peanut skins might be an interesting source of proanthocyanidins for the use in food supplements. To determine the biofunctional potential of this source, the

proanthocyanidin composition is needed. Therefore, we characterized the molecular diversity of A-type proanthocyanidin dimers to heptamers present in peanut skin (**Chapter 2**). Often monomers are present in the extracts that are used to analyze the bioavailability or bioactivity of proanthocyanidins, which might interfere with the outcomes of these studies. To prevent this for our studies on bioavailability and metabolism, we isolated and purified several A- and B-type procyanidin dimers from peanut skin and grape seeds (**Chapter 3**). Care was taken to specifically exclude monomers from these isolates. Subsequently, the absorption and metabolism of these purified dimers, and fractions containing trimers and tetramers was studied by an *in situ* perfusion rat model (**Chapter 4**). A major fraction of the procyanidins ingested will reach the colon where they become substrates for microbiota. The microbial metabolites formed might (partly) explain the health effects found for proanthocyanidins. Therefore, an *in vitro* fermentation model with human microbiota was used to identify metabolites of B-type dimers (**Chapter 5**). Because intervention studies showed that proanthocyanidin intake seems to favorably affect endothelial function, the potential of various proanthocyanidins and their microbial metabolites to increase nitric oxide production of endothelial cells was studied (**Chapter 6**). Finally, the results from this thesis are discussed in a broader sense (**Chapter 7**).

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Chapter 2

Combined normal-phase and reversed-phase liquid chromatography/ESI-MS as a tool to determine the molecular diversity of A-type procyanidins in peanut skins

Based on: Maaike M. Appeldoorn, Jean-Paul Vincken, Mark Sanders, Peter C.H. Hollman and Harry Gruppen, Combined normal-phase and reversed-phase liquid chromatography/ESI-MS as a tool to determine the molecular diversity of A-type procyanidins in peanut skins, *submitted*

Abstract

Peanut skins, a by-product of the food industry, are a rich source of proanthocyanidins, which might be used in food supplements. Data on the molecular diversity of proanthocyanidins in peanut skins is limited and conflicting with respect to the ratio of double (A-type) versus single linked (B-type) flavan-3-ol units.
We combined NP and RP-LC-MS to analyze the molecular diversity of proanthocyanidins in a 20% (v/v) MeOH extract of peanut skins. Monomeric to pentameric fractions were pre-purified using NP-HPLC and further separated by RP-HPLC-MS. With this method, 83 different proanthocyanidin molecular species were characterized and quantified. A-type PC oligomers were predominant and represented 97.6 % (w/w) of the extract. In addition, the position of the A-linkages in 16 trimers and 27 tetramers was determined and appeared to occur at all possible positions. The majority of trimers and tetramers with one or more A-linkage(s), always had an A-linkage at the terminal unit.

Key words: peanut skins, proanthocyanidins, A-type, quantification, molecular diversity, NP-HPLC, RP-HPLC

Abbreviations used: DP, degree of polymerization; E, extension unit; HRF, heterocyclic ring fission; PA, proanthocyanidin; PC, procyanidin; PD, prodelphinidin; PP, propelargonidin; QM, quinine methide fission; RDA, retro-Diels-Alder fission; T, terminal unit

Introduction

Proanthocyanidins (PAs), present in a wide variety of food products and beverages, are the second most abundant class of phenolic compounds in our diet (*1*). Intervention studies with PA-rich extracts and products like cocoa and wine suggest protective effects of PAs against cardiovascular diseases (*2, 3*). Peanut skins, a by-product from the peanut butter industry, might be an excellent source for the production of PA-rich extracts for use in food supplements, like the ones already available from grape seed (Vitaflavan, DRT Nutraceutics, France) and pine bark (Pycnogenol, Horphag Research, Switzerland). To explore the full potential of peanut skins as a food supplement, it is important to know the molecular diversity of the PAs present.

PAs can be divided into several groups. The most common PAs, occurring in food sources such as peanut skins, are the procyanidins (PCs) (*1*), exclusively consisting of (epi)catechin units. Besides PCs, prodelphinidins (PDs), a heterogeneous group consisting of at least one (epi)gallocatechin unit and additional (epi)catechin units, and propelargonidins (PPs), a heterogeneous group consisting of at least one (epi)afzelechin unit and additional (epi)catechin units, occur in food sources.

The monomeric units of PAs are linked through a C4-C8 or C4-C6 bond (B-type), which can coexist with an additional C2-O-C7 linkage (A-type). Each proanthocyanidin oligomer contains one terminal unit of which the C-ring is not connected to another monomeric unit. All other units are called extension units. The most well-known sources of A-type PAs, mainly PCs, are peanuts, plums, cranberries and cinnamon (*1, 4*), but data about the molecular diversity of PCs are limited. The position of A-linkages in several tetramers and pentamers from plums and cinnamon has been identified by mass spectrometric (MS) analysis on the basis of their product ions (*5*). The position of the A-linkage can be source dependent. In plums, it was located at the terminal unit, while in cinnamon it was located between the extension units. Similarly, the monomeric composition within prodelphinidins and propelargonidins, ((epi)catechin, (epi)gallocatechin and (epi)afzelechin), and positions of the monomers in the molecule has been determined with MS analysis (*5-7*).

Peanut skins have a high PA content (17% (w/w)) (*8*). The degree of polymerization (DP) of PAs in peanut skins has been reported to range from 2 to 8, while only low amounts of monomers are present (*9, 10*). In the skins, both C4-C8 and C4-C6 linked A and B-type PCs have been identified (*8, 11, 12*). Reports concerning the ratio of double (A-linkage) / single (B-linkage) linked flavan-3-ol units in peanut oligomers are contradictory. Lazarus and coworkers (*10*) analyzed a peanut PC extract, containing monomers up to octamers, with NP-HPLC-UV-MS to determine the abundance of A-linked oligomers. They found that the ions, detected with MS, corresponding to A-linked

oligomers predominated over the ions corresponding to B-linked oligomers, but the positions of the A-linkages were not determined. Furthermore, their analytical technique (NP-HPLC) underestimated the molecular diversity, because it poorly separates oligomers of the same DP. In contrast to Lazarus and coworkers (*10*), NMR data of Karchesy and Hemingway (*8*) showed that the A-linkages were rare in a polymer fraction of peanut skins (linkage ratio of A:B was 1:15). The position of the A-linkages in peanut skins has only been identified in one trimer and one tetramer (*12*).

In the present study, we combined NP and RP-LC-MS to be able to analyze molecular PA species that are present in peanut skins. NP-HPLC has been used before to separate and characterize peanut PCs (*10*), but we used it as a first step to obtain fractions of monomers to pentamers. Subsequently, each fraction was further separated by RP-LC-MS, which enabled us to determine molecular diversity of peanut skins PCs, which appeared to be much larger than reported before. Additionally, the most abundant molecular species were determined and the positions of A-linkages in a large number of trimers and tetramers were established.

Materials and Methods

Materials

Peanut skins were kindly provided by Imko-The Nut Company BV (Doetinchem, The Netherlands). Organic solvents used for extractions were of analytical grade. Organic solvents used for HPLC analysis were all of HPLC grade. Hexane, dichloromethane, acetonitrile and acetone were purchased from Sigma-Aldrich (Steinheim, Germany), methanol from Mallinckrodt Baker B.V. (Deventer, The Netherlands) and ethyl acetate, sulfuric acid, hydrochloric acid and glacial acetic acid from Merck (Darmstadt, Germany). Other chemicals were of analytical grade and purchased from Merck (Darmstadt, Germany). Milli-Q water from a Millipore system was used.

Extraction

Peanut skins (75 g) were defatted with hexane using soxhlet extraction. The residue was air dried. The defatted skins (59.6 g) were extracted successively three times with 1L of 20% (v/v) aqueous MeOH, three times with 1L of 70% (v/v) aqueous MeOH, and three times with 1L of 70% (v/v) aqueous acetone. After each extraction the suspension was filtered over a 595 ½ filter (Schleicher & Schuell, Dassel, Germany) after which the retentate was subjected to the next extraction. The three 20% (v/v) MeOH fractions were combined, as well as the three 70% (v/v) MeOH and the three 70% (v/v) acetone fractions. Subsequently, they were concentrated with a rotary evaporator and lyophilized, resulting in three extracts

referred to as 20MeOH, 70MeOH and 70Acetone. During the extractions and evaporation, light was excluded as much as possible by the use of marquees and tinfoil. The three extracts were subjected to solvent partitioning for further purification. The 20MeOH extract was put in a separation funnel to which 500 mL water and 500 mL ethyl acetate was added, and placed in an ultrasonic bath for 10-15 min at room temperature. After vigorous mixing, the water phase and ethyl acetate phase were collected separately. The water phase was re-extracted twice following the same solvent partitioning procedure, resulting in three ethyl acetate phases and one water phase. The ethyl acetate phases were combined and subsequently extracted with water (1:1, v/v) twice. At the end of the partitioning procedure, one ethyl acetate phase and three water phases were obtained. For the 70MeOH and the 70Acetone extracts the same procedure was followed. The ethyl acetate was evaporated with a rotary evaporator, and the material was dissolved in water and lyophilized, resulting in three final extracts referred to as: 20MeOH-EA, 70MeOH-EA and 70acetone-EA. The water phases of each extract were analyzed for their total neutral saccharide contents.

Analysis of the total neutral saccharide content

The total neutral saccharide content was determined according to the Dubois method (*13*) with some alterations. A stock solution of 150 µg glucose/mL water was used to make a standard curve: 0, 50, 100 and 200 µL of the glucose stock was filled up to 1000 µL with a 2.5% (w/v) phenol (Fluka Chemie GmbH, Buchs, Switzerland) solution in water (R^2= 0.9965). From the water phases, obtained after extraction, 100-200 µL was taken and filled up to 1000 µL with the 2.5% (w/v) phenol solution. After addition of 2.5 mL sulfuric acid (96% w/w) and cooling down, the absorbance was measured at 490 nm in an UV 1601 spectrophotometer (Shimadzu Benelux B.V, ‘s Hertogenbosch, The Netherlands).

Determination of PA oligomer composition by NP-HPLC-UV-MS

The presence of PA oligomers in the 20MeOH-EA, 70MeOH-EA and 70acetone-EA extract was determined by NP-HPLC-UV-MS (*14*). A Thermo Spectra system was used containing a P 4000 pump, an AS 300 autosampler and an UV 3000 detector (Thermo Separation products, Fremont, CA, USA). Analysis was performed on a Luna Silica (2) column (4.6 mm ID x 250 mm, 5 µm particle size, Phenomenex, Torrance, CA) operated at room temperature. Samples were dissolved in methanol. The mobile phase consisted of (A) dichloromethane, (B) methanol and (C) acetic acid/H_2O (50% v/v). The flow rate was 1 mL/min and detection was performed at 280 nm. The elution profile was as follows; C was kept constant at 4% throughout the elution; 0-20 min, B: linearly 14%-23.5%; 20-50 min, B: linearly 23.5%-35% followed by a washing step at 96% B for 10 min and reconditioning of the column.

An LCQ Classic equipped with an ESI source was coupled to the HPLC system by means of a splitter (Accurate, LC Packings, Amsterdam, The Netherlands), which reduced

the flow rate 20 times. The MS detector was controlled by Xcalibur software (Thermo Finnigan, San Jose, CA). Measurements were performed in the negative mode with an ion spray voltage of 4.5 kV, a capillary voltage of -5.0 V and a capillary temperature of 270 °C. The scan range was set from *m/z* 100-2000. The MS^2 function was performed in the data dependent mode. The collision energy value was 27%.

Preparative isolation of monomers to pentamers by NP-HPLC

Fractionation by DP was performed on a normal phase column, Inertsil PREP-SIL 30 mm ID x 250 mm with a 10 μm particle size (GL sciences Inc., Tokyo, Japan). A Waters system equipped with a 2767 sample manager, a 2525 binary gradient module, a 2996 photodiode array detector (PDA) and an UV fraction manager was used (Waters Inc., Etten-Leur, The Netherlands). The binary mobile phase consisted of (A) hexane and (B) acetone. The elution profile was as follows: 0-30 min, B linearly 40%-60%; 30-50 min, B isocratic on 60%; 50-70 min, B linearly 60%-75% followed by a washing step at 98% B for 3 min and reconditioning of the column for 10 min. The flow rate was 27.2 mL/min and PDA spectra from 210-300 nm were recorded. Each run, 10 mL (~ 50 mg/mL acetone/hexane/ethanol (7:3:2) 20MeOH-EA extract, in total 5 runs: 2654 mg) was applied. Fractions (18 mL) were collected during 90 min and pooled into 9 fractions: F1 (13.6-22.3 min: 55 mg), F2 (22.3-28.2 min: 16 mg), F3 (28.2-38.1 min: 447 mg), F4 (38.1-46.8 min: 82 mg), F5 (46.8-62.0 min: 346 mg), F6 (62.0-64.0 min: 59 mg), F7 (64.0-71.2 min: 304 mg), F8 (71.2-77.2: 257 mg) and F9 (77.2-90.0: 651 mg). These fractions (total of 2217 mg = 84% (w/w) recovery) were rotary evaporated, lyophilized and weighted prior to further analysis.

Characterization of PA oligomers by RP-UPLC-UV-MS

Fractions F1 to F4

These fractions were separated on an XterraRP dC18, 4.6 mm ID x 150 mm column with a 3.5 μm particle size (Waters) (*14*). A Thermo Spectra system was used containing a P 4000 pump, an AS 300 autosampler and an UV 3000 detector (Thermo Separation products, Fremont, CA, USA). Samples were dissolved in methanol. The mobile phase was composed of (A) H_2O + 0.1% (v/v) acetic acid and (B) acetonitrile + 0.1% (v/v) acetic acid. The flow rate was 0.7 mL/min and detection was performed at 280 nm. The elution profile was as follows; the first 5 min isocratic on 10% B; 5-35 min, B: linearly 10%-30%; 35-40 min, B: linearly 30%-90% and reconditioning of the column. MS analysis was similar as with NP-HPLC-UV-MS.

Fractions F5 to F9

These fractions were analyzed by RP-UPLC-UV-MS on an Accela system equipped with an Accela pump, column oven (50 °C), autosampler and PDA detector (Thermo Scientific),

and controlled by Xcalibur software (Waters). Analysis was performed on an Acquity UPLC-BEHC18 Shield, 2.1 mm i.d. x 150 mm column with 1.7 µm particle size (Waters). The different oligomeric fractions were dissolved (1 mg/mL) in 0.1% (v/v) aqueous acetic acid and 5 µL was injected. The mobile phase was composed of (A) H_2O + 0.1% (v/v) acetic acid and (B) acetonitrile + 0.1% (v/v) acetic acid. The flow rate was 0.6 mL/min and the column oven temperature was set on 50 °C. Detection was performed at 280 nm. The elution profile was as follows: The first 0.25 min isocratic on 9% B; 0.25-18 min, B: linearly 9%-32.5%; 18-19 min, B: linearly 32.5%-90%; 19-23 min, B: isocratic on 90% followed by reconditioning of the column for 3 min.

The UPLC was connected to an MS detector by means of a splitter (Analytical Scientific Instruments (ASI), El Sobrante, CA), which reduced the flow rate to 0.3 mL/min. An LTQ XL equipped with an ESI Source was used and controlled by Xcalibur software (Thermo Scientific, Waltham, MA). Measurements were performed in the negative mode with an ion spray voltage of 4.5 kV, a capillary voltage of 5.0 V and a capillary temperature of 200 °C. Optimal collision energy values (CID) were obtained by infusion of the different oligomeric mixtures and were: *m/z* 575, CID 40%; *m/z* 863, CID 35%; *m/z* 1149, 1151, 1439 and 1437, CID 40% and *m/z* 1435, CID 45%. For each oligomeric fraction, a full scan was made within the range of *m/z* 150-2000 and MS^2 scans were recorded for further characterization and to determine the position of the A-linkage.

Results

Oligomeric composition of the extracts

Successive extraction of defatted peanut skins with 20% MeOH, 70% MeOH and 70% acetone yielded 15.8, 6.2 and 5.1 g of dry material, respectively. Additionally, ethyl acetate partitioning resulted in a final yield of 2.8 g 20MeOH-EA and 0.9 g 70MeOH-EA extract. The ethyl acetate partitioning of these two fractions removed 82-86% (w/w) dry material, including 34-49% (w/w) glucose equivalents. For the 70Acetone-EA extract 27% (w/w) glucose equivalents were removed by ethyl acetate partitioning, and a final yield of less than 0.1 g was obtained.

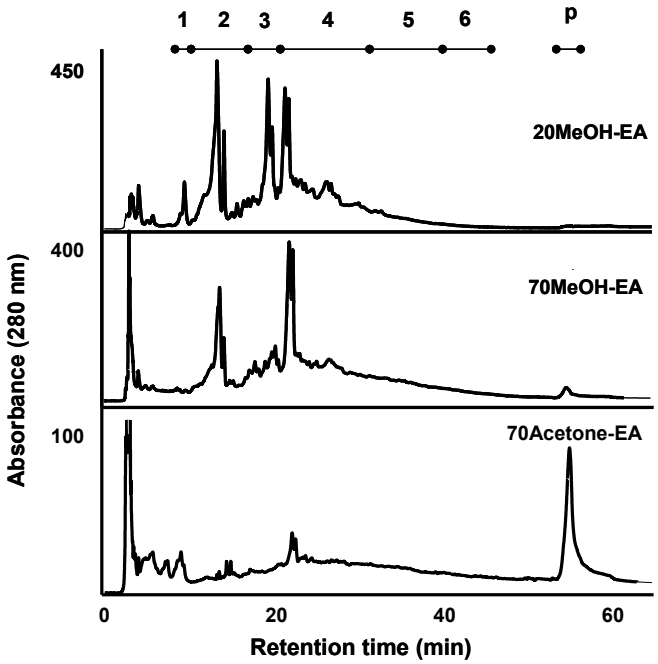

Figure 1. Normal phase chromatograms of the extracts 20MeOH-EA, 70MeOH-EA and 70Acetone-EA. The numbers 1 to 6 represent the DP, determined by MS analysis, and p represents polymeric material.

The oligomeric composition of each extract was determined by NP-HPLC-UV-MS. NP-HPLC enables separation by DP (*15*) (**Figure 1**). The DP, depicted as numbers in **Figure 1**, was assigned based on the *m/z* values. As an example, **Figure 2** shows the precursor ions present in the 20MeOH-EA extract, which contained a range of monomers (*m/z* 289) to hexamers (*m/z* 1725). On the basis of NP-HPLC-MS, only precursor ions of A-linked PCs were present (*m/z* 575, 861, 863, 1151, 1149, 1437 and 1725), which can be distinguished from B-linked oligomers (e.g. *m/z* 577, 865, 1153, 1441, and 1729) by the 2 Da difference for each A-linkage present. The 20MeOH-EA extract was richer in small oligomers than the other extracts (**Figure 1**). The acetone extract contained mainly polymeric material, eluting at the end as one unresolved peak. The high yield of the 20MeOH-EA extract and its large variety in PCs with different DPs made it a good source to determine the molecular diversity of A-type oligomers present in peanut skins. Nine fractions, F1 up to F9 (**Figure 3**), were obtained by separation using preparative NP-HPLC.

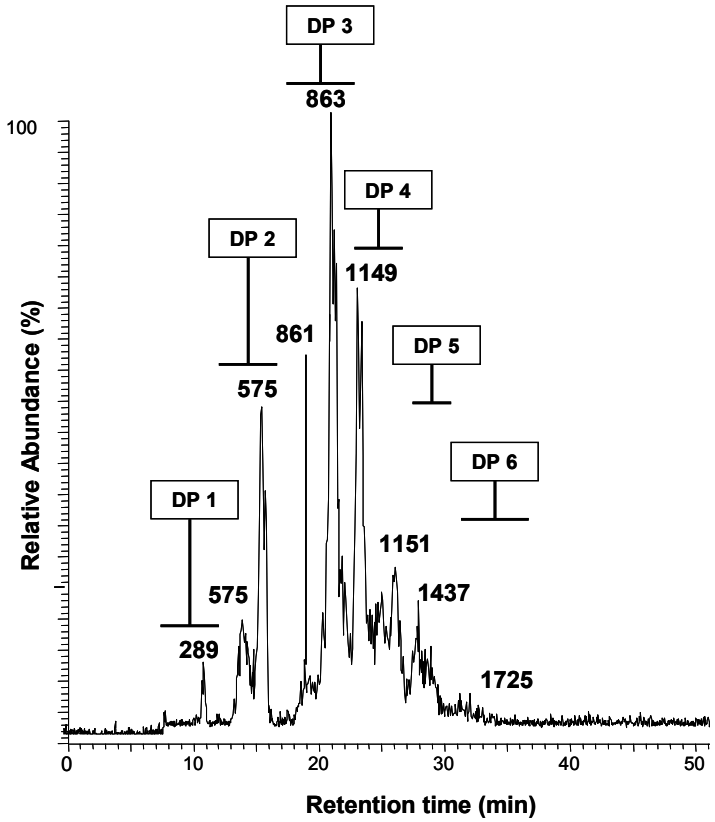

Figure 2. Precursor ions that were detected in the 20MeOH-EA extract, analyzed by NP-HPLC-UV-MS, using identical conditions as in **Figure 1**. DP represents the degree of polymerization.

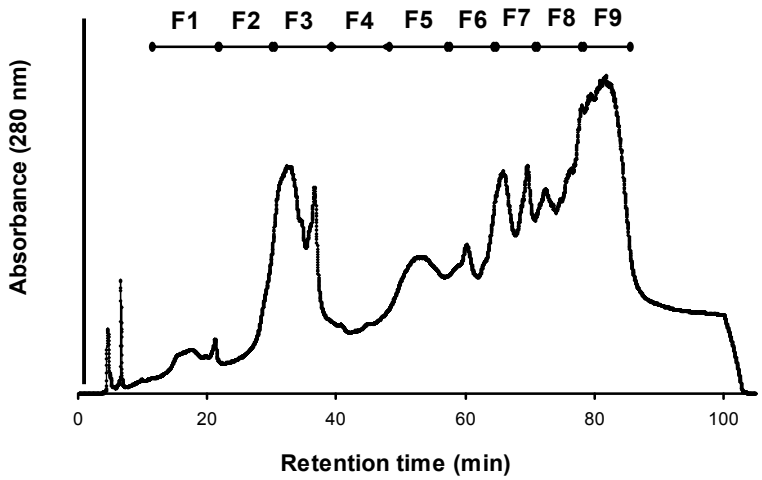

Figure 3. Separation of the 20MeOH-EA extract with preparative normal-phase HPLC. Nine fractions were collected: F1-F9.

Molecular diversity of procyanidins in peanut skins

Monomers and dimers

Fractions F1 to F4 were characterized with RP-HPLC-UV-MS (**Figure 4**). By comparison with standards it could be shown that fraction F1 exclusively contained catechin (**1**) and epicatechin (**2**) in a molar ratio of 5:1. Only A-type dimers were detected in fraction F2. The composition of each peak, which was determined by their precursor ions ($[M-H]^-$) and product ions, is given in **Table 1**. Peaks **3-6** in F2 represented A-type PCs (*m/z* 575). Furthermore, peak **7** (*m/z* 589) might represent an A-type methylated PC dimer, but no MS^2 data were obtained to confirm this finding. Besides PC dimers, fraction F2 also contained an A-type propelargonidin dimer, consisting of one (epi)afzelechin and one (epi)catechin unit (**8**) (*m/z* 559).

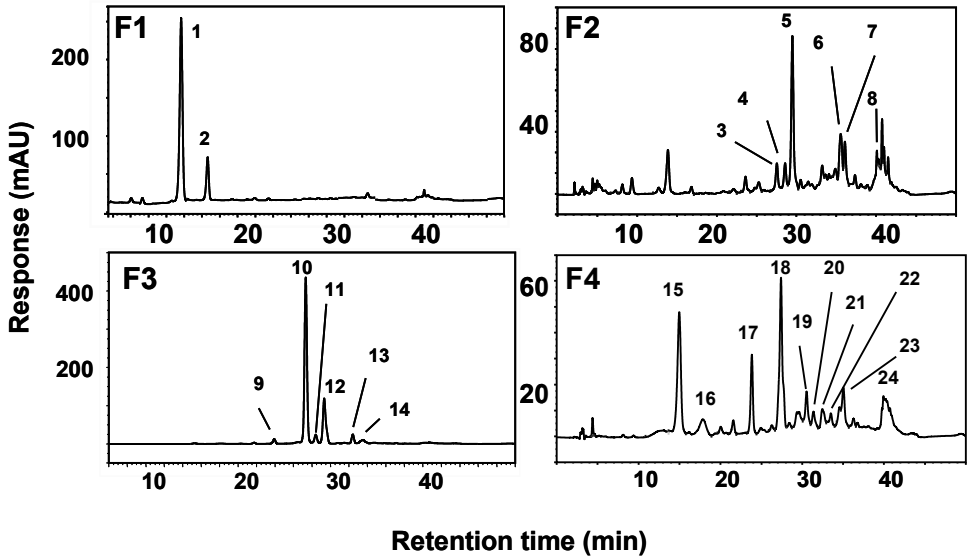

Figure 4. RP-HPLC profiles of fractions F1-F4 (**Figure 3**). Compositional information on the peaks is given in **Tables 1** and **2**.

Table 1. Compositional information of the peaks observed in **Figures 4** and **7** based on MS analysis.

DP	nr of A-bonds	composition[a]	$[M-H]^-$	product ions	peak numbers	% (w/w) of 20 MeOH-EA
1	0	(epi)cat	289	245	1	2.5
2	0	(epi)cat	577	451, 425, 407, 289, 287	9, 14-17	2.4
2	1	(epi)afz-A-(epi)cat	559	433, 407, 289, 269	8	< 0.1
2	1	(epi)cat	575	449, 423, 407, 289, 285	3-6, 10-13	19.9
2	1	(epi)cat + CH_3	589		7	< 0.1
3	0	(epi)cat	865	739, 713, 577, 575	57	< 0.1
3	1	(epi)cat	863	737, 711, 693, 575, 451, 411	18-20, 25-37, 39-47, 56, 60	17.0
3	2	(epi)cat	861	735, 709, 575, 571	21-23, 38, 55	1.6
3	2	(epi)cat + (epi)afz	845	719, 693, 677, 575, 555, 289	24	0.5
4	1	(epi)cat	1151	1025, 999, 863, 575	59, 65, 71-75, 77, 79, 80, 82, 89	4.3
4	2	(epi)cat	1149	863, 861, 859, 575, 573	48-51, 53, 58, 61-64, 66-69, 76, 78, 83, 85, 90	18.8
4	3	(epi)cat	1147		54, 70	0.1
4	2	(epi)cat + (epi)afz	1133		52	< 0.1
5	2	(epi)cat	1437	1311, 1285, 1149, 863, 861, 573	81, 84, 86-94, 97, 99-103, 105-108, 110	26.0
5	3	(epi)cat	1435		95, 96	0.4
6	1	(epi)cat	1727		98, 104	2.2
6	3	(epi)cat	1723		110-114	3.5
7	3	(epi)cat	2011		109	0.8

[a] The A represents a double (A-type) bond, (epi)cat = (epi)catechin, (epi)afz= (epi)afzelechin, CH_3 = an additional methyl group

A tentative structure of this dimer, with (epi)afzelechin as the extension unit (E) and (epi)catechin as the terminal unit (T), was drawn based on the product ions detected (**Figure 5**). Three characteristic fragmentation routes have been described for PAs: Quinone methide fission (QM), retro-Diels-Alder fission (RDA) and heterocyclic ring fission (HRF) (*5, 16*). The product ions resulting from QM cleavage of the terminal ($_T$) and extension ($_E$) unit, $[M_T - H]^-$ and $[M_E - 5H]^-$ for A-type dimers, provided information about the monomeric sequence within propelargonidins and prodelphinidins (*5, 17*). The QM product ions of the dimer (*m/z* 559) were *m/z* 289 $[M_T - H]^-$ and *m/z* 269 $[M_E - 5H]^-$ (**Figure 5**), representing an (epi)catechin [290 Da - H = 289]$^-$ and (epi)afzelechin unit [274 Da - 5H= 269]$^-$, respectively.

RDA fragmentation for A-type dimers has been reported to occur mainly at the terminal unit (*5*). The RDA product ion of *m/z* 407 indicated a loss of 152 Da, confirming that the terminal unit is an (epi)catechin unit. Also the RDA *m/z* 391 (407 - 16 Da) was present, which has been reported for A-type dimers (*18*). Based on both the QM and RDA product ions, we concluded that (epi)catechin was the terminal unit, and (epi)afzelechin the extension unit. Product ions derived from RDA, QM and HRF fragmentation could only be used to determine the position of (epi)afzelechin and (epi)catechin units, but not to distinguish between the epimers.

The dimers in fraction F3 have been identified as described in detail elsewhere (*14*). In brief, F3 contained four A-type PC dimers (**10-13**), of which A1 (**10**) and A2 (**12**) were most abundant, and two B-type PC dimers (**9** and **14**). Fraction F4 contained some B-type PC dimers (**15-17**).

Trimers to heptamers

Besides B-type PC dimers, fraction F4 also contained A-type PC trimers with one (**18-20**) or two (**21-23**) A-type linkages. Additionally to the PC trimers present, an A-type propelargonidin trimer was detected (peak **24**, *m/z* 845). In a similar way as described for the propelargonidin dimer (peak **8**, **Figure 5**), a tentative structure on the positions of the monomeric units is given in **Figure 6** based on the product ions detected. The RDA product ion of *m/z* 693 indicated a loss of 152 Da. Therefore, the terminal unit was annotated as (epi)catechin. Also the RDA product ion *m/z* 677 (693 -16 Da) was present. The identified QM product ions had *m/z* values of 575, 289 $[M_T - H]^-$ and *m/z* 555 $[M_E - 5H]^-$ (**Figure 6**). Based on these QM and RDA product ions, the trimer was characterized as (epi)afzelechin-A-(epi)catechin-A-(epi)catechin, in which A represents an A-linkage.

To improve resolution of the complex, multi-component chromatograms, fractions F5 to F9 were analyzed with RP-UPLC-UV-MS (**Figure 7**). Fraction F5 exclusively contained A-type PC trimers. Fractions F6 and F7 also contained A-type PC trimers, and one B-type PC trimer (*m/z* 865) (**57**) was detected.

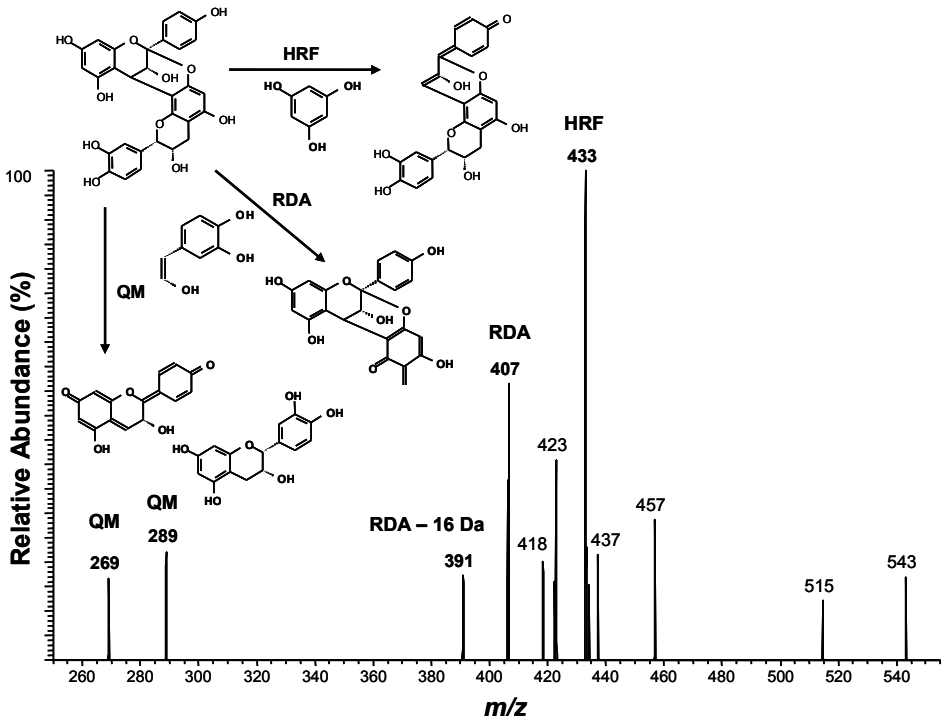

Figure 5. Fragmentation pattern of peak 8 (*m/z* 559; **Figure 4**), consistent with a structure composed of (epi)afzelechin (extension unit) and epi)catechin (terminal unit).

A-type PC tetramers with one (*m/z* 1151), two (*m/z* 1149) or three (*m/z* 1147) A-linkages were detected in fractions F6 to F8. B-type PC tetramers (*m/z* 1153) did not occur in any fraction. One peak (*m/z* 1133) (**52**) was tentatively identified as a propelargonidin tetramer, consisting of one (epi)afzelechin unit, three (epi)catechin units and two A-linkages. No MS^2 data could be obtained to confirm this finding. PC pentamers, hexamers and one heptamer (**109**), containing up to three A-linkages, were detected in fractions F8 and F9.

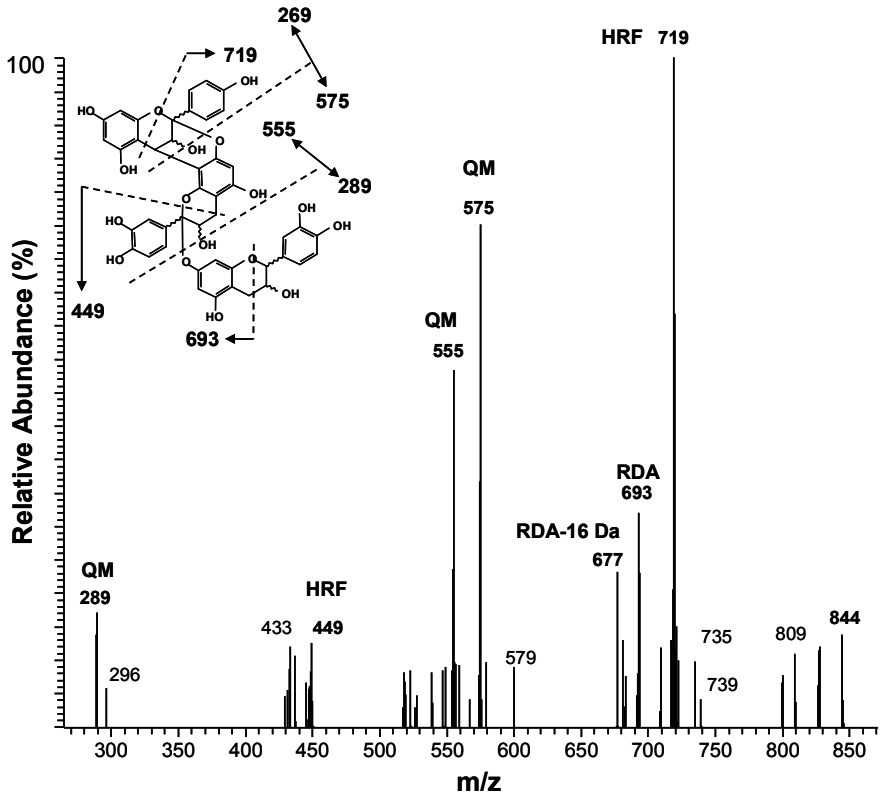

Figure 6. Fragmentation pattern of peak 24 (*m/z* 845; **Figure 4**), consistent with a structure composed of one (epi)afzelechin and two (epi)catechin units.

