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Analysis of proanthocyanidins

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1 Introduction

Plant phenolics are aromatic secondary metabolites that are ubiquitously spread throughout the plant kingdom. They comprise more than 8000 substances with highly diverse structures and molecular masses ranging from <100 Da for simple phenolic compounds to >30 000 Da for highly polymerized structures. Plant phenols can be subdivided in the following classes: phenolic acids, flavanones, flavones and flavonols, anthocyanidins, flavan-3-ols and proanthocyanidins (PAC). For flavonoids, more than 5000 different kinds have been described [1].

The colorless PAC are also known as oligoflavanoids; they comprise condensed polyphenols or condensed tannins. Apart from lignin, they represent the most abundant class of natural phenolic compounds. The PAC obtained their name from the characteristic oxidative depolymerization reaction in acidic medium, which yields colored anthocyanidins. PAC consist of monomeric flavan-3-ol units, which are mainly linked through C4 → C8 or sometimes C4 → C6 bonds. These linkages are both called B-type linkages. When an additional ether linkage is formed (mainly) between C2 and C7, compounds are called A-type PAC (Fig. 1); this subclass lacks two hydrogens compared to the B-type. In addition to the 2β → O7 linkage, A-types with a 2β → O5 linkage are also found, *e.g.*, in cocoa [2].

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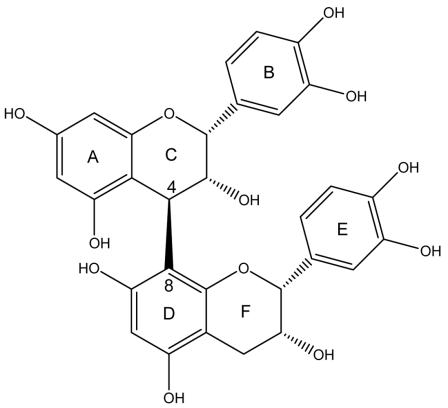
Abbreviations: **DMACA**, dimethylaminocinnamaldehyde; **DPm**, mean degree of polymerization; **FD**, fluorescence detection; **HPTLC**, high-performance TLC; **NP-HPLC**, normal-phase HPLC; **PAC**, proanthocyanidins; **SEC**, size-exclusion chromatography

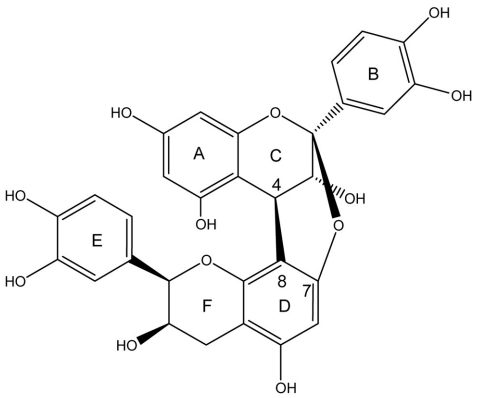
There are a variety of different classes of PAC depending on the substitution pattern of the monomeric flavan-3-ol units; the most common monomeric units are represented in Fig. 2 [3]. The PAC that exclusively consist of (epi)catechin units are designated procyanidins, the most abundant type of PAC in plants. The less common PAC containing (epi)afzelechin or (epi)gallocatechin subunits are termed propelargonidin or prodelphinidin, respectively. 5-Deoxy subunits [(epi)robinetinidol or (epi)fisetinidol] are also known. Pecan nut (*Carya illionensis*) pith tannin is predominatly a prodelphinidin tannin (prodelphinidins: procyanidin = 6:1), pine (*Pinus radiate*) bark tannin an entirely procyanidin of high mean degree of polymerization (DPm), quebracho (*Schinopsis balansae* var. *chaqueno*) wood tannin a predominantly profisetinidin/prorobinetinidin tannin, and apple (*Malus silvestris*) tannin, for the most part, a procyanidin of low molecular mass. In red wines colored PAC with anthocyan units in polymeric alliance have also been identified [4].

The flavan-3-ol subunits may carry acyl or glycosyl substituents. The most common substituent bound as an ester is gallic acid to form 3-*O*-gallates. Several glycosylated PAC oligomers have been identified, with the sugar linked to the C3 or the C5 position as the most widely distributed glycosylation.

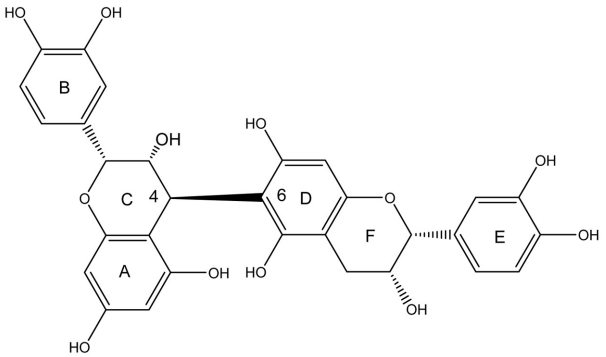
Procyanidins have a high structural diversity that is based on the four possible monomer units, (2*R*, 3*S*)-catechin, (2*S*, 3*R*)-ent-catechin, (2*R*, 3*R*)-epicatechin and (2*S*, 3*S*)-ent-epicatechin, with the diastereomeric monomers (2*R*, 3*S*)-catechin and (2*R*, 3*R*)-epicatechin being predominant (Fig. 2). Regarding the stereochemistry of their C2-C3 bond, they exhibit *cis* (2*R*, 3*R*; epicatechin) or *trans* (2*R*, 3*S*; catechin) configuration. Monomers with 2*S*-configuration are marked by the prefix ent- (enantio). The bond and its direction are displayed in brackets and configuration of the



Procyanidin B2
[epicatechin-(4β-8)-epicatechin]



Procyanidin A2
[epicatechin-(4β-8,2β-O7)-epicatechin]



Procyanidin B5
[epicatechin-(4β-6)-epicatechin]

Figure 1. Examples of A- and B-type procyanidins.

interflavanoid bond at C4 is specified by the IUPAC α,β nomenclature. More common trivial nomenclature for procyanidins was first introduced by Weinges *et al.* [5] and later extended by Thompson *et al.* [6]. The current valid nomenclature for oligomeric procyanidins was introduced by Hemingway *et al.* [7]. The monomers including their linkage of the most common PAC are shown in Table 1.

The high complexity of procyanidins is based on the four monomers mentioned above, the different types of interflavanoid bonds, and the various lengths of chains, known as degree of polymerization (DPm). Higher molecular mass procyanidins are usually of moderate size (>3000 Da) [7]; however, polymers with very high molecular masses (20 000 – 150 000 kDa) have also been reported [8].

Oligomeric and polymeric procyanidins are synthesized in the growing fruit and are colorless in their original unoxidized form. Although usage of the term "oligomeric" varies somewhat, dimers, trimers, and up to heptamers are generally referred to as oligomeric (n = 2∼5), whereas larger chains are generally referred to as polymeric procyanidins or tannins (n = 6∼∞) [9].

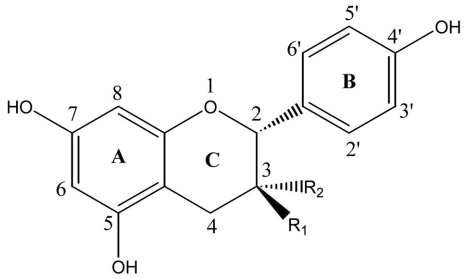
The taste of the procyanidins, an important quality factor for many fruits and their processed products, consists of a mixture of bitterness and astringency [10 – 12]; its balance strongly depends on the molecular mass. Sensory studies have demonstrated that tetrameric procyanidins exhibit strongest bitterness, whereas the astringent effect is described to increase with a higher degree of polymerization [13].

Importantly, a number of pharmacological effects have been reported for procyanidins, *e.g.*, antiviral, antimicrobial, anti-HIV, radical scavenging, anti-oxidative, anti-complementary and anti-tumor promoting properties, as well as cardiotonic and anti-arteriosclerotic activities [14 – 25]. Recently, Zessner *et al.* [26] demonstrated a positive correlation between the content of oligomeric procyanidins in apple juice extract and aromatase inhibition as well as inhibition of the diphenylpicrylhydrazyl (DPPH) assay and indicated a negative correlation with the oxygen radical absorbance capacity (ORAC) units. In addition, the procyanidins play an important role in the plant's defense mechanism against predators and microorganisms [27, 28].

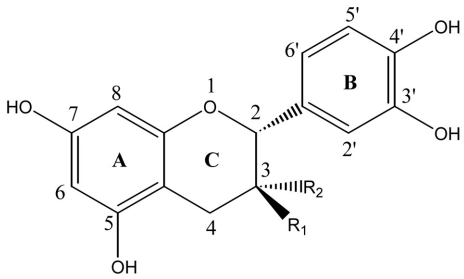
Since it is evident that procyanidins show such a broad range of different effects, their reliable qualitative and quantitative analysis is an attractive challenge. Here, an overview of the established analytical methods used for qualification and quantification of procyanidins or PAC in general is given.

2 Chemical properties

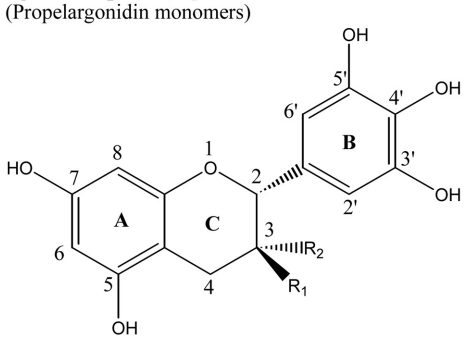
The monomers, (2*R*, 3*R*)-epicatechin and (2*R*, 3*S*)-catechin, are stable in aqueous acidic solution up to pH ~5. With increasing pH an isomerization at the C2 position to yield (2*S*, 3*S*)-ent-epicatechin and (2*S*, 3*R*)-ent-catechin has been observed, (2*R*, 3*S*)-catechin being more stable than the (2*R*, 3*R*)-epicatechin [29, 30]. The oligomeric procyanidins decompose and isomerize at pH 2. In the course of procyanidin B_2 [(−)-epicatechin-(4β-8)-(−)-epicatechin] decom-



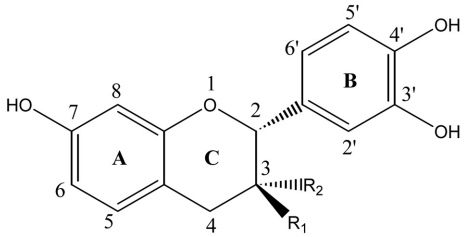
R_1 = OH; R_2 = H, Afzelechin
R_1 = H; R_2 = OH, Epiafzelechin
(Propelargonidin monomers)



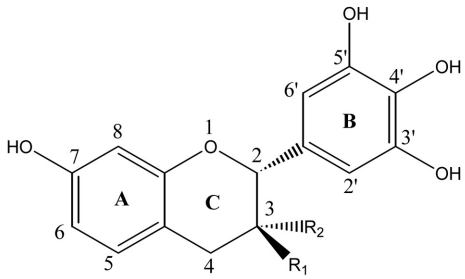
R_1 = OH; R_2 = H, Catechin
R_1 = H; R_2 = OH, Epicatechin
(Procyanidin monomers)



R_1 = OH; R_2 = H, Gallocatechin
R_1 = H; R_2 = OH, Epigallocatechin
(Prodelphinidin monomers)



R_1 = OH; R_2 = H, Fisetinidol
R_1 = H; R_2 = OH, Epifietinidol
(Profisetinidin monomers)



R_1 = OH; R_2 = H, Robinetinidol
R_1 = H; R_2 = OH, Epirobinetinidol
(Prorobinetinidin monomers)

Figure 2. Common flavan-3-ol units of proanthocyanidins (PAC) [3].

position, both epicatechin and procyanidin B_5 [(−)-epicatechin-(4β-6)-(−)-epicatechin] were observed [31]. In addition, it has been shown that the stability depends not only on the pH but also on the linkage of the two monomers and the degree of polymerization. Thus, the 4β-8 bond is more stable than the 4β-6 linkage. Monomers and dimers were rapidly degraded at pH > 9.0 within several minutes; the degradation products (not identified to date) showed a brown color in the incubation solution [31]. Kondo *et al.* [32] have reported that the single linked B-type procyanidins were transformed *via* radical oxidation at pH 7 or *via* oxidation with oxygen into the corresponding double linked A-type procyanidins.

3 Sample preservation and preparation

PAC are very sensitive to oxygen, light, acid and alkaline; in addition, the activity of 'polyphenol oxidase' can also rapidly reduce the amount of PAC in plant tissues [33]. Hence, care should be taken not to damage the plant material when collecting it. Fresh material has a high moisture content and is endangered by growth of mold if it is stored without any treatment. Therefore, the material should be dried to inactivate 'polyphenol oxidase', or, better, stored in a refrigerator to protect it from oxidation [34]. Freeze-drying seems to be the most suitable method; studies with PAC from several pine species (*Pinus* sp.) did not show any dif-

Table 1. Linkage of the most common proanthocyanidins (PAC)

Trivial name		CAS
Procyanidin B1	Epicatechin-(4β → 8)-catechin	20315-25-7
Procyanidin B2	Epicatechin-(4β → 8)-epicatechin	29106-49-8
Procyanidin B3	Catechin-(4α → 8)-catechin	23567-23-9
Procyanidin B4	Catechin-(4α → 8)-epicatechin	29106-51-2
Procyanidin B5	Epicatechin-(4β → 6)-epicatechin	12798-57-1
Procyanidin B6	Catechin-(4α → 6)-catechin	12798-58-2
Procyanidin B7	Epicatechin-(4β → 6)-catechin	12798-59-3
Procyanidin B8	Catechin-(4α → 6)-epicatechin	12798-60-6
Procyanidin C1 (Cinnamtannin A1)	Epicatechin-(4β → 8)-epicatechin-(4α → 8)-epicatechin	37064-30-5
Procyanidin C2	Catechin-(4α → 8)-catechin-(4β → 8)-catechin	37064-31-6
Procyanidin A1	Epicatechin-(4β → 8, 2β → O → 7)-catechin	12798-56-0
Procyanidin A2	Epicatechin-(4β → 8, 2β → O → 7)-epicatechin	41743-41-3
Procyanidin A4	Epicatechin-(4β → 8, 2β → O → 7)-ent-epicatechin	111466-29-6
Prodelphinidin B1	Epigallocatechin-(4β → 8)-gallocatechin	78362-04-6
Prodelphinidin B2	Epigallocatechin-(4β → 8)-epigallocatechin	87392-61-8
Prodelphinidin B3	Gallocatechin-(4α → 8)-gallocatechin	78362-05-7
Prodelphinidin B4	Gallocatechin-(4α → 8)-epigallocatechin	68964-95-4
Cinnamtannin B1	Epicatechin-(4β → 8, 2β → O → 7)-epicatechin-(4β → 8)-epicatechin	88082-60-4
Cinnamtannin B2	Epicatechin-(4β → 8)-epicatechin-(4β → 8, 2β → O → 7)-epicatechin-(4β → 8)-epicatechin	88038-12-4

ference between freshly collected samples and freeze-dried ones [28]. On the other hand, sun drying led to a decrease of the yield of procyanidins of up to 68% [35]. However, a major problem with freeze-dried material is its hygroscopic nature. Since the cell membranes are broken on freezing, exclusion of moisture during storage of the samples is very important to avoid degradation reactions [36]. In general, samples should not be stored too long before analysis. In coffee pulp (*Coffea* sp.), for example, only 46% of the original content of PAC could be detected after a storage period of 1 year. In addition, a shift to higher molecular mass compounds has been noticed [37].