Position of the A-linkages in trimers and tetramers from peanut skins

The position of the A-linkages of several trimers and tetramers (**Table 2**) could be determined via the product ions derived from QM fission (**Figure 8**). The position of the A-linkage within trimers was detected at both possible positions; between the internal and terminal unit and between the two extension units. When tetramers contained one A-linkage (*m/z* 1151), this linkage was not located between the two internal units. When tetramers contained two A-linkages (*m/z* 1149), these linkages were present at all possible positions. The position of the A-linkages within higher DP oligomers could not be determined, because insufficient amounts of product ions were obtained to draw conclusions.

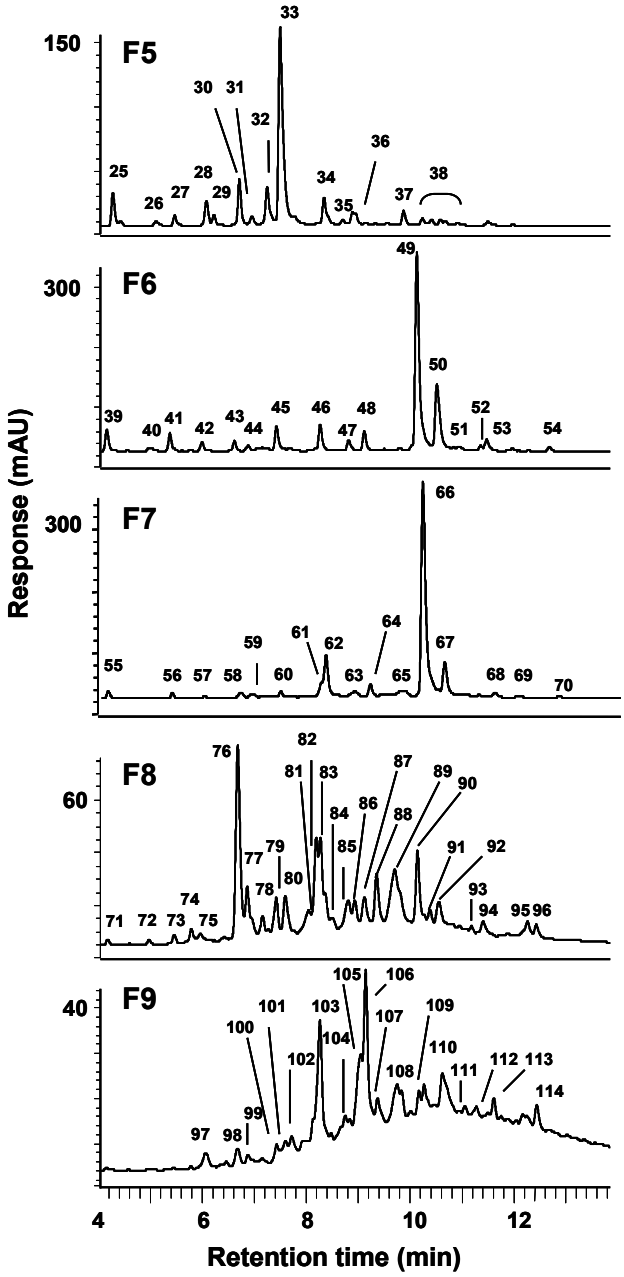

Figure 7. RP-UPLC profiles of fractions F5-F9 (**Figure 3**). Compositional information on the peaks is given in **Tables 1** and **2**.

Table 2. Position of A-linkages in trimers and tetramers separated by RP-UPLC (**Figure 7**) based on MS analysis. **A** denotes the position of the A-linkage.

connection sequence[a]	$[M-H]^-$	product ions[b]	peak numbers	% (w/w) of 20 MeOH-EA
(epi)cat-**A**-(epi)cat-(epi)cat	863	573, 289	26, 27, 34, 35, 40, 41, 46, 47	1.9
(epi)cat-(epi)cat-**A**-(epi)cat	863	575, 287	25, 28-33, 36, 37, 39, 42-45	13.6
(epi)cat-**A**-(epi)cat-**A**-(epi)cat	861	575, 571, 289, 285	38, 55	1.1
(epi)cat-**A**-(epi)cat-(epi)cat-(epi)cat	1151	861, 577, 573	73, 75	0.3
(epi)cat-(epi)cat-**A**-(epi)cat-(epi)cat	1151	863, 861	-	-
(epi)cat-(epi)cat-(epi)cat-**A**-(epi)cat	1151	863, 575	59, 65, 77, 80, 82, 89	3.4
(epi)cat-**A**-(epi)cat-**A**-(epi)cat-(epi)cat	1149	859	78	0.2
(epi)cat-(epi)cat-**A**-(epi)cat-**A**-(epi)cat	1149	861	58, 61, 63, 69, 83	1.7
(epi)cat-**A**-(epi)cat-(epi)cat-**A**-(epi)cat	1149	575, 573	48-50, 53, 62, 64, 66-69, 76, 83, 85, 90	17.3
(epi)cat-**A**-(epi)cat-**A**-(epi)cat-**A**-(epi)cat	1147		54, 70	0.1

[a] the monomeric unit on the right side represents the terminal unit
[b] product ions derived from quinine methide fission

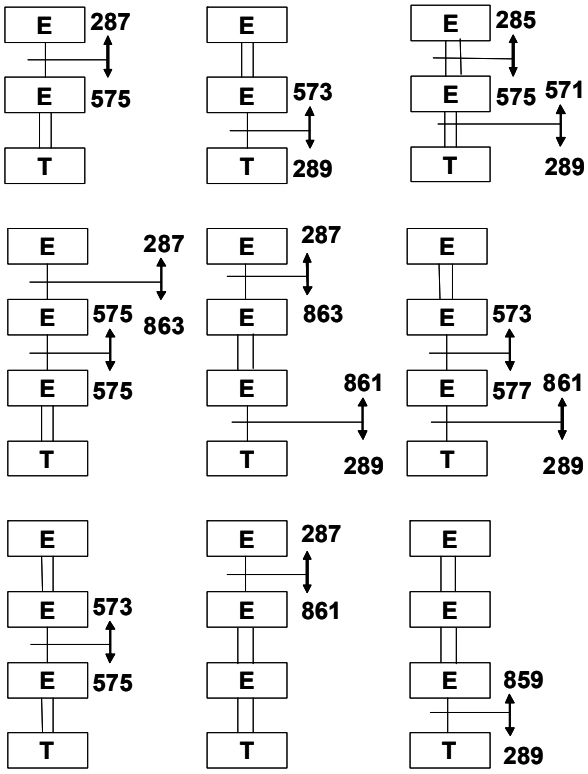

Figure 8. A schematic representation of QM fragmentation (*m/z* values) of trimers and tetramers to determine the position of the A-linkage. E represents the extension units and T the terminal unit of the oligomer.

Quantification of PC oligomers

Similar molar extinction coefficients (at 280 nm) have been reported for epicatechin (ε 3750 $M^{-1}cm^{-1}$) and catechin (ε 4050 $M^{-1}cm^{-1}$) (*19*). Because mainly PCs were present (96.7% (w/w) of the 20MeOH-EA extract), it was assumed that the oligomers present within one fraction, which are of similar DP, also had a similar molar extinction coefficient. For each peak, its relative contribution to the total peak area of that fraction was determined. For example, F1 (**Figure 4**) contained peak **1** and **2**, representing 83.1 and 16.9% of the total area, respectively. Subsequently, the relative mass contribution of each peak to the 20MeOH-EA extract was determined. For example, 55 mg of F1 was obtained after preparative NP-HPLC, which represents 2.5% (w/w) of the total amount of 20MeOH-EA extract (2217 mg). Therefore, peak **1** was calculated to represent (2.5 * 0.831) 2.1% (w/w) of the 20MeOH-EA extract. Similar calculations were performed for each peak in fractions F2-F9 (**Tables 1** and **2**). If a peak represented e.g. two different structures, based on MS data, the area was divided by two. Based on these calculations the A-type oligomers represented 95.0% (w/w) of the 20MeOH-EA extract (**Table 1**). Only 2.4% (w/w) B-type PC dimers and < 0.1% (w/w) B-type PC trimers were present. The tentatively identified A-type properlargonidins (**8**, **24**, **and 52**) represented < 0.7% (w/w) of the total amount of A-type oligomers (95.0% (w/w)).

PC trimers with one A-linkage (*m/z* 863) represented 17.0% (w/w) while PC trimers with two A-linkages (*m/z* 861) represented only 1.6% (w/w) (**Table 1**). The position of the A-linkage in the majority of the PC trimers with one A-linkage (13.6% (w/w) with **30, 32** and **33** as main peaks of a total of 17.0% (w/w)) was between the internal and terminal unit (**Table 2**). Of all the tetramers (23.3% (w/w) of the 20MeOH-EA extract), those with two A-linkages (*m/z* 1149) were the most abundant and accounted for 18.8% (w/w) of the 20MeOH-EA extract (**Table 1**). The main peaks were detected in fractions F6 (**49** and **50**), F7 (**61**, **62**, **66** and **67**) and F8 (**76**). Similarly as with the trimers, tetramers that contained at least one A-linkage between the internal and terminal unit predominated and represented 22.5% (w/w) of the 20MeOH-EA extract from a total of 23.0% (w/w) of A-type tetramers in which the A-linkage position was determined (**Table 2**). Most of the pentamers, hexamers and heptamers contained two or more A-linkages (**Table 1**).

Discussion

Diversity of PAs in peanut skins

PCs were the main group of PAs present in a 20% MeOH extract of peanut skins. This is in line with data reported for acetone and water extracts of peanut skins (*8, 10-12*). Catechin

and epicatechin, in a molar ratio of 5:1, were the exclusive free monomers present, which is in line with the ratio of 9:1 reported previously (*8*).

Novel findings were the presence of propelargonidins (**8**, **24** and **52**), containing one (epi)afzelechin unit as an extension unit, and the possible presence of a methylated dimer (**7**). However, these molecular species were a minority and represented only < 0.8% (w/w) of the 20MeOH-EA extract. Methylated dimers are not commonly present in food, although methylated A-type PAs have been detected in *Cassipourea gummiflua* (*20*).

We could separate 114 different peaks. However, peaks with the same *m/z* values and retention times that were detected in successive fractions might represent the same proanthocyanidin molecule. For example, peak **9** (fraction F3) and **17** (fraction F4) both had an *m/z* value of 577 and similar retention times. Therefore, these two peaks were considered to represent the same B-type PC dimer. In the same way, all peaks with similar *m/z* values and retentions times in successive fractions have been compared. In addition, the positions of the A-linkages were accounted for. For example, the A-linkage of the PC trimers in peak **36** (fraction F5) and peak **47** (fraction F6), having the same *m/z* value (863) and retention time, occurred at different positions. Thus, peak **36** and **47** represent different molecules. Based on these considerations, the 114 peaks appeared to account for 83 different PA molecular species, which were characterized with respect to number and composition of subunits, as well as to number and position of A-linkages.

Peanut skins contain predominantly A-type PCs

Depending on the extraction solvent a different oligomeric composition was obtained, as expected (*21*). We continued with the 20MeOH-EA extract, which yielded most PAs. Previous studies dealing with the identification of peanut skins PAs were performed with 70% acetone extracts (*8, 10, 11*). Our results showed that the 20MeOH-EA extract was rich in lower DP oligomers; oligomers of DP >7 were not present.

Conflicting results have been obtained on the abundance of A- and B-type PAs in peanut skins. Lazarus and coworkers (*10*) reported that A-type PCs dominated over B-type PCs. In contrast, Karchesy and Hemingway (*8*) reported a ratio of 1:15 of A- versus B-linkages. These latter observations were based on mass spectrometric data only, without separation of oligomers, which limits both accurate qualification and quantification of the molecular diversity. The combination of NP and RP-HPLC enabled us to estimate the contribution of each peak to the total extract, based on their UV responses. Our data support the finding of Lazarus and coworkers (*10*). We predominantly identified A-type PCs (95.0% (w/w) of the 20MeOH-EA extract); only few B-type PC dimers (2.4% (w/w) of the 20MeOH-EA extract) and one B-type PC trimer (< 0.1% (w/w) of the 20MeOH-EA extract) was present. No B-type tetramers to heptamers were detected.

The main trimers and tetramers in peanut skins contained a terminally located A-linkage

Different positions of the A-linkages for several trimers to pentamers have been reported for plum and cinnamon PCs (*5*). Theoretically, a precursor ion corresponding to a PC trimer with one A-linkage (*m/z* 863) could represent many different structures, but the molecular diversity was not addressed in that study.

The positions of the A-linkages in peanut PCs were only reported for a purified trimer and tetramer (*12*). Using the same considerations for preventing overestimation of the number of compounds as described for the determination of molecular diversity, we were able to determine the position of A-linkages in 16 trimers and 27 tetramers. Our data showed that they were present at all positions possible: At the extension, internal and terminal units. Separation by RP-LC prior to MS analysis enabled quantification of the main peaks. These results showed that the majority of trimers and tetramers with one or more A-linkage(s) (13.6% (w/w) and 22.5% (w/w) of the 20MeOH-EA extract, respectively) always had an A-linkage at the terminal unit. In plums, the A-linkage within tetramers is also located at the terminal unit (*5, 22*). On the other hand, in cinnamon the A-linkage was detected between the extension units, but not at the terminal unit (*5, 23*). Therefore, our data provide further support for the concept that the location of the A-linkages seems to be source-dependent.

In conclusion, by combining NP and RP-HPLC, we were able to demonstrate the presence of a large molecular diversity of proanthocyanidins present in a 20% MeOH extract of peanut skins. Furthermore, this method enables estimation of the most abundant molecular proanthocyanidin species that were present. These data may contribute to the evaluation of the health potential of peanut skins based supplements.

Acknowledgements

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Chapter 3

Efficient isolation of major procyanidin A-type dimers from peanut skins and B-type dimers from grape seeds

Based on: Maaike M. Appeldoorn, Mark Sanders, Jean-Paul Vincken, Véronique Cheynier, Christine Le Guernevé, Peter C.H. Hollman and Harry Gruppen, Efficient isolation of major procyanidin A-type dimers from peanut skins and B-type dimers from grape seeds, *in press, Food Chem.*, **2009**.

Abstract

In order to fully explore the biofunctional potential of proanthocyanidins (PA), isolated and well-characterized PA dimers are of great importance. Current methods to obtain pure A- and B-type dimers are laborious, because they comprise multiple chromatographic steps, often yielding only one or two specific dimers. In the current study, an efficient isolation procedure is described, to isolate a large variety of A-type dimers from peanut skins and B-type dimers from grape seeds. Yields increased 20-400 times for A-type dimers and about 10 times for B-type dimers compared to other methods with a lesser number of chromatographic steps. Dimers isolated from peanut skins were identified as; epicatechin-(2-O-7, 4-8)-catechin (A1), epicatechin-(2-O-7, 4-8)-epicatechin (A2), epicatechin-(2-O-7, 4-6)-catechin, epicatechin-(2-O-7, 4-8)-*ent*catechin, isolated from peanut skins for the first time, and epicatechin-(4-6)-catechin (B7). Dimers from grape seeds were identified as; epicatechin-(4-8)-catechin (B1), epicatechin-(4-8)-epicatechin (B2), catechin-(4-8)-catechin (B3) and catechin-(4-8)-epicatechin (B4).

Key words: grape seeds, peanut skins, isolation, procyanidin dimers, reversed-phase HPLC

Abbreviations used: CV, column volume; 1D, one dimensional; 2D, two dimensional; DP, degree of polymerization; HRF, heterocyclic ring fission; HSCCC, high speed counter current chromatography; IFL, interflavanic linkage; NP, normal phase; PA, proanthocyanidin; PC, procyanidin; QM, quinine methide fission; RDA, retro-Diels-Alder fission; SPE, solid phase extraction

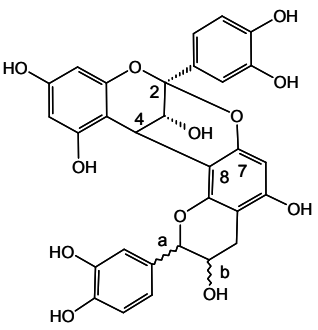
Introduction

Proanthocyanidins (PAs), also referred to as condensed tannins, are present in a wide variety of food products. PAs can be divided into several subclasses, of which the procyanidin (PC), exclusively consisting of (epi)catechin units and their galloyl derivatives, is the most abundant. The monomeric units of PCs are linked through a C4-C8 or C4-C6 bond (B-type), which can coexist with an additional C2-O-C7 or the less abundant C2-O-C5 bond (A-type) (**Figure 1**).

The biofunctional potential of PAs, including PCs, has been established through human intervention studies (*1*), animal studies and *in vitro* assays (*2*). However, the effects found cannot be solely ascribed to PAs, because in these studies mostly foods or crude extracts have been used that also contained monomers ((epi)catechin) or other phenolic and bioactive compounds. Therefore, bioactivity studies performed with pure PAs are important, especially with dimers, as these are believed to be reasonably well bioavailable. Furthermore, A- and B-type PA dimers should be compared much more extensively with respect to their bioactivity and bioavailability.

Several studies reported anti-oxidative properties for both A- and B-type dimers. For example, B-type dimers were reported to inhibit LDL oxidation (*3, 4*) and were more potent than ascorbic acid and trolox (*5*). Furthermore, an A-type dimer from *Geranium niveum* showed a higher scavenging ability for ABTS, O_2^-, OH^- and HOCl on a molar basis, compared to resveratrol and/or ascorbic acid (*6*). Because these studies used different test systems, it can not be concluded which type of dimer is the better antioxidant. Other studies reported that A-type dimers stimulated endothelial dependent relaxation (*7*) and suppressed allergic responses against ovalbumin (*8*), but no B-type dimers were included in those studies. Only few studies directly compared the bioactivity of A- and B-type dimers. The antiviral activity and inhibitory activity against microsomal lipid peroxidation was similar for A- and B-type dimes (*9*), but A-type dimers were better protectants of a lipid bilayer against disruption by Triton-X compared to B-type dimers (*10*). Thus, several activities have been tested but the information is insufficient to conclude which of the dimers, A- or B-type, have more bioactive potential.

To bring about a biological effect, sufficient amounts of dimers should reach the target tissues. Although intact oligomers with a degree of polymerization (DP) >2 are believed to be unabsorbable, data on the absorption of dimers are not consistent (*11-14*). Furthermore, A-type PAs have not been included in these bioavailability studies. In order to fully explore the biofunctional potential of PAs, research should focus on the relation between their structure, bioavailability and bioactivity. Isolated and well-characterized PA dimers are, therefore, of great importance.



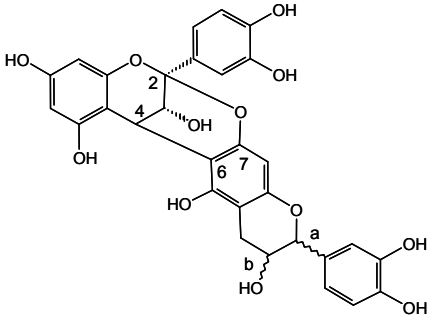
A-type dimer (2-O-7, 4-8) linkage

1) EC - C (a = ▌ , b = ⦙)

2) EC - EC (a = ▌ , b = ▌)

3) EC - entC (a = ⦙ , b = ▌)

4) EC - entEC (a = ⦙ , b = ⦙)



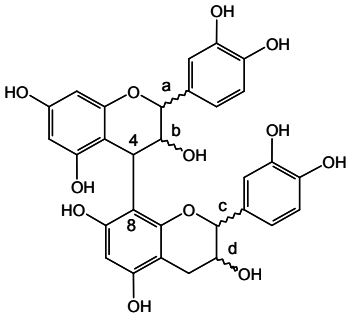
A-type dimer (2-O-7, 4-6) linkage

5) EC - C (a = ⦙ , b = ▌)

6) EC - EC (a = ⦙ , b = ⦙)

7) EC - entC (a = ▌ , b = ⦙)

8) EC - entEC (a = ▌ , b = ▌)



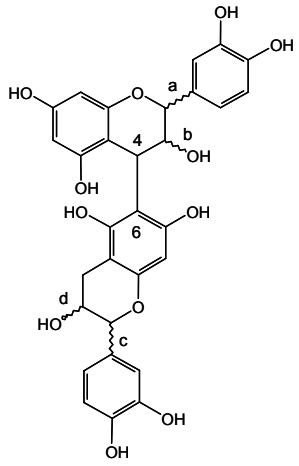
B-type dimer (4-8) linkage

9) EC - C (a = ⦙ , b = ⦙, c = ▌ , d = ⦙)

10) EC - EC (a = ⦙ , b = ⦙, c = ⦙, d = ⦙)

11) C - C (a = ▌ , b = ⦙, c = ▌, d = ⦙)

12) C -EC (a = ▌, b = ⦙, c = ⦙, d = ⦙)



B-type dimer (4-6) linkage

13) EC - C (a = ⦙, b = ⦙, c = ▌, d = ⦙)

14) EC - EC (a = ⦙, b = ⦙, c = ⦙, d = ⦙)

15) C - C (a = ▌, b = ⦙, c = ▌, d = ⦙)

16) C -EC (a = ▌, b = ⦙, c = ⦙, d = ⦙)

Figure 1. The structures of A-type and B-type dimers present in grape seeds and peanut skins, respectively. EC=epicatechin, C= catechin and *ent* = enantiomer.

Efficient isolation of different types of PA dimers requires sources containing high amounts and a large diversity of dimers. Commonly used sources for the isolation of B-type PCs are apple and cocoa, which primarily consist of epicatechin units (*15, 16*) that are mainly 4-8 linked (*16, 17*). Grape seeds are also rich in B-type PCs (*18*) with relatively more catechin units (*19*) compared to apple and cocoa. Additionally, 4-6 linkages and galloylated derivatives are present (*16, 20, 21*). Therefore, grape seed is a promising source to isolate a large diversity of B-type dimers. A-type PCs can be found in peanuts, plums, cranberries and cinnamon (*18*). Peanut skins contains up to 17% (w/w) PCs (*22*) and both C4-C8 and C4-C6 linked A- (with an additional C2-O-C7 linkage) and B-types have been identified (*22, 23*).

The methods reported to obtain pure A- and B-type dimers are laborious as they include multiple chromatographic steps. The first step in isolation usually consists of a separation based on DP. Previously, size exclusion materials like Sephadex LH-20 (*17, 24*) and Toyopearl (*20, 25*) were used for this purpose. A combination of five different columns, including four size exclusion columns, was required to isolate several A-type dimers from peanut skins (*23*). The high diversity of B-type PC dimers in grape seeds makes it a good but also challenging source for the current chromatographic methods to isolate the dimers. Recently, a high speed counter current chromatographic (HSCCC) technique was used to isolate PC dimers from grape seeds (*26*). However, an elution of many hours was needed and additional Reversed-phase (RP) HPLC was a prerequisite to obtain pure dimer fractions. Nowadays, more specific silica-based stationary phases are used (*16, 27-30*) and separations of PAs from several sources up to DP 10 have been described (*27*). Additionally, fractions up to DP 8 have been purified from apple (*31*). Nevertheless, when pure dimer fractions are to be isolated, often size exclusion chromatography, instead of normal phase (NP) HPLC, in combination with RP-HPLC has been used. Advantages to use NP-HPLC followed by RP-HPLC are: 1) only one HPLC system is required instead of the multiple systems needed for HSCCC, 2) peak shape on NP and thereby purity of the different DP fractions is better than that on size exclusion or HSCCC, 3) a baseline separation of dimers on NP-HPLC facilitates isolation of a fraction containing only all dimers while size exclusion or HSCCC often results in inefficient isolation of dimers to prevent contamination with monomers or trimers due to overlap of peaks.

The combination of NP- and RP-HPLC to obtain pure dimers has rarely been used and little is known about the behavior of A-type PCs on such columns. In this study an efficient method for the isolation of both A- and B-type dimers is described by combining NP- and RP-HPLC chromatography, yielding sufficient amounts of material for bioavailability studies.

Materials and Methods

Materials

Sources to isolate PCs were peanut skins, kindly provided by Imko-The Nut Company BV (Doetinchem, The Netherlands) and a commercially available procyanidin extract from grape seeds (Vitaflavan, DRT, Levita Chemical International NV, Antwerpen, Belgium). PC dimer standards B1, B2, B3 and B4 were obtained at Apin Chemicals (Abingdon, UK). Organic solvents used for HPLC analysis were all HPLC-grade. Milli-Q water from a Millipore system was used.

Extraction of peanut skins

Peanut skins (~75 g) were defatted with hexane using soxhlet extraction. The residue was air dried. The defatted skins (~60 g) were extracted three times with 1L of 20% (v/v) aqueous methanol. After each extraction the suspension was filtered over a 595 ½ filter (Schleicher & Schuell, Dassel, Germany), after which the pellet was subjected to the next extraction. The three 20% (v/v) methanol fractions were combined, concentrated by evaporation under vacuum and lyophilized. During the extractions and evaporation, light was excluded as much as possible by the use of marquees and tinfoil. Solvent partitioning was used for further purification. The lyophilized extract was put into a separation funnel to which 500 mL H_2O and 500 mL ethyl acetate was added. This was placed in an ultrasonic bath for 10-15 min at room temperature. After vigorous mixing, the H_2O phase and ethyl acetate phase were collected separately. The H_2O phase was put back into the funnel and the solvent partitioning procedure was repeated twice resulting in three ethyl acetate phases and one H_2O phase. The ethyl acetate phases were combined and subsequently extracted with H_2O (1:1, v/v) twice. The ethyl acetate phase was vacuum evaporated, dissolved in H_2O and lyophilized, resulting in the final extract referred to as: peanut skin extract.

Removal of gallic acid from grape seed procyanidins

To enable preparative HPLC isolation of PC dimers, gallic acid substituents were enzymatically removed. Vitaflavan was dissolved (2 g/L) in a sodium acetate buffer (0.1 M, pH 5.0), after which tannase (Gamma Chemie, Darmstadt, Germany) was added (80 mg/L). The mixture was incubated at 30 °C for 24 h after which freshly prepared tannase was added. After another 24 h of incubation, free gallic acid was removed by SPE. The C18 cartridge (Sep-Pak Vac 200 cc, 5g, Waters, Millford, MA) was conditioned following the supplier's instructions. The sample was loaded and washed with 2 column volumes (CV) H_2O. Subsequently, PCs were eluted with methanol (2 CV). The methanol was evaporated under vacuum. The sample was dissolved in H_2O, frozen, and lyophilized.

Preparative isolation of A- and B-type dimers

Normal phase HPLC

Fractionation by DP was performed on an Inertsil PREP-SIL 30 mm ID x 250 mm column with a 10 µm particle size (GL sciences Inc., Tokyo, Japan). A Waters system equipped with a 2767 sample manager, a 2525 binary gradient module, a 2996 photodiode array detector (PDA) and an UV fraction manager was used. The binary mobile phase consisted of (A) hexane and (B) acetone. The elution profile was as follows: 0-30 min, 40%-60% B; 30-50 min, isocratic at 60% B; 50-70 min, 60%-75% B, followed by a washing step at 98% B for 3 min, and reconditioning of the column. The flow rate was 27.22 mL/min and PDA spectra from 210-300 nm were recorded. In a run, 10 mL (~70 mg/mL in acetone/hexane/ethanol (7:3:2)) peanut skin extract or the tannase-treated grape seed extract was applied. The fraction containing dimers was collected from 22.3-28.2 min for the peanut skin extract and from 28.2 - 38.1 min for the grape seed extract. The fractions were evaporated under vacuum and lyophilized.

Reversed phase HPLC

An XterraRP 50 mm ID x 100 mm column with a 5 µm particle size (Waters) was used with a flow rate of 82.7 mL/min. The fractions containing dimers (~70 mg), which were obtained with preparative NP-HPLC, were dissolved in methanol using ultrasonic treatment if needed and filtered over a Spartan ® 0.45 µm filter (Schleicher & Schuell, Dassel, Germany) before injection. The binary mobile phase consisted of (A) H_2O + 0.1% (v/v) acetic acid and (B) acetonitrile + 0.1% (v/v) acetic acid. The elution profile for the separation and isolation of the individual peanut skin dimers was as follows: the first 3 min isocratic on 0% B; 3-23 min, B: linearly 0%-22%; 23-28 min, B: linearly 22%-89% followed by reconditioning of the column. The elution profile for the grape seed dimers was as follows: the first 10 min isocratic on 10% B; 10-23 min, B: linearly 10%-50%; 23-27 min, B: linearly 50%-95% followed by reconditioning of the column. PDA spectra were recorded from 210-300 nm. The fractions that were collected after injecting peanut skin dimers were; I_P (15.7-16.6 min), II_P (17.6-18.7 min), III_P (18.7-19.6 min), IV_P (19.6-20.5 min), V_P (21.2-22.0 min) and VI_P (22.0-22.8 min). The fractions that were collected after injecting grape seed dimers were; I_G (10.0-12.2 min), II_G (13.0-13.7 min), III_G (13.9-14.2 min), IV_G (14.8-15.5 min), V_G (15.7-15.9 min), VI_G (16.1-16.3 min), VII_G (16.5-17.0 min) and $VIII_G$ (17.4-17.8 min). Subsequently, the fractions were evaporated under vacuum and either lyophilized or solubilized in methanol prior to further analysis. Fraction IV_G consisted of two PC dimers, which were further purified on an Atlantis dC18 (19 mm ID x 100 mm, 5 µm particle size; Waters). The fraction (~16 mg) was dissolved in 10 mL 50% (v/v) aqueous methanol, filtered over a Spartan ® 0.45 µm filter and injected. The flow rate was 17.1 mL/min. The binary mobile phase consisted of (A) H_2O + 0.1% (v/v) acetic acid

and (B) methanol + 0.1% (v/v) acetic acid combined in the following elution profile: 0-16.7 min, B: linearly 5%-45%; 16.7-20 min, B: linearly 45%-60%; 20-23.3 min, isocratic at 60% B, followed by reconditioning of the column. Fractions were collected from 9.5-10.5 min (IV_{G1}) and from 10.8-12.0 min (IV_{G2}).

Analytical HPLC

Normal phase HPLC

A Thermo Spectra system was used containing a P 4000 pump, an AS 300 autosampler and an UV 3000 detector (Thermo Separation products, Fremont, CA, USA). Analysis was performed on a Luna Silica (2) 4.6 mm ID x 250 mm column with a 5 µm particle size (Phenomenex, Torrance, CA) operated at room temperature. Samples were dissolved in methanol. The mobile phase consisted of (A) dichloromethane, (B) methanol and (C) acetic acid/H_2O (50% v/v). The flow rate was 1 mL/min and detection was performed at 280 nm. The elution profile was as follows; C was kept constant at 4% throughout the elution; 0-20 min, B: linearly 14%-23.5%; 20-50 min, B: linearly 23.5%-35% followed by a washing step at 96% B for 10 min and reconditioning of the column.

Reversed phase HPLC

The same Thermo Spectra system was used and analysis was performed on an XterraRP dC18, 4.6 mm ID x 150 mm column with a 3.5 µm particle size (Waters). Samples were dissolved in methanol. The mobile phase was composed of (A) H_2O + 0.1% (v/v) acetic acid and (B) acetonitrile + 0.1% (v/v) acetic acid. The flow rate was 0.7 mL/min and detection was performed at 280 nm. The elution profile for peanut skin dimers was as follows; the first 5 min isocratic on 10% B; 5-35 min, B: linearly 10%-30%; 35-40 min, B: linearly 30%-90% and reconditioning of the column. The elution profile for grape seed dimers was as follows; the first 15 min isocratic on 10% B; 15-35 min, B: linearly 10%-50%; 35-40 min, B: linearly 50%-95% followed by reconditioning of the column. Samples were compared to retention times of B-type dimer standards B1, B2, B3 and B4.

Characterization of B-type dimers by phloroglucinolysis

Fractions I_P and VI_P, obtained after preparative HPLC, were analyzed on RP-HPLC before and after acid-catalyzed degradation as described by Fournand and coworkers (*32*). Briefly, intact dimers were analyzed on RP-HPLC-UV. After acid-catalyzed degradation of the dimers in the presence of excess phloroglucinol, released terminal units and extension unit-phloroglucinol adducts were analyzed by RP-HPLC-UV. Dimers were identified based on the retention time of the intact dimers and the retention time of (epi)catechin and their phloroglucinol adducts, released after acid-catalyzed degradation.

Spectroscopic analysis

ESI-MS detection

An LCQ Classic equipped with an ESI source was used and controlled by the software Xcalibur (Thermo Finnigan, San Jose, CA). The detector was coupled to an HPLC system by means of a splitter (Acurate, LC Packings, Amsterdam, The Netherlands), which reduced the flow rate 20 times. Measurements were performed in the negative mode with an ion spray voltage of 4.5 kV, a capillary voltage of -5.0 V and a capillary temperature of 270 °C. The scan range was set from *m/z* 100-2000. The MS/MS function was performed in the data dependant mode. The collision energy value was 27%.

Maldi-TOF-MS

Samples were analyzed on an Ultraflex instrument equipped with a nitrogen laser of 337 nm and controlled by the software Flexanalysis (Bruker Daltonics B.V., Wormer, The Netherlands). A laser intensity of 29% was applied and data were collected within a mass range of 400-2000 Da. The apparatus was operated in both the positive and the negative mode with a delayed extraction time of 100 ns and an acceleration voltage of 20 kV. Samples were mixed with the matrix 2,5-dihydroxybenzoic acid (Bruker Daltonics) (10 mg/mL H_2O) (1:1) and 2 µL was put on a stainless steel metal plate, crystallized under a blow drier and analyzed.

NMR spectroscopy

NMR experiments were performed on a Varian UNITY INOVA 500 MHz spectrometer (Varian NMR instruments, Palo Alto, CA, USA) equipped with a 3 mm indirect detection probe operating at 500 MHz for ^{1}H and 125.7 MHz for ^{13}C. One-dimensional (1D) ^{1}H, two dimensional (2D) ^{1}H TOCSY and ROESY, and 2D (^{1}H-^{13}C) HSQC and HMBC spectra of samples dissolved in methanol-d4 (fractions I_P, II_P and IV_P) or dimethyl sulfoxide-d6 (fractions III_P, V_P and VI_P) were recorded at 298 °K. Chemical shifts (δ) are given in ppm and coupling constant (*J*) values are given in Hz. Spectra signals were referenced to the solvent signals, either DMSO (^{1}H signal at 2.5 ppm and ^{13}C signal at 39.5 ppm) or methanol (^{1}H signal at 3.3 ppm and ^{13}C signal at 49.0 ppm). Spectra were processed and analyzed with VNMR software (Varian, CA, USA).