Isolation is usually done by extraction. During this step, high temperature and purging the extract with air has to be avoided. Adding an antioxidant to the solvent can be helpful to prevent oxidation [38]. Sodium metabisulfite has been used in concentrations of 0.5–2% in extraction solvents, *e.g.*, analyzing apple (*Malus sylvestris*) [39] and grape tissues [40]. Ascorbic acid can also be used; concentrations described in the literature range from 0.1 to 5% [41, 42]. Peng and Jay-Allemand [38] added different antioxidants to the extraction solvent. The effect was determined using the protein precipitating activity of the procyanidins. In the samples containing ascorbic acid or sodium metabisulfite the authors measured an increase of 30–75% of precipitating activity. Thus, by adding antioxidative agents to the extraction solvent the recovery of native plant phenolics can be enhanced, but total phenol determination methods based on redox reaction, *e.g.*, Folin-Ciocalteau, can no longer be used. It is also possible that *in vitro* studies using different cell lines can be influenced by the antioxidants.

The selection of the extraction solvent is not as easy as it seems. Anhydrous organic solvents release mainly monomers and low molecular mass compounds, whereas most of the oligomeric and polymeric PAC are partially insoluble in water [43, 44]. Therefore, aqueous organic solvents were used; initially aqueous acetone [45] and, recently, acidified acetone were employed [46, 47]. In 1986, Galletti and Self [48] especially recommended acidification for procyanidins that were bound strongly to polar fibrous matrices, as the increased polarity of the acidic solvent shifts the equilibrium of the PAC towards the solvent medium. However, the usage of acidified extraction solvents is a double-edged sword. Because of the acid sensitiveness of the interflavanoid bond, structural modifications of the compounds have to be assumed. However, the esterification with gallic acid is stable under acidic conditions [49]. Another possibility to release the PAC from cell wall constituents is the addition of urea to the extraction solvent [50].

Nonetheless, procyanidins cannot be extracted exhaustively. The amount of non-extractable compounds depends strongly on the matrix, the extraction solvent and the DPm; for instance, the 97% of total PAC of the outer bark of European conifers consists of non-extractable PAC [28]. Furthermore, prodelphinidins are much less extractable than procyanidins.

Due to the high amount of protein, PAC analysis in body fluids is a difficult task. For purification, Shoji and coworkers [51] added 8 M urea to plasma to solubilize the protein-procyanidin complex. The procyanidins were then extracted using C_{18} Sep-Pak cartridges. Before applying the plasma, the cartridges were preconditioned with 5% formic acid in methanol; afterwards the procyanidins were eluted with 1 N HCl in methanol. Low molecular mass procyanidins can be extracted directly with acid methanol [52, 53] or used directly for analysis [54]. For subsequent HPLC analysis an

additional purification step using polyamide, Sephadex LH-20 or C_{18} cartridges is recommended in the literature [19, 51].

In conclusion, the extraction is the most critical step in analyzing PAC. In addition, preservation procedures, storage conditions of the samples and the conditions during homogenization and extraction have an impact on the amount and the composition of the extractable PAC. If possible, the addition of an antioxidant, either metabisulfite or ascorbic acid, is advisable.

4 Analysis of procyanidins

Various methods are used for procyanidin analysis. Most techniques are non-selective and analyze all phenolics simultaneously. In general, the methods can be divided into three main categories: (i) methods based on biological effects; (ii) conventional methods; and (iii) chromatographic methods.

4.1 Methods based on biological effects: Protein precipitation

The ability of PAC to form protein precipitates is applied for their detection and characterization from different plant extracts [38, 55 – 57]. Such precipitation reactions depend on the concentration and structure of the plant phenols as well as proteins involved; for instance, higher affinity was found for conformationally loose proteins than for tightly coiled globular ones. Proteins are most efficiently precipitated by PAC at pH values near their p*I*. Based on their strong hydrogen bond acceptor ability, proline-rich proteins exhibit high affinities for tannin [58]. Therefore, bovine serum albumin (BSA) and the collagen-containing dermal powder are the proteins used most often for precipitation assays. The Pharmacopoea Europaea [59] combines the classical dermal powder method with spectral photometry using pyrogallol as a single reference for tannin quantification. However, this method does not account for differences in basic structure and reduction capacity within the tannin class. Moreover, this method is not suitable for tannin analysis of colored solutions, *e.g.*, red wine or grape juice. For the parallel determination of tannins and polymeric pigments (tannins containing a covalent bound anthocyanin moiety) in grape extracts and wines, Harbertson *et al.* [60] developed a combined method using protein precipitation and bisulfite bleaching.

Due the absorption maximum of tannins at about 280 nm, spectral quantitation before and after protein precipitation seems to be a promising option. However, interference caused by excess precipitation of proteins limits the practical application. A new precipitation assay for the quantitation of tannins in red wine uses methylcellulose, which was found to precipitate tannins selectively [61]. Measurement of the absorbance at 280 nm before and after tannin precipitation allows a selective determination of the tannins. The assay is claimed to be both robust and specific for PAC, as it does not suffer from interference of other phenolics absorbing at 280 nm [62].

4.2 Conventional methods

In general, conventional methods are cheap and fast, as in the majority of cases no extensive clean-up procedure is necessary. However, they lack specificity for individual compounds and show poor reproducibility between different kinds of samples (and laboratories). This phenomenon does not depend on the procyanidins but on the matrices that influence the yield on colored reaction products [42, 63]. An overview of different unspecific conventional methods described in the literature is given in Table 2. They are not discussed in detail because of the mentioned limitations.

Specific conventional methods use particular structure properties of procyanidins. The most common assays are: (i) acid butanol assay; (ii) dimethylaminocinnamaldehyde (DMACA) assay; and (iii) vanillin assay.

4.2.1 Acid butanol assay

The acid butanol assay uses the characteristic reaction that gave the PAC their name. In diluted mineral acids PAC are cleaved into the carbocation unit (extension units) and the flavan-3-ol unit (terminal units). The carbocations are rapidly converted into anthocyanidins by autoxidation. The formation pathway according to Porter *et al.* [63] is shown in Fig. 3. Side reactions, which lead to the formation of not well-characterized polymeric material with λ_{max} = 450 nm, commonly known as phlobaphenes, are reported to be the reason for relative low yields [63]. The problem with this assay is that the procyanidins with a higher DPm produce more anthocyanidin than dimers (theoretical yield = max. 50%) because of the existence of more extension units. Absorbance information of procyanidins after conversion with the acid butanol assay is given in Table 3.

Another problem is that the PAC structure influences the yield and the kinetics of the reaction. For example, Bate-Smith [44] observed that prodelphinidins gave a higher yield than procyanidins. In addition, the 4β-8 linkage between the monomer units results in a higher cyanidin recovery than the 4β-6 linkage, which may be due to different reaction kinetics. To increase the reproducibility and the yield of cyanidins, Porter *et al.* [63] recommended adding ferric ions to accelerate autoxidation. However, the observed increase in yield was shown not to be constant and led to higher but more variable contents [64, 65]. Correspondingly, results of quantification strongly depend upon the standard used for preparation of the calibration plot. If cyanidin is used as standard, values will be underestimated, as only a particular percentage of cyanidin is produced

Table 2. Some unspecific conventional methods to analyze PAC

Method	Principle	Detection
Folin-Denise	Reduction of complex polymeric ions formed from phosphomolybdic and phosphotungstic heteropolyacids to complex molybdenum-tungsten blue	725 – 770 nm
Folin-Ciocalteu	See Folin Denis (addition of HCl and Li_2SO_4)	725 – 770 nm
Prussian blue method	Reduction of Fe(III) to Fe(II) followed by formation of the Prussian blue complex $[Fe^{(III)}Fe^{(II)}(CN)_6]$	720 nm
Reaction with molybdate	Oxidation and complexing with molybdate	420 nm
Formaldehyde assay	Precipitation of proanthocyanidins with formaldehyde in acidic solution *via* the formation of a methylene bridge; phloroglucinol improves precipitation	Gravimetric
Ytterbium	Precipitation of phenolics with ytterbium acetate	Gravimetric

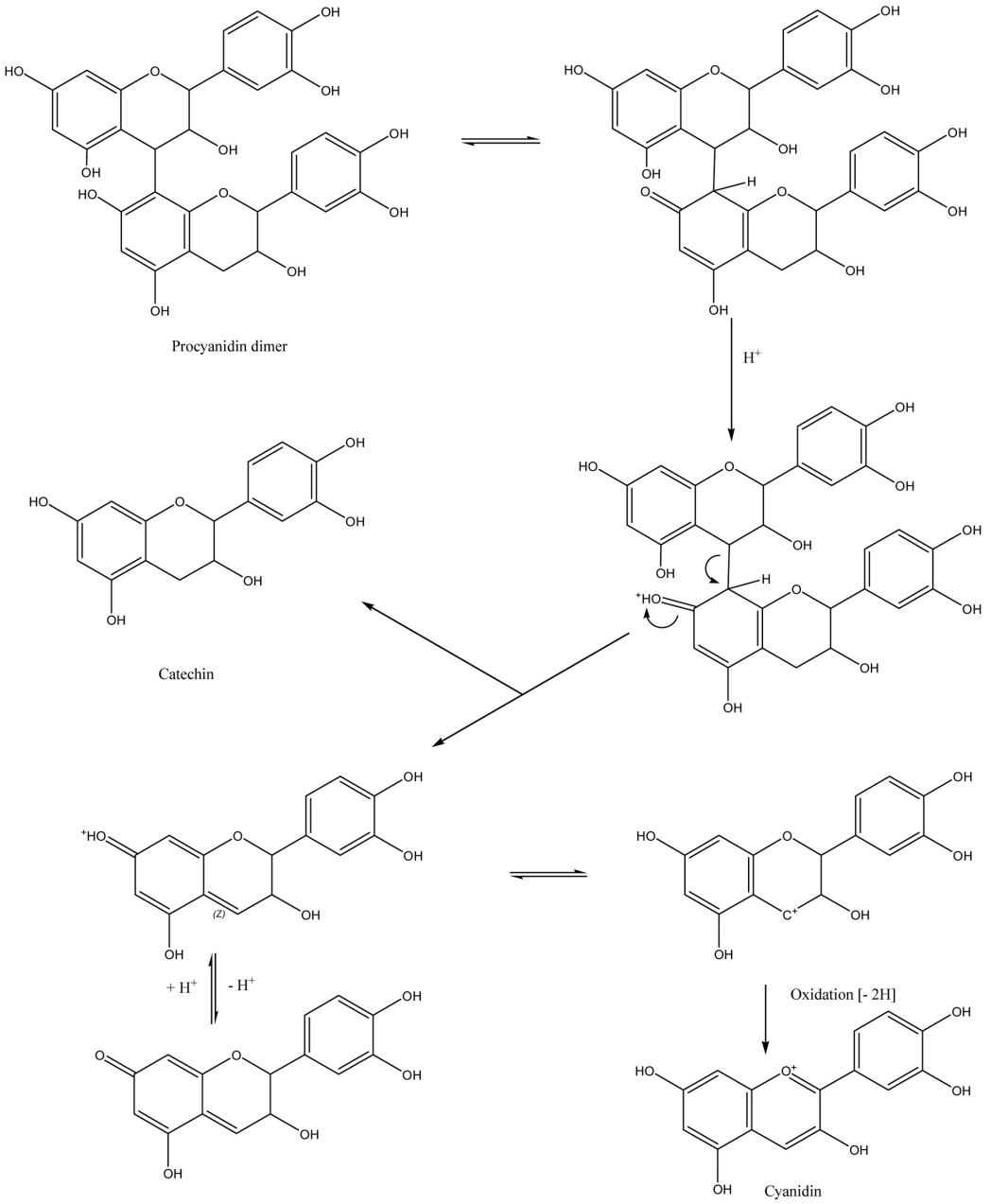


Figure 3. Formation pathway of procyanidin dimer to red-colored cyanidin and catechin.

Table 3. Absorbance data of cyanidin and procyanidins using the acid butanol assay

Compound	$A^{1\%}$	Solvent	Reference
Cyanidin chloride	1027	BuOH/ HCl	[97]
Cyanidin	1053	BuOH/ HCl	[97]
Procyanidin B2	90–106	BuOH/ 5% HCl	[140]
Procyanidin B1, B3, B4 mixture	136	BuOH/5% HCl	[140]
A-type procyanidins	600	BuOH/ 5% HCl	[44]
Trimer	170	BuOH/ 5% HCl	[140]
Tetramers	140–180	BuOH/5% HCl	[140]
Higher oligomers	200	BuOH/ 5% HCl	[140]
Polymeric procyanidins	243	BuOH/ 5% HCl	[141]
Procyanidin B1	270	BuOH/ 5% HCl ferric ions added	[63]
Procyanidin B3	280	BuOH/ 5% HCl ferric ions added	[63]
Procyanidin B7	225	BuOH/ 5% HCl ferric ions added	[63]
Trimer	345	BuOH/ 5% HCl ferric ions added	[63]
Polymeric procyanidins	450–490	BuOH/ 5% HCl ferric ions added	[63]

$A^{1\%}$, specific absorbance (coefficient of absorbance for a 1% solution; BuOH = 1-Butanol)

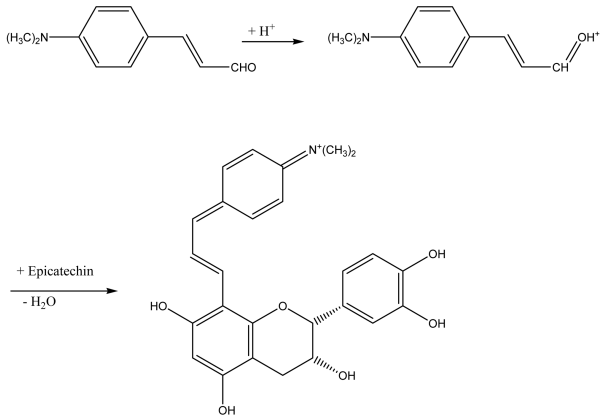


Figure 4. Condensation of epicatechin with 4-dimethylaminocinnamaldehyde.

(Table 3). As well defined procyanidins are not commercially available, the isolation from the matrix in the course of analysis is the most recommendable method to obtain reliable results.

4.2.2 DMACA assay

The fundamental principle of the DMACA assay is the condensation of the nucleophilic site of the phloroglucinol structure (A-ring) with aldehydes, forming a colored product detectable at 640 nm. In strong acidic solution, aldehydes are protonated at their carbonyl oxygen, forming electrophilic carbocations. As a result of the delocalization of the positive charge in substituted benzaldehydes, the electrophilicity is reduced. Therefore, reactions only occur with phenolic compounds that show a phloroglucinol or resorcinol type oxygenation pattern [43, 66, 67]. Electron-withdrawing substituents considerable reduce the reactivity [67].

The reaction scheme (Fig. 4) is similar to that of the vanillin assay. DMACA prefers the C8 of the A-ring and reacts only with the terminal units [68]. Phloroglucinol, resorcinol and other compounds with a 5,7-dihydroxylation pattern of the aromatic A-ring form condensation products, similar to the vanillin assay dihydrochalcones. The affinity for (−)-epicatechin is a few fold higher [69]. The advantage compared to the vanillin assay is reported to be the five fold higher sensitivity and the lower interferences with anthocyanins. The assay is also easier to perform, as the reaction is carried out at room temperature [42, 69]. However, the resulting dye is not regarded to be stable [70]. Nonetheless, this assay is well established and has received higher attention for post column derivatization in HPLC analysis [71, 72].