Results and discussion

Preparative isolation of A- and B-type dimers

Use of NP-HPLC for the isolation of a dimeric fraction

To be able to isolate the grape seed dimers, gallic acid substituents were enzymatically (tannase) removed, which improved the resolution especially between the dimers and trimers, as described previously (*16*). The dimers from the tannase-treated grape seed extract, as well as those from the peanut skin extract, were isolated with preparative NP-HPLC (**Figure 2**), which separates by DP.

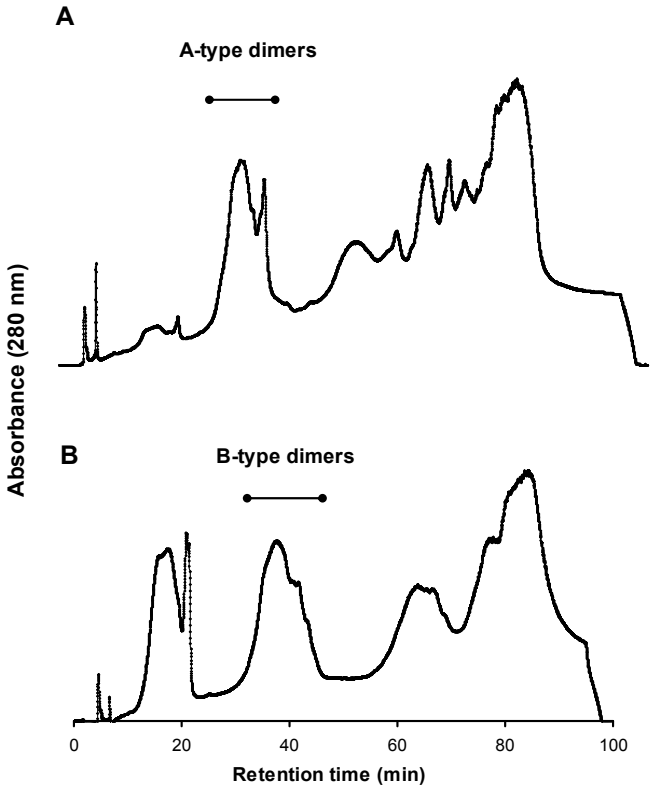


Figure 2. Isolation of A-type dimers from peanut skins (panel **A**) and B-type dimers from grape seeds (Panel **B**) by preparative NP-HPLC. The fractions containing the A- or B-type dimers were collected, evaporated under vacuum and lyophilized.

The total recovery of all material after NP-HPLC was 85% (w/w) (n=1) for the peanut skin extract and 78% (w/w) ± 4.8 (n=4) for the grape seed extract. This is comparable to recoveries obtained for apple extracts obtained with the same method (*31*). The dimers represented 16.8% (w/w) (n=1) of the peanut skin extract and 21.2% (w/w) ± 2.1 (n=5) of the grape seed extract, similar to previously reported amounts in grape seeds (*33*). Both dimer fractions were free of monomers and trimers as their masses, *m/z* 289 $[M-H]^-$ and *m/z* 865, 863 (1 A-interflavanic linkage (IFL)), and 861 $[M-H]^-$ (2 A-IFL), respectively, were not detected by NP-HPLC-UV-MS. The A- and B-type dimers could be distinguished by their parent ions (*m/z* 575 $[M-H]^-$ and *m/z* 577 $[M-H]^-$ respectively) and accompanying MS/MS spectra. PCs have three characteristic fragmentation routes, which have been described as: Quinone methide (QM), retro-diels-alder (RDA) and heterocyclic ring fission (HRF) cleavage (*34*). A lower retention of the peanut skin dimers compared to grape seeds dimers on the silica stationary phase (**Figure 2)** could hint at the presence of A-type dimers, which are less polar than the B-type dimers due to the additional bond.

This was confirmed by the MS/MS spectra, which showed parent ions of *m/z* 575 for the peanut skin dimers with *m/z* 289 + 285 (QM), *m/z* 423 + 407 (RDA) and *m/z* 449 (HRF) as fragment ions. The grape seed dimers had parent ions of *m/z* 577, and *m/z* 289 + 287 (QM), *m/z* 425 + 407 (RDA) and *m/z* 451 (HRF) as fragment ions. The observation that the fragmentation of A-type dimers, just like the B-type, yields a fragment ion of 407 rather than 405 has been reported earlier (*35*).

Use of RP-HPLC for purification of individual dimers

The fractions containing the dimers were further separated on an XterraRP dC18 column. The total recovery of all material after RP-HPLC was 65.1% (w/w) (n=1) for the peanut skin dimers and 68.7% (w/w) ± 5.3 (n=3) for the grape seed dimers.

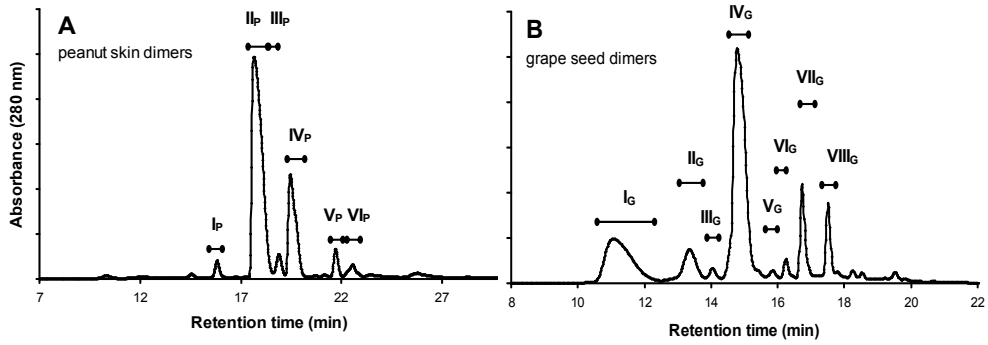


Figure 3. Isolation of dimers from peanut skins (**A**) and grape seeds (**B**) on a preparative XterraRP C18 column. Fractions were collected as indicated by I through VIII. The origin is indicated by P (peanut skins) and G (grape seeds).

The peanut skin dimers contained 6 peaks in total indicated by I_P till VI_P (**Figure 3A**). Separation of the grape seed dimers resulted in 8 peaks, I_G till $VIII_G$ (**Figure 3B**). Peak IV_G consisted of 2 components based on reinjection on an Atlantis dC18 column. Preparative separation on this column resulted in two pure fractions: IV_{G1} and IV_{G2} (**Figure 4**). The total recovery was 66.4% (w/w) (n=1).

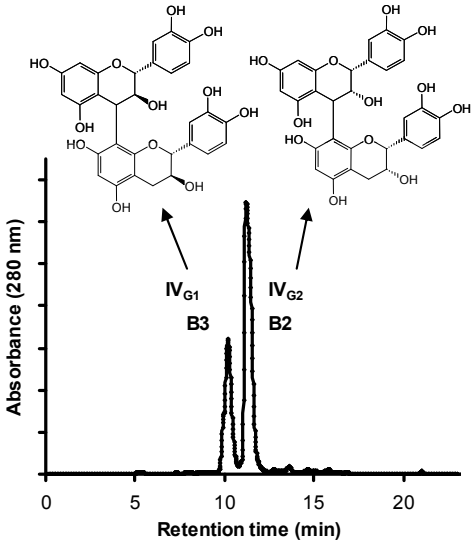


Figure 4. Isolation of dimers B2 (IV_{G2}) and B3 (IV_{G1}) on a preparative Atlantis dC18 column from fraction IV_G, previously isolated on XterraRP (**Figure 3B**).

Structure elucidation of isolated A-type dimers

Four peaks (II_P to $V_{P)}$ were characterized as A-type dimers based on their parent ions (*m/z* 575 $[M-H]^-$) and MS/MS fragments. Because no commercial standards were available for A-type dimers, NMR spectroscopy was performed for structural identification (**Tables 1** and **2**)

Determination of interflavanic linkage (IFL)

The NMR spectra of the fractions II_P, III_P, IV_P and V_P were very similar and typical for A-type dimers as described previously (*23*). In short, an isolated AB system in the heterocyclic proton region (δ 4.0-4.5 ppm, J=2.8-3.5 Hz) was attributed to protons at carbon 3 and 4 of the C-ring (C3-C and C4-C). Two meta-coupled doublets and a singlet in the aromatic region (δ ± 6 ppm) were assigned to the protons of the A-ring and the residual proton of the D-ring, respectively. Two AMX systems in the aromatic downfield region (δ 6.5-7.5) were detected as B- and E-ring protons. The other signals were unambiguously attributed to the F-ring protons. Therefore, the A-IFL involved both the C4 and C2 of the

Table 1. ^{1}H NMR chemical shifts (δ in ppm) and multiplicity (*J* in Hz) of compound of fractions I_P to V_P in either MeOH-d4 (fractions I_P, II_P and IV_P) or DMSO-d6 (fractions III_P and V_P) at 298 °K.

Ring	proton	I_P	II_P	III_P	IV_P	V_P
C	2	4.91*	-	-	-	-
	3	4.01 (bs)	4.06 (d, 3.5)	3.94 (d, 3.0)	4.06 (d, 3.4)	3.97 (d, 2.8)
	4	4.55 (bs)	4.23 (d, 3.5)	4.19 (d, 3.0)	4.40 (d, 3.4)	4.12 (d, 2.8)
A	6	5.99	5.95 (d, 2.3)	5.85[a] (m)*	6.07 (d, 2.3)	5.90 (d, 1.6)
	8	6.04 (bs)	6.06 (d, 2.3)	5.86[a] (m)*	6.00 (d, 2.3)	5.83 (d, 1.6)
B	2	6.88 (bs)	7.13 (d, 2.2)	6.98 (d, 1.9)	7.14*	7.03 (d, 1.9)
	5	6.71 (m)*	6.80 (m)*	6.73 (m)*	6.85*	6.74 (d, 8.3)
	6	6.68 (m)*	7.01 (dd, 8.4, 2.2)	6.83 (m)*	7.02 (dd, 8.3, 2.2)	6.89 (dd, 8.3, 1.9)
F	2	4.57 (d, 7.7)	4.73 (d, 7.9)	4.61 (d, 6.8)	4.92 (bs)	4.50 (d, 6.8)
	3	3.97 (m)	4.14 (m)	3.88 (m)	4.23 (bs)	3.84 (m)
	4α	2.46 (m)*	2.57 (dd, 16.3, 8.4)	2.41 (dd, 17.2, 8.4)	2.76 (dd, 17.3, 2.2)	2.44 (dd, 16.4, 7.4)
	4β	2.75 (m)*	2.93 (dd, 16.3, 5.7)	2.78 (dd, 17.2, 4.9)	2.94 (dd, 17.3, 4.9)	2.66 (dd, 16.4, 5.3)
D	6	-	6.09 (s)	5.98 (s)	6.09 (s)	-
	8	5.99*	-	-	-	5.78 (s)
E	2	6.83 (bs)	6.91 (bs)	6.85 (m)*	7.15*	6.69*
	5	6.76 (m)*	6.82 (m)*	6.73 (m)*	6.81*	6.67*
	6	6.71 (m)*	6.82 (m)*	6.72 (m)*	6.98 (dd, 8.2, 1.9)	6.56 (dd, 8.1, 2.1)

[a] in the same column assignments may be interchanged.
* multiplicity undetermined due to overlaps

Table 2. ^{13}C NMR chemical shifts (δ in ppm) of compound of fractions I_P to V_P in either MeOH-d4 (fractions I_P, II_P and IV_P) or DMSO-d6 (fractions III_P and V_P) at 298 °K.

ring	carbon	I_P	II_P	III_P	IV_P	V_P
C	2	77.0	99.6	98.6	99.5	99.0
	3	72.5	67.4	65.6	67.4	65.3
	4	37.3	28.9	27.7	28.5	28.3
A	5	157.8	156.1[a]	155.6[a]	156.3[a]	155.8[a]
	6	96.6	97.9[b]	96.2[b]	95.9	96.1
	7	159.2	157.4	156.3	157.5	156.3
	8	95.9	96.2[b]	94.3[b]	97.6	93.6
	4a	102.4	103.3	102.4	103.7	103.7
	8a	159.2	153.5[a]	152.6[a]	153.6[a]	152.5[a]
B	1	132.1	131.5	130.4	131.2	130.4
	2	114.7	115.4	114.6	114.5	114.7
	3	145.8	144.9	144.1[c]	144.9	144.2
	4	145.4	146.1	145.4[c]	146.2	145.3
	5	115.4	115.4	114.8	114.9	114.5
	6	119.0	119.4	117.6	118.7	117.8
F	2	82.4	84.0	81.5	81.1	80.7
	3	68.6	67.8	65.8	66.2	66.1
	4	28.6	28.6	28.4	29.2	27.7
D	5	155.6	155.5	154.2	156.0	153.2
	6	108.0	96.2	94.3	95.6	107.7
	7	159.2	151.4	150.4	151.7	150.0
	8	96.6	106.1	104.9	106.6	92.8
	4a	102.4	102.4	101.2	101.8	102.3
	8a	154.7	150.8	149.8	151.5	153.2
E	1	131.9	129.8	129.9	130.5	130.4
	2	115.1	115.4	114.6	115.2	114.1
	3	145.9	146.0	145.0	145.9	144.7
	4	145.8	146.0	145.0	145.7	144.7
	5	115.4	115.9	114.8	115.3	114.9
	6	119.9	120.3	118.3	119.7	117.9

[a, b, c] in the same column assignments may be interchanged.

upper unit. The junctions at the lower unit were determined through the analysis of both ^{1}H-^{13}C long-range HMBC and ^{1}H through space ROESY correlations.

The bond positions of fractions II_P, III_P and IV_P were determined in a similar way as illustrated in **Figure 5A**. First, C8a-D was attributed through its correlation with the proton at C2-F, whereas C5-D was identified through its correlations with both protons at C4-F. The residual proton of the D-ring showed correlations with two deshielded carbons (δ > 150 ppm). One could easily be determined to be the C5-D, while the other one was deduced to be C7-D, since its chemical shift was different from that of C8a-D. Therefore, the residual proton was determined to be at C6-D. Consequently, the IFL implied attachment to C8 of the D-ring. Also the long range correlation between the proton at C4-C and C8a-D and ROESY spectra, which showed correlations between the proton at C4-C and E-ring protons, were in agreement with a C4-C8 linkage.

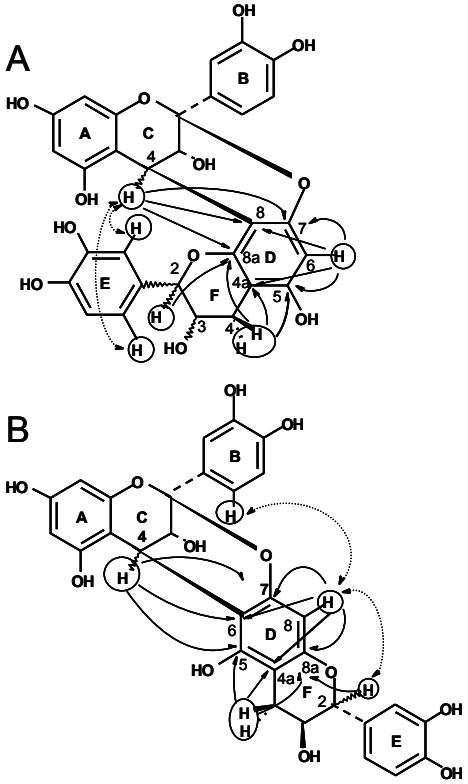


Figure 5. Main HMBC (⟶) and ROESY (⟷) correlations allowing linkage positions to be established, in either (C2-O-C7, C4-C8) **A**) or (C2-O-C7, C4-C6) **B**) dimers.

The ether bond was deduced to be C2-O-C7, as it is the only possibility in case of a C4-C8 linkage. Therefore, the components of fractions II_P, III_P and IV_P were identified to be (C2-O-C7, C4-C8) double linked dimers.

In the case of fraction V_P, no conclusive evidences of the IFL positions could be found based on long range HMBC correlations. The residual D-ring proton could not be determined, because C8a-D and C5-D overlapped. However, ROESY correlations between the proton at C4-C and protons of E-ring were clearly lacking, which indicated a C4-C6 linkage (*23*) (**Figure 5B**). In this case, the ether linkage can be either C2-O-C7 or C2-O-C5. Since a weak, but clear, correlation occurred between the proton at C8-D and the proton at C2-F, as well as one between the proton at C8-D and protons at C2 and C6 of the B-ring, the linkage can be deduced to be C2-O-C7. The C2-O-C7 linkage was also in accordance with the ^{13}C chemical shift of C7-D, which was upfield (δ 150) compared to free hydroxylated C7-A (δ 156.3). Based on all the data the component of fraction V_P was identified as a (C2-O-C7, C4-C6) double linked dimer.

NMR spectra of fraction VI_P were not conclusive because of its low purity. The major compound, which, based on NMR signals, represented about 35% of fraction VI_P, exhibited some typical NMR signals (an ABC-ring system) of an A-type dimer. However, structural identification was difficult due to numerous overlaps and poor signal intensities. Phloroglucinolysis of fraction VI_P did not result in depolymerization, which supported the hypothesis of the presence of an A-type dimer in this fraction.

Monomeric composition

In the case of fractions II_P, III_P, and V_P, the large values observed for the J_{23} F-ring coupling constants (J ~7Hz) were characteristic for catechin. In contrast, for fraction IV_P this value was relatively low (~1Hz), which is characteristic for an epicatechin unit. The upper units of fractions II_P-V_P were supposed to be epicatechin, as Lou and coworkers (*23*) found that A-type dimers from peanut skins always contained epicatechin as upper units. Therefore, the two major peaks, II_P and IV_P, were identified as epicatechin-(2-O-7, 4-8)-catechin (**1**), also referred to as A1, and epicatechin-(2-O-7, 4-8)-epicatechin (**2**), referred to as A2. Fraction III_P showed similar NMR spectra compared to fraction II_P (A1). Dimer A1 has been identified in peanut skins before and seems to be abundantly present (*22, 23*). Therefore, the major fraction II_P was annotated to be A1, while the less abundant fraction III_P, containing a similar NMR spectrum, was tentatively identified as the enantiomer of A1 (**3**). The presence of enantiomers in peanut skins was also found by Lou and co-workers (*23*), who identified the enantiomer of dimer A2. However, the enantiomer of A1 has not been identified in peanut skins before. Fraction V_P was characterized as epicatechin-(2-O-7, 4-6)-catechin (**5**).

Structure elucidation of isolated B-type dimers

The fractions obtained from grape seeds all represent B-type dimers (*m/z* 577 $[M-H]^-$) based on RP-HPLC-UV-MS and their fragment products with MS/MS. Comparison with standards resulted in the identification of I_G as B1 (**9**), II_G as B4 (**12**) and IV_G, which consisted of both B2 (**10**) and B3 (**11**). The remaining peaks could not be identified further. Peak IV_G was further separated on an Atlantis dC18 column, resulting in pure fractions of B2 (**10**) (IV_{G2}) and B3 (**11**) (IV_{G1}) (**Figure 4**).

One peak isolated from peanut skins (I_P, **Figure 3A**) was identified as a B-type dimer, which was B7 (epicatechin-(4-6)-catechin) (**13**) based on its retention time before phloroglucinolysis and its products after phloroglucinolysis. This was confirmed with NMR (**Tables 1** and **2**). Both 1D and 2D homonuclear ^1H and 2D heteronuclear ^1H-^{13}C experiments were performed. The main difference compared to the spectra of an A-type dimer is the presence of a proton at C2-C. The structure of this dimer was determined as follows: C8a-D (δ 154.7) was first identified through its long range correlation with the proton at C2-F. The bond location was determined from the long range correlations of the proton at C4-C. This proton had no correlation with C8a-D at δ 154.7 but exhibited correlations with several deshielded carbons at δ 155.6, 159.2, 159.2 and 157.8, which were attributed to C5-D, C7-D, C8a-A and C5-A. A long range correlation of the proton at C4-C with C5-D and the lack of a correlation with C8a-D indicated that C4-C is linked to C6-D (*36*). The J_{23} F-ring coupling constant values allowed to discriminate between epicatechin (J ~1Hz) and catechin (J ~7Hz) (2,3 *cis* or *trans*) configuration of the lower unit (*37*) and was determined to be catechin since the J_{23} coupling constant value was 7.7 Hz.

Conclusion

The main A-type dimers, A1 (**1**) and A2 (**2**), were efficiently isolated. In comparison to a previously published method (*23*), the required chromatographic methods were reduced from five to two, while simultaneously the yields of A1 and A2 increased 20-400 times. To compare both methods, the yields of A1 and A2 were expressed as weight percentage of the total amount of peanut skins that was used. Lou and coworkers (*23*) used skins of 279 kg peanuts. The amount of skins they used was calculated to be ~11 kg, assuming that the skins represent 4% (w/w) of the total peanut (*38*). Differences in extraction procedure and the origin of peanut skins might also have contributed to the pronounced yield differences found. Next to A1 and A2, four other peanut skin dimers were isolated including the tentatively identified enantiomer of A1 (**3**), not previously detected in peanut skins, epicatechin-(2-O-7, 4-6)-catechin (**5**), B7 (**13**) and one unidentified dimer.

Although grape seeds have been used before as a source to isolate PC dimers, data on the efficiency of those isolations are not well documented. Dimers isolated with NP-HPLC resulted in about 21% (w/w) of the PC extract, which is in accordance with compositional LC-MS analysis of PC dimers in grape seed extracts performed by others (*21, 33*). The yield for B1-4 from the grape seed extract using NP/RP-HPLC was about 10 times higher compared to a recently published HSCCC method (*26*). Recoveries for the additional RP-HPLC that was performed after HSCCC were not given, but would likely lower their total yield. Furthermore, pre-purification steps like precipitation or SPE to remove polymers and improve resolution, prior to HSCCC, were not needed.

The combination of NP/RP-HPLC is suitable to efficiently isolate a large variety of both A- and B-type dimers with a limited number of chromatographic steps. **Table 3** summarizes the purity, yield and structure of each peak that was isolated. Sufficient amounts of material were obtained to study the bioavailability of both A- and B-type dimers, which is subject of further research in our laboratory.

Table 3. Characteristics of the isolated A- and B-type dimers obtained after successive preparative normal and reversed phase HPLC

source	fraction[a]	composition/structure[b]	purity[c]	% ($^{w}/_{w}$)[d]
peanut skin extract	I_P	EC-(4-6)-C (**13**)	93	0.2
	II_P	EC-(2-O-7, 4-8)-C (**1**)	99	6.9
	III_P	EC-(2-*O*-7, 4-8)-*ent* C (**3**)	81	0.3
	IV_P	EC-(2-*O* -7, 4-8)-EC (**2**)	98	2.1
	V_P	EC-(2-*O*-7, 4-6)-C (**5**)	99	0.3
	VI_P	-	< 75	0.2
grape seed extract	I_G	EC-(4-8)-C (**9**)	95	3.2
	II_G	C-(4-8)-EC (**12**)	81	1.2
	III_G	-	<75	0.2
	IV_{G1}	C-(4-8)-C (**11**)	88	1.5
	IV_{G2}	EC-(4-8)-EC (**10**)	99	7.1
	V_G	-	80	0.1
	VI_G	-	65	0.2
	VII_G	-	93	1.2
	$VIII_G$	-	96	0.9

[a] the fractions represent the peaks as shown in **Figures 4** and **5**.
[b] the numbers represent the structures in **Figure 1**.
[c] based on the peak area (280 nm), assuming equal molar response factors, analyzed on analytical RP-HPLC
[d] calculated as % (w/w) of the peanut skin extract and grape seed extract.

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Chapter 4

Procyanidin dimers A1, A2 and B2 are absorbed without conjugation or methylation from the small intestine of rats

Based on: Maaike M. Appeldoorn, Jean-Paul Vincken, Harry Gruppen, and Peter C. H. Hollman, Procyanidin dimers A1, A2 and B2 are absorbed without conjugation or methylation from the small intestine of rats, *accepted, J. Nutr.* **2009**.

Abstract

Intervention studies with procyanidin-rich extracts and products like cocoa and wine suggest protective effects of procyanidins against cardiovascular diseases. However, there is no consensus on the absorption and metabolism of procyanidin dimers. Interestingly, nothing is known about the absorption of A-type procyanidins. In the current study, the absorption and metabolism of purified procyanidin dimers A1 (epicatechin-(2-O-7, 4-8)-catechin), A2 (epicatechin-(2-O-7, 4-8)-epicatechin), and B2 (epicatechin-(4-8)-epicatechin), A-type trimers, and monomeric epicatechin were compared by *in situ* perfusion of the rat small intestine. Potential enhancement of the absorption of dimers by oligomers with a higher degree of polymerization was also tested. Unmodified and methylated metabolites were distinguished from their conjugates by analyzing the difference in the amount of unmodified and methylated compounds in samples before and after treatment with β-glucuronidase. Our results showed for the first time that A1 and A2 dimers are absorbed from the small intestine of rats, and that they are better absorbed than dimer B2. Absorption of the A-type dimers was only 5-10% of that of monomeric epicatechin. None of the dimers were conjugated or methylated in contrast to epicatechin, which was partly methylated and 100% conjugated. A-type trimers were not absorbed. Furthermore, we confirmed that the presence of tetrameric procyanidins enhanced the absorption of B2. On the contrary, the tetramers did not affect the absorption of A1. Epicatechin, methylated epicatechin or their conjugates were no metabolites of any of the procyanidins tested.

Key words: Bioavailability; A-type procyanidin dimers; rat; intestinal perfusion

Abbreviations used: DMSO, dimethyl sulfoxide; DP, degree of polymerization; EC, epicatechin; ECD, electro-chemical-detection; LOD, limit of detection; 3OMeEC, 3'-O-methyl-epicatechin; 4OMeEC, 4'-O-methyl-epicatechin; PC, procyanidin; UPLC, Ultra-Performance-liquid-Chromatography

Introduction

Proanthocyanidins, also known as condensed tannins, are one of the most abundant subclasses of phenolic compounds in our diet (*1)*. Many foods and beverages that are regularly consumed contain high amounts of proanthocyanidins, e.g. apples, berries, nuts, chocolate, wine and cranberry juice (*1*). The most common subgroup of proanthocyanidins is the procyanidins (PCs). They are oligomers of exclusively (epi)catechin units and their galloyl derivatives. PC monomeric units are connected through a single C4-C8 or C4-C6 linkage. These PCs are referred to as B-type. Besides B-type, also A-type PCs exist, which have an additional C2-O-C7 or C2-O-C5 linkage (**Figure 1**). PC sources always contain the B-type. In some sources A-type PCs are additionally present, like in cranberries, peanuts, plums and spices like cinnamon (*1*).

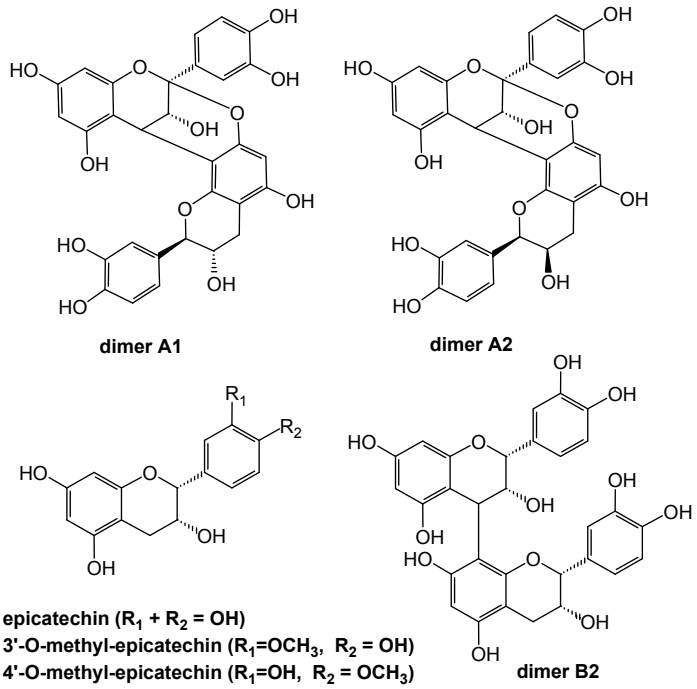

Figure 1. Structures of A1, A2, B2, epicatechin, 3'-*O*-methyl-epicatechin and 4'-*O*-methyl-epicatechin, that were tested for their bioavailability or detected as metabolites.

Intervention studies with PC-rich extracts and products like cocoa and wine suggest protective effects of PCs against cardiovascular diseases (*2, 3*). *In vitro* studies reported an increased antioxidant (*4*) and antitumor activity (*5, 6*) and an inhibitory effect on pancreatic lipase (*7*), with increasing degree of polymerization (DP) on the basis of molarities. However, it is unlikely that the high DP PCs will reach the plasma. After consumption of cocoa (*8*) and a grape seed extract (*9*), from the PCs only dimers B2 (epicatechin-(4-8)-epicatechin) and B1 (epicatechin-(4-8)-catechin) have been detected in human plasma. In humans and rats, besides monomers only PC dimers seem to be absorbed intact (*8-10*), but with a much lower efficiency than the monomers (*11*). In a study performed by Shoji and coworkers (*12*), also PC trimers were detected in rat plasma, which might be explained by the high dose of 1000 mg/kg that was administered. Their data also showed that PC oligomers with a DP ≥ 8 increased the absorption of PC dimers and trimers. Only few studies used single pure PC dimers to study their absorption (*10, 13-15*). No consensus has been obtained on the absorption and metabolism of B-type PC dimers. Some studies report the presence of epicatechin and its methylated and/or conjugated forms as B-type PC dimer metabolites (*10, 13*), while others did not detect any absorption at all (*14, 15*). All these studies only included B-type dimers. Nothing is known about the absorption of A-type PC.

In the current study, absorption and metabolism of epicatechin and purified PC dimers B2, A1 (epicatechin-(2-O-7, 4-8)-catechin) and A2 (epicatechin-(2-O-7, 4-8)-epicatechin) (**Figure 1**) are compared. Absorption of A-type PC might be different from that of B-type PC due to the presence of an additional linkage that alters its structure. Also two mixtures containing only A-type trimers or tetramers were tested. To investigate the influence of the presence of higher DP oligomers on the absorption of dimers, perfusion of dimers was also performed together with a mixture of A-type tetramers. Absorption was measured by *in situ* perfusion of the rat small intestine, using the protocol of Arts and coworkers (*16*). Already upon absorption, phenolic compounds can be conjugated by glucuronidation and/or sulfation and in addition methylation can take place (*17*). Samples were analyzed for the presence of unmodified, methylated and/or conjugated metabolites by measuring their unmodified and methylated forms before and after treatment with a β-glucuronidase/sulfatase.

Materials and Methods

Chemicals

Procyanidin dimers B2 (from grape seeds), A1, A2, a mix of A-type trimers (A-type DP3) and a mix of A-type tetramers (A-type DP4) (from peanut skin) were purified as described

in **Chapters 2** and **3**. (-)-Epicatechin and β-glucuronidase with sulfatase activity (*Helix pomatia*, G1512) were purchased from Sigma (St Louis, MO, USA). 3'-*O*-methyl-epicatechin (3OMeEC) and 4'-*O*-methyl-epicatechin (4OMeEC) (**Figure 1**) were purchased from Gerbu Biochemicals (Gaiberg, Germany). All other chemicals used were of analytical grade.

Animals and diets

The experimental protocol was approved by the Animal Welfare Committee of Wageningen University, Wageningen, The Netherlands. Male Wistar rats (Harlan, Horst, The Netherlands; specific pathogen free, n= 49), mean body weight ~ 200 g at arrival, were housed in groups of two. The room was temperature controlled (19-22 °C) with 55% relative humidity and a 12 h light-dark cycle (lights on at 6 am). After arrival, the rats were fed a standard diet for 4 days to acclimatize, followed by 7 days on a commercially available radiated flavonoid free diet (adjusted NIH-31 diet, Arie Blok, Woerden, The Netherlands, (see **Table 1** for composition). Rats had *ad libitum* access to water and feed. In total seven compounds and/or combinations were tested for their bioavailability, namely epicatechin, B2, A1, A2, A-type DP3, A-type DP4 and a mixture consisting of A-type DP4, B2 and A1 (**Table 2**). For each compound or mixture the perfusion was performed in six rats. There was no significant difference in average weight between the different treatment groups (**Table 2**).

Table 1. Composition of the radiated flavonoid free diet, which was fed to the rats for 7 days before perfusion experiments were performed.

composition	percentage (w/w)
glucose (Cerelose ™)	43.79
D,L-methionine	0.20
salt	0.16
choline 50%	0.25
KCl	0.49
KH_2PO_4	0.67
MgO	0.04
$CaHPO_4.2*H_2O$	1.10
$CaCO_3$	0.90
corn starch	10.00
cellulose	5.00
acid casein	22.00
$MgSO_4.7*H_2O$	0.40
corn oil	14.50
vitamin premix NIH-31	0.25
trace element premix NIH-31	0.25
sum	**100**

Table 2. The average weight and bile flow of the rats that were used for perfusion and the recovery of each test compound from the perfusion buffer.

compound/mixture		perfusion buffer concentration (µM ± SE) t=0	t=30		rats (n=6)[a] mean weight (g ± SE)	bile flow (n=5)[a] mean (mg/min ± SE)
epicatechin		100	100	± 2	273.7 ± 2.3	7.7 ± 1.2
B2		100	96	± 3	273.3 ± 3.1	7.9 ± 0.8
A1		100	108	± 8	271.4 ± 3.0	7.3 ± 1.0 [b]
A2		100	96	± 5	276.7 ± 3.3	9.3 ± 0.7
A-type DP3		100	102	± 2	274.3 ± 2.7	8.4 ± 0.6
A-type DP4		100	105	± 7	278.8 ± 4.3	7.3 ± 1.0 [b]
mixture	A1	100	96	± 2	277.7 ± 3.3	8.1 ± 0.7
	B2	100	94	± 3		
	A-type DP4	100	94	± 2		

[a] the average weight and bile flow of the rats were not significantly different (One-way Anova, $P \leq 0.05$)
[b] n=6

In situ perfusion of the small intestine

The perfusion was performed as described by Arts and co-workers (*16*). In short, rats were anaesthetized and the bile duct, portal vein and small intestine (duodenum, jejunum and ileum) were cannulated and the small intestine gently rinsed with 60 mL saline (0.9% (w/v) NaCl) (37 °C). Each test substance was dissolved in dimethyl sulfoxide (DMSO) and diluted to a final concentration of 100 µM (0.1% (v/v) DMSO) with potassium phosphate buffer (5 mM, pH 6.7) containing 100 mM NaCl, 20 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$ and 0.5 mM sodium taurocholate. The saline in the intestine was replaced with 15 mL of the test solution. Subsequently, the cannula of the intestine was immediately connected to the pump (Meyvis, Minipuls 3, Gilson), after which perfusion started (1 mL/min, 30 min). Samples were taken of bile (t=0, 0-15 and 15-30 min) and portal vein blood (t=0, 15 and 30 min). In addition, samples were taken from the perfusion solution before entering the cannulated intestine (t=0 and 30 min) and after passing the cannulated intestine (t=10, 20 and 30 min). The bile flow was determined by weighing the samples taken (**Table 2**) and was shown not to be significantly different between the treatment groups.