4.2.3 Vanillin assay

The vanillin assay was used predominantly in the last two decades of the 20th century, but is nowadays mostly replaced by the acid butanol assay and the thiolytic degradation. The vanillin assay is similar to the DMACA assay. In strong acidic solution the protonated vanillin carbocation reacts as an electrophile with the flavanol to form a colored compound, which is measured at 500 nm (Fig. 5). Due to their UV absorption, anthocyanidins may substantially interfere, and a correction must be made by subtracting a suitable blank assay [73]. Chlorophyll may also affect the color development [74]. As with the acid butanol assay, differences in molar absorption coefficients also occur in the vanillin assay. For example, catechin gives a higher molar extension coefficient (ε-value) than its epimer [75]. In addition, absorbance values are generally higher for procyanidins than for monomers (Table 4), as the reaction does not take place at all flavan-3-ol extension units or only at the terminal units [76]. For higher sensitivity Butler *et al.* [77] recommended the usage of 2,4-dimethoxybenzaldehyde and a mixture of HCl and glacial acetic acid as suitable solvent.

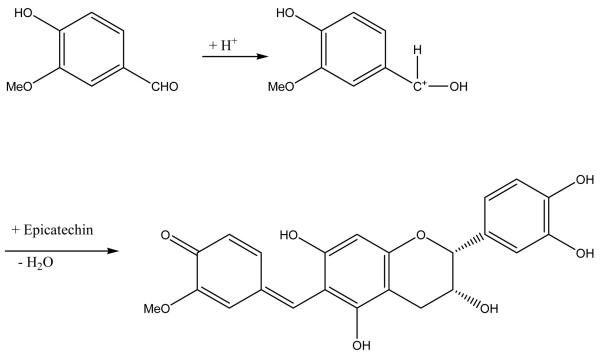


Figure 5. Condensation of epicatechin with vanillin.

In conclusion, this assay is not really suitable for anthocyanin containing samples. Nonetheless, it is can be used to quicky specify the flavan-3-ol content of a sample. In general, the vanillin reaction depends on timing and temperature. Therefore, performing a vanillin assay is more difficult than an acid butanol assay [78]. The usage of (+)-catechin instead of purified PAC as a standard for quantification may lead to a considerable overestimation.

4.3 Chromatographic methods: Separation

The separation and determination of low molecular mass flavan-3-ols can easily performed using RP-HPLC; however, a large proportion of phenolic compounds in fruit and vegetables consists of highly condensed polymeric procyanidins [79]. In general, polymeric procyanidins are very polar compounds, as the hydrophobicity decreases with increasing DPm. Separation and determination of these substances is very difficult because of the exponentially increasing variety of isomers and oligomers exhibiting different DPm. Therefore, the kind of chromatography selected depends on the procyanidins expected to be present in the matrix to be analyzed. In general, all classical chromatographic methods have been applied for procyanidin analysis.

4.3.1 Gas chromatography

GC is not really the method of choice for analyzing procyanidins. To improve volatility, the use of derivatization is inevitable, and trimethylsilyl derivatives have been described previously [80]. Recently, a mixture of trimethylchlorsilane and bis-(trimethylsilyl)-trifluoroacetamide at a ratio 1:3 as derivative agent has also been reported [81]. Pyrolysis GC-MS methods have been reported as an alternative method, in which the procyanidin mixture is pyrolyzed at 500°C. The main pyrolysis products of catechin and epicatechin are catechol and 4-methylcatechol, respectively, which are generated from the B-ring. This method was claimed to be suitable for qualitative and quantitative procyanidin analysis in tannin-rich samples [82]. In general, GC requires the use of an apolar phase, *e.g.*, DB-1.

4.3.2 Size exclusion chromatography

Size exclusion chromatography (SEC) is typically used to separate procyanidins in a preparative way, although this method has also been used to determine the molecular mass of procyanidins. For this, the system was calibrated with polystyrene standard or procyanidin oligomer acetate [8, 83]; however, since the advent of high performance mass spectrometers, this method is not longer 'up-to-date'. As procyanidins are too polar to be separated on a typical gel permeation chromatography (GPC) column, the separation of procyanidins is usually performed on methyl ether or acetyl derivatives using an organic or aqueous mobile phase. In general, chromatography on Sephadex LH-20 or Toyopearl HW-40, using elution with aqueous alcohol or aqueous acetone (sometimes with increasing amounts of organic solvents), has been commonly applied to the separation of procyanidins from plant extracts [19, 84 – 87]. Addition of urea has been suggested to give a better separation of polymers, but the separation of procyanidins of high

Table 4. Absorbance data of procyanidins using the vanillin assay according to [77]

Compound	$A^{1\%}$	Solvent	wavelength (nm)
Epicatechin	11.8	MeOH/ 4% HCl/ 0.5% Vanillin	500
Catechin	8.2	MeOH/ 4% HCl/ 0.5% Vanillin	500
Procyanidin B2	34.9	MeOH/ 4% HCl/ 0.5% Vanillin	500
Procyanidin B3	25.2	MeOH/ 4% HCl/ 0.5% Vanillin	500
Procyanidin A2	2.8	MeOH/ 4% HCl/ 0.5% Vanillin	500
Polymers from sorghum grain	22.8	MeOH/ 4% HCl/ 0.5% Vanillin	500
epicatechin	271	CH3COOH/ 4% HCl/ 0.5% Vanillin	510
Catechin	243	CH3COOH/ 4% HCl/ 0.5% Vanillin	510
Procyanidin B2	155	CH3COOH/ 4% HCl/ 0.5% Vanillin	510
Procyanidin B3	170	CH3COOH/ 4% HCl/ 0.5% Vanillin	510
Procyanidin A2	134	CH3COOH/ 4% HCl/ 0.5% Vanillin	510
Polymers from sorghum grain	56	CH3COOH/ 4% HCl/ 0.5% Vanillin	510

$A^{1\%}$, specific absorbance (coefficient of absorbance for a 1% (w/v) solution

Table 5. NP-HPLC methods described in literature for procyanidin analysis

Stationary phase	Mobile phase	Matrix	Reference
Silica	Dichloromethane-methanol-water-formic acid	Cocoa beans/grape seed	[90]
Silica	Dichloromethane-methanol-water-acetic acid	Tamarind seed (*Tamarindus indica*)	[47]
Silica	Dichloromethane-methanol-water-acetic acid	Birch leaves (*Betula spp.*)	[91]
Silica	Hexane-acetone	Apple (*Malus pumila*)	[92]
Silica	Hexane-methanol-ethyl acetate	Apple (*Malus pumila*)	[92]
Diol	Acetonitrile-methanol-water acetic acid	Cocoa (*Theobroma cacao L.*)	[93]

DPm value usually remains incompletely [88]. Therefore, SEC can be used to pre-separate procyanidins on a preparative scale, but is not suitable as a quantitative analysis method.

4.3.3 Normal-phase-HPLC

Many years ago, Lea [89], for the first time, successfully separated procyanidins according to their DPm on SiO_2 thin layer plates. Procyanidin dimers up to heptamers isolated from apple were separated using high-performance TLC (HPTLC) with toluene-acetone-formic acid (30:30:10) as the mobile phase. On the basis of this HPTLC method, Rigaud *et al.* [90] developed a normal-phase (NP)-HPLC gradient of dichloromethane and methanol using a constant amount of aqueous formic acid (1:1; v/v). Their development is still important in current NP-HPLC. Sudjaroen *et al.* [47] were able to separate procyanidins up to undecamers from tamarind and Karonen *et al.* [91] successfully separated procyanidins from birch leaf up to a DPm of ten.