Preparation of samples

Plasma, bile and perfusion buffer samples were similarly treated as described by Donovan and coworkers (*14*). The amounts of unmodified (not metabolized) epicatechin, A1, A2, B2 or DP3 and their methylated and/or conjugated (glucuronides and sulfates) forms were determined. The amounts of conjugates were determined by the measurement of unmodified and methylated forms before and after treatment with a β-glucuronidase/sulfatase, which hydrolyses the conjugates. Bile (20 µL) was mixed with β-glucuronidase (10 µL, ~60.000 U/ mL sodium acetate buffer, 0.5 M, pH 5.0 containing 7

g/L ascorbic acid) and incubated for 1 h at 37 °C. Subsequently, samples were deproteinized with 100 µL methanol containing 200 mM hydrochloric acid and centrifuged (12000 g; 10 min; 4 °C). Plasma (80 µL) was incubated with 35 µL β-glucuronidase and perfusion buffer samples (50 µL) were incubated with 35 µL β-glucuronidase and 20 µL sodium acetate buffer (7 g/L ascorbic acid) to reach a pH of 5.0. Both plasma and perfusion buffer samples were incubated similarly, but for 2 h at 37 °C, and subsequently deproteinized with 200 µL methanol containing 200 mM hydrochloric acid. The stability of each test compound (100 µM) incubated for 2 h at similar conditions as used during the enzyme incubation was ≥ 90% in the absence of β-glucuronidase and ≥ 89% in the presence of β-glucuronidase except for A-type DP3 (82%).

Verification of β-glucuronidase activity under various test conditions

To test if the enzyme activity was altered by the presence of bile and plasma, 20 µL of plasma, bile or sodium acetate buffer was mixed with 8.5 µL β-glucuronidase (~ 8000 U/mL sodium acetate buffer, 0.5 M, pH 5.0) and 7 µL phenolphthalein-glucuronide (3 mM in demineralized water) and incubated for 2 h at 37 °C. Subsequently, 145 µL sodium glycine buffer (200 mM, pH 10.4) was added and the color was measured at 540 nm. Samples were incubated in duplicate. A blank, containing inactive β-glucuronidase (the enzyme was added after addition of glycine buffer) served as a control. β-Glucuronidase activity was not significantly affected by the presence of plasma or bile and was 110% (SD 12) and 89% (SD 6), respectively, compared to the activity measured in the absence of plasma or bile. Each time samples were incubated with β-glucuronidase, a control sample with phenolphthalein-glucuronide was analyzed to confirm β-glucuronidase activity. In a similar way the effect of 1.5 -170 µM PC dimers on β-glucuronidase activity was tested. No significant decrease in activity was detected due to the presence of the dimers.

HPLC-Electro-Chemical-Detection (ECD)

Quantitative determination of PCs and monomers was performed with HPLC-ECD, as described previously (*18*). The cells of the coularray detector were set on 250, 350, 450 and 600 mV. The mobile phase was composed of (A) sodium phosphate buffer (100 mM, pH 3.35) containing 10 mg/L SDS and (B) 60% (v/v) acetonitrile, 10% (v/v) methanol in sodium phosphate buffer (30 mM, pH 3.45) containing 15 mg/L SDS. The flow rate was 1 mL/min. The most optimal column was selected for each analyte and matrix combination. Analysis was performed on four different columns. An Inertsil ODS-3, 4.6 mm i.d. x 150 mm, 5 µm column (GL Sciences) was used for the detection of EC, 3OMeEC, 4OMeEC in bile and plasma (before and after β-glucuronidase treatment) and for all compounds in the perfusion fluid samples (before and after β-glucuronidase treatment). An XterraRP dC18, 4.6 mm i.d. x 150 mm, 3.5 µm column (Waters) was used to analyze A1, A2 and B2 in plasma (before and after β-glucuronidase treatment) and bile (before β-glucuronidase

treatment). An Atlantis dC18, 4.6 mm i.d. x 150 mm, 3 µm column (Waters) was used for the analysis of A-type DP3 in bile (before β-glucuronidase treatment) and A-type DP4 in bile and plasma (before β-glucuronidase treatment) and a Symmetry Shield 4.6 mm i.d. x 150 mm, 5 µm column (Waters) for A-type DP3, A1, A2 and B2 in bile (after β-glucuronidase treatment) and A-type DP3 in plasma (before and after β-glucuronidase treatment). The elution profile was as follows: 0-20 min, B: linearly 20%-75%; 20-25 min, B: linearly 75%-100%; 25-28 min, isocratic at 100% B; 28-30 min, B: linearly 100%-20%, and reconditioning of the column for 3 min. For each sample 50 µL was injected. The limit of detection (LOD) for EC, 3OMeEC, 4OMeEC, B2, A1 and A2 was about 20 nM and for A-type DP3 60 nM.

UPLC-MS analysis

UPLC-MS analysis was used to identify methylated and/or glucuronidated dimers and to confirm the presence of epicatechin, A1, A2, B2, DP3, 3OMeEC and 4OMeEC. A Micromass Quattro Ultima system (Waters), equipped with an ESI source and controlled by Masslynx software was coupled to an Acquity Ultra Performance LC system (Waters). Analysis was performed on an Acquity UPLC-BEHC18, 2.1 mm i.d. x 100 mm, 1.7 µM column (Waters) with a flow rate of 0.4 mL/min, which was reduced to 0.2 mL/min by capillaries before it entered the MS. The column oven temperature was 40 °C. The mobile phase was composed of (A) 10% (v/v) aqueous acetonitrile adjusted to pH 3.35 with formic acid and (B) 60% (v/v) aqueous acetonitrile with 10% (v/v) methanol adjusted to pH 3.45 with formic acid. The elution profile was as follows: 0-1 min, isocratic on 10% B; 1-5 min, B: linearly 10%-50%; 5-8 min, B: linearly 50%-100%; 8-10 min isocratic at 100% B; 10-10.1 min, B: linearly 100%-10%, and reconditioning of the column for 0.4 min. For each sample 15 µL was injected.

Measurements were performed in the negative ion mode with a capillary voltage of 2.0 kV, a cone voltage of 25 V, a capillary temperature of 300 °C and a source temperature of 120 °C. The collision energy values were optimized by infusion of standards. For each perfused compound/mixture, two plasma samples and two bile samples, both before and after β-glucuronidase treatment, were analyzed for the presence of EC, 3OMeEC, 4OMeEC, B2, A1, A2 and A-type DP3 in the Multiple-Reaction-Monitoring (MRM) mode with each 2-3 precursor/product ion pairs as indicated in **Table 3**. Identification was performed on the basis of MS data and retention times of standards. The LOD for each compound was about 10 nM. Furthermore, samples were screened for the presence of glucuronidated and/or methylated A- and B-type dimers (*m/z* 589, 591, 751, 753, 765, 767 $[M-H]^-$) in the Select-Ion-Recording (SIR) mode and by product ion scans (**Table 3**).

Table 3. MS detection of the test compounds and their metabolites.

compound [a]	precursor ion $[M-H]^-$	product ion [b]	CE (V) [c]
epicatechin	289.1	109.2	20
		203.2	20
		245.2	15
3OMeEC/4OMeEC	303.0	137.1	20
		244.1	15
		285.2	15
A1/A2	575.1	285.1	25
		289.1	25
		449.2	25
B2	577.1	289.1	20
		407.2	20
		425.2	15
DP3	863.1	575.2	20
		711.2	20
methylated B2	591.0	-	25
B2-glucuronide	753.0	-	25
methylated B2-glucuronide	767.0	-	20
methylated A1/A2	589.0	-	25
A1/A2-glucuronide	751.0	-	25
methylated A1/A2-glucuronide	765.0	-	20

[a] 3OMeEC= 3'-O-methyl-epicatechin and 4OMeEC= 4'-O-methyl-epicatechin.
[b] the most abundant product ions were determined by infusion with standards.
[c] CE= collision energy in Volts

Statistical Analysis

A Student's *t* test was performed to determine significant decreases of each component in perfusion buffer or a significant increase in plasma or bile. One-way Anova using SPSS software (SPSS for windows, version 15.0, SPSS Inc., Chicago, IL) was used to test for statistical differences of weight and bile flow of the rats (**Table 2**) between treatment groups. A probability of ≤ 0.05 was considered significant. Error bars are depicted as mean ± SE.

Results

Perfusion fluid

Perfusion fluid was sampled before it entered the cannulated intestine (t=0 and t=30 min) to test the stability of the test compounds in the buffer used. No significant decline was

detected for any of the test compounds during the 30 min of perfusion (**Table 2**). Incubation with β-glucuronidase of the perfusion fluid that had passed the cannulated intestine showed that no conjugates were present in any of the treatments. Thus conjugation by epithelial cells and subsequent excretion of conjugates back into the lumen did not take place.

Plasma

Perfusion with procyanidins

MS analysis (see **Table 3** for precursor and product ions) showed that methylated and/or conjugated dimers and unmodified, methylated and/or conjugated epicatechin monomers were absent after perfusion with any of the PCs. Perfusion with pure B2 did not result in its (< 10 nM) absorption in plasma. After perfusion with dimer A1 or A2, both were detected in plasma by LC-MS analysis (no further results shown). Unmodified A1 increased from 0.05 (SE 0.02) μM after 15 min to an average of 0.11 (SE 0.04) μM after 30 min, quantified with ECD (**Figure 2A**). Dimer A2 could not be quantified due to disturbance of peaks derived from the matrix.

Perfusion with A-type trimers (DP3) did not result in their absorption (< 10 nM) and no monomers or dimers were detected. Perfusion of the mixture (containing DP4, B2 and A1) resulted in the absorption of unmodified A1 and B2, which was confirmed with LC-MS (**Figures 3A** and **B**). A1 significantly ($P \leq 0.03$) increased from 0.02 (SE 0.002) μM at 15 min to 0.06 (SE 0.01) μM at 30 min, and 0.05 (SE 0.02) μM B2 was detected only after 30 min (**Figure 3B**). Perfusion with A-type DP4 was performed as a control to rule out depolymerization into monomers, dimers or trimers. No monomers, dimers or trimers were detected.

Perfusion with epicatechin

No unmodified or methylated epicatechin was detected in plasma, but only their conjugated forms (determined as epicatechin and methylated epicatechin after enzymatic hydrolysis of the conjugates) were found. After perfusion with epicatechin for 30 min, the concentration of epicatechin conjugates was 0.7 (SE 0.1) μM (**Figure 2C**). Furthermore, 3'-*O*-methyl-epicatechin conjugates were present, which reached a concentration of 0.3 (SE 0.04) μM after 30 min. MS analysis confirmed the identity of both epicatechin (*m/z* 289.1 $[M-H]^-$) and 3'-*O*-methyl-epicatechin (*m/z* 303.0 $[M-H]^-$) and their retention times were equal to those of the standards (**Figure 3C**). 4'-*O*-Methyl-epicatechin was not detected. The molar ratio between epicatechin and 3'-*O*-methyl-epicatechin in plasma was 2.3 (after 30 min).

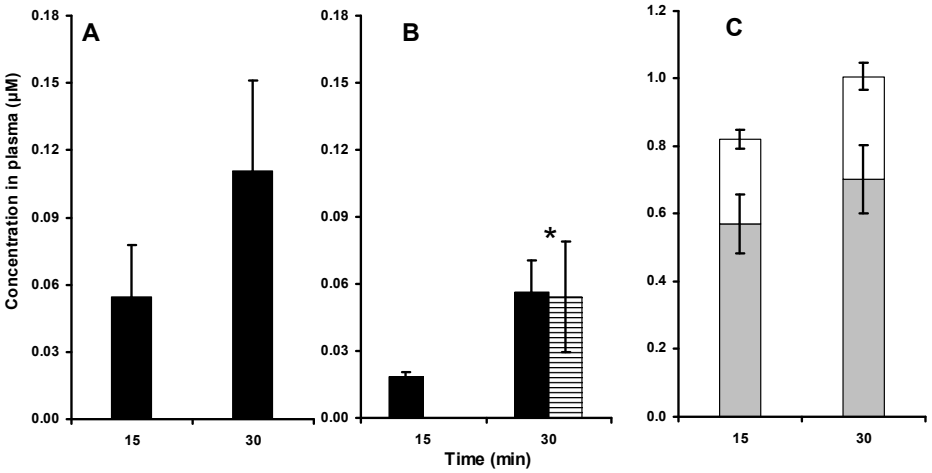

Figure 2. Plasma concentrations of A1 (■), B2 (⊟), epicatechin conjugates (■) and 3'-*O*-methyl-epicatechin conjugates (□) after perfusion with: A1 (panel **A**); a mixture of A1, B2 and A-type DP4 (panel **B**) and epicatechin monomer (panel **C**). Note that the scale of the Y-axis in panel C is different. Error bars represent an average of n ≥ 4 ± SE except for B2 (n= 2). Significant increases in time are indicated with *.

Bile

Perfusion with procyanidins

Similarly as found in plasma, no methylated and/or conjugated dimers were detected in bile (MS analysis, see **Table 3** for precursor and product ions). Treatment with β-glucuronidase introduced many interfering peaks, so quantification of dimers by HPLC-ECD was not always possible. No unmodified, methylated or conjugated monomeric epicatechin was detected after perfusion with any of the PCs. Perfusion with the dimers B2 and A2 did not result (< 10 nM) in the excretion of unmodified B2 or A2 by bile. After perfusion with the mixture only unmodified A1 could be quantified. An average concentration of 0.20 (SE 0.06) and 0.34 (SE 0.06) µM was present after 0-15 and 15-30 min, respectively (**Figure 4A**). However, the presence of unmodified A1 could not be confirmed with LC-MS analysis. After perfusion with both pure A1 and the mixture, the presence of A1 in bile was confirmed with MS analysis, but only after treatment with β-glucuronidase. This might indicate that A1 was excreted by bile as its conjugate, although no A1-glucuronide [*m/z* 751.0 M-H]$^-$ could be identified. As with plasma, perfusion with trimers (DP3) did not result in their absorption.

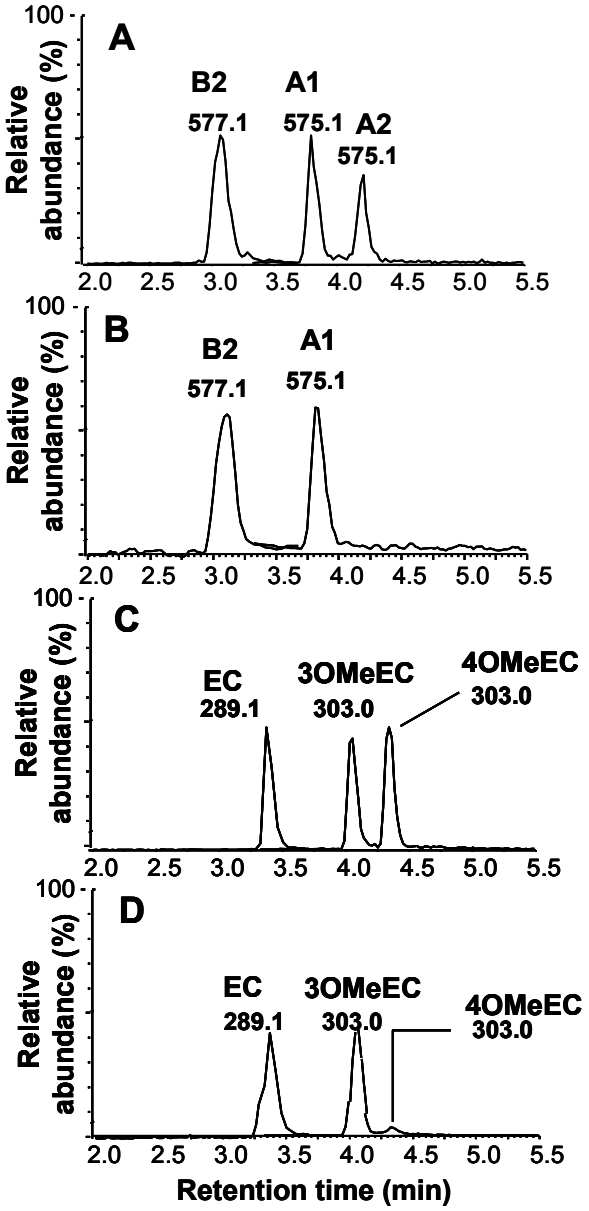

Figure 3. MS detection of specific masses in the MRM (negative) mode after separation by UHPLC-MS of **A**) standard of B2, A1 and A2; **B**) B2 and A1 in plasma after perfusion with a mix (B2, A1 and DP4); **C**) standard of epicatechin (EC), 3'-*O*-methyl-epicatechin (3OMeEC) and 4'-*O*-methyl-epicatechin (4OMeEC); and **D**) EC, 3OMeEC and 4OMeEC in bile (after deconjugation with β-glucuronidase) after perfusion with EC.

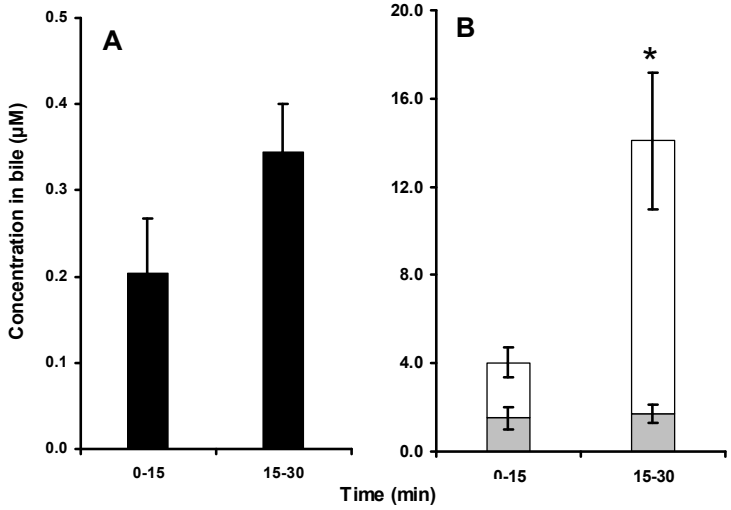

Figure 4. Bile concentrations of A1 (■), epicatechin conjugates (■) and 3'-*O*-methyl-epicatechin conjugates (□) after perfusion with: a mixture of A1, B2 and A-type DP4 (panel **A**) and epicatechin monomer (panel **B**). Note that the scale of the Y-axis is different. Error bars represent an average of n=5 ±SE. Significant increases in time are indicated with *.

Perfusion with epicatechin

No unmodified epicatechin was detected in bile. 3'-*O*-Methyl-epicatechin conjugates were the major metabolites present in bile. The concentration increased significantly ($P \leq 0.01$) from 2.5 (SE 0.7) µM after 0-15 min to a concentration of 12.4 (SE 3.1) µM after 15-30 min (**Figure 4B**). The concentration of epicatechin-conjugates was 1.7 (SE 0.4) µM after 15-30 min. The presence of epicatechin-conjugate and 3'-*O*-methyl-epicatechin-conjugate, determined as epicatechin (*m/z* 289.1) and 3'-*O*-methyl-epicatechin (*m/z* 303.0), respectively, after treatment with β-glucuronidase, was confirmed with MS analysis (**Figure 3D**). After 30 min, the molar ratio 3'-*O*-methyl-epicatechin-conjugate / epicatechin-conjugate was 7.3. Also the presence of 4'-*O*-methyl-epicatechin-conjugate (determined as 4'-*O*-methyl-epicatechin (*m/z* 303.0)) was established on the basis of MS analysis (**Figure 3D**), but could not be quantified with ECD.

Discussion

We showed for the first time that procyanidin dimers A1 and A2 can be absorbed from the rat small intestine. Additionally, we showed that they were better absorbed than dimer B2. The absorption of dimers was 10-20 times less than that of epicatechin (epicatechin

conjugates + 3'-*O*-methyl-epicatechin conjugates). This is a smaller difference than found in a human study after the consumption of flavanol-rich cocoa (*8*), where about 50 times less dimer was absorbed than epicatechin. High DP oligomers seemed to enhance the absorption of B2, but did not affect the absorption of A1. In contrast to epicatechin, conjugation or methylation of procyanidin dimers did not occur. The A-type trimers were not absorbed.

Bioavailability of procyanidin dimers

Our results showed that A1, A2 and B2 were absorbed as unmodified compounds, which is in line with the results of Shoji and coworkers (*12*), who predominantly (90%) detected unmodified dimers in rat plasma. Therefore, in contrast to epicatechin (*19-21*), conjugation of procyanidins seemed to be of minor importance. Neither epicatechin nor its metabolites were detected in plasma and bile after perfusion with any of the dimers, A-type trimers and tetramers. This does not correspond to data obtained by Spencer and coworkers (*13*). These authors detected epicatechin as the main metabolite after *ex vivo* perfusion of the rat small intestine with B2 and B5 (epicatechin-(4-6)-epicatechin).

Procyanidin oligomers affect the absorption of B2

The presence of tetramers enhanced the absorption of B2, but not that of A1. No A1, B2 or trimers were detected after perfusion of tetramers only. The results on B2 are in line with the findings of Shoji and coworkers (*12*) who detected elevated concentrations of B1, B2 and trimer C1 when low DP oligomers (DP 2-5) were fed in combination with high DP oligomers (≥ DP 8). Their hypothesis was that the high DP oligomers bind to mucosal proteins facilitating a better absorption of the lower DP oligomers. The influence of high DP oligomers on the absorption of low DP oligomers should be examined in more detail as high DP oligomers usually coexist with low DP oligomers in foods (*1*). Therefore, bioavailability of some procyanidins might be underestimated in studies performed with only low DP oligomers.

Metabolism of dimers is of minor importance

Methylation

In vitro, the enzyme catechol-O-methyl-transferase, responsible for the methylation of different flavonoids *in vivo* (*22*), has been demonstrated to be able to methylate PC dimers (*13*). However, in contrast to epicatechin, no methylated and/or conjugated dimers were detected in plasma or bile in our *in vivo* study. The lack of methylated and/or conjugated procyanidin standards complicates the analysis of such metabolites. Retention times and product ions to optimize MS detection are not known. Therefore, the settings might not have been optimal to obtain a sufficiently low detection limit. Small amounts of methylated

B-type dimers have been detected after an *ex vivo* perfusion of the rat small intestine (*13*). Also methylated dimers were detected in plasma with MS analysis after administration of 1000 mg/kg B1 and B2 to rats (*12*). In both studies, the methylated dimers were not dominating. In contrast, in a rat study performed with specific dimers from wine (*23*), composed of two epicatechin units linked by an ethyl bridge, tetramethylated dimers were found as the only metabolites in plasma (*24*). However, the additional ethyl bridge between the monomeric units might have changed the metabolism of these dimers. Our *in vivo* study together with others (*12, 13*) showed that methylation is of minor importance for the absorption of B1, A1 and A2.

Conjugation

Glucuronidation of phenolic compounds has been described to occur both in the enterocytes of the small intestine and the liver (*22*). For several flavonoids like quercetin (*25*), genistein and apigenin (*26*), the glucuronides formed in the enterocytes were excreted back into the lumen. Levels of excretion of flavonoids ranging between 0-52% of the initial absorbed dose have been reported (*22*). In our study, the perfusion fluid that had passed the cannulated intestine did not contain any conjugates for any of the compounds tested. Therefore, conjugation and subsequent excretion back into the lumen did not occur for epicatechin, procyanidin dimers B2, A1 and A2, and A-type trimers and tetramers. This is in line with results obtained for catechin (*27*). Also in plasma, conjugates of the procyanidin dimers A1, A2 and B2 or trimers were absent.

In bile, A1 was detected (with LC-MS) only after treatment with β-glucuronidase, which suggests its conjugation in the liver. However, the presence of glucuronidated A1 in bile could not be confirmed with LC-MS. As described above, the lack of appropriate standards complicates the analysis of such metabolites. Dimers B2 and A2 were not detected in bile, nor their metabolites. Considering that bile contained about 6 times more A1 and 14 times more epicatechin metabolites than plasma and that B2 was found in plasma after perfusion with the mixture, this suggests that B2 and A2 are excreted by bile to a very low extent, if at all.

Absorption and metabolism of epicatechin

Epicatechin was used as a reference compound because its absorption has been described in many animal (*19-21*) and human studies (*28-30*). The detection of conjugated epicatechin and conjugated 3'-*O*-methyl-epicatechin (molar ratio: 2.3) as main metabolites is in good agreement with that of catechin absorption (molar ratio: 2.8) tested previously in the same perfusion system (*27*). Our results on the metabolism of epicatechin are in line with the findings in humans: Epicatechin is completely conjugated, and methylated epicatechin occurs (*28-30*).

In conclusion, our results show that dimers A1 and A2 can be absorbed, but their absorption is 10-20 fold lower than that of epicatechin. In contrast to epicatechin, these dimers are not conjugated or methylated, thus conserving their biological activity inside the body. Because bioactivity is mostly altered by conjugation, the conserved activity of the free dimers *in vivo* might partly compensate their lower absorption as compared to epicatechin. Furthermore, bioavailability of procyanidins might be underestimated as synergy between low and high DP oligomers seems to take place. As procyanidin contents of foods are relatively high, procyanidin dimers might contribute to a protective effect of polyphenol-rich foods.

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Chapter 5

Procyanidin dimers are metabolized by human microbiota with 2-(3,4-dihydroxyphenyl)acetic acid and 5-(3,4-dihydroxyphenyl)-γ-valerolactone as the major metabolites

Based on: Maaike M. Appeldoorn, Jean-Paul Vincken, Anna-Marja Aura, Peter C.H. Hollman and Harry Gruppen, Procyanidin dimers are metabolized by human microbiota with 2-(3,4-dihydroxyphenyl)acetic acid and 5-(3,4-dihydroxyphenyl)-γ-valerolactone as the major metabolites, *J. Agric. Food Chem.*, **2009**, 57 1084-1092

Abstract

Procyanidins (PCs) are highly abundant phenolic compounds in our diet and might be responsible for the health effects of chocolate and wine. Due to low absorption of intact PCs, microbial metabolism might play an important role. Sofar, only a few studies, with crude extracts, rich in PCs but also containing a multitude of other phenolic compounds, have been performed to reveal human microbial PC metabolites. Therefore, the origin of the metabolites remains questionable. We performed an *in vitro* fermentation of purified PC dimers with human microbiota. The main metabolites identified were 2-(3,4-dihydroxylphenyl)acetic acid and 5-(3,4-dihydroxyphenyl)-γ-valerolactone. Other metabolites detected were: 3-hydroxyphenylacetic acid, 4-hydroxyphenylacetic acid, 3-hydroxyphenylpropionic acid, phenylvaleric acids, monohydroxylated phenylvalerolactone and 1-(3',4'-dihydroxyphenyl)-3-(2",4",6"-trihydroxyphenyl)propan-2-ol. Metabolites that could be quantified accounted for at least 12 mole % of the dimers, assuming 1 mole of dimers is converted into 2 moles of metabolite. A degradation pathway, which is partly different from that of monomeric flavan-3-ols, is proposed.

Key words: degradation pathway, human microbial metabolites, hydroxyphenylacetic acids, proanthocyanidins, valerolactones

Abbreviations used: PCs, procyanidins; CV, column volume; ECD, coularray detector; BA, benzoic acid; PPA, 3-(phenyl)propionic acid; 3,4-diHPA, 2-(3,4-dihydroxyphenyl)acetic acid; 3,4-diHPP, 3-(3,4-dihydroxyphenyl)propionic acid; 3-HPP, 3-(3-hydroxyphenyl)propionic acid; 3-HPA, 2-(3-hydroxyphenyl)acetic acid; 4-HPA, 2-(4-hydroxyphenyl)acetic acid; 3,4-diHPP-2-ol, 1-(3',4'-dihydroxyphenyl)-3-(2",4",6"-trihydroxyphenyl)propan-2-ol; 3,4-diHPVal, 5-(3,4-dihydroxyphenyl)-γ-valerolactone; 3,4-diHPV, 5-(3,4-dihydroxylphenyl)valeric acid; HPP-2-ol, 1-(3' or 4'-hydroxyphenyl)-3-(2",4",6"-trihydroxyphenyl)propan-2-ol; 3 or 4-HPVal, 5-(3 or 4-hydroxyphenyl)-γ-valerolactone; 3 or 4-HPV, 5-(3 or 4-hydroxyphenyl)valeric acid; PVal, 5-phenyl-γ-valerolactone; SPE, solid phase extraction.

Introduction

The intestinal microbiota have been shown to be important for the bioavailability (*1, 2*) and bioactivity (*3*) of certain phenolic compounds that are present in many daily consumed food products, such as fruits, vegetables and nuts (*4*). Proanthocyanidins (PAs) are one of the most abundant phenolic compounds in our diet (*5*). They potentially are responsible for the cardioprotective properties of cocoa products and wine (*6, 7*). PAs are thought to be poorly absorbable. Hence, microbiota of the colon play an important role in their digestion. However, knowledge on the fate of PAs in the body is still incomplete. The most common subclass of PAs is the procyanidins (PCs), which exclusively consist of (epi)catechin units and their galloyl derivatives. The monomeric units of PCs are linked through a C4-C8 or C4-C6 bond (B-type), which can coexist with an additional C2-*O*-C7 bond (A-type). Only PC dimers are believed to be absorbed intact (*8-10*), albeit at much lower efficiency than the monomeric units (*11*). Thus, high amounts of PCs will reach the colon intact as shown by Kahle and co-workers (*12*)

The monomeric units of PCs, (epi)catechin, are known to be degraded into several phenolic acids namely: phenylvaleric acids, phenylpropionic acids, phenylacetic acids, benzoic acids and hippuric acids (**Figure 1**), all mainly hydroxylated at the *meta* position (*13-16*).

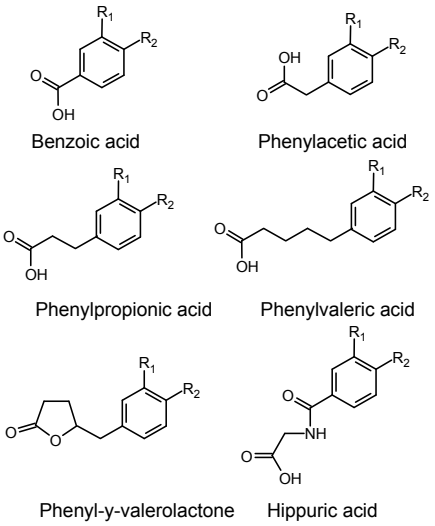


Figure 1. Basic structures of microbial metabolites from various species formed out of (epi)catechin, which are usually hydroxylated at the positions indicated by R_1 and/or R_2.

Furthermore, 5-(3,4-dihydroxyphenyl)-γ-valerolactone and 5-(3-hydroxyphenyl)-γ-valerolactone have been identified as metabolites of both epicatechin and catechin in various species including man (*13, 17-22*).

Ingestion of PC-rich products resulted in increased urinary excretion of most of the phenolic acids mentioned above (*14-16, 23, 24*). Therefore, phenolic acids seem to be major metabolites of PCs. However, often PC-rich mixtures were used, which not exclude the possibility that the metabolites found actually originates from other phenolic compounds that are also present in those mixtures. The microbial metabolic pathways of PCs are still not completely characterized. Knowledge on the formation and identity of these metabolites is important as they could contribute to the potential health effects of PCs in e.g. cocoa and wine.

A limited number of *in vivo* and *in vitro* studies, mostly in rats, have been performed to characterize microbial metabolites of PCs (*14, 15, 23-26*). Studies performed with animals can result in substantially different bioconversions compared to humans, as has been shown for catechin and epicatechin (*19, 27*). *In vitro* fermentations are easier to perform than *in vivo* studies. They have the additional advantage that relatively high concentrations of metabolites, not yet affected by absorption and metabolism, are present, which facilitates detection. Sofar, only one *in vitro* fermentation of PC oligomers with an average degree of polymerization (DP) of 7 has been performed with microbiota of one human subject (*28*). The use of large PC oligomers might inactivate the microbiota as they have pronounced antimicrobial properties (*29, 30*), likely due to their high interaction with proteins (*31, 32*). Therefore, in the present study, a well-defined and pure PC dimer fraction was subjected to an *in vitro* fermentation with human feces as a source of microbiota. Feces of 4 volunteers was pooled, as commonly done by others (*27, 33*), to adjust for inter-individual variations. The main objective of this study was to identify human microbial metabolites originating from PCs. Based on the metabolites that were detected a possible degradation route is proposed.

Materials and Methods

Materials

All organic solvents used for HPLC analysis were HPLC grade. Vitaflavan (DRT, Levita Chemical International NV, Antwerpen, Belgium), a commercially available grape seed extract, was used as PC dimer source. PC standards B1 (epicatechin-(4-8)-catechin), B2 (epicatechin-(4-8)-epicatechin), B3 (catechin-(4-8)-catechin) and B4 (catechin-(4-8)-epicatechin) were purchased from Apin Chemicals (Abingdon, UK). Standards of (-)-epicatechin and (+)-catechin and the phenolic acids 3-(3-hydroxyphenyl)propionic acid (3-

HPP), 3-(3,4-dihydroxyphenyl)propionic acid (3,4-diHPP), 2-(3-hydroxyphenyl)acetic acid (3-HPA), 2-(4-hydroxyphenyl)acetic acid (4-HPA), 2-(3,4-dihydroxyphenyl)acetic acid (3,4-diHPA), 3-(phenyl)propionic acid (PPA), and benzoic acid (BA) were purchased from Sigma-Alldrich chemie (Steinheim, Germany).

Isolation and characterization of grape seed procyanidins

Removal of gallic acid substituents

To enable preparative HPLC isolation of PC dimers, gallic acid substituents were enzymatically removed as described in **Chapter 3**. The percentage of galloylation before and after tannase treatment was determined by thiolysis. To be able to distinguish between the monomeric flavan-3-ols that are formed during thiolysis and the ones already present in the extract, the monomeric flavan-3-ols were removed with SPE before thiolysis as described by Vidal and coworkers (*34*). The samples were loaded and washed with H_2O (3 CV), after which the monomeric flavan-3-ols were removed with diethylether (3 CV). The PCs were eluted with MeOH (3 CV), evaporated, and dissolved in 2 mL dry MeOH to a concentration of approximately 8 mg/mL. Thiolysis was performed as described by Guyot and coworkers (*35*) with some adjustments. In short, PC samples (250 µL) were mixed with dry MeOH (500 µL) containing 5% (v/v) benzylmercaptan, and 3.3% (v/v) HCl in dry MeOH (250 µL) and incubated at 40 °C for 90 min. After incubation, the samples were dried under air, dissolved in 1 mL dry MeOH, and analyzed on HPLC equipped with UV and MS detection.

RP-HPLC analysis

A Thermo Spectra system was used containing a P 4000 pump, an AS 300 autosampler and an UV 3000 detector (Thermo Separation products, Fremont, CA, USA). Separation was performed on an XterraRP dC18, 4.6 mm ID x 150 mm column with a 3.5 µm particle size (Waters). The mobile phase was composed of (A) H_2O + 0.1% (v/v) HOAc and (B) ACN + 0.1% (v/v) HOAc. The elution profile was as follows: 0-10 min, of 15%-25% B; 10-15 min, 25%-40% B; 15-20 min, isocratic on 40% B; 20-25 min, 40%-95% B; 25-30 min, 95%-15% B, and reconditioning of the column. The flow rate was 0.7 mL/min and detection was performed at 280 nm.

ESI/MS detection

An LCQ Classic equipped with an ESI source was used and controlled by Xcallibur software (Thermo Finnigan, San Jose, CA, USA). The detector was coupled to the HPLC system by means of a splitter (Accurate, LC Packings, Amsterdam, The Netherlands), which reduced the flow rate 20 times. Measurements were performed in the negative mode with an ion spray voltage of 4.5 kV, a capillary voltage of -5.0 V, and a capillary

temperature of 270 °C. The scan range was set from *m/z* 100-2000. The MS/MS function was performed in the data dependent mode. The collision energy value was 27%.

Preparative isolation of procyanidin dimers

Fractionation by DP was performed on a normal phase (NP) column, Inertsil PREP-SIL, 30 mm ID x 250 mm with a 10 µm particle size (GL Sciences, Tokyo, Japan). A Waters system equipped with a 2767 sample manager, a 2525 binary gradient module, a 2996 photodiode array detector and an UV fraction manager was used. The binary mobile phase consisted of (A) hexane and (B) acetone. The elution profile was as follows: 0-30 min, 40%-60% B; 30-50 min, isocratic at 60% B; 50-70 min, 60%-75% B, followed by a washing step at 98% B for 3 min, and reconditioning of the column. The flow rate was 27 mL/min and PDA spectra from 210-300 nm were recorded. The tannase treated PC fraction was dissolved in 10 mL (~70 mg/mL) acetone/hexane/ethanol (7:3:2) and injected. The eluate containing the dimers was collected (28.2-38.1 min), evaporated under vacuum and lyophilized prior to further analysis.

Recovery of phenolic acids and PC dimers from the fermentation buffer

Seven phenolic acids were used to determine their extraction efficiency from a fecal suspension: 3-HPP, 3,4-diHPP, 3-HPA, 4-HPA, 3,4-diHPA, PPA and BA. A 25% (w/v) fecal suspension was made as described previously (*36*) with some alterations. Freshly passed feces were immediately suspended in the buffer and homogenized with a stomacher (Seward, Worthing, UK) operating for 60 s and sieved (1 mm). Part of it was autoclaved to inactivate the microbiota. The active and the inactive suspension (200 µL) were each combined with carbonate-phosphate buffer (pH 5.5) (*37*) containing the phenolic acids (800 µL) in a Kimax tube (n=3 for both active and inactive suspension), resulting in a 5% (w/v) suspension and 200-400 µg/mL phenolic acids. Samples containing the active suspension were immediately acidified to a pH below 2 by adding 60 µL HCl (12.1 M) and 5 mL ethyl acetate to inactivate the microbiota and prevent conversions. The time between the collection of the feces and acidification did not exceed 10 min. Samples were vortexed vigorously and centrifuged at 14000 rpm (Eppendorf 5417C, rotor F45-30-11, Hamburg, Germany) for 10 min at RT. The ethyl acetate layer was pipetted into another Kimax tube. The remaining H_2O phase was re-extracted twice with 5 mL ethyl acetate as described above. The ethyl acetate phases were individually evaporated with a TurboVap (Zymark, Russelsheim, Germany) operated at 50 °C under a stream of nitrogen. The samples were subsequently dissolved in 1 mL methanol and diluted 40 times before analysis with HPLC-UV-ECD as described later. Extraction recoveries are shown in **Table 1**. The third extraction removed only 10% or less of each phenolic acid.

Table 1. Recovery of several phenolic acids after evaporation only, or after extraction from the fermentation suspension including subsequent evaporation

compound	recovery (%) after evaporation	after extraction and subsequent evaporation							
		5% (w/v) active suspension				5% (w/v) inactive suspension			
		1st	2nd	3rd	total	1st	2nd	3rd	total
3,4-diHPA	92 ± 2	64 ± 9	31 ±14	10 ± 0	105 ±12	58 ±12	28 ± 7	9 ± 1	95 ±16
4-HPA	92 ± 1	64 ± 8	26 ± 7	7 ± 2	97 ± 2	75 ±10	28 ± 9	6 ± 1	109 ±11
3-HPA	89 ± 1	60 ± 8	25 ± 5	6 ± 3	91 ± 5	78 ±11	27 ± 8	5 ± 1	110 ± 7
3,4-diHPP	97 ± 1	60 ± 8	23 ± 7	7 ± 1	90 ± 3	69 ± 9	24 ± 8	6 ± 1	99 ± 9
3-HPP	105 ± 1	66 ± 9	25 ± 6	6 ± 3	97 ± 4	86 ±12	27 ± 9	5 ± 1	118 ± 9
PP	120 ± 8	62 ±11	6 ±10	0	68 ± 0	66 ±12	0	0	66 ±12
BA	71 ± 3	42 ± 9	12 ± 9	2 ± 3	56 ± 3	57 ±12	14 ± 8	0	71 ±11

[a] data (average of n=3 ± SD) are based on UV measurements at 270 nm except for the hydroxylated acids, which were detected with coularray. Three subsequent extractions were performed (1st - 3rd), resulting in a total extraction recovery for each phenolic acid.

To test the stability of the dimers during extraction they were dissolved in carbonate-phosphate buffer (pH 5.5) (100 µg/mL) without microbiota and extracted twice as described above. The total ethyl acetate phase (10 mL) was evaporated and the samples were dissolved in 500 µL H_2O/ACN/MeOH (86:12:2), acidified with 8 µL H_3PO_4 (14.8 M) and filtered over a 0.22 µm Acrodisc membrane (PALL corporation, MI, USA). Samples were analyzed with HPLC-UV-ECD. Extraction recovery of dimers was 15-30%. Furthermore, degradation into catechin and epicatechin took place, which together accounted for ~0.6 mole % of the originally added dimers. This was observed based on injection of standards and MS analysis (described later). No other degradation products were detected. Catechin and epicatechin were stable in this system (*38*).

Fermentation of procyanidin dimers by human microbiota

In vitro fermentation process

The dimeric fraction was subjected to fermentation with a pool of human microbiota obtained from four volunteers who usually ingested a normal diet, presented no digestive disease, and had not received antibiotics for at least 3 months as previously described (*36*). Freshly passed feces were immediately taken in an anaerobic chamber, pooled and homogenized in carbonate-phosphate buffer (pH 5.5). The mixture of dimers (5 µmol in total) was fermented by 1% (w/v) and 5% (w/v) fecal suspensions (10 mL) at 37 °C. Samples were taken after 0, 1, 2 and 4 h for the 1% (w/v) suspension and after 0, 1, 2, 4, 6, 8 and 24 h for the 5% (w/v) suspension. Stability of the dimers to the applied conditions was studied by incubating them in the buffer without microbiota or with heat inactivated microbiota. Furthermore, fermentations with active fecal suspensions without added dimers were conducted as negative controls for each time point. All incubations were performed in

triplicate at all time points mentioned above. At each time point, the content of the fermentation bottle was divided over four eppendorfs (2 mL each), frozen rapidly in liquid nitrogen and stored at -20 °C.

Short chain fatty acid analysis

Suppression of SCFA production in the presence of PC dimers could indicate their possible inhibitory effects on microbial enzymes involved in SCFA formation. Analysis of SCFA was performed with capillary gas chromatography as described previously (*39*). The sum of the amounts of acetic acid, propionic acid, 2-methyl-propionic acid and butyric acid was taken and expressed as total SCFA production.

Sample preparation before HPLC analysis

One eppendorf for each time point was put in an ice bath, slowly thawed, transferred into a Kimax tube, acidified and extracted with ethyl acetate twice as described for the analysis of the extraction recovery. The total ethyl acetate phase (10 mL) was evaporated under nitrogen to a small volume, transferred into HPLC vials and evaporated to dryness. Samples were stored at -20 °C.

Analysis of microbial metabolites

HPLC-UV-ECD analysis

An Hitachi system (Tokyo, Japan) composed of L-2100 pumps and a L-2200 autoinjector extended with a Spark Mistral column oven set at 30 °C, an UV and a CoulArray detector (ECD) (ESA, Chelmsford, MA, USA) was used. Analysis was performed on an Inertsil ODS-3, 4.6 mm ID x 150 mm, 5 µm column (GL Sciences). The mobile phase was composed of (A) 10% ACN in sodium phosphate buffer (100 mM, pH 3.35) containing 10 mg/L SDS and (B) 60% ACN, 10% MeOH in sodium phosphate buffer (30 mM, pH 3.45) containing 15 mg/L SDS. The flow rate was 1 mL/min. Detection was performed at 270 nm and 50, 450, 600 and 700 mV. The elution profile was as follows: 0-15 min, 20%-57.5% B; 15-20 min, 57.5%-100% B; 20-30 min, isocratic at 100% B; 30-32 min, 100%- 20% B, and reconditioning of the column. Samples were dissolved in 500 µL of H_2O/ACN/MeOH (86:12:2), acidified with 8 µL H_3PO_4 (14.8 M) and filtered over a 0.22 µm Acrodisc membrane (PALL). For each sample 10 µL was injected.

Phenolic acid concentrations were calculated based on calibration curves (1-20 µg/mL) and corrected with the average amounts found in the active controls (microbiota without dimers) (20-80 nmol, except 4-HPA gave backgrounds of 0.03-0.14 µmol) and extraction recoveries (**Table 1**). Dimer contents were calculated based on calibration curves (3-1000 µg/mL) and peak areas of dimer peaks 1, 3 and 4 (**Figure 2**) at 600 mV and dimer

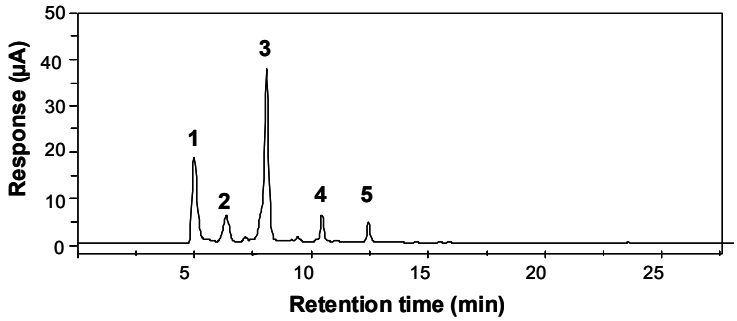


Figure 2. RP-HPLC profile of the dimer fraction purified by NP-HPLC. The dimers 1 (B1), 2 (B4) and 3 (B2 + B3) were identified with standards. Peaks 4 and 5 were also identified as PC dimers (*m/z* 577) but could not be identified further.

peak 2 at 450 mV. Dimer peak 5 could not be monitored because of co-elution with other peaks. The amount of dimers detected at t=0 h was expressed as 100%.

MS analysis

To screen the samples for the presence of phenolic acids and additional metabolites, such as valerolactones, HPLC coupled to a LCT Premier quadruple TOF spectrometer equipped with an ESI source (Waters/Micromass) was used. An Acquity Ultra Performance HPLC system (Waters) was used with the same gradient as used with ECD analysis. The eluents were adjusted for MS analysis. Sodium phosphate and SDS were omitted and the pH was adjusted with formic acid. After passing the HPLC the flow rate was reduced to 0.2 mL/min for MS analysis. The LCT Premier was operated in the negative mode at a resolution of 10500 (FHWM), with a source temperature of 120 °C, desolvation temperature of 350 °C, a capillary voltage of 2250 V and a sample cone voltage of 50 V. Measurements were performed in the W mode. Data were collected in the full scan mode from *m/z* 100-750.

A Waters Micromass Quattro Micro 3 system, equipped with an ESI source and controlled by Masslynx software was coupled to an HPLC system (Agilent technologies, 1200 series, Santa-Clara, CA, USA) with the same conditions as used with the LCT Premier quadruple TOF spectrometer. Measurements were performed in the negative mode with a capillary voltage of 2.0 kV, a capillary temperature of 300 °C and a source temperature of 120 °C. The cone voltage and collision energy were optimized for the phenolic acids and dimers by infusion experiments of standards (**Table 2**). Selected samples were analyzed for the presence of phenolic acids and dimers in the MRM mode. Furthermore, daughter scans were made for the following selected masses, representing intermediate structures and valerolactones: 581, 579, 475, 416, 414, 305, 291, 289, 275, 221, 209, 207, 193, 191, 175 and 125 at a cone voltage of 20 V and a collision energy value of 20%.

Table 2. The formation of the phenolic acids over time was confirmed by MS/MS analysis of specific product ions originating from their parent ions. The collision energy used is indicated by Coll. And the Cone voltage applied with Cone.

phenolic compound	parent ion $[M-H]^-$	product ion $[M-H]^-$	coll. (V)	cone (V)
2-(3,4-dihydroxyphenyl)acetic acid	166.9	122.9	10	20
		94.7	20	20
2-(3-hydroxyphenyl)acetic acid	150.9	106.9	10	20
		64.7	25	20
2-(4-hydroxyphenyl)acetic acid	150.9	106.9	10	20
		64.7	25	20
3-(3-hydroxyphenyl)propionic acid	164.9	121.0	10	25
		105.9	20	25
		76.7	10	20
procyanidin dimer	577.1	289.0	25	35
		124.9	30	35

Statistical analysis

The Student's t-Test was used to test: if the amount of dimers significantly reduced in time, if SCFA production was altered due to the presence of PC dimers and if there were significant differences in phenolic acid recovery after extraction. A probability < 0.05 was considered significant. Error bars are depicted as mean ±SD.

Results

Composition of the procyanidin dimer substrate

Enzymatic removal of gallic acid substituents improved the resolution upon preparative NP-HPLC, especially between the dimers and trimers (data not shown). The percentage of galloylation was calculated based on the peak areas of benzylthioethers and monomeric flavan-3-ols. Previously published response factors (*40*) were used to correct the area's of the different peaks because no benzylthioether standards are available. Each response factor (RF) was divided by the RF of catechin. For example, 4015870 (RF epicatechin) / 3724170 (RF catechin) = 1.08. Subsequently, the area found for epicatechin was divided through 1.08. The degree of polymerization was calculated as; (area monomeric flavan-3-ols (terminal units) + area benzylthioethers)/ area monomeric flavan-3-ols (terminal units). The proportion of gallic acid decreased from 14% (w/w) to 1% (w/w). After preparative purification, the dimer fraction was free of monomeric flavan-3-ols and trimers as their masses, *m/z* 289 $[M-H]^-$ and *m/z* 865 $[M-H]^-$, respectively, were not detected by NP-HPLC-UV-MS. Three peaks out of 5 could be identified based on RP-HPLC with standards namely peak 1 was characterized as B1, peak 2 as B4 and peak 3 consisted of both B2 and

B3, based on separation on a different RP column (unpublished results). PC dimer peaks 4 and 5 (*m/z* 577 $[M-H]^-$) could not be further identified (**Figure 2**). Only B-type dimers (*m/z* 577 $[M-H]^-$) were detected to be present in the mixture.

Quantitative analysis of microbial metabolites originating from procyanidin dimers

PC dimers were not completely stable over 8 h of incubation in the absence of microbiota (buffer control) (**Figure 3A**). Peaks 1 and 2 were rather stable, but peaks 3 and 4 showed a decline of 40-60% compared to t=0 h (not significant, based on concentrations). After 1 h, no further decline was detected. Despite extraction recovery of dimers was low, a significant decrease in the dimer content in the presence of microbiota was detected for the first 4 h (**Figure 3B**).

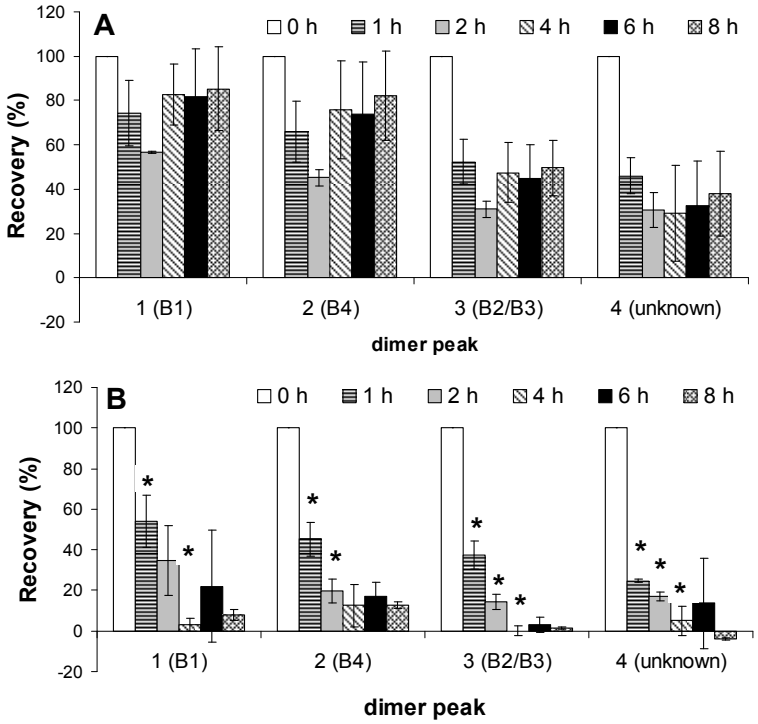


Figure 3. Recovery of dimers 1 (B1), 2 (B4), 3 (B2/B3) and 4 (unknown) (see **Figure 2**) from the buffer control (Panel **A**) and the 5% (w/v) active suspension (Panel **B**) over 8 h of incubation. The amount detected at t=0 h was expressed as 100%. The bars represent the fermentation time as indicated in the legend. Error bars represent the SD of triplicates and a significant decline in comparison to the previous time point was calculated by Student's t-test based on the concentrations ($P < 0.05$) and indicated by an asterisk.

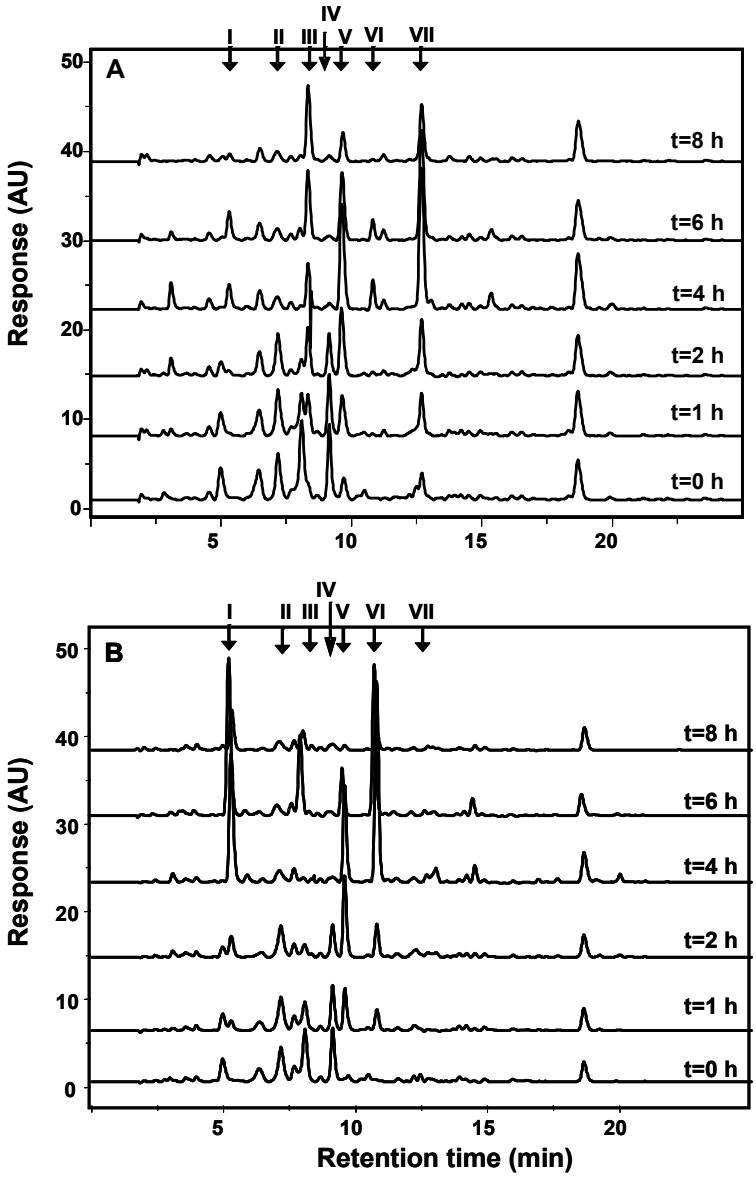


Figure 4. HPLC-ECD detection at 600 mV (Panel **A**) and 450 mV (Panel **B**) of the dimer mixture fermented with a 5% (w/v) fecal suspension over 8 h. Several phenolic acids and flavonoids could be identified with standards and one peak (**VI**) remained to be identified. Compounds are indicated by: **I** = 3.4-diHPA, **II** = catechin, **III** = 4-HPA, **IV** = epicatechin, **V** =3-HPA, **VI** = ? and **VII** = 3-HPP.

While the dimers disappeared, 3,4-diHPA, 3-HPA, 3-HPP and 4-HPA appeared (**Figure 4A**), which were identified based on retention time and relative ECD response (at 450 and 600 mV) of standards. Except for 3,4-diHPA and an unknown peak (VI), which were better detected at 450 mV (**Figure 4B**), the phenolic acids were optimally detected at 600 mV. The identity of the four phenolic acids was confirmed with HPLC-MS/MS analysis (**Table 2**). The additional unknown product (VI) (**Figure 4B**) appeared after 1 h of fermentation with a maximum response around 4 h.

The concentrations of the phenolic acids identified all increased during 4 to 6 h of fermentation, after which they decreased rapidly (**Figure 5**). After 6 h, the 4 identified phenolic acids accounted for ~1.2 µmol in a ratio of 1:6:12:21 (4-HPA:3-HPA:3-HPP:3,4-diHPA) originating from 5 µmol PC dimers. BA proved to be absent and the concentration of PPA was similar to that found in the active suspension without dimers. Additionally, both catechin and epicatechin were detected within the first 2 h of fermentation. However, their total amount was similar as detected at all time points of the buffer control, which was 1.5 - 2 mole % of the dimers added. This excludes the possibility that catechin and epicatechin were formed by microbial degradation of dimers during incubation. As described earlier, catechin and epicatechin were formed during the extraction in similar amounts as found in the samples (total of ~0.06 mole %). No phenolic metabolites were detected in fermentations performed with a 1% (w/v) suspension.

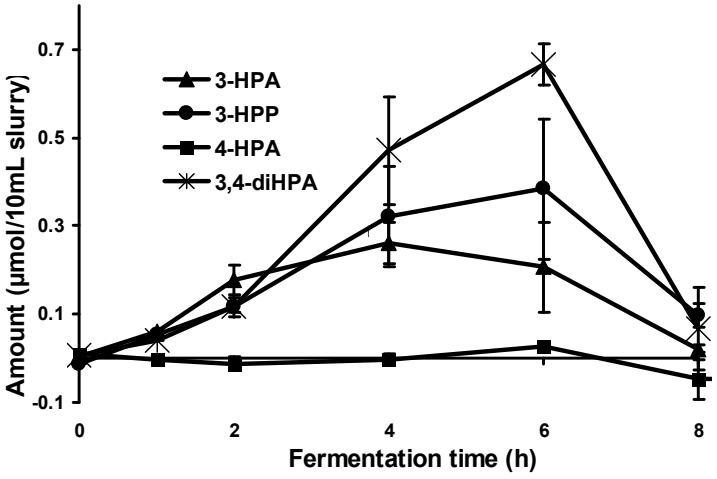


Figure 5. The formation of 4 phenolic acids over 8 h of fermentation of the dimer mixture with a 5% (w/v) human fecal suspension. Error bars represent the SD of triplicates.

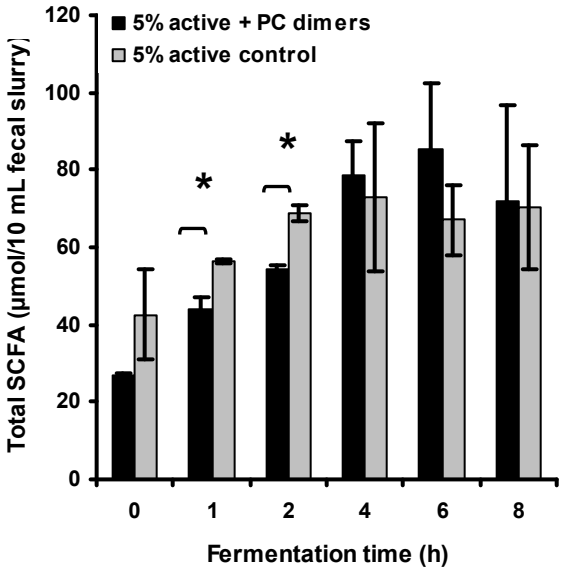


Figure 6. Total SCFA (acetic, propionic, 2-methylpropionic and butyric acid) production of a 5% (w/v) human fecal suspension in the presence or absence (control) of the dimer mixture. Significant differences ($P \leq 0.05$) are indicated by an asterisk.

SCFA analysis was performed to monitor the activity of the microbiota in the presence of dimers. Initially (1-2 h), the presence of dimers inhibited SCFA formation significantly in the 5% (w/v) suspension (**Figure 6**). However, no significant difference in maximum amounts of total SCFA produced was found in the presence or absence of dimers. In the 1% (w/v) suspension no significant differences in SCFA production were found and levels were 6-7 times lower (data not shown) than those in the 5% (w/v) suspension.

Identification of microbial metabolite VI as 5-(3,4-dihydroxyphenyl) -γ-valerolactone

Additional analysis with HPLC-qTOF-MS was performed to identify the unknown peak VI. Two major peaks appeared in the total ion current (TIC) trace after 6 h at RT=8.3 and 10.0 min, and some additional ones at RT= 11.4, 12.2 and 15.3, which were absent or small at t= 0 h (**Figure 7A**). In addition, these peaks were not detected in the active suspension without dimers (data not shown). A shift in retention times was observed with HPLC-qTOF-MS (**Figure 7A**) compared to the HPLC-UV-ECD profiles (**Figure 4**). Based on the retention times and masses analyzed for the standards, the peak at 10.0 min was identified as 3-HPP. Based on the relative elution order of the phenolic acids, (epi)catechin and dimers, the peak

at t=8.3 min most probably was metabolite VI. The average mass spectrum recorded for metabolite VI consisted of three *m/z* values namely 207, 305 and 415 (**Figure 7B**). The $[M-H]^-$ 207 pointed to 5-(3,4-dihydroxyphenyl)-γ-valerolactone (3,4-diHPVal), which has a mass of 208 Da and has been detected previously as a metabolite of catechin and epicatechin (*13, 17-20*). The elemental composition, calculated by Masslynx software, supported this hypothesis. The *m/z* 415 corresponded to 208 plus 207, which is most likely an adduct of 207, and annotated as $[2M-H]^-$. Similar adducts were observed for most of the phenolic acid standards, including 3-HPP (**Figure 7B**). The adduct *m/z* 305 $[M+H_3PO_4-H]^-$, showed a product ion of *m/z* 97 when analyzed with MS/MS and arises through the interference of phosphate originating from the samples. The MS/MS spectrum of *m/z* 207 analyzed with triple Quad MS contained *m/z* 163 as the main product ion, which is typical for 3,4-diHPVal (*17, 20*). Therefore, metabolite VI was annotated as 3,4-diHPVal and its presence was confirmed at all time points except at t=0 h (**Figure 8**). No 3,4-diHPVal was detected in the control samples.

Identification of precursors of 5-(3,4-dihydroxyphenyl)-γ-valerolactone and other metabolites

Precursors

3,4-diHPVal has been described to be preceded by the formation of 1-(3',4'-dihydroxyphenyl)-3-(2",4",6"-trihydroxyphenyl)propan-2-ol (3,4-diHPP-2-ol) (Mw 292) by reductive cleavage of the (epi)catechin C-ring (*19, 20*). HPLC-MS/MS of the samples taken at 0 - 8 h of the active suspension, incubated with and without dimers, were analyzed for the presence of these metabolites (m/z 291) (**Figure 8**). The monomeric units (epi)catechin (*m/z* 289 $[M-H]^-$) were confirmed to be present between 0 to 2 h of fermentation (also based on RT of standards). As mentioned earlier, this was attributed to the extraction procedure. Furthermore, *m/z* 291 $[M-H]^-$ (3,4-diHPP-2-ol) was present after 1 and 2 h, but absent after 6 h.

Other metabolites

Analysis with HPLC-qTOF indicated the presence of several metabolites other than 3,4-diHPVal (**Figure 7A**). At RT 11.4, 12.2 and 15.3 *m/z* values of 209, 191 and 193, respectively were identified. The elemental composition of these compounds was calculated by Masslynx software to represent mono- and dihydroxylated phenylvaleric acids (*m/z* 209 and 193) and monohydroxylated phenylvalerolactone (*m/z* 191). Results of daughter scans of these and other selected parent ions are given in **Figure 8**.

No masses corresponding to a dimer that underwent reductive cleavage in one or both of the C-rings of the monomeric units (*m/z* 579 and 581 $[M-H]^-$) were detected. Based on qTOF data 3,4-diHPVal seemed to be dehydroxylated into 5-(3 or 4-hydroxyphenyl)-γ-

valerolactone (3 or 4-HPVal, *m/z* 191 $[M-H]^-$) (**Figure 7**), which was confirmed by MS/MS analysis (**Figure 8**).No 5-phenyl-γ-valerolactone (PVal) (*m/z* 175 $[M-H]^-$) was detected. Both monohydroxylated phenylvaleric acid (3 or 4-HPV) and dihydroxylated phenylvaleric acid (3,4-diHPV) were present after 6 h of fermentation. None of the selected parent ions were detected in the active suspension without dimers. Other potential metabolites, phloroglucinol (*m/z* 125), a flavan-3-ol with a phloroglucinol substituent (*m/z* 414), 3,4-diHPP-2-ol with a phloroglucinol substituent (*m/z* 416), a flavan-3-ol with a carboxylated phloroglucinol substituent (*m/z* 475), a monohydroxylated HPP-2-ol (*m/z* 275) and a mono or dimethylated valerolactone (*m/z* 221 and 235) were not present in any sample.

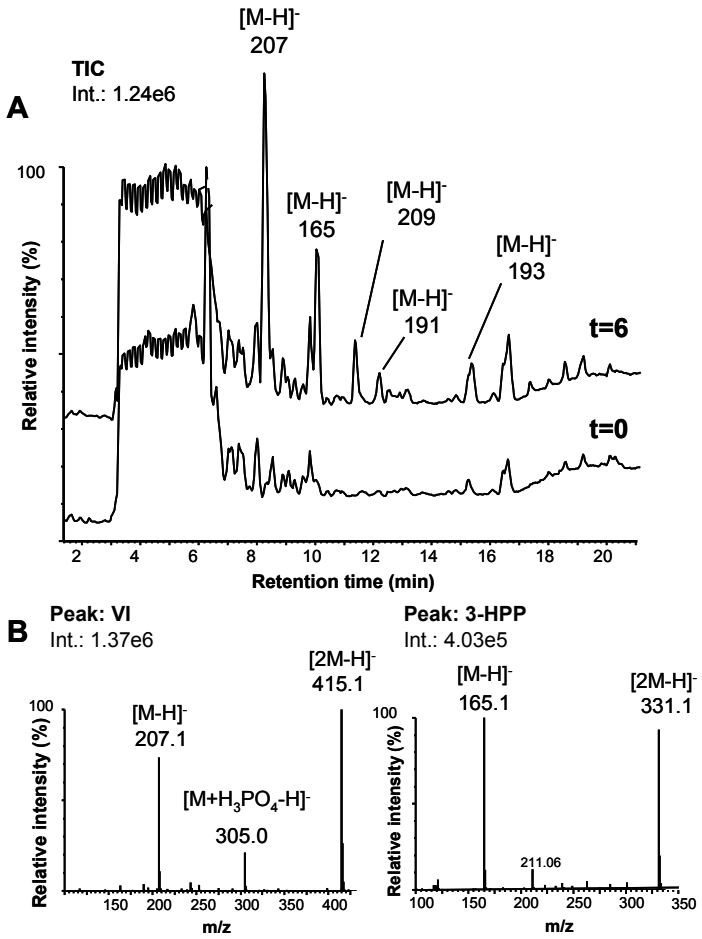
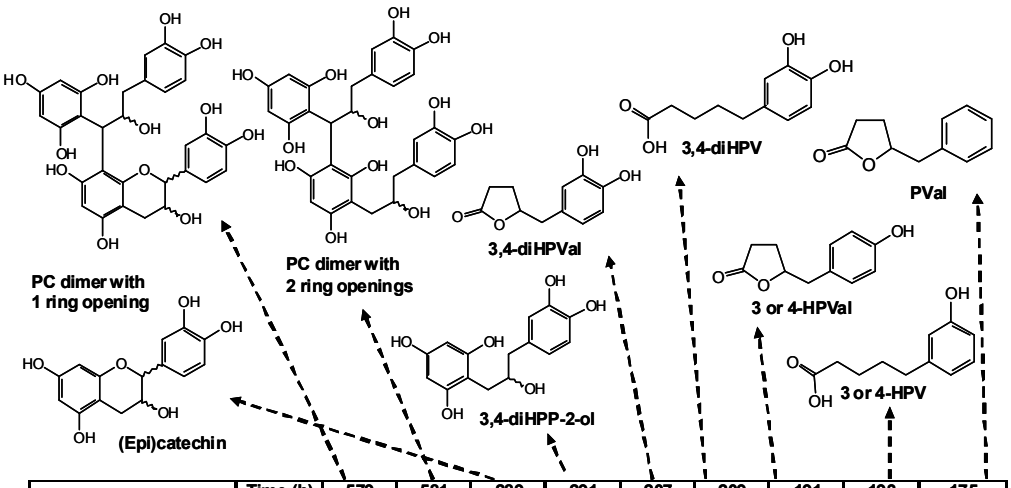


Figure 7. HPLC-qTOF analysis after 0 and 6 h of fermentation of the dimer mixture. The TIC of t=0 and t=6 h are compared (**A**) and spectra of metabolite VI (RT=8.3 min) and 3-HPP (RT=10 min) are given (**B**).