Methods containing hexane can also be applied. Shoji *et al*. [92] presented a method using hexane-acetone as the mobile phase, resulting in the separation of procyanidins up to a DPm of five. However, these authors observed a poor resolution of the peaks. Therefore, an optimized method with hexane-methanol-ethyl acetate as solvent was developed, leading to the separation of procyanidins up to octamers with a good resolution. Recently, also an apparently powerful method was published in which separation of procyanidins with a DPm up to 14 on a diol stationary phase was achieved [93]. An overview of different NP methods described in the literature is given in Table 5. In contrast to SEC, in NP-HPLC the procyanidins elute in the order of increasing molecular mass (increasing polarity). However, care must be taken as the highly polymerized procyanidins are often not completely eluted from a silica-based NP column. The cause is the strong adsorption to the silica stationary phase and the relatively low solubility in the organic mobile phase. Acid degradation can also take place because of the high concentrations of organic acids used for most separation methods, which is a tremendous disadvantage for preparative operations. A solution for this could be the application of the newly described method using a diol stationary phase [93]. The procyanidins did not adsorb irreversibly to this phase and the acid ratio was lower at a higher separation capacity.

4.3.4 RP-HPLC

RP-HPLC is the most common technique for separating plant phenols. PAC can also be separated on C18 columns but resolution is limited up to tetrameric structures. The reasons why higher PAC cannot be resolved are manifold. Basically, the concentration of individual compounds decreases with increasing DPm and a higher number of possible isomers. In addition, the peak capacity of the stationary phase is reduced in complex samples. The result is an unresolved hump or a drift of the baseline, which is typical for high polymeric PAC [94]. Low molecular mass PAC, which can be subjected to chromatography as individual peaks on C18 material, are eluted – in contrast to NP chromatography – not according to their degree of polymerization. The main factor influencing the retention time is the stereochemistry and the overall polarity [95]. For example, epicatechin and its oligomers were more strongly retarded than the monomer and procyanidins consisting of catechin [95, 96], and procyanidins with $4 \rightarrow 6$ linkage as well as doubly linked procyanidins were eluted later than $4 \rightarrow 8$-linked ones [34]. In general, the elution order of the dimeric and the trimeric procyanidins C1 and C2 remained nearly constant and was more or less unchanged by the type of eluent, acid and stationary phase, whereas the retention times of procyanidin B1 and B4 were slightly affected by changes of the chromatographic conditions [97].

The chromatographic behavior of procyanidins is also affected by the concentration of the injected sample solution. With increasing amount of procyanidins, their peaks shifted towards lower retention times. Putman and Butler [98] referred to on-column reactions, which were thought to be caused by trace metal impurities. Therefore, the authors recommended the addition of a chelator.

In general, gradients are applied starting at a high percentage of aqueous phase using methanol, ACN and acetic acid or formic acid as organic constituent of the eluent [8, 34, 50, 76, 91, 94, 99, 100]. 3,4,5-Trimethoxycinnamic acid can be used as an internal standard, as this substance elutes very late and does not interfere with the polymeric procyanidins [96, 101].

4.3.5 Derivatization

Derivatization methods can be distinguished into pre- and post-column techniques. Post-column derivatization is not often reported as it requires specific equipment. In the past,

dimethylaminocinnamaldehyde has mostly been used for the analysis of different fruits or beverages [70, 102, 103].

Nowadays, pre-column derivatization is more usual. Two methods have been established. The first involves the degradation of procyanidins in the presence of benzyl mercaptan, and was introduced by Rigaud *et al.* [104] and enhanced by Guyot *et al.* [105]. Koupai-Abyazani *et al.* [95] developed a similar method using the acid degradation of procyanidins in the presence of the nucleophilic agent phloroglucinol and subsequent RP-HPLC separation. More recently, Karonen *et al.* [84] reported a limitation of the thiolysis method as some polymeric PAC are resistant to degradation with thiols (notably when PAC originate from aged tissues, *e.g.*, outer bark). Therefore, the method using a solution of 0.1 M HCl in methanol containing 50 mg/mL phloroglucinol and 10 mg/mL ascorbic acid seems to be more suitable [84]. During this reaction, the extension units of the PAC are transformed into benzylthiol or phloroglucinol adducts, respectively (Fig. 6). The terminal units are liberated as flavan-3-ols. The DPm of the procyanidins in the sample can be calculated by estimating the proportion of adducts to free flavan-3-ols. However, it is essential to consider the kind of PAC present. Galloylation of a procyanidin may decrease the cleavage of the interflavanoid linkage under mild conditions (5 min, 30°C, 0.1 M methanolic HCl). Koehler *et al.* [106] observed a degradation of 60% for 4 → 8 connected procyanidin dimer when the terminal unit was galloylated and 25% when the upper unit was galloylated. A 4 → 6 linkage with galloylation of the upper unit reduced the cleavage up to 5%. For complete cleavage the authors recommend a temperature of about 50°C. The cleavage of the ester bondage of procyanidin-3-*O*-gallates has not been observed under the strong acidic conditions during thiolysis [49]. Therefore, incubation of the ester with tannase in 0.1 M acetate buffer at pH 5.0 was recommended [107].

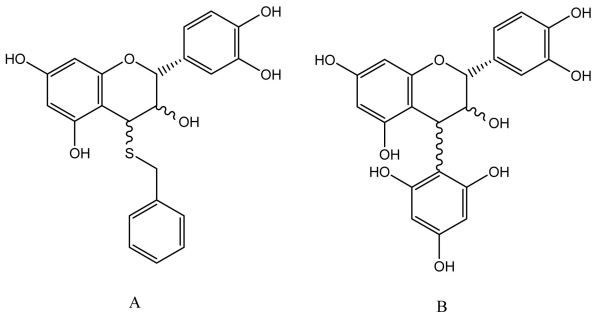


Figure 6. Structures of thiolysis (A) and phloroglucinol (B) degradation adducts of procyanidins.

Table 6. Absorbance data of procyanidins and their monomers at 280 nm

Compound	ε (l/mol cm)	Solvent	Reference
(+)-catechin	3300	MeOH	[68]
(−)-epicatechin	3780	MeOH	[97]
Procyanidin B2	7100	MeOH	[68]
Procyanidin B3	6700	MeOH	[68]
Procyanidin B4	8170	MeOH	[97]
Procyanidin B5	7810	MeOH	[97]
Procyanidin C1	11800	MeOH	[97]
	10600	MeOH	[68]
Procyanidin C2	9800	MeOH	[68]
Procyanidin A2	5200	MeOH	[69]

ε, molar coefficient of absorbance

5 Detection

Different methods exist to detect procyanidins after HPLC separation. The most common are UV-DAD, fluorescence and MS detection.

5.1 UV and DAD

The UV spectra of PAC exhibit two maxima, the first at about 200 – 220 nm and the second at 278 nm [108]. A third band occurs between these two maxima if aromatic substituents are conjugated to the PAC, *i.e.*, gallic acid [97]. The effect of substituents on the UV spectrum depends more on their position and interaction than on their chemical nature. The ortho and meta positions give bathochromic shifts of the UV maxima at longer wavelengths [109]. In addition, a symmetrical UV spectrum, relating to the maximum at 280 nm, is characteristic for a homogeneous procyanidin polymer [110], whereas symmetry is disturbed in mixed procyanidin-prodelphinidin polymers. Prodelphinidin polymers also show lower specific absorption coefficients than procyanidin polymers [97]. Since most other plant phenolics exhibit different characteristic absorption maxima, procyanidins can be easily identified using UV-DAD. Higher sensitivity in quantification of procyanidins can be reached at 210 nm, because of the higher molar absorption coefficients of procyanidins in comparison to 280 nm. UV detection of individual procyanidins after chromatographic separation implies that molar absorbance depends on the structure. In the series catechin, procyanidin dimer and procyanidin trimer the molar absorbance increases (Table 6). The molar absorbance of the twofold-linked procyanidin A2 is much higher than that of the monomers [69]. Nonetheless, a disadvantage of UV-DAD in the analysis of procyanidins is that the spectra of monomers, procyanidin oligomers and even of some of their degradation products do not exhibit any obvious differences, and even the diastereomers (2*R*, 3*R*)-epicatechin and (2*R*, 3*S*)-catechin cannot be distinguished properly [68]. As commercially available procyanidins are rather scarce, the fact that molar absorbance of procyanidin oligomers is roughly the sum of the absorbance of the monomer units could be used to estimate, as a first

Table 7. *m/z* signals of procyanidins recorded by ESI-MS in the negative mode [119]

Degree of polymerization	*m/z*		
	$[M-H]^-$	$[M-2H]^{2-}$	$[M-3H]^{3-}$
1	289.0		
2	577.0		
3	865.2	432	
4	1153.8	576	
5	1441.0	720.2	
6	1729.5	864.9	576
7	2017.5	1008.3	672
8	2305.5	1153.1	768
9	2593	1297.0	864
10	2881	1441.0	960.3

approximation, the content of low molecular mass procyanidins.