	Time (h)	579	581	289	291	207	209	191	193	175
5% active + PC dimers	0	no	-	yes	no	no	-	-	-	-
	1	no	no	yes	yes	yes	-	no	-	-
	2	no	-	yes	yes	yes	-	-	-	no
	4	-	-	no	no	yes	no	no	no	-
	6	no	-	-	no	yes	yes	yes	yes	no
	8	no	-	-	no	yes	no	no	yes	no
5% active control	0	no	-	no	no	no	-	-	-	-
	1	no	-	no	no	no	-	no	-	-
	2	no	-	no	no	no	-	-	-	-
	4	-	-	no	no	no	no	no	-	-
	6	no	-	-	no	no	no	no	no	-
	8	no	-	-	no	no	no	no	no	-

Figure 8. Several metabolites were detected with HPLC-MS/MS in the negative mode. Daughter scans of the parent masses ($[M-H]^-$) of each metabolite were collected. Data of the fermented dimer mixture (only B-types) with a 5% (w/v) microbial suspension for 8 h were compared with the control samples. Samples that were not analyzed are indicated with -. Yes and no indicates the presence or absence of the metabolite.

Discussion

The four quantified ethyl acetate soluble metabolites, 3,4-diHPA, 3-HPA, 4-HPA, and 3-HPP, accounted for 1.2 µmol originating from 5 µmol B-type PC dimers (**Figure 5**). Additional metabolites found were various hydroxylated phenylvaleric acids and phenylvalerolactones, and 3,4-diHPP-2-ol. However, these could not be quantified because of the lack of standards. The metabolite 3,4-diHPA has not been detected as a human metabolite in studies performed with pure catechin (*21, 38*). Therefore, a direct release of

3,4-diHPA from PC dimers is suggested, without preceding conversion of PCs into monomers.

Quantification of reaction products

PC dimers (B-type) were converted into smaller molecules without preference for a specific dimer (**Figure 3B**) as they all decreased within the same time scale. It was estimated that the metabolites found after 6 h accounted for at least 12 mole % conversion, assuming that 1 mole of dimer is converted to 2 moles of metabolites. *In vitro* fermentation of PA oligomers performed by Deprez and coworkers (*28*) resulted in only 2.7 % ethyl acetate soluble products, based on radioactive labeling. This difference might indicate that the dimers are easier accessible for microbiota than higher oligomers. A high protein affinity of the oligomers might have contributed to low accessibility or antimicrobial effects.

Identified metabolites compared to literature

The most abundant human metabolite of PC dimers was 3,4-diHPA. In rats the urinary excretion of this metabolite also increased after the intake of PC dimer B3 (*15*). An *in vitro* fermentation of PA oligomers with human microbiota did not result in this metabolite (*28*). Also ingestion of PC rich grape seed tablets did not result in increased urinary excretions of 3,4-diHPA in humans (*24*), in contrast to ingestion of PC rich chocolate where 3,4-diHPA was observed in urine of healthy human subjects (*16*). This variation in metabolism could have resulted from differences in composition (degree of galloylation and/or degree of polymerization) of the oligomers or extracts used. Furthermore, an *in vitro* fermentation with tea extracts rich in monomeric flavan-3-ols resulted in the formation of 3,4-diHPA (*33*). However, quercetin, which is also present in tea (*41*), is also known to be degraded into 3,4-diHPA (*36, 42*) and therefore it remains unclear where 3,4-diHPA, after fermentation of the tea samples, originate from. Studies performed with pure catechin and human microbiota (*21, 38*) did not result in 3,4-diHPA.

The formation of 3-HPP as a main metabolite is consistent with previous studies (*23, 28*). The high amount of 3-HPA in comparison to 4-HPA suggests that 3,4-diHPA is preferably dehydroxylated at the *para* position. A preference to dehydroxylate the *para* position of several flavonoids has been described previously for human microbiota (*36, 38, 43*). Therefore, the monohydroxylated phenylvalerolactone that was detected (**Figure 8**) is suggested to be 3-HPVal, formed out of 3,4-diHPVal, through dehydroxylation at the *para* position.

Tentative degradation pathway

An important question is whether cleavage of the interflavan bond of dimeric PCs into monomeric flavan-3-ols is the first step in their metabolism. The detection of 3,4-diHPP-2-ol within 2 h suggests that dimers are cleaved into their monomeric units. However, no

additional catechin or epicatechin other than that formed during extraction was detected. It might be argued that a slow conversion of dimers into flavan-3-ols followed by a rapid conversion into 3,4-diHPP-2-ol, phenylvalerolactones and phenolic acids explains that the flavan-3-ol intermediates are not detected. Indeed, Aura and coworkers (*38*) found that monomeric flavan-3-ols are rapidly metabolized; 1 µmol monomeric flavan-3-ols was completely fermented within 2 h by a 10 mL 5% fecal suspension within the same fermentation system. To our knowledge monomeric flavan-3-ols have never been detected as microbial metabolites of PCs in humans. Their absence (indicated by us and others) and our finding of 3,4-diHPA (not found as a common human metabolite in studies performed with pure monomeric flavan-3-ols (*21, 38*) as the main metabolite does not hint at conversion of dimers into monomeric flavan-3-ols as the main degradation route as previously suggested (*26*). An alternative route could be that the interflavan bond is split after reductive cleavage of one (Mw 580) or both C-rings (Mw 582) of the dimer resulting in the release of 3,4-diHPP-2-ol. However, this route also seems unlikely as *m/z* 579 and 581 (**Figure 8**) were not detected.

Figure 9 shows a tentative degradation pathway for PC dimers (B-type). The formation of both phenolic acids and phenylvalerolactones suggests that cleavage of the C- and A-ring occurs at the points indicated in **Figure 9**. The C-ring cleavage has previously been shown to be an important mechanism for human microbial degradation of several flavonoids (*44*). Our data (**Figure 8**) imply that phenylvalerolactones are slowly degraded into phenylvaleric acids, which were only detected after 6 h of fermentation while 3,4-diHPVal was already present after 2 h. The amount of phenylvaleric acid produced varies between species (*45*). It is not often detected in high amounts as metabolite of monomeric flavan-3-ols or PCs, while 3-HPP is often detected as a main metabolite (*24, 28*). Therefore, phenylvaleric acids seem to be rapidly converted into 3-HPP, which was present in high amounts. This fits with the results obtained by Meselhy and coworkers (*19*), who obtained phenylvaleric and phenylpropionic acids after fermentation of phenylvalerolactones with human microbiota. Previously, rat microbiota were shown to cleave (+)-[*ring A* - ^{14}C]catechin through the A-ring to form phenylvalerolactone and CO_2. The valerolactone was again further metabolized into 3-HPP (*22, 46*). Detection of phenylvalerolactones after fermenting PC dimers shows that human microbiota are also capable to cleave the A-ring.

Human microbiota convert PC dimers into a number of smaller metabolites. Our findings suggest a direct degradation of dimers as depicted in **Figure 9** instead of cleavage into flavan-3-ols first. The health potential of these metabolites should be studied as the original PAs are only present in plasma in low amounts.

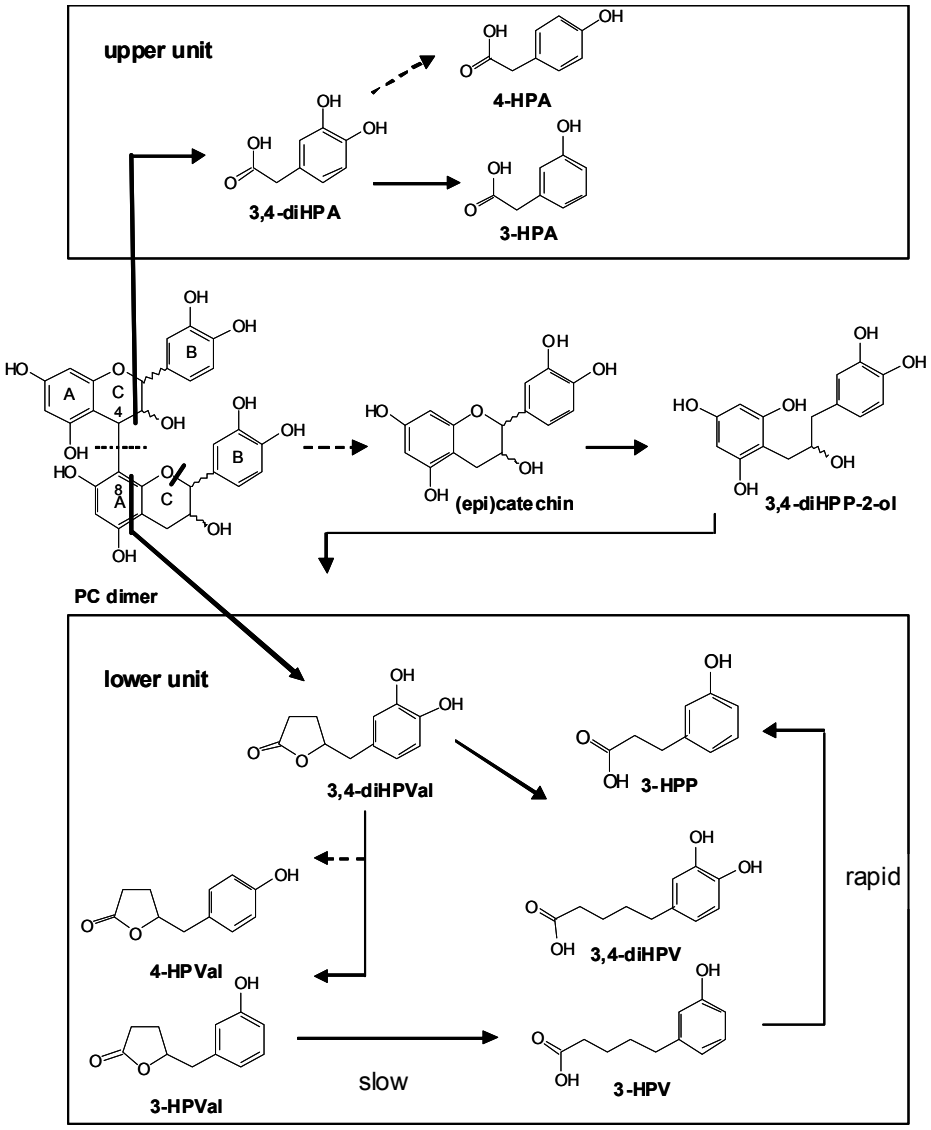


Figure 9. Tentative pathway for human microbial degradation of PC dimers (B-type). The preferred route is indicated with ⟶ and metabolites derived from the upper and lower unit are grouped within rectangles.

Acknowledgements

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Chapter 6

Some phenolic compounds enhance nitric oxide production in endothelial cells *in vitro*

Based on: Maaike M. Appeldoorn, Dini P. Venema, Theodorus H.F. Peters, Marjorie E. Koenen, Ilja C.W. Arts, Jean-Paul Vincken, Harry Gruppen, Jaap Keijer, and Peter C. H. Hollman, Some phenolic compounds enhance nitric oxide production in endothelial cells *in vitro*, *submitted*.

Abstract

The blood pressure lowering and vasorelaxing properties of chocolate and wine, might relate to the presence of phenolic compounds. One of the mechanisms involved might be stimulation of nitric oxide (NO) production by endothelial cells, as NO is a major regulator of vasodilatation. This study aimed to develop an *in vitro* assay based on the hybrid human endothelial cell line EA.hy926, to rapidly screen phenolic compounds for their NO stimulating potential. The assay was optimized, and a selection of 33 phenolics, viz. procyanidins, monomeric flavan-3-ols, flavonols, a flavone, a flavanon, a chalcone, a stilbene, and various phenolic acids were tested for their ability to enhance endothelial NO production. Of the 33 polyphenols tested, only resveratrol (285% increase in NO production), quercetin (110% increase), epicatechingallate (ECg) (85% increase), and epigallocatechingallate (EGCg) (60% increase) were significant ($P \leq 0.05$) enhancers. Several phenolics, e.g. procyanidins, showed a non-significant tendency to elevate the NO production. Concentration dependent correlations between enhanced NO production and eNOS expression were demonstrated for the three polyphenols tested (resveratrol, ECg and EGCg). Thus, an easy screening tool for cellular NO production was developed. Using this assay we showed that only a limited number of phenolic compounds might enhance NO production with an increased amount of eNOS enzyme as a possible contributing mechanism.

Key words: nitric oxide, polyphenols, vasodilatation, cardiovascular diseases, EA.hy926 cells

Abbreviations used: DP, degree of polymerization; ECg, epicatechingallate; EGC, epigallocatechin; EGCg, epigallocatechingallate; eNOS, endothelial nitric oxide synthase; FBS, fetal bovine serum; H_2O_2, hydrogen peroxide; HUVEC, human umbilical vein endothelial cells; L-NAME, N^{ω}-nitro-L-arginine methyl ester hydrochloride; NO, nitric oxide; SOD, superoxide dismutase

Introduction

Compromised endothelial function plays an important role in the growing occurrence of age- and obesity-related cardiovascular diseases (*1*). Healthy vasculature regulates blood pressure and blood distribution to different tissues via vasodilatation and vasoconstriction. Nitric oxide (NO) is one of the main mediators of vasodilatation. In mammalians, endothelial NO is produced by the enzyme endothelial nitric oxide synthase (eNOS), which converts L-arginine in the presence of O_2 and NADPH into L-citruline and NO (*2*). A decreased NO level plays a central role in endothelial dysfunction (*1*).

Several studies showed that products rich in phenolics, like red wine and cocoa, favorably affect endothelial function (*3, 4*). Taubert (*5*) reviewed intervention studies with cocoa products, which consistently showed reductions in blood pressure. Furthermore, cocoa drinks rich in flavan-3-ols improved the flow-mediated dilation, a measure of endothelial function, in human subjects. This coincided with an increase of NO in plasma (*6*). All together, these studies suggest that phenolic compounds could play a beneficial role in endothelial function.

Cocoa and wine contain a multitude of phenolic compounds and it would be impossible to test all candidates in intervention studies. Therefore, several *in vitro* and *ex vivo* systems have been developed to screen the vasoactive potency of compounds. Most systems involve isolated arteries and aortas to study vasorelaxing properties (*7-11*) and relaxation of porcine coronary arteries was shown to correlate strongly with NO levels (*9*). The use of fresh tissue to screen many compounds would require a lot of animals. Cultured human umbilical vein endothelial cells (HUVEC) could provide a simpler alternative to screen for vasoactive compounds. However, HUVECs have to be freshly isolated and lose their ability to produce NO already after a limited number of passages (*12*). Edgell (*13*) developed the hybrid cell line EA.hy926 by fusing HUVEC cells with the permanent cell line A549. This hybrid cell line stably produces NO, even after a large number of cell divisions, and thus may be used as an efficient screening tool. EA.hy926 cells have been used before by Wallerath and coworkers (*14*) to measure eNOS expression after exposure to only a limited number of phenolic compounds without measuring NO production (*14*) except for resveratrol (*15*).

Because NO is directly linked to endothelial function we used it as a parameter to measure the vasorelaxing potential of polyphenols. Knowledge on the ability of individual polyphenols to increase NO production is limited. Therefore, we used EA.hy926 cells to screen 33 phenolic compounds for their potential to stimulate NO production. Phenolic compounds, known to be present in chocolate and wine, were tested for their vasoactive potential including (epi)catechin and proanthocyanidins. Furthermore, microbial metabolites of proanthocyanidins and other flavonoids, phenolic acids (*16, 17*), were tested

as the bioavailability of proanthocyanidins is limited. The selection was extended with a number of monomeric flavan-3-ols, flavonols, flavones, flavanones and one chalcone.

Materials and Methods

Materials

All organic solvents used for HPLC analysis were of HPLC grade. Phenolic compounds were obtained as indicated; hippuric acid (Alldrich), 4-hydroxyhippuric acid (Bracher), 2-hydroxy hippuric acid (kindly provided by P. Kroon, IFR, Norwich), 4-hydroxyphenylpropionic acid, phenylpropionic acid, 3-hydroxyphenylacetic acid, 3,4-dihydroxyphenylacetic acid, vanillic acid, *p*-coumaric acid, caffeic acid, benzoic acid, kaempferol (Fluka), 3-hydroxyphenylpropionic acid, 3,4-dihydroxyphenylpropionic acid, procyanidin dimers B1 till B4 (Apin chemicals), phloretin (Extrasynthèse). All other phenolics were obtained from Sigma. Mixtures composed of procyanidin dimers or tetramers were isolated from both peanut skin (kindly provided by Imko-The Nut Company BV, Doetinchem, The Netherlands) as well as a commercially available grape seed extract (Vitaflavan DRT, Levita Chemical International NV, Belgium, Antwerpen) as described in **Chapters 2** and **3**. Vitaflavan, mainly composed of B-type dimers, trimers and tetramers with gallic acid side chains, will be further referred to as B-type DP2-4+ gallic acid (DP = degree of polymerization). Chemicals and enzymes used were obtained from Sigma unless stated otherwise. Epicatechinglucuronide was prepared as described by Vaidyanathan and co-workers (*18*), subsequently purified with HPLC and freeze dried.

Cell culture

EA.hy926 cells, kindly provided by Dr. Edgell, were cultured according to their instructions (*13*). In short, cells were grown in DMEM (Gibco) containing 10% Fetal Bovine Serum (FBS) (Gibco), 25 mM HEPES and 2% penicilline/streptomycine. Cells were seeded in a 24-well plate at a density of 1.75 x 10e5 cells/well and incubated at 37 °C and 5% CO_2 (CO_2 medical, Hoek-Loos, the Netherlands). After 24 h the cells were confluent and after 48 h they were used to screen for potential bioactive phenolic compounds.

The CO_2 was purified with a sulfur trap (Valco Instruments Company Inc, Switzerland) to remove NO traces before it entered the incubator. Water in the reservoir of the incubator was refreshed before every experiment. Without this refreshment, NO accumulated from 0 nM to 17000 nM in a couple of weeks.

In vitro assay for NO production

Exposure conditions

Differences between inner and outer wells of 24-well plates can amount to 1000 nM NO due to contamination with NO from ambient air. Therefore, only the 8 inner wells were used to screen the phenolic compounds. The outer wells contained 1 mL water each to trap NO. Phenolic compounds were dissolved in DMSO (100 mM) and diluted in DMEM containing 25 mM HEPES, 150 U/mL SOD and 300 U/mL catalase, to a final concentration of 0.1% DMSO. Cells were exposed to 300 µL cell culture media, containing the phenolic compound (100 µM), for 24 h whereafter supernatant was removed and stored at -80 °C to measure the stability of the phenolic compounds. Subsequently, fresh cell culture medium (300 µL) containing calcium ionophore A23187 (5 µM) was added to the cells to increase the sensitivity of the assay (*19*) (addition of 5 µM A23187 for 1 h significantly increased the NO production 6 fold by cells exposed to 100 µM resveratrol for 24 h). After 1 h of incubation with A23187, medium was removed and stored in tightly closed cryovials (Simport Plastics Ltd., Beloeil, Canada) at -80 °C until NO measurement. Each exposure was tested in triplicate (3 wells) and repeated on at least 3 different days. Thus, each exposure generated 3 x 3 = 9 values. In each series, 100 µM resveratrol was taken as a positive control (average SD of 34 nM NO between the 3 wells). A control was included composed of 0.1% DMSO in DMEM and identically treated as the samples, (average SD of 18 nM NO between the 3 wells). Measurements were corrected for NO produced by cells exposed to only DMEM. As a negative control, cells were exposed for 24 h to resveratrol as described above and subsequently incubated with fresh culture medium containing calcium ionophore and the eNOS inhibitor N^{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME) (10 µM).

NO_x-measurement

NO_x-analysis was performed as described by Feelisch and coworkers (*20*) with a few alterations. In short, a water-jacketed reaction vessel, kept at 60 ºC, was filled with 20 mL freshly prepared Browns solution. Browns solution, stored in the dark on ice until use, was made as follows: 45 mM KI and 10 mM I_2 in milli-Q water (shake 5 min at 250 rpm) was subsequently mixed with glacial acetic acid (1: $12^1/_3$) followed by ultrasonic treatment for 5 min. Samples were injected into the reaction vessel in triplicate (50 µL) through a septum that was replaced after each series of measurements. Inside the reaction vessel Browns solution reduced NO_x (NO_2, NO_2^-, and nitrosated and nitrosylated species) to NO (g). NO (g) was transported by helium through a condenser (3 ºC) followed by a 1 M NaOH solution, which was kept on ice, to remove traces of acids. Subsequently, the NO (g) passed a 0.22 µm filter before it entered a chemiluminiscence detector (CLD 88 et., Eco Physics, Duernten, Switzerland). The detection range was set at 0-50 ppb. The whole system was

kept at a constant overpressure of 1-1.05 bar throughout the measurements. Browns solution was refreshed when peak broadening appeared or large bubbles were generated in the reaction vessel. Calibration curves were made with potassium nitrite (0-1000 nM) dissolved in a physiologic saline solution (0.9% (w/v) NaCl) (R^2> 0.99).

To prevent NO_x-contamination, tubes and equipment that were used were carefully screened and exposure of samples to air was minimized. NO_x measurements were only performed when air NO_x concentrations (expressed as NO_2) in the Wageningen region were below 40 μg/m^3 (measured by the Dutch National Institute for Public Health and the Environment, (*21*)) . A plasma sample, which was stored in small batches at -80 °C, was used to determine the reproducibility of the NO measurements. This control sample had an average NO content of 145 nM (14 measurements of duplicates). The CV was calculated to be 6.9% within days (14 duplicates) and 12.4% between days (n=14). Results of a series of analysis were rejected when the value obtained in this control sample exceeded ± 2 x $SD_{between}$ from the average level.

Expression of results

The mean increase in NO production caused by a compound was calculated as the difference between the mean of the sample values of at least 3 exposures (≥ 9 values) and the mean of the DMSO blank values measured within the same exposures, and expressed as a percentage of the DMSO blank.

HPLC analysis to measure stability of the phenolic compounds during exposure conditions

The cell culture media, which were collected after 24 h, were filtered through a 0.45 μm filter and analyzed on HPLC. For the procyanidin fractions a Thermo Spectra system was used containing a P 4000 pump, an AS 300 autosampler and an UV 3000 detector (Thermo Separation products). Analysis was performed on an XterraRP dC18, 4.6 mm ID x 150 mm, 3.5 μm column (Waters) at room temperature. The mobile phase was composed of (A) water + 0.1% (v/v) acetic acid and (B) acetonitrile + 0.1% (v/v) acetic acid. The flow rate was 0.7 mL/min and detection was performed at 280 nm. For the fractions isolated from peanut skin the elution profile was as follows; the first 5 min isocratic on 10% B; 5-35 min, B: linearly 10%-30%; 35-40 min, B: linearly 30%- 90% followed by reconditioning of the column. Another elution profile was used to analyze the fractions isolated from grape seeds; 15 min isocratic on 10% B; 15-35 min, B: linearly 10%-50%; 35-40 min, B: linearly 50%-95% followed by reconditioning of the column. For each sample 20 μL was injected.

Phenolic acids were analyzed on a system composed of L-6200 and L-6000A pumps (Hitachi), a 234 autoinjector (Gilson), a Kratos Spectroflow 783 UV detector (Kratos Analytical Instruments) and a Spark Mistral column oven set at 30 °C (Separations Analytical Instruments B.V). Analysis was performed on an XBridge C18, 3.0 mm ID x

250 mm, 5 μm column (Waters). The mobile phase was composed of (A) 2% (v/v) acetonitrile and (B) 40% (v/v) acetonitrile in sodium phosphate buffer (0.01 M, pH 1.5). The flow rate was 0.42 mL/min and detection was performed at 220 nm. The elution profile was as follows; 0-20 min, B: linearly 3%-100%; 20-24 min, isocratic on 100% B; 24-25 min, B: linearly 100%- 3% followed by reconditioning of the column. For each sample 10 μL was injected.

Epicatechin, catechin, ECg, epigallocatechin (EGC) and EGCg were analyzed on a Hitachi system composed of L-2100 pumps, a L-2200 autoinjector a CoulArray detector (ESA, Inc., Chelmsford, MA) and a Spark Mistral column oven set at 30 °C. Analysis was performed on an Inertsil ODS-3, 4.6 mm ID x 150 mm, 5 μm column (GL Sciences). The mobile phase was composed of (A) 10% (v/v) acetonitrile in sodium phosphate buffer (25 mM, pH 2.4) and (B) 30% (v/v) acetonitrile in sodium phosphate buffer (25 mM, pH 2.4). The flow rate was 1 mL/min and detection was performed at -70, -10, 70 and 150 mV. The elution profile was as follows; 0-20 min, B: linearly 0%-80%; 20-23 min, B: linearly 80%-100%; 23-25 min, isocratic on 100% B; 25-26 min, B: linearly 100%- 0% followed by reconditioning of the column. For each sample 10 μL was injected. Resveratrol was analyzed with the same conditions except detection was performed with UV detection. Apigenin, naringenin, quercetin, kaempferol and phloretin were analyzed on the same system as described for phenolic acids and an inertsil-ODS3 column as described for the flavan-3-ols. An isocratic elution was performed with 31% (v/v) acetonitrile in sodium phosphate buffer (25 mM, pH 2.4). Recoveries were calculated based on peak areas compared to the original DMSO stocks that were diluted with water instead of medium.

Assessment of eNOS expression by quantitative real time PCR analysis

Total RNA from EA.hy926 cells, exposed 24 h to resveratrol, EGCg or ECg (0 to 200 μM) with or without 10 μM L-NAME, was isolated to asses eNOS expression levels by quantitative RT-PCR ($\Delta\Delta$ C_T method) (*22*). All exposures were performed in triplicate on 3 different days and for each condition cells from 4 wells were pooled. After an exposure of 24 h, as described above, the medium was removed and the cells were washed with 1 mL ice-cold PBS. Subsequently, the cells were incubated for 15 min in 0.5 mL TRIzol (Invitrogen), resuspended, pooled and stored at -80 °C. After thawing, RNA was extracted with 1.2 mL chloroform and precipitated with 1.2 mL isopropanol according Invitrogen's instruction. RNA quality and quantity was verified on a Bio-Rad Experion and Nanodrop spectrophotometer (Nanodrop technologies) and accepted when $OD_{260/280}$ >1.8.

For each sample, cDNA was synthesized (iScript cDNA Synthesis kit, Bio-Rad) and eNOS expression levels were assessed in triplicate on a MyIQ5 single color real time cycler (Bio-Rad) using iQ SYBR-Green Super mix (Bio-Rad) and eNOS specific primers (see **Table 1**). Quantitative RT-PCR data were analyzed with iQ5 optical system software

(version 2), normalized to ribosomal protein L32 and β-actin reference genes and results were accepted when the standard curve (of all analyzed genes), of serial dilutions from pooled cDNA samples, showed good efficiencies and linear amplification (R^2 >0.99).

Table 1. Primers used for quantitative RT-PCR.

gene symbol	sequence ID	forward primer (5'-3')	reverse primer (5'-3')	product length (bp)
eNOS	NM_000603	GAGACTTCCGAATCTGGAACAG	GCTCGGTGATCTCCACGTT	102
RP L32	NM_000994.3	GCTGGAAGTGCTGCTGATGTG	CGATGGCTTTGCGGTTCTTGG	83
β-actin	NM_001101.2	CCACCCCACTTCTCTCTAAGGAG	GCATTACATAATTTACACGAAAGCAATG	94

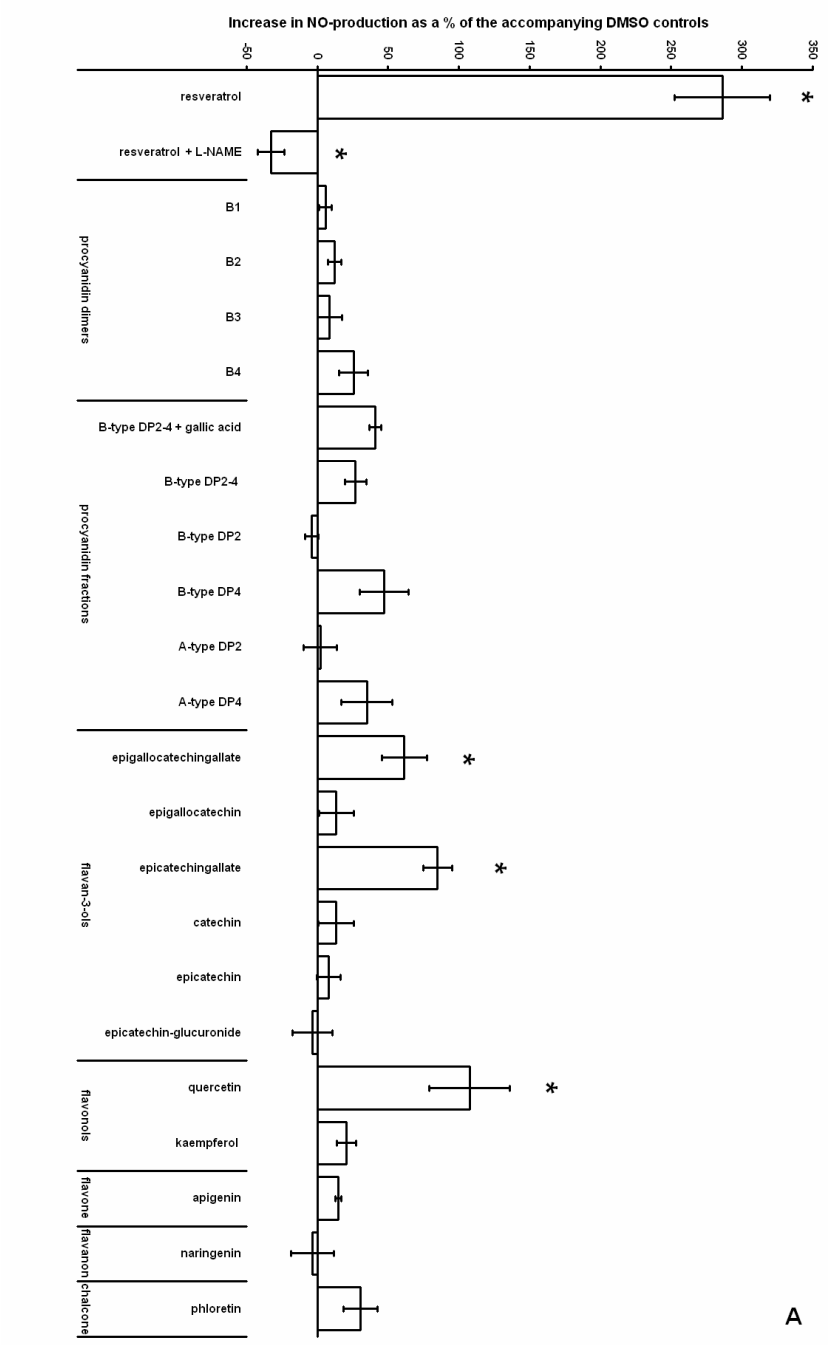
Statistical analysis

The Cochran's test (ISO 5725-2, 1994) was used to determine if the highest within-day variation that was measured for each phenolic compound on at least 3 different days could be considered as an outlier. If the test statistic exceeded the 5% critical value, the measurement was excluded from further statistical analyses. The ability of each phenolic to significantly enhance NO production or eNOS expression compared to the DMSO controls that were measured on the same days was tested by Student's t-Test. A probability < 0.05 was considered significant. Error bars are depicted as mean ±SEM of at least 3 exposures. To determine if enhanced NO production correlated with eNOS fold changes, the NO production was expressed as the concentration of NO detected in exposed cells (0-100 μM phenolic compound) divided by the concentration of NO detected in non-exposed cells (0.1% DMSO).

Results

Vasoactive potency of a large set of phenolic compounds

In total 33 phenolic compounds were tested for their ability to stimulate NO production (**Figure 1**). Resveratrol had the largest significant effect (285% increase in NO production), followed by quercetin (110% increase), ECg (85% increase), and EGCg (60% increase). A number of other phenolic compounds also seemed to enhance NO production, although this did not reach statistical significance. As opposed to their galloylated forms, epicatechin and catechin did not enhance NO production. Like epicatechin, epicatechin-glucuronide, a human metabolite of epicatechin, did not enhance NO production either. Procyanidins showed a tendency to increase NO production, which seemed to be related to their degree of polymerization. However, a selection of their colonic metabolites, phenolic acids, had no effects.


Increase in NO-production as a % of the accompanying DMSO controls
-50
0
50
100
150
200
250
300
350
resveratrol
resveratrol + L-NAME
B1
B2
B3
B4
procyanidin dimers
B-type DP2-4 + gallic acid
B-type DP2-4
B-type DP2
B-type DP4
A-type DP2
A-type DP4
procyanidin fractions
epigallocatechingallate
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epicatechin-glucuronide
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kaempferol
flavonols
apigenin
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naringenin
flavanon
phloretin
chalcone
A

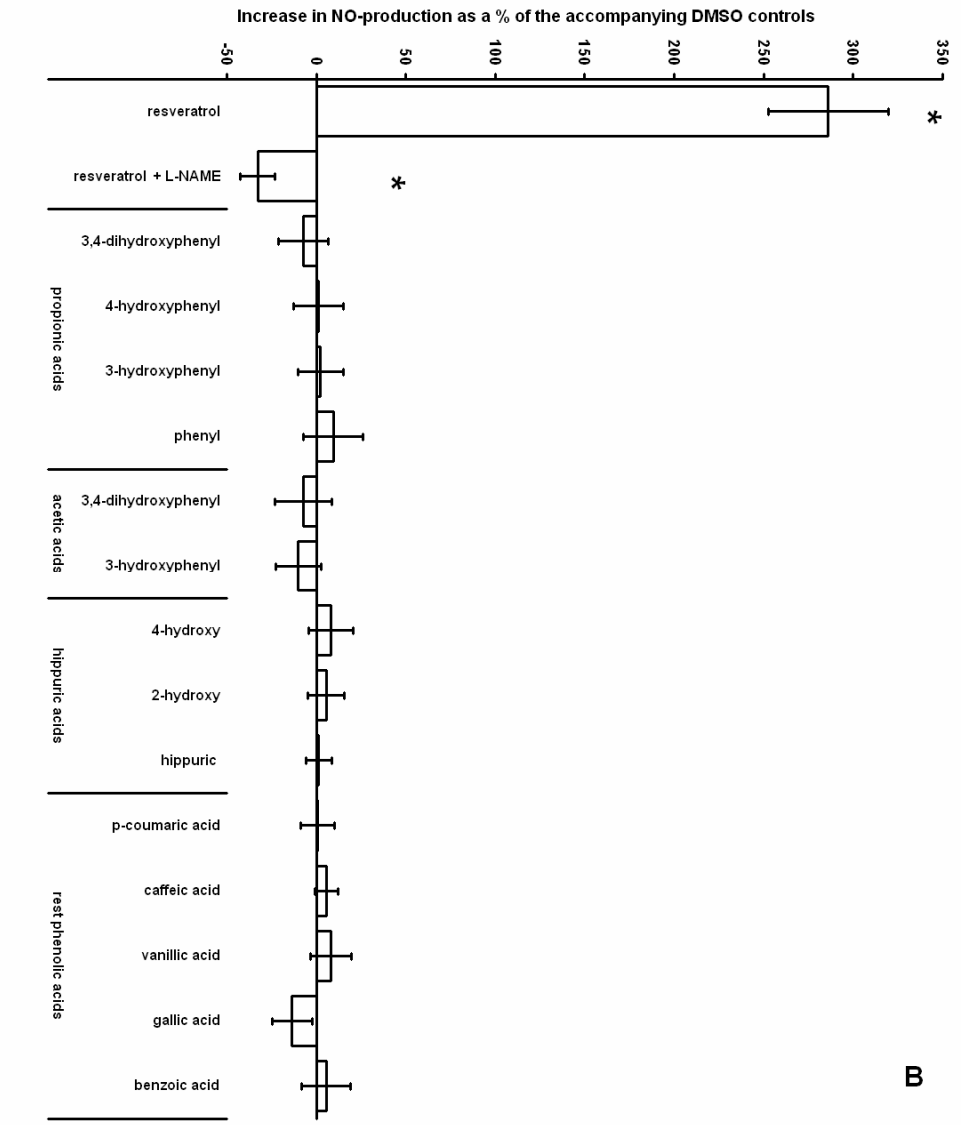

Figure 1. Average increase in NO production as a percentage of the DMSO control after exposing EA.hy926 cells to 100 µM of flavonoids (**A**) or phenolic acids (**B**) for 24 h. *Trans*-resveratrol served as a positive control. Data represent mean ± SEM of 3 or more independent exposure experiments. Significant differences were calculated with a Students t-test by comparing the NO concentrations (nM) after exposure to a phenolic compound with the NO concentrations after exposure to the control (0.1% DMSO) measured on the same days.

After exposure to 100 µM resveratrol the addition of L-NAME, a competitive inhibitor of eNOS, decreased NO production (**Figure 1**). Similar results were obtained for epicatechin, ECg and EGCg (data not shown).

Dose dependant increase in both NO_x production and eNOS expression

Exposure of EA.hy926 cells to resveratrol, EGCg, and ECg resulted in a significant dose dependant increase in NO production (**Figure 2**). For resveratrol and EGCg, production of eNOS mRNA was significantly enhanced and paralleled NO production. The increase in eNOS mRNA level after exposure to ECg did not reach statistical significance (**Figure 2C**). NO production expressed as the ratio between exposed (0-100 µM, phenolic compound) and non-exposed (0.1% DMSO) cells, correlated linearly in a dose-dependent manner with the changes in eNOS mRNA expression levels. Addition of L-NAME did not affect eNOS expression but only inhibited NO production (**Figure 3**). Epicatechin (100 µM) did not elevate eNOS mRNA levels (data not shown), consistent with its failure to increase NO production (**Figure 1**).

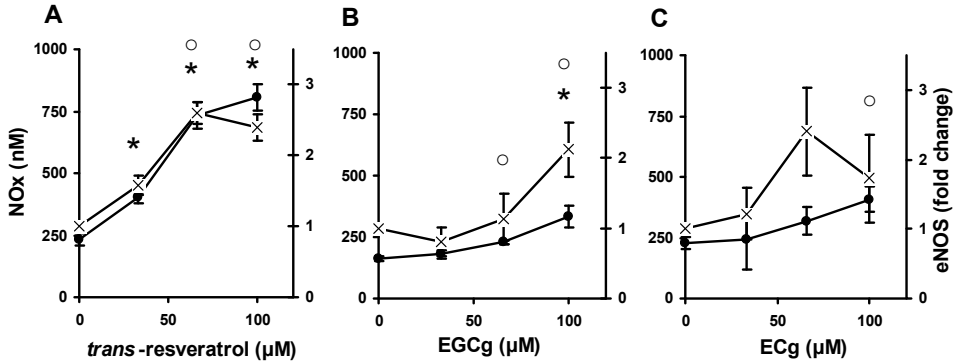

Figure 2. Dose-dependant increase in NO production (●) and eNOS expression (x) after exposing EA.hy926 cells for 24 h to **A**) *trans*-resveratrol, **B**) EGCg, and **C**) ECg. Each exposure consisted of 3 triplicates repeated on at least 3 different days. Error bars represent ± SEM and significant differences are indicated by ○ for NO production and * for eNOS expression ($P < 0.05$) analyzed by Student's t-Test.

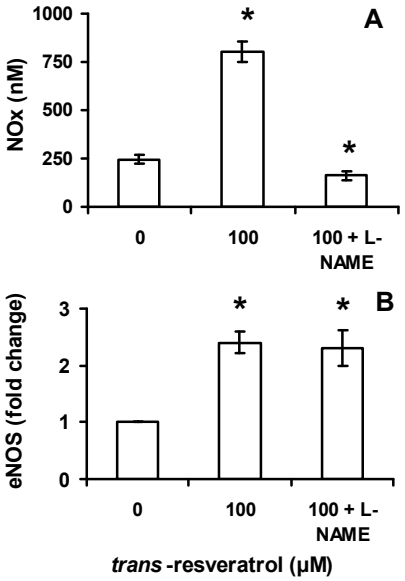

Figure 3. The effect on NO production (**A**) and eNOS expression (**B**) of exposing L-NAME together with *trans*-resveratrol to EA.hy926 cells for 24 h. Error bars represent ± SEM of n ≥ 14 for NO measurements and n=3 for expression data. Significant differences compared to the exposure without *trans*-resveratrol was calculated with Student's t-test ($P < 0.05$) and indicated by an asterisk.

Stability of the phenolic compounds

The presence of the originally added phenolic compounds (**Table 2**) in the medium was analyzed after 24 h by HPLC. Addition of HEPES, to stabilize the pH, and the enzymes SOD and catalase, to prevent oxidative reactions, improved the stability of compounds like 3,4-dihydroxyphenylpropionic acid, ECg and EGCg. These additions did not affect NO production in the DMSO control sample and were included in the protocol. All phenolic acids showed good recoveries (> 80%) with the exception of 3,4-dihydroxyphenylpropionic acid (69%) and caffeic acid (56%). Catechin and epicatechin had recoveries of about 50%. Epicatechin was partly epimerized (~25%) towards catechin. Catechin did not epimerize. EGC, ECg, EGCg, all procyanidins (grape DP2-4, A- and B-type dimers/tetramers and the dimers B1 till B4), quercetin and kaempferol showed low recoveries (< 50%). The low recoveries obtained for procyanidins might be caused by their complexation with proteins and peptides from the medium *(23)*. Therefore, both A- and B-type tetramers were tested in medium with and without FBS. Although recoveries of 27-40% were obtained after 24 h of exposure in the presence of FBS (**Table 2**), the stability of both A- and B-type tetramers decreased dramatically in the absence of FBS with recoveries of only 5% and 18% respectively, already shortly after exposure.

Table 2. Recovery of the phenolic compounds after 24 h of incubation with EA.hy926 cells in the culture medium. Effects of the addition of HEPES, SOD and catalase were tested (A=without, B= with). Measurements are n=1, unless indicated otherwise.

phenolic compound	% recovery	
	A	B
trans-resveratrol	-	79
3,4-dihydroxyphenyl propionic acid	13	69
4-hydroxyphenyl propionic acid	91	92
3-hydroxyphenyl propionic acid	101	116
phenyl propionic acid	-	-
4-hydroxyhippuric acid	98	95
2-hydroxyhippuric acid	90	87
hippuric acid	103	106
p-coumaric acid	87	84
caffeic acid	31	56
vanillic acid	103	129
gallic acid	-	-
benzoic acid	-	-
procyanidin B1	-	22
procyanidin B2	-	30
procyanidin B3	-	15
procyanidin B4	-	9
procyanidin B-type DP2-4 + gallic acid	-	46[b]
procyanidin B-type DP2-4	-	39[b]
procyanidin B-type DP2	-	36[b]
procyanidin A-type DP2	-	41[b]
procyanidin B-type DP4	-	40
procyanidin A-type DP4	-	27[b]
epigallocatechingallate	0	12[a]
epigallocatechin	0	-
epicatechingallate	23	43[a]
catechin	-	51
epicatechin	68	48
quercetin	-	12[b]
kaempferol	-	41[b]
apigenin	-	52[b]
naringenin	-	66[b]
phloretin	-	32

- = not analyzed

[a] n=3

[b] n=2

Discussion

The *in vitro* assay developed offers a simple tool to screen phenolic compounds for their ability to increase NO production. Out of 33 phenolic compounds tested at a level of 100 µM, only resveratrol, quercetin, EGCg and ECg significantly enhanced NO production.

Dose dependent correlations between NO stimulation and eNOS upregulation were found for all three polyphenols tested, resveratrol, EGCg and ECg, suggesting that stimulation of eNOS expression contributes to the increased NO production.

Vasoactive potential within several phenolic classes

Representatives of different structural classes of phenolics were tested: procyanidins, monomeric flavan-3-ols, flavonols, a flavone, a flavanon, a stilbene, a chalcone and phenolic acids. Resveratrol, a stilbene, had by far the highest activity, which fits with the results of Wallerath who showed a comparable enhancement in both NO production (*15*) and eNOS expression (*14, 15*) in EA.hy926 cells (1-100 µM). Of the flavonols, only quercetin significantly enhanced NO production, whereas kaempferol was inactive. Possibly, multiple hydroxyl groups at the B-ring are favorable as suggested by Taubert (*9*). However, our data on the monomeric flavan-3-ols do not support this hypothesis. Like catechin and epicatechin, the more hydroxylated EGC did not stimulate NO production, as would be expected based on that hypothesis. Conflicting results have been reported for epicatechin, which was classified as weak/nonactive in several studies with porcine coronary arteries (*9, 24*) or rat aorta (*25, 26*). However, in humans epicatechin induced flow mediated vasodilatation. ECg and EGCg were the only monomeric flavan-3-ols that significantly stimulated NO production in our assay. EGCg has been classified as a potent vasoactive compound in various systems such as bovine aortic endothelial cells and rat vasculature (*27, 28*). The galloyl moiety seemed critical for the activity of ECg and EGCg, because epicatechin and EGC, lacking this moiety, had no activity. Gallic acid itself was not active at all, which is in accordance with other studies performed with EA.hy926 cells (*14*) and arteries (*9, 25*).

Effects of isolated proanthocyanidins on NO production have never been studied before. Except for dimers, procyanidins with a higher degree of polymerization showed a tendency to increase NO production, but no significance was reached. A range of monomers up to decamers were tested with rabbit aorta and similarly only tetramers and higher oligomers caused relaxation (*8*). The interflavanic linkage between the flavan-3-ol units (A or B-type) seemed to have no effect in our study in contrast to previous observations where only A-type dimers and trimers showed significant relaxation of rabbit aorta rings (*8, 29*).

Because procyanidins are very poorly absorbed, the vasodilating properties of their colonic metabolites, phenolics acids, are of interest. However, these phenolics did not enhance NO production. Wallerath (*14*) showed that some phenolic acids like benzoic acid, vanillic acid, *p*-coumaric acid and caffeic acid increased eNOS mRNA production. However, no data on NO production were presented. The same phenolic acids (up to 2 mM) were not able to cause relaxation of rat aortas (*25*).

In our study apigenin, naringenin and phloretin, representing a flavone, flavanon and a chalcone, respectively, did not enhance the NO production. In porcine arteries,

conflicting effects for apigenin were reported (*9, 24*). In agreement with our results, naringenin (12-100 μM) was not able to enhance NO production and eNOS expression in HUVEC cells (*30*). However, results on porcine arteries are conflicting, because naringenin has been classified as a moderate (*24*) as well as a weak/nonactive (*9*) vasoactive compound. Phloretin has shown moderate relaxation in porcine coronary arteries (30-100 μM) (*24*) but to our knowledge nothing is known about its ability to affect NO production.

Correlation between NO production and eNOS expression

The dose dependent correlations between NO production and eNOS expression as found for resveratrol, ECg and EGCg, suggested that NO production was enhanced via a larger amount of eNOS enzyme. This was also observed by Wallerath and coworkers (*15*) EA.hy926 cells exposed to up to 100 μM resveratrol, and in addition, the amount of eNOS protein increased.

Stability of the phenolic compounds

Many phenolic compounds are unstable at the pH usually required in the test systems used. Until now, stability has not been taken into account. In this study it was shown that several phenolic compounds were unstable during the 24 h of incubation. To prevent oxidation of phenolic compounds by superoxide and hydrogen peroxide (H_2O_2) released by autoxidation, the enzymes SOD and catalase were added. In addition, H_2O_2 can either increase eNOS activity (*31*) or decrease NO bioactivity (*32*). Generation of H_2O_2 might partly explain differences in the vasoactive potency found for several phenolics within different test systems. Our data confirm the beneficial effects of SOD/catalase on the stability of a number of phenolics. Furthermore, proteins in the medium or on the cell surface (*33*) might interfere during the assay when procyanidins are tested, as was shown by Aldini and co-workers (*10*). Incubations without proteins in the medium could diminish procyanidin-protein interactions. However, in the absence of 10% FBS in the medium, which represents a less physiological condition, recoveries were even lower suggesting a protective effect of the proteins. The influence of such interactions on the bioactivity of phenolic compounds *in vivo* remains to be investigated.

Physiological relevance of the assay

Physiological concentrations in humans of the phenolics tested are usually well below 10 μM (*34*). Even for the strongest enhancer, resveratrol, we could only detect a significant effect above 30 μM. However, conditions *in vivo* are quite different from those in the cell culture. The mere fact that NO had to be measured in a relatively large volume of medium against a high background, only permitted relatively large changes (~60 nM) in NO to be detected. However, Taubert (*9*) showed that an increase of only 8.5 nM NO already induced vasorelaxation *ex vivo* in porcine coronary arteries. Still, results of our screening assay

generally fit with this *in vivo* assay: quercetin, but not kaempferol, induced NO production *in vitro* as well as vasorelaxation; naringenin, apigenin, catechin, epicatechin, gallic and coumaric acid were not able to enhance NO production *in vitro*, and also failed to increase *in vivo* NO production or relaxation in the porcine arteries (*9*). In contrast with our study, ECg and resveratrol were not active in that study. But, resveratrol (*35, 36*), EGCg (*27, 28*) and quercetin (*24, 37*) induced vasorelaxation in various other artery models. Thus, results obtained with our *in vitro* assay correspond with those of the *ex vivo* artery models.

Metabolism of phenolic compounds inside the body, resulting in methylation and conjugation of phenolic hydroxyl groups with glucuronic acid and sulfate, could also affect their activity. We showed that glucuronidation of epicatechin did not change its negative effect in this assay. Modulation by glucuronidation, sulfatation and/or methylation on the NO production *in vitro* of phenolics still needs to be studied.

In conclusion, this assay showed to be an easy tool to screen large sets of phenolic compounds for their potency to enhance endothelial NO production. Only 4 out of 33 phenolics, resveratrol, quercetin, ECg, and EGCg, were able to enhance NO production significantly. No clear relation between their structure and activity was apparent.

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Chapter 7

General discussion

The main goal of this thesis was to study the mechanisms that could explain the potential health effects of proanthocyanidins (PAs). As a first step, the absorption and biotransformation of procyanidins (PCs) were studied. Subsequently, the potential of PCs and their microbial metabolites to improve endothelial function were determined by assessing their ability to influence NO production, using an endothelial cell line. The use of purified PCs for these studies was necessary to produce unambiguous results on their bioavailability, biotransformation and bioactivity. Therefore, an efficient isolation procedure was developed to obtain both pure A- and B-type PCs. Appropriate analytical techniques were set-up for the analysis and characterization of PAs needed for all experiments. The main achievements and findings of this thesis are:

Analytical methods (**Chapters 2+3**)

i) A protocol to efficiently purify A- and B-type dimers
ii) A powerful method to characterize PA molecular diversity, and quantify individual PAs

Absorption (**Chapter 4**)

iii) A-type PC dimers were better absorbed than B-type PC dimers
iv) Oligomeric PCs enhanced the bioavailability of B-type PC dimers

Biotransformation (**Chapters 4+5**)

v) A-type and B-type PC dimers were exclusively absorbed as unmodified compounds
vi) 3,4-Dihydroxyphenyl-γ-valerolactone and 3,4-dihydroxyphenylacetic acid were the main metabolites of B-type PC dimers upon exposure to human microbiota

Bioactivity (**Chapter 6**)

vii) A- and B-type PC dimers to pentamers had insignificant vasorelaxing properties as measured by nitric oxide production of endothelial cells
viii) Hydroxylated phenylpropionic/phenylacetic acids, the main microbial metabolites of PCs, had no vasorelaxing properties as measured by nitric oxide production of endothelial cells

The biofunctional potential of PAs will be discussed based on the main findings of this thesis.

Quantification of PAs in our diet

Without doubt, PAs are an important part of the total amount of ingested phenolic compounds from our diet (*1*). The methods to quantify PAs in food have improved over time, but accurate quantification is still a challenging task. Two main techniques have been used for the quantification of PAs: The butanol/HCl assay and NP-HPLC-UV-MS.

Quantification by the butanol/HCL assay

The butanol-HCl assay is a colorimetric assay to quantify the total amount of PAs based on the anthocyanidin formation from the extension units of PAs. However, the color formation depends on many factors, amongst others the presence of water and metal ions (*2, 3*). In addition, PA characteristics like the type of interflavanic linkage and the monomeric composition influence the rate and intensity of color development. Monomeric units connected by an A-linkage are relatively resistant to acid-catalyzed cleavage (*4, 5*) compared to B-linkages. Furthermore, the C4-C6 bond is more resistant to cleavage than the C4-C8 bond (*2*). Color formation also depends on the type of anthocyanidins formed, as their UV responses differ (*2*). When the above is taken into account, the type of standard that is used can either overestimate or underestimate the amount of PAs and, therefore, quantification by the butanol-HCl assay should be approached with caution.

Quantification by NP-HPLC

Besides the butanol/HCl assay, quantification based on NP-HPLC-UV-MS has been performed (*1, 6*). The advantage of NP-HPLC-UV-MS analysis compared to the butanol/HCl assay is that the different oligomeric groups up to a DP of approximately 7, depending on the source, can be quantified separately. For quantification purposes, oligomeric standards of different degrees of polymerization (DP) have been isolated, often from cocoa, by preparative NP-HPLC. These standards are subsequently used to quantify PAs in foods and beverages as done by Gu and coworkers (*1*). This method only enables quantification of the total amount of each oligomeric group, e.g. dimers, trimers, etc. Besides UV detection, additional MS analysis enables the determination the presence of A- and B-type PAs and the different PA subgroups. MS analysis has also been used for quantification (*6*). However, quantification of A- versus B-type PAs on the basis op precursor ions might not be very accurate as in our experience optimal MS settings for the detection of A- and B-type PAs differ. Therefore, NP-HPLC is not suitable to determine the proportion of A- versus B-type within dimers (or larger oligomers).

Quantification by combining NP- and RP-HPLC

With respect to the difference we observed in the bioavailability of A- and B-type dimers (**Chapter 4**), data on their proportion in food sources, which is currently lacking, would be desirable. From the above, it can be concluded that the butanol/HCl assay and NP-HPLC are not suitable to separately quantify A- and B-type PCs. Therefore, in this thesis we combined NP-HPLC and RP-HPLC to characterize and quantify individual molecular PA species in a 20% (v/v) methanol extract of peanut skins (**Chapter 2**). Pre-purification of fractions with different DPs by NP-HPLC followed by the analysis of each fraction on RP-HPLC enabled us to quantify single oligomers of similar DP. Subsequently, the proportions of A- versus B-type PCs could be determined. The peanut skins extract appeared to be

mainly composed of A-type PCs (> 95.0% (w/w) of the 20MeOH extract). Furthermore, the number and position of the A-linkage in the most abundant molecular species was established. Determination of the molar extinction coefficients of individual PA oligomers might further improve this method as a quantification technique.

PA composition of food: The influence of processing

With the improvement of the methods for the quantification and characterization of the PA composition in food, the next step would be to determine the factors that can change PA composition in foods, e.g. processing. Some processes are known to alter the PA composition of food, particularly the ratio between A-type and B-type PAs. For example, the fermentation duration of cocoa beans was negatively correlated with the analyzable amount of PCs because they were (enzymatically) oxidized (*7*). Furthermore, the blanching of peanuts extensively decreased the PC content, in which A-types were more affected (94-100 % (w/w) decrease) compared to B-types (53-90% (w/w) decrease) (*8*). It was hypothesized that the decrease in PC content resulted from their high solubility in hot water (*8*) and based on their results A-type PC seem more hot water soluble compared to B-type PCs. Another explanation for the difference between A- and B-type PCs might be a higher affinity of A-type PCs to bind to denatured (due to the conditions) proteins compared to B-type PCs. Besides peanut processing, maceration and subsequent cold/hot pressing of grapes followed by pasteurization resulted in an increase of PAs in grape juice (*9*). Furthermore, enzymatic treatments might affect PC composition. For example, laccase is an enzyme that is used by the food industry for the stabilization of beer, wine and fruit juices (*10*). This enzyme oxidizes phenolic compounds, which will polymerize and subsequently the aggregates can be removed by e.g. filtration. This possibly also results in removal of PAs. The activity of laccase towards different PA subgroups remains to be established. In addition, the use of laccase might change PA composition, because this enzyme has been reported to convert B-type dimers into A-type dimers (*11*).

Absorption of PCs

Possible mechanisms of absorption

To date, one of the main questions is: To which extent are PAs absorbed by the human body and via which mechanisms? Based on the current knowledge, the bioavailability of PAs is relatively low compared to other flavonoids (*12*) and their mechanism of absorption remains unknown.

The absorption mechanisms that have been reported for phenolic compounds involve paracellular and transcellular passive diffusion, and transcellular active transport by

specific transporters (**Figure 1**). Passive transport is mainly related to the hydrophobicity of the compounds. Due to the somewhat lipophilic character of many phenolic compounds, they can be transported transcellularly via passive diffusion through the lipid bilayer of the cell membrane. PAs, which are hydrophilic due to the many hydroxyl groups, might not be able to pass the lipid bilayer and, therefore, they might be preferentially absorbed via paracellular diffusion.

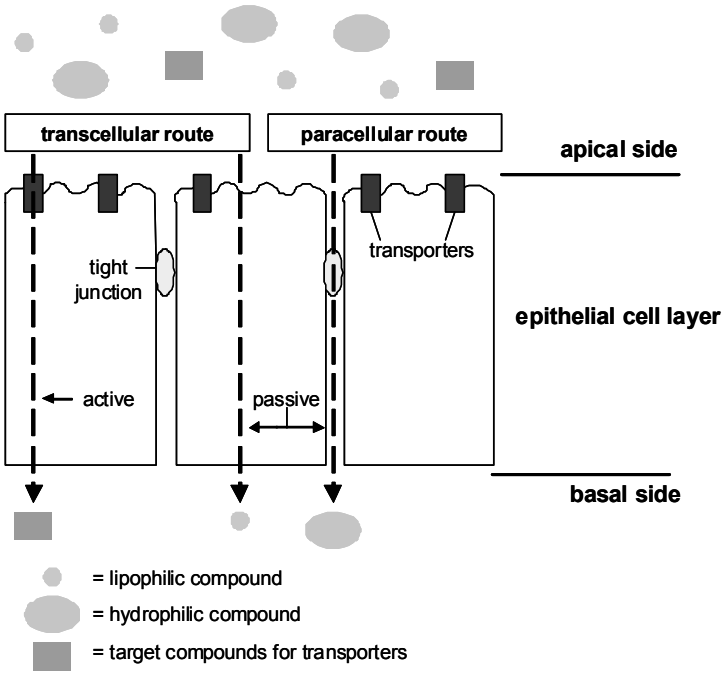

Figure 1. Transport mechanisms through the epithelial layer of the small intestine.

Paracellular diffusion is regulated by the tight junctions and is limited by large molecular size and poor water solubility of the compounds (*13*). In Caco-2 cells the pore size of the junctions was determined to be 5.2 Å, but this could be extended to ~ 22 Å by the use of perturbants that open tight junctions by a Ca^{2+} independent (palmitoyl-DL-carnitine) or Ca^{2+} dependent (ethyleneglycol-*bis*-(*β*-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid) mechanism (*14*).

Table 1 summarizes studies performed with caco-2 cells from which transport routes for phenolic compounds have been deduced. For example, the flavonoid hesperitin was shown to be transported transcellularly, while its corresponding hydrophilic glycoside hesperidin was absorbed paracellularly (*15*).

Table 1. Proposed transport routes for several phenolic compounds[1] .

Transcellular[1]	**Data that pointed to a specific transport route**[2]	**Ref.**
naringenin (active)[2]	transport not affected by TER[5], transport affected by a proton gradient	(*16*)
epicatechingallate (active)	accumulated in Caco-2 cells, transport decreased by adding substrates of the MCT[6] transporter	(*17*)
p/m-coumaric acid (active)	transport of a MCT substrate was inhibited, transport decreased by adding substrates of the MCT transporter, compound concentration not limiting	(*18-20*)
hesperetin (active)	transport not affected by TER, transport affected by a proton gradient	(*15*)
caffeic acid (active)	transport decreased by adding substrates of the MCT transporter	(*18*)
ferulic acid (active)	transport decreased by adding substrates of the MCT transporter	(*21*)
genistein	compound concentration not limiting , accumulated in Caco-2 cells	(*22*)
3-HPP (active)[3]	transport of a MCT substrate was inhibited, transport decreased by adding substrates of the MCT transporter, compound concentration not limiting	(*18, 20*)
3,4-diHPP[4]	transport decreased by adding target compounds of the MCT transporter	(*20*)
Paracellular		
(epi)catechin	transport inversely related to TER	(*23*)
epicatechingallate	transport inversely related to TER	(*23*)
epigallocatechin(gallate)	transport inversely related to TER	(*23*)
hesperidin	transport inversely related to TER	(*15*)
caffeic acid	transport inversely related to TER, transport increased linearly with concentration	(*18*)
chlorogenic acid	transport inversely related to TER, transport increased linearly with concentration	(*18*)
gallic acid	transport increased linearly with concentration	(*19*)
3,4-diHPP	transport inversely related to TER, transport increased linearly with concentration	(*20, 24*)

[1] based on data obtained from the transport across the cell membrane of Caco-2 cells.
[2] if data indicated that active transport was involved this is indicated between brackets.
[3] 3-HPP= 3-hydroxyphenylpropionic acid
[4] 3,4-diHPP= 3,4-dihydroxyphenylpropionic acid
[5] TER= transepithelial electrical resistance, also abbreviated as TEER, which is a measure of paracellular transport
[6] MCT= monocarboxylic acid transporter

From studies that report transcellular transport of phenolic compounds as the main transport route, it is not always clear if transporters are involved and to which extent. However, it can be concluded that transcellular transport of phenolic acids, containing a carboxylic acid group, involves the monocarboxylic acid transporter (MCT). Interestingly,

epicatechingallate, which does not contain a free carboxylic group, also seems to be transported by MCT.

For several phenolic compounds, paracellular diffusion has been reported as transport mechanism (**Table 1**). This does not necessarily mean that the transport mechanism reported is the exclusive or the most important absorption mechanism involved. From **Table 1** it can be concluded that paracellular and transcellular transport can coexist. For example, caffeic acid was reported to be mainly absorbed paracellularly, but it is also, to a lesser extent, transported transcellularly by the MCT transporter (*18*).

Besides studies performed with Caco-2 cells, a number of perfusion experiments have been performed with the small intestine of rats. Within these studies, detection of methylated and/or glucuronidated metabolites in portal vein plasma or at the apical or basal side of the intestine also hints at transcellular transport. Methylation and glucuronidation already occurs inside epithelial cells, and detection of these metabolites in the portal vein (before liver metabolism can occur) proves that the parent compound has entered the epithelial cells. For example, glucuronidated metabolites were detected at the basal side of the intestine after perfusion with hesperetin, indicating transcellular transport (*25*), which is in accordance with data obtained with Caco-2 cells (*15*) (**Table 1**). Based on data obtained by perfusion studies, epicatechin (**Chapter 4**), quercetin (*25, 26*), luteolin and kaempferol (*25*) are also transported transcellularly. The absence of methylated and or conjugated metabolites in perfusion systems does not necessarily indicate paracellular transport as substrate specificity of the enzymes involved should also be considered. One of those enzymes is catechol-O-methyl-transferase (COMT), and PC dimers have been reported to be substrates for this enzyme (*27*). Based on our results obtained on the absorption of PC dimers (**Chapter 4**), a possible absorption mechanism will be discussed below.

Absorption of A- and B-type procyanidin dimers

Prior to our study, only data on the absorption of B-type PCs were available. The results presented in **Chapter 4** showed that the A-type dimers A1 and A2 were absorbed in the rat small intestinal perfusion model. In contrast, the B-type dimer B2 was not absorbed. This suggests that A-type dimers are better absorbed than B-type dimers. This observation was supported by comparing the absorption of both A- and B-type dimers with that of epicatechin. The A-type dimers were 10-17 times less absorbed than epicatechin (on a molar basis) (**Chapter 4**), while B-type dimers were about 50 times (corrected for their molar ratios in the cocoa beverage) less absorbed than epicatechin, analyzed in humans, upon consumption of a cocoa beverage (*28*). The concentration of PC B-type dimers, only detected twice in human plasma, did not exceed 0.05 µM (*28, 29*). Our data on the absorption of A-type dimers in rats should still be confirmed in human studies. This data could be relevant with respect to the intake of e.g. cranberry supplements or other sources rich in A-type PCs.

A study performed with a Caco-2 cell line supports the hypothesis that PCs are transported by paracellular diffusion (*30*). Absorption of PCs was shown to be inversely related to the transepithelial electrical resistance (TER) value, which is a measure of paracellular transport (*31, 32*). The hydrophilic character of PCs and the absence of methylated metabolites in our perfusion study (**Chapter 4**) hints at paracellular transport. This was based on the finding that PC dimers are substrates of COMT (*27*) and therefore are expected to be methylated when transported transcellularly. If transported by the paracellular route, absorption of PAs is limited by the pore size of the paracellular route. The three dimensional structures of PC dimers B1 to B4 have been reported to exist in two conformations that are slowly interchanged: Compact and extended (*33*). The compact form dominated (B1: 92%, B3: 95%) when catechin was present as terminal unit. When epicatechin instead of catechin was present as terminal unit, the proportion of the extended form was higher (B2: 45%, B4: 17%). For B2-3-O-gallate the compact form was dominating (≥ 98%). So the gallyol moiety on B2 strongly influences its preferred three dimensional structure. **Figure 2** shows the compact and extended 3D structure for dimer B2 (*33*).

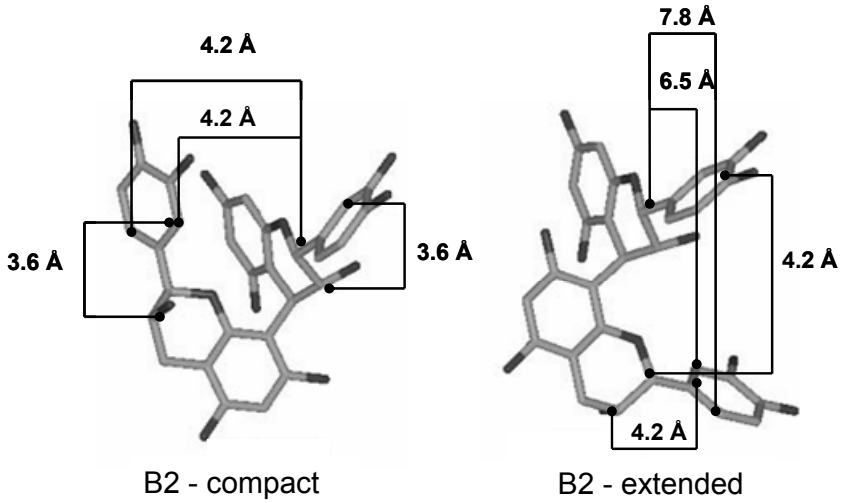

Figure 2. The 3D representation of the compact and extended form of PC dimer B2 (*33*, with permission). The length of the lines is not correlated with the length in angstrom (Å).

The hydrodynamic radii of dimers B1-B4 in D_2O have been calculated to be between 7.4-8.0 Å (*34*). Besides the type of solvent, the radius is also depending on the preferred confirmation of the dimer, compact or extended, because the largest distance that was reported in the extended form of B2 was already 7.8 Å (**Figure 2**). Based on these data, paracellular transport seems to be possible for PA dimers as the pore size of tight junctions

was 5.2 Å, which could be maximized to 22 Å (*14*). It can be hypothesized that A-type dimers are more compact than B-type dimers, due to the additional ether bond, which might explain their better absorption compared to B-type dimers.

The presence of oligomers enhances the absorption of dimers

We found that dimer B2 was not absorbed from the rat small intestine when tested as a single compound. In contrast, it was clearly absorbed in the presence of tetramers (**Chapter 4**). A similar positive effect of high DP oligomers on the absorption of low DP oligomers has been reported by Shoji and coworkers (*35*). The A-type trimers were not absorbed (**Chapter 4**), which supports the hypothesis that only dimers are absorbed. Although the hydrodynamic radius of the B-type trimer C2, calculated to be 8.7 Å (*34*), is not much larger than those of dimers, this might have been above the cut-off of the tight junction pores in our test system.

The lack of absorption of high DP oligomers has resulted in an increased interest in foods and extracts rich in low DP oligomers. Also, processes that depolymerize high DP oligomers have been developed (*36, 37*), in order to increase the bioavailability of PCs. However, based on the finding that high DP oligomers enhance the absorption of low DP oligomers, the removal or depolymerization of high DP oligomers could also counteract the bioavailability of PAs.

The mechanisms responsible for the PA dimer bioavailability-enhancing effect of higher DP oligomers are unknown. Shoji and coworkers (*35*) hypothesized that the larger oligomers (DP $\geq$ 8) would preferentially bind to mucosal proteins compared to the small oligomers, which would leave the small oligomers available for absorption. Another explanation might be that PCs could decrease the free [Ca^{2+}]. Calcium ions play an important role in the formation (extracellular Ca^{2+}) and regulation (intracellular Ca^{2+}) of the inter-epithelial junctions. These include tight junctions (*38, 39*), which restrict paracellular transport (*38, 39*). When calcium concentrations decrease, the tight junctions will relax and intestinal permeability increases (*38, 40*). PAs have been shown to be able to affect calcium homeostasis within different cell types. For example, pretreatment of platelet cells of diabetic patients with an A-type trimer (10 μM) reduced free [Ca^{2+}] (*41*). In another study with T-lymphocyte cells (Jurkat T-cells), epicatechin, PC dimer B2 and PC trimer C1 prevented K^+ stimulated Ca^{2+} mobilization with IC_{50} values of 10, 24 and 196 nM, respectively, (*42*). This effect was obtained with physiological relevant concentrations. Furthermore, the ability of PCs to decrease Ca^{2+} mobilization was inversely related to the DP. With respect to the PAs in food sources, in which the relative proportion of oligomers (DP > 2) is high (*1*), the concentration of trimers or higher DP oligomers that reach the small intestine might still be relevant.