5.2 Fluorescence

RP-HPLC with UV detection at 280 nm is helpful for analyzing and quantifing PAC in food samples (*cf.* UV and DAD). The limitation of this method is its ineffective separation of higher oligomers. Using NP-HPLC separations (especially employing dichloromethane), the detection at 280 nm is insufficient. Fluorescence detection has been shown to be more suitable, as it is able to increase the selectivity for procyanidins and to reduce the signal from other absorbing compounds. For example, to detect procyanidins and prodelphinidins a fluorescence detector was set at an excitation wavelength of 276 nm and an emission wavelength of 316 nm [100, 111–113]. The detection limit of procyanidins using 280 nm is in the range of 9.0–11.0 ng (on-column) in comparison to 20–23 ng (on-column) reported by Rohr *et al.* [114] and Donovan *et al.* [115] considering catechin and epicatechin, respectively. According to the information provided by Gu *et al*. [100], the detection limits using fluorescence were in a range of 23–64 pg (on-column), *i.e*., nearly one thousand times lower than that observed by UV detection. A much higher fluorescence detection limit for catechin (3 ng/on-column) was described by Donovan *et al.* [115] using different excitation and emission wavelengths at 280 and 310 nm. Adamson *et al.* [111] reported a limit of detection for procyanidins between 0.01 mg/ml and 0.03 mg/ml for monomers to decamer flavan-3-ols in cocoa, respectively.

5.3 LC-MS and LC-MS/MS

In LC-MS ions are generated from the eluting molecules of an HPLC device. They are subsequently separated according their *m/z* ratio. Different interfaces for generating the ions are currently available, *e.g*., thermospray (TSP), atmospheric pressure chemical ionization (APCI), or ESI [116, 117]. ESI is a gentle and sensitive and the most used method to date [118]. Analyzing procyanidins in grape extracts Wu *et al.* [117] determined a limit of quantification of about 40 ng/ml. For higher sensitivity, additional experiments using the mass spectrometer can be performed that lead to specific reaction products, *i.e*., using a triple quadrupol (MS/MS) or an ion trap for MS^2 or MS^n experiments, respectively. Table 7 gives an overview of specific ions detected in procyanidin analysis. Of course, misinterpretations have to be avoided. Cheynier *et al.* [119] detected double and triple charged ions that could be interpreted as a lower degree of polymerization. In addition, a general problem arises from the decreasing ionization efficiency observed with increasing degree of polymerization [120, 121].

Friedrich *et al.* [122] described a technique for elucidating the structures of unknown oligomeric procyanidins (Table 8). Multiple MS experiments using an LCQ IT was used and a fragmentation pathway was proposed. Here $[M_B\text{-}H]^-$, which represents the base unit with only one interflavanoid linkage at C8 or C6, and $[M_T\text{-}3H]^-$ ions, which represent the top unit with only one C-C-bond at C4, were derived by cleavage of the interflavanic bond. The retro Diels-Alder fission in the T-unit was found to be the most important fragmentation and resulted in the $[M\text{-}C_8H_8O_4\text{-}H]^-$ ion [122, 123].

5.4 MALDI-TOF-MS

The application of the MALDI-TOF-MS for analysis of oligomeric procyanidins has been introduced within the last 10 years. In the first publication, the range of polymerization of PAC in apples from dimer to pentadecamer was determined [124]. Most recently, Es-Safi *et al.* [125] detected polymers up to a DPm of 25. Yang and Chien [126] have investigated procyanidins from grapes, and recommend using MALDI-TOF not only for characterization but also for quantification. Dihydroxybenzoic acid (DHB) is the matrix best suited for procyanidin analysis in the reflectron mode. Compared with other commonly used matrix systems, such as *trans*-3-indolacrylic acid (IAA), *R*-cyano-4-hydroxycinnamic acid (CCA), sinapinic acid (SA), 9-nitroanthracene (9NA), 5-chlorosalicylic acid (5CSA), 2-(4-hydroxyphenylazo)benzoic acid (HABA) and dithranol, DHB provides the broadest mass range with the least background noise. Although IAA matrix provides a mass range similar to DHB, it tends to generate a very high background of noise in the mass range below 500, blocking out the signals from the monomers and the dimers [125, 126]. In various publications, cesium trifluoroacetate or sodium chloride was added to the matrix/sample solution to promote the formation of a single ion adduct $[M+ Cs/Na]^+$ [127–129]. Several authors have stressed the difficulty in preparing standards to develop response factors for each group of oligomers [124, 126].

Table 8. General scheme to elucidate the sequence of monomeric units in dimeric proanthocyanidins by negative ESI and trap CID [122]

$[M-H]^-$	$[M-C_8H_8O_4-H]^-$ (RDA-product	$[M-C_6H_6O_3-H]^-$	$[M_B-H]^-$	$[M_T-3H]^-$	Sequence of monomeric units
577	425	451	289	289	c-c
593	425	467	289	303	gc-c
593	441	467	305	287	c-gc
609	441	483	305	303	gc-gc

c, catechin; gc, gallocatechin, RDA, Retro Diels-Alder; M_B, molecular mass of the base unit ; M_T, molecular mass of the top unit

In conclusion, the HPLC/MS methods have wide-ranging utility for analysis of multiple types of PAC in a large variety of food and beverage matrices. Obviously, fluorescence detection is superior to UV detection in samples with complex matrices containing low concentrations of procyanidins. Due to the high sensitivity and selectivity of the fluorescence detector, these methods will become increasingly more importance for quantifying procyanidins in food samples. Among the MS methods, MALDI-TOF is a potent tool for qualitative analysis of the procyanidins present in the matrix without extensive sample preparation; however, quite high concentrations are necessary. ESI, as the most sensitive ionization method, is a useful technique for structure elucidation when used in combination with MS/MS. However, only low molecular mass procyanidins can be quantified due to the lack of ionization of highly condensed procyanidins.

Analytical methods that eliminate the need for sample preparation are in progress. The most recent development in MS, the nanostructured initiator mass spectrometry (NIMS) [130], which combines the molecular specificity of MALDI with the resolution of secondary ion MS (SIMS), is expected to have advantages in the analysis of small molecules and may open the way for future highly selective and sensitive analysis in biological matrices.

6 Structure elucidation

Nuclear magnetic resonance spectroscopy (NMR) analysis is a well-established technique for structure elucidation of molecules. To investigate the absolute configuration circular dichroism (CD) spectroscopy can be applied.

6.1 NMR

NMR is a powerful and theoretically complex analytical tool for structure elucidation. Basic principles are the nuclear spin and the splitting of energy levels in a magnetic field. All nuclei that contain odd numbers of protons or neutrons posses an intrinsic magnetic moment and the mechanical property of spin. The overall spin of the nucleus is determined by spin quantum number I, with the most commonly measured nuclei ^1H and ^{13}C having a spin of I = 1/2. Nuclei with such a spin can only adopt two discrete states according to the magnetic field, parallel and antiparallel, also referred to as up and down, respectively. Transitions between these states can be caused and registered *via* radiation or emission of electromagnetic waves. The magnetic field and the molecule structure determine the resonance frequency. Further structural data can be elucidated by observing spin-spin coupling, a process by which the precession frequency of a nucleus can be influenced by the magnetization transfer from nearby nuclei [131].

This technique is well established in organic chemistry. However, it is difficult to determine the structure of procyanidins by NMR, especially the position of the interflavanoid bond ($4\beta \rightarrow 8$ or $4\beta \rightarrow 6$). One reason is that the procyanidins show very broad ^1H signals at room temperature due to atropisomerism. This results from steric interactions in the vicinity of the interflavanoid bond, which allows the flavonoid units to rotate almost freely. Tarascou *et al.* [132] reported a ratio of up to 55:45 of two isomeric rotamers observed *via* ^1H-NMR spectroscopy depending on the structure of the procyanidin dimers. To handle the problem of atropisomerism, different approaches have been adopted, *e.g.*, derivatization to phenolic permethyl ethers or to 3-*O*-acetates [133] to limit the free rotation. Another possibility is the cleavage of the interflavanoid bond by thiolysis [104] or reaction with phloroglucinol [134] in combination with the comparison of ^{13}C-NMR chemical shifts of each unit. Evaluation of the position of the interflavanoid bond of underivatized procyanidins has been proposed previously by Nonaka *et al.* [135]. The authors used the chemical shift of the H2 of the terminal unit (lower unit) of a dimeric procyanidin with the 2,3-*trans* configuration. In case of a $4 \rightarrow 6$ bond the chemical shift of this proton amounted to 4.58 ppm, while for a $4 \rightarrow 8$ bond it was 4.91 – 4.97 ppm. A similar method was recommended by Vdovin *et al.* [136]. They used the chemical shift of H6 or H8 (E-ring) of an acetylated or methylated procyanidin obtained under conditions of rotation about the interflavanoid bond at temperatures of 100 – 170°C. The H6 (E-ring) exhibits a chemical shift of 6.06 – 6.16 ppm in the $4 \rightarrow 8$-linked dimers, whereas the H8 (E-ring) shows a 6.20 – 6.38 ppm shift in the $4\beta \rightarrow 6$-linked dimers. In contrast, Shoij *et al.* [137]

Table 9. Chemical shifts of procyanidin B1-B4 and A4 [132, 137, 142]

		Procyanidin B1		Procyanidin B2		Procyanidin B3		Procyanidin B4		Procyanidin A4	
	Solvent and temperature	Methanol-d_4 (−40°C)		Methanol-d_4 (−40°C)		H_2O/D_2O (22°C)		$H_2O/[D_6]$-ethanol (22°C)		Acetone-d_6 Room temperature	
Ring	Position	Chemical shift	J [Hz]	Chemical shift	J	Chemical shift	J	Chemical shift	J	Chemical shift	J
C	2	5.1 br s		5.06 br s		4.39 d	10	4.31 d	9.7		
	3	3.92 br s		3.79 br s		4.29 m		4.13 dd		4.10 d	4
	4	4.65 br s		4.61 br s		4.36 d	10.5	4.28 d	8.5	4.27 d	4
A	6	5.92 br s		5.92 d	2	6.00 s		5.89 s		6,13 d	2
	8	5.94 br s		5.94 d	2	5.71 s		5.82 s		6,08 d	2
B	2′	6.85 br s		6.83 d	2	6.80 d	<1	6.57 d	1.6	a)	
	5′	6.68 d	8	6.68 d	8	6.83 d	8.2	6.63 d	8.2	a)	
	6′	6.67 d	8	6.60 dd	8, 2	6.61 dd		6.34 dd		a)	
F	2	4.96 d	5	4.95 br s		4.58 d	7.9	4.7 d	<1	b)	
	3	4.16 m		4.24 br t		3.92 m		4.00 m		4.67 d	8
	4	2.53 d	17	2.79 d	17	2.85 dd	15.8,	2.78	17.2,	4,00 m	
		2.58 dd	17, 4	2.94 dd	17 , 4	2.49 d	5.3	2.55	4.7		
							8.5		<1		
D	6	5.82 s		5.88 s		6.15 s		6.02 s		6,04 s	
	8										
E	2′	6.82 br s		7.10 d	2	6.61 d	<1	6.55 d	1.8	a)	
	5′	6.69 d	8	6.72 d	8	6.87 d	8.2	6.68 d	8.2	a)	
	6′	6.88 d	8	6.85 dd	8, 2	6.46 dd		6.29 dd		a)	

br, broad; d, doublet; dd, doublet of doublet; m, multiplet; s, singlet.
a) not given in literature [142].
b) overlapped by solvent.

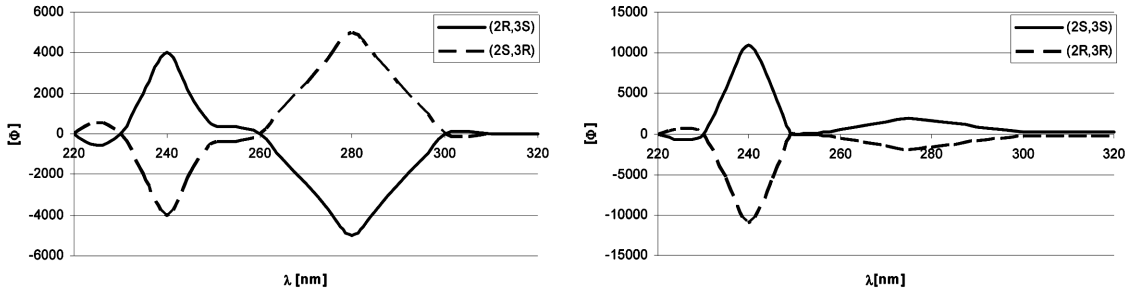


Figure 7. CD-spectra of *cis*-[(2*S*, 3*S*); (2*R*, 3*R*)] and *trans*-[(2*R*, 3*S*); (2*S*, 3*R*)] flavan-3-ols [139].

showed that low temperature ^{1}H-NMR resulted in sharper signals than the spectra recorded at ambient temperature. In addition, this method was found to be more sensitive to procyanidins. An overview of the chemical shifts of different procyanidin dimers is given in Table 9.

6.2 CD spectroscopy

CD is a powerful tool for identifying the absolute configuration of optically active molecules. The basic principle is that the plane of polarized light rotates at an angle α when absorbed by an optically active molecule. Moreover, the optically active matter differentially absorbs the left and the right hand circular polarized light. The resulting light is therefore elliptically polarized and the medium exhibits CD. The absolute configuration and conformation of a molecule can be determined with the obtained CD spectra in combination with NMR spectroscopy.

Any optically active compound exhibits an optical rotatory dispersion curve, which gives the optical activity (Φ) in the function of wavelength (λ in nm). If the compound under investigation has no chromophores that absorb in the wavelength range being used, its curve is plain or normal without any extrema. Anomalous curves exhibit a maximum, a minimum, or both, and are observed when the molecule possesses an absorption band in the region being investigated (Fig. 7). These anomalous dispersion effects are known as Cotton effects. For detailed information see [138].

Flavan-3-ols exhibit a chroman chromophore. This chroman derivate belongs to the benzene chromophores with a chiral *O*-heterocyclic ring (second sphere) and the substituent (C-ring, third sphere) [138], resulting in the observed Cotton effects at 200 – 240 nm (^1L_a) and 260 – 280 nm (^1L_b). Van Rensburg *et al.* [139] found that the (2*R*, 3*S*)- and (2*S*, 3*R*)-*trans* enantiomers show Cotton effects of opposite sign and the (2*R*, 3*R*)- and (2*S*, 3*S*)-*cis* enantiomers exhibit Cotton effects of the same sign for the ^1L_a transition