A possible mechanism involved in the regulation of calcium homeostasis by PAs might be their calcium binding properties (*43-45*). This might result in a decreased [Ca^{2+}],

and subsequent opening of the tight junctions, which could result in increased absorption of dimers. In order to explain the enhanced absorption of dimers in the presence of oligomers, the calcium binding capacity should increase with DP. Flavonoids are known to chelate metal ions (*46*) and three binding sites have been identified: The catechol moiety and between the 3-hydroxy or 5-hydroxy and the 4-keto group (*47*). The catechol moiety on the B-ring was shown to be an important feature for metal ion chelation (*48*). This moiety is also present in PAs and increases with DP on the basis of molarities. Additional binding sites for calcium might arise from hydroxyl/oxygen groups in different monomeric units, which are forced in each other's proximity, when the molecules adopt their most favorable conformation (**Figure 3**).

When calcium is indeed bound by the catechol moiety and between the monomeric units of PAs as indicated in **Figure 3**, the calcium binding capacity will not only increase on the basis of molarities, but also on the basis of weight. As an example, 1 g of dimers or tetramers will bind 2 to 2.5 times more calcium, respectively, than 1 g of monomers. The increased calcium binding capacity of oligomers compared to monomers will eventually reach a plateau value of ~3. Research in this area would benefit from the determination of the Ca^{2+} binding capacity of PAs as one of the mechanisms that could affect calcium availability. Additionally, *in vitro* studies with e.g. Caco-2 cells could be used to monitor free [Ca^{2+}] in the presence of PA oligomers in relation to the absorption of dimers.

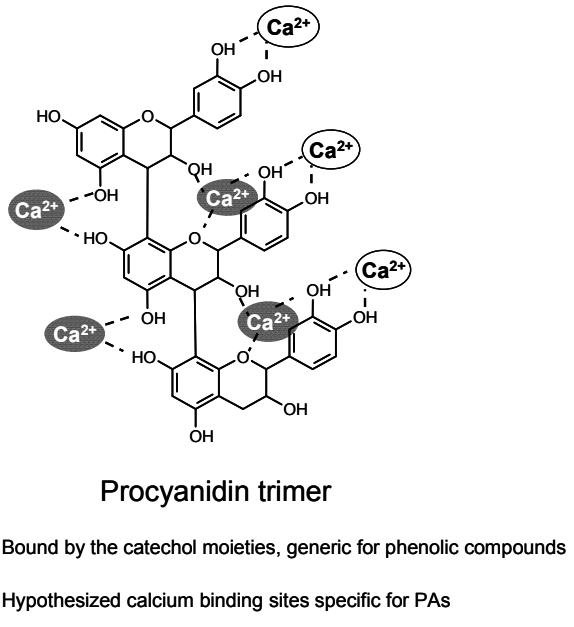

Figure 3. Proposed calcium binding sites within PAs, including the catechol moieties.

Microbial metabolism of PAs

Due to the limited absorption of PAs, large quantities will reach the colon (*49*) and are exposed to the microbiota. Therefore, microbial metabolites represent an important fraction of the ingested PAs, and might (partly) be responsible for the health effects attributed to PAs.

We detected hydroxylated phenylpropionic and phenylacetic acids and hydroxylated phenylvalerolactone as the main microbial metabolites derived from B-type PC dimers upon incubation with human feces (**Chapter 5**). Our finding of 3,4-dihydroxyphenylacetic acid (3,4-diHPA) as a major metabolite was used to suggest a tentative degradation pathway for B-type PC dimers (**Chapter 5**). This metabolite had not been detected in studies performed with pure (epi)catechin and human microbiota (*50, 51*). Therefore, it was suggested to originate directly from the B-type dimers without their prior cleavage into monomeric units.

Based on our pathway proposed, it is expected that the fermentation of higher DP oligomers will result in increased amounts of 3,4-diHPA correlated to the number of extension units (**Figure 4**). The fate of parts of the PC, the A-rings and part of the C-rings, remains unknown. It might be hypothesized that these parts will be converted into phloroglucinol and CO_2. The CO_2 was released after fermentation of [ring A-^{14}C]-catechin in guinea pigs and rats (*52*), and represented 20 % of the ingested dose. Phloroglucinol has also been postulated to originate form the A-ring of several flavonoids, like epicatechin, quercetin, luteolin and naringenin (*53-55*), but its detection is difficult. Phloroglucinol seems to be quickly converted into butyrate and acetate (*56*), or it binds to cysteine or other compounds in the growth medium (*57*), which complicates its detection. Our experiments can not exclude the possibility that phloroglucinol is a metabolite of PC dimers, despite our attempts to detect it with MS analysis.

A study in which microbial metabolites of low and high DP oligomers are directly compared might confirm the proposed pathway for PCs degradation. Data on the microbial conversion of A-type PAs are lacking. Possibly, A-type PAs are more resistant to microbial degradation, because of the additional ether bond. This additional bond might also affect the type of metabolites formed.

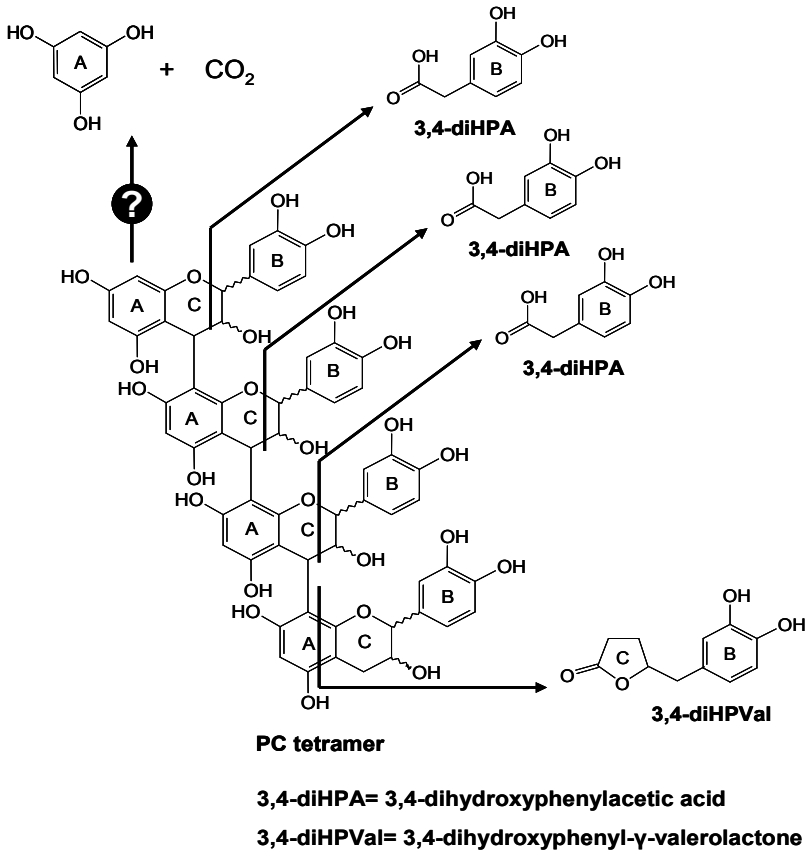

Figure 4. Expected degradation pathway for PC oligomers based on the proposed pathway given in **Chapter 5**.

PAs and decreased blood pressure: Possible modes of action

Numerous human intervention studies with PA-rich foods, beverages and supplements reported changes in biomarkers, mainly related to a decreased risk for CVD (*58*). Especially studies reporting decreased blood pressure and increased flow mediated dilatation after the intake of cocoa, both in healthy (*59*) and hypertensive (*60, 61*) human subjects, are promising. PAs are considered to be responsible for those beneficial effects, but despite extensive research to confirm this, unambiguous proof is still lacking.

Nitric oxide (NO), which is produced by eNOS in endothelial cells, is known to be a key signaling molecule, regulating vasodilatation. Therefore, we tested the ability of PCs and their microbial metabolites to stimulate NO production (**Chapter 6**) as a possible mechanism for the observed decrease in blood pressure after cocoa intake. PCs only showed a small insignificant effect, which seems to be positively related to the DP. For the microbial metabolites of PCs, no enhanced NO production was detected. Besides phenolic acids, valerolactones are also important human microbial metabolites of PCs. Valerolactones are structurally different from PCs and other phenolic acids, and thus it might be worthwhile testing them with respect to vasorelaxing potential. Valerolactones can be synthesized, but the synthesis protocols are quite complex (*62, 63*). They can also be purified after microbial fermentation (*64*), but this is laborious and requires a large amount of starting material. Therefore, efficient methods to generate sufficient quantities of valerolactones for bioactivity studies are of great interest.

Our test system was designed to detect an increase in NO-production, but the NO-level also depends on the stability of NO itself, which can be degraded by reactive oxygen species (ROS). PAs acting as antioxidants (*65*) might scavenge ROS or target other mechanisms involved in ROS production (*66, 67*), thereby maintaining high NO levels. For example, PC dimer B2 was potent in inhibiting NADPH oxidase and scavenging O_2^-, as tested in human umbilical endothelial cells (*67*), thereby reducing overall ROS production. Due to the use of HEPES, SOD and catalase in our test system, the effect of antioxidative actions of the test compounds could not be detected.

Health potential of PA-rich dietary sources: Which are the bioactive compounds?

PCs as bioactive compounds

The low plasma amounts of A- and B-type PC dimers, were not methylated and/or conjugated (**Chapter 4**), which is in contrast to most phenolic compounds (*68*). Therefore, PCs conserve their biological activity, which facilitates the translation of their *in vitro* activity to *in vivo* situations.

As mentioned earlier, plasma concentrations of PCs in humans (< 0.05 μM) (*28, 29*) are relatively low compared to other phenolic compounds. Flavonols, flavanols and isoflavones have been reported to reach concentrations of up to about 5 μM (*12*) and total epicatechin concentrations in human plasma ranged from 0.25-6 μM, after chocolate consumption (*28, 69-71*). However, the conserved structures of PCs *in vivo* might partly compensate their lower absorption. The proportion of unmetabolized versus metabolized compounds could be important with respect to their bioactivity. For example epicatechin

was completely methylated and conjugated upon absorption upon absorption from the rat small intestine (**Chapter 4**) and some studies reported a decreased bioactivity of methylated and glucuronidated epicatechin (*72, 73*). Also, in humans unmodified epicatechin represented only < 10% of the total epicatechin metabolites (*71*). Therefore, plasma concentrations of unmetabolized PC dimers (0.05 μM) are similar to, or only 10 times lower than, the concentrations of unmetabolized epicatechin (0.03-0.6 μM, 10 % of total concentration in humans).

Human microbial metabolites of PCs as bioactive compounds

Besides PCs themselves, their microbial metabolites might contribute to the beneficial health effect of cocoa and wine. Microbial conversions are known to play an important role in the absorption of several other phenolic compounds. For example, quercetin-3-rutinoside is not absorbed in the small intestine (*26*), but it is absorbed from the colon after microbial removal of the sugar moiety (*74*). Other examples are high plasma concentrations of enterodiol and enterolactone (*75*), which are microbial products of lignans (*76, 77*).

Data on the bioavailability of the PC metabolites, hydroxyphenylpropionic acids, hydroxyphenylacetic acids and hydroxyphenylvalerolactones is limited and the extents of their absorption in humans remain to be investigated. Phenolic acids are known to be extensively excreted in urine (*62, 78, 79*), and some have been detected in plasma (*80*) of human subjects.

Concerning the bioactivity of microbial PC metabolites, none of the tested metabolites positively influenced NO-production (**Chapter 6**). A limited number of studies determined the bioactivity of the microbial metabolites of PCs. Of these studies only few were related to cardiovascular health, including anti-platelet activity of 3,4-diHPA and 4-HPA (*81*) and anti-oxidant activity of 3,4-diHPA (*82*). Up to now, not enough data are available to conclude whether microbial metabolites of PCs are (partly) responsible for the decreased blood pressure found after cocoa and wine consumption.

PCs as stimulators for the absorption of other health beneficial compounds

In **Chapter 4** the stimulation of dimer absorption by oligomers was described. Based on this finding, it might be hypothesized that PAs also stimulate the absorption of other compounds, targeting *in vivo* processes involved in maintaining endothelial function. One study performed (*83*) supports the hypothesis that PCs can increase the bioavailability of other phenolic compounds. This study showed that the bioavailability of hypericin in rats increased with 58% when administered together with PC dimer B2. In addition, an earlier study (*84*) the antidepressant activity of hypericin, an anthraquinone derivative, measured in the forced swimming test in rats, also increased when administered together with B2.

Summarizing, PC oligomers (DP > 4) enhanced the absorption of dimers (**Chapter 4**, (*35*), and dimer B2 increased hypericin bioavailability (*83*). Therefore, PAs have the potential to improve the bioavailability of other phenolic compounds, which might also include compounds with blood pressure lowering properties.

This thesis increased our knowledge on the absorption, biotransformation and bioactivity of A- and B-type PCs. A possible interaction between high and low DP oligomers, influencing absorption processes has been discussed, which suggests that until now the biofunctional potential of PAs has been underestimated.

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Summary

Intervention studies showed that the intake of proantocyanidins (PAs) is associated with beneficial health effects, mainly related to the prevention of cardiovascular diseases. For example, several studies showed that a high intake of cocoa and wine, which are rich in PAs, decreased blood pressure. PAs are considered to be the bioactive compounds responsible for those beneficial health effects.

PAs are phenolic compounds that are abundant in our diet. Well known PA food sources are cocoa, apple, grape seeds, wine, nuts, and cinnamon. PAs are oligomers of monomeric flavan-3-ols and belong to the class of flavonoids. The monomeric units can be linked to each other by a single C4-C8 or C4-C6 linkage, which is referred to as B-type. Besides these single linkages an additional ether bond can be present, C2-O-C7 or C2-O-C5, which is referred to as A-type. PA is a general name that includes several subgroups. With respect to food, three subgroups are relevant: Procyanidins (PCs), prodelphinidins (PDs) and propelargonidins (PP). PCs form a homogenous group that exclusively exists of (epi)catechin units. The other two subgroups, PDs and PPs, are heterogeneous groups that, besides (epi)catechin, exist of (epi)gallocatechin and (epi)afzelechin units, respectively.

To determine whether PAs are the bioactive compounds responsible for the blood lowering effects of cocoa and wine, research on their bioavailability, bioconversion and bioactivity is needed. Therefore, the aims of this thesis were: **i)** to determine the bioavailability and metabolism of A-type in comparison to B-type procyanidins, **ii)** to determine the human microbial metabolites of B-type procyanidins, and **iii)** to determine the potential of A- and B-type procyanidins or their microbial metabolites to improve vascular function by influencing NO-release of endothelial cells. To achieve these aims techniques for the isolation, characterization and quantification of PAs were developed. This thesis focused on the bioactive potential of A- and B-type PCs, which were isolated from peanut skins and grape seeds, respectively.

Considering the reported health effects of PAs, food supplements enriched in PAs are of interest for the food industry. Peanut skins, as a by-product of the peanut butter industry, are rich in A-type PAs and might be a potential source for food supplements. To determine the biofunctional potential of this source, the PAs present were characterized and quantified as described in **Chapter 2**. The combination of NP and RP-HPLC was used as a tool to determine the PA molecular diversity. Fractions (9 in total), containing different oligomers, were first isolated with NP-HPLC from a 20% (v/v) aqueous methanol extract from peanut skins. Subsequently, each fraction was separated on RP-HPLC and peaks were identified with mass spectrometric analysis. Quantification was based on UV responses, which were assumed to be similar for the oligomers within each fraction. The extract mainly contained A-type PCs (95.0 % (w/w)) and out of a total of 114 peaks, we could distinguish 83 different PA molecular species. In the majority of the trimers and tetramers, the A-linkage was positioned at the terminal unit, and often more than one A-linkage

occurred. This method also enabled determination of the ratio of A- versus B-type dimers (19.9 % (w/w) vs 2.4 % (w/w) of the extract).

In **Chapter 3**, we describe the efficient isolation of both B-type dimers form grape seeds and A-type dimers from peanut skins by combining NP- and RP-HPLC. Previously used methods often included multiple chromatographic steps and focused on the isolation of one or two specific dimers. We reduced the number of chromatographic steps and obtained a yield of 20-400 times more A-type dimers and of about 10 times more B-type dimers compared to other methods. Most of the dimers (9 out of 15) were identified with NMR spectroscopy and mass spectrometry. For B-type dimers we additionally used a combination of thiolysis and available standards. We isolated six dimers from peanut skins. The main dimers were A1 (epicatechin-(2-O-7, 4-8)-catechin) and A2 (epicatechin-(2-O-7, 4-8)-epicatechin). Furthermore, epicatechin-(2-O-7, 4-6)-catechin, epicatechin-(2-O-7, 4-8)-*ent*catechin, which was isolated from peanut skins for the first time, and B7 (epicatechin-(4-6)-catechin) were identified. One dimer could not be identified. From grape seeds eight B-type dimers were isolated of which four were identified as B1 (epicatechin-(4-8)-catechin), B2 (epicatechin-(4-8)-epicatechin), B3 (catechin-(4-8)-catechin) and B4 (catechin-(4-8)-epicatechin). The method described delivered enough purified PAs, which were used to study their bioavailability, microbial conversion and blood pressure lowering potential.

After obtaining pure A- and B-type dimers and fractions of A-type trimers and tetramers, their absorption was tested by an *in situ* perfusion model of the small intestine of rats (**Chapter 4**). We for the first time reported absorption of A-type PC dimers and showed that they were better absorbed than B-type PC dimers. Furthermore, A-type PC tetramers stimulated the absorption of B-type PC dimers. In contrast to epicatechin, both A- and B-type PC dimers were absorbed unmetabolized. A-type PC trimers were not absorbed. Neither epicatechin nor its methylated and/or conjugated metabolites were detected as a result of perfusion with any of the PCs.

Because only a small amount of the PAs is absorbed, a large amount will reach the colon where microbial conversions might take place. The microbial metabolites of PAs might be (partly) responsible for the beneficial effects of PAs. Thus, identification of these PA microbial metabolites is necessary to determine their bioactivity. In **Chapter 5** the *in vitro* fermentation of B-type PC dimers with human microbiota is described. Care was taken that the PC substrate was free of (epi)catechin, because the presence of (epi)catechin would have obscured the origin of the metabolites. The main metabolites identified were 2-(3,4-dihydroxylphenyl)acetic acid and 5-(3,4-dihydroxyphenyl)-γ-valerolactone. Based on the type of metabolites that were detected after different fermentation times, a tentative degradation route for B-type dimers has been proposed. In the literature, 2-(3,4-dihydroxylphenyl)acetic acid has not been reported as a metabolite after fermentation of

pure epicatechin. Because we detected it as the main metabolite of B-type PC dimers, this suggests its direct release from B-type dimers without prior cleavage of the interflavanic bond of the dimer.

The potential vasorelaxing properties of PAs might relate to the stimulation of nitric oxide (NO) production by endothelial cells, as NO is a major regulator of vasodilatation. A hybrid human endothelial cell line EA.hy926 was optimized to rapidly screen phenolic compounds for their NO stimulating potential. Dose-dependent correlations between NO stimulation and eNOS upregulation were found for selected phenolic compounds, suggesting the stimulation of eNOS expression as a possible mechanism of increased NO-production. Most A- and B-type procyanidins showed a non-significant tendency to elevate NO production, and microbial metabolites were inactive (**Chapter 6**). Besides phenolic acids, valerolactones were also important human microbial metabolites of procyanidins (**Chapter 5**). Due to a lack of standards we were not able to test valerolactones for their vasorelaxing potential.

In the last chapter (**Chapter 7**), the mechanisms involved in the biofunctional potential of PAs are discussed in relation to the results obtained. More particularly, we hypothesized that PAs are transported via paracellular diffusion and that the oligomer-enhanced bioavailability of B-type dimers might involve calcium-mediated mechanisms. Furthermore, the progress in PA analysis and the new insights in microbial conversion routes of higher DP PA oligomers were elaborated, as well as mechanisms, besides NO stimulation, that might be considered as PA targets.

Samenvatting

Interventie studies hebben laten zien dat de inname van proanthocyanidinen (PA's) geassocieerd kan worden met positieve gezondheidseffecten die hoofdzakelijk gerelateerd zijn aan het voorkomen van hart en vaatziekten. Een aantal van die studies liet zien dat een hoge inname van cocoa en wijn, beide rijk in PA's, resulteerde in een verlaagde bloeddruk. PA's worden gezien als de bioactieve componenten die verantwoordelijk zijn voor deze positieve gezondheidseffecten.

PA's zijn fenolische componenten die rijkelijk in ons dieet voorkomen. Voedsel bronnen die bekend staan PA's te bevatten zijn cocoa, druivenpitten, wijn, noten en kaneel. PA's zijn oligomeren van monomere flavan-3-olen en behoren tot de klasse van de flavonoïden. De monomere bouwstenen kunnen aan elkaar gekoppeld worden door middel van een enkelvoudige C4-C8 of C4-C6 binding, wat aangeduid wordt als B-type. Naast deze enkelvoudige binding kan er een extra ether binding aanwezig zijn, C2-O-C7 of C2-O-C5, wat aangeduid wordt als A-type. PA's is een algemene naam die verschillende subgroepen omvat. Met het oog op voeding zijn er drie relevante subgroepen: procyanidinen (PC's), prodelphinidinen (PD's) en propelargonidinen (PP's). PC's vormt een homogene groep die exclusief uit (epi)catechine bouwstenen bestaat. De twee andere groepen, PD's en PP's, zijn heterogene groepen die naast (epi)catechine, bestaan uit respectievelijk (epi)gallocatechine en (epi)afzelechine.

Om na te kunnen gaan of PA's bioactieve componenten zijn en verantwoordelijk voor de bloeddruk verlagende werking van cocoa en wijn is er onderzoek nodig naar hun biobeschikbaarheid, bioconversie en bioactiviteit. Dit resulteerde in de volgende doelen van dit proefschrift: **i**) bepaling van de biobeschikbaarheid en het metabolisme van A-type in vergelijking tot B-type PC's, **ii**) bepaling van de door menselijke darm microbiota gevormde metabolieten uit B-type PC's en **iii**) bepaling van de potentie van A- en B-type PC's en hun microbiële metabolieten om de vasculaire functie te verbeteren door de NO-productie van endotheel cellen te stimuleren. Om deze doelen te bereiken werden er eerst technieken ontwikkeld om de PA's te isoleren, te karakteriseren en te kwantificeren. Dit proefschrift richt zich op het bioactieve potentieel van A- en B-type PC's die opgezuiverd werden uit respectievelijk pindavliesjes en druivenpitten.

Op basis van alle gerapporteerde gezondheidseffecten gerelateerd aan PA's, zijn voedingssupplementen die verrijkt zijn in PA's interessant voor de levensmiddelen industrie. Pindavliesjes, een bijproduct van de pindakaas productie, zijn rijk in A-type PC's en daardoor een mogelijke bron voor voedingssupplementen. Om het biofunctionele potentieel van deze bron te bepalen werden de aanwezige PA's gekarakteriseerd en gekwantificeerd (**Hoofdstuk 2)**. Er werd een combinatie van NP en RP-HPLC gebruikt om de moleculaire diversiteit van PA's te kunnen bepalen. Eerst werden er 9 fracties, elk bestaande uit een verschillende oligomere samenstelling, uit een 20% (v/v) waterig methanol extract van pindavliesjes geïsoleerd met behulp van NP-HPLC. Vervolgens werd

elke fractie apart met RP-HPLC geanalyseerd en werden de pieken geïdentificeerd met behulp van massa spectrometrische detectie. De kwantificering was gebaseerd op UV metingen waarin werd aangenomen dat oligomeren binnen één fractie een vergelijkbaar UV signaal zouden geven. Het extract bestond voornamelijk uit A-type PC's (95.0 % (w/w)) en van de in totaal 114 geanalyseerde pieken konden we 83 verschillende moleculaire structuren onderscheiden. De meerderheid van de trimeren en tetrameren bevatte een A-binding aan de terminale kant van het oligomeer en vaak waren er meer dan één A-binding aanwezig. Deze methode maakte het mogelijk om de proportie A- versus B-type dimeren (19.9 % (w/w) vs 2.4 % (w/w) van het extract) te bepalen.

In **Hoofdstuk 3** wordt een methode beschreven om zowel B-type dimeren uit druivenpitten en A-type dimeren uit pindavliesjes op een efficiënte manier te zuiveren waarbij NP- en RP-HPLC gecombineerd werd. De methoden die voorheen gebruikt werden omvatte vaak meerdere chromatografische stappen en waren vaak gericht op het zuiveren van één of twee specifieke dimeren. Wij hebben het aantal chromatografische stappen verminderd terwijl de opbrengst 20-400 keer toenam voor A-type dimeren en ongeveer 10 maal voor B-type dimeren in vergelijking met andere methoden. Het merendeel van de dimeren (9 van de 15) kon worden geïdentificeerd met NMR spectroscopie en massa spectrometrie. Daarnaast werden B-type dimeren ook geïdentificeerd op basis van thiolyse en standaarden. Uit pindavliesjes werden zes dimeren geïsoleerd. De voornaamst aanwezige dimeren waren A1 (epicatechine-(2-O-7, 4-8)-catechine) en A2 (epicatechine-(2-O-7, 4-8)-epicatechine). Verder werden ook epicatechine-(2-O-7, 4-6)-catechine, epicatechine-(2-O-7, 4-8)-*ent*catechine, die voor het eerst uit pindaschil geïsoleerd werd, en B7 (epicatechine-(4-6)-catechine) geïdentificeerd. Eén dimeer kon niet geïdentificeerd worden. Uit druivenpitten werden totaal acht dimeren geïsoleerd waarvan er vier geïdentificeerd konden worden als B1 (epicatechine-(4-8)-catechine), B2 (epicatechine-(4-8)-epicatechine), B3 (catechine-(4-8)-catechine) and B4 (catechine-(4-8)-epicatechine). Deze isolatie methode resulteerde in genoeg PA's om te gebruiken in studies naar hun biobeschikbaarheid, microbiële omzetting en bloeddruk verlagend potentieel.

Nadat er zuivere A- en B-type dimeren plus fracties van A-type trimeren en tetrameren verkregen waren, werd hun absorptie getest in een *in situ* perfusie model met de dunne darm van ratten (**Hoofdstuk 4**). Met dit model konden we de absorptie van A-type PC dimeren aantonen wat nog niet bekend was. Bovendien werden A-type PC dimeren beter geabsorbeerd dan B-type PC dimeren. Verder werd de absorptie van B-type PC dimeren gestimuleerd door de aanwezigheid van A-type PC tetrameren. In tegenstelling tot epicatechine werden zowel A- als B-type PC dimeren geabsorbeerd zonder te worden gemetaboliseerd. A-type PC trimeren werden niet geabsorbeerd. Epicatechine en zijn gemethyleerde en/of geconjugeerde metabolieten werden niet gedetecteerd na perfusie met PC's.

Omdat maar een klein gedeelte van de PA's geabsorbeerd wordt zal een groot gedeelte de dikke darm bereiken waar microbiële omzettingen plaats kunnen vinden. De microbiële metabolieten van PA's zouden (gedeeltelijk) verantwoordelijk kunnen zijn voor de voordelige effecten die aan PA's gerelateerd zijn. Daarom is identificatie van die metabolieten nodig om hun bioactiviteit te kunnen testen. **Hoofdstuk 5** beschrijft de *in vitro* fermentatie van B-type PC dimeren met humane microbiota. Het PC substraat bevatte geen (epi)catechine omdat in aanwezigheid van (epi)catechine de oorsprong van de metabolieten onduidelijk zou zijn. De hoofd metabolieten werden geïdentificeerd als 2-(3,4-dihydroxylphenyl)acetic acid en 5-(3,4-dihydroxyphenyl)-γ-valerolactone. Gebaseerd op het type metabolieten die werden gedetecteerd na verschillende tijden van fermentatie, werd er een hypothetische afbraak route voor B-type dimeren voorgesteld. In de literatuur wordt 2-(3,4-dihydroxylphenyl)acetic acid niet genoemd als metaboliet na fermentatie van zuivere epicatechine. Omdat wij het als één van de hoofd metabolieten van B-type dimeren hebben gedetecteerd is het aannemelijk dat dit metaboliet direct vrijgemaakt wordt uit B-type dimeren zonder dat de interflavan binding van het dimeer verbroken wordt.

Het bloeddruk verlagend potentieel van PA's zou o.a. gerelateerd kunnen zijn aan de mogelijke stimulatie van stikstof oxide (NO) productie door endotheel cellen aangezien NO een belangrijke regulator is van vaatverwijding. Het gebruik van een hybride humane endotheel cellijn (EA.hy926) werd geoptimaliseerd om fenolische componenten snel te kunnen screenen voor hun NO stimulerende werking. Voor enkele fenolische componenten werden er correlaties gevonden tussen de stimulatie van NO productie en eNOS expressie wat gemeten werd met verschillende doses. Dit suggereert dat de stimulering van eNOS expressie een mogelijk mechanisme is waardoor de NO productie toeneemt. De meeste A- en B-type PC's lieten een niet significante toename zien in NO productie en de microbiële metabolieten waren inactief (**Hoofdstuk 6**). Naast fenolzuren waren ook valerolactonen belangrijke microbiële metabolieten die vanuit PC's werden gevormd (**Hoofdstuk 5**). Echter, door het gebrek aan standaarden konden we valerolactonen niet testen voor hun vaatverwijdend potentieel.

In het laatste hoofdstuk (**Hoofdstuk 7**) worden de mechanismen die betrokken kunnen zijn in het biofunctionele potentieel van PA's in relatie tot de verkregen resultaten bediscussieerd. In het bijzonder bespreken we de hypothese dat PA's via paracellulaire diffusie door de darmwand worden getransporteerd. Verder wordt de stimulerende werking van de aanwezigheid van tetrameren op de absorptie van B-type dimeren besproken en daarbij de mogelijke betrokkenheid van een calciumafhankelijk mechanisme. Ook werd er uitgeweid over de vooruitgang in PA analyse, de nieuw verkregen inzichten in de microbiële omzetting van grotere oligomeren en de mechanismen (naast stimulering van NO productie) die betrokken zijn in vaatverwijding en die door PA's beïnvloed zouden kunnen worden.

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Op naar de volgende uitdaging!

About the author

Curriculum Vitae

Maaike Marieke Appeldoorn was born on the 9th of April 1981 in Woudenberg. In 1998 she finished her Higher General Secondary Education at the Johannes Fontanus College in Barneveld. In the same year she started the bachelor study Laboratory Science at Larenstein International Agricultural College in Velp, with the specialization Food Analysis. During this study she performed two internships. At Royal Numico in Wageningen she studied the effect on pre-operatieve food on the energy status of rats that were subjected to surgery. During the second internship performed at the Agrotechnological Research Institute (ATO) in Wageningen she studied the allergenicity of different apple cultivars. In 2002 she received her BSc degree and continued with the master study Food Technology at Wageningen University. She followed the specialization Product Functionality and did her thesis on the absorption of flavours in packaging material at the Laboratory of Food Chemistry. In April 2004 she received her MSc degree. In November 2004 she was appointed at the Laboratory of Food Chemistry of Wageningen University to conduct the PhD project described in this thesis. From the 1st of April Maaike started as Biochemical Researcher at Royal Nedalco, located in Bergen op Zoom.

Publications

Maaike M. Appeldoorn, Mark Sanders, Jean-Paul Vincken, Véronique Cheynier, Christine Le Guernevé, Peter C.H. Hollman and Harry Gruppen, Efficient isolation of major procyanidin A-type dimers from peanut skins and B-type dimers from grape seeds, *in press, Food Chem.*, **2009**.

Maaike M. Appeldoorn, Jean-Paul Vincken, Anna-Marja Aura, Peter C.H. Hollman and Harry Gruppen, Procyanidin dimers are metabolized by human microbiota with 2-(3,4-dihydroxyphenyl)acetic acid and 5-(3,4-dihydroxyphenyl)-γ-valerolactone as the major metabolites, *J. Agric. Food Chem.*, **2009**, 57 1084-1092.

Maaike M. Appeldoorn, Jean-Paul Vincken, Mark Sanders, Peter C.H. Hollman and Harry Gruppen, Combined normal-phase and reversed-phase liquid chromatography/ESI-MS as a tool to determine the molecular diversity of A-type procyanidins in peanut skins, *submitted*.

Maaike M. Appeldoorn, Jean-Paul Vincken, Harry Gruppen, and Peter C. H. Hollman, Procyanidin dimers A1, A2 and B2 are absorbed without conjugation or methylation from the small intestine of rats, *accepted, J. Nutr.* **2009**.

Maaike M. Appeldoorn, Dini P. Venema, Theodorus H.F. Peters, Marjorie E. Koenen, Ilja C.W. Arts, Jean-Paul Vincken, Harry Gruppen, Jaap Keijer, and Peter C. H. Hollman, Some phenolic compounds enhance nitric oxide production in endothelial cells *in vitro*, *submitted*.

Overview of completed training activities

Discipline specific activities

Conferences
Health Ingredients, Amsterdam, The Netherlands, 2004
European workshop Improving the Health Value of Food Plants – Phytochemical Optimization (COST926), Egmond aan Zee, The Netherlands, 2005
XXIII International Conference on Polyphenols, Winnipeg, Canada, 2006
NWO-nutrition meeting, Deurne, The Netherlands, 2007
XXIV International Conference on Polyphenols, Salamanca, Spain, 2008
NOW-nutrition meeting, Deurne, The Netherlands, 2008

Courses
Ecophysiology of the GI-tract, VLAG, Wageningen, The Netherlands, 2005
Pharmacokinetics, LACDR, Oss, The Netherlands, 2005
Vertebrate; structure and functioning, Experimental Zoology, Wageningen, The Netherlands, 2007

General courses
Supervision of Undergraduate Students, OWU, Wageningen, The Netherlands, 2005
PhD Introduction Week, VLAG, De Bilt, 2005
Scientific writing, CENTA, Wageningen, The Netherlands, 2007
Career Perspectives, VLAG, Wageningen, The Netherlands, 2008

Additional activities
Preparation PhD research proposal, Wageningen, The Netherlands, 2004
PhD Study trip, Japan, 2004
PhD Study trip, Belgium, France and England, 2006
Food Chemistry Seminars, Wageningen, The Netherlands, 2004-2006
Food Chemistry Colloquia, Wageningen, The Netherlands, 2004-2006
RIKILT Seminars, Wageningen, The Netherlands, 2007-2008

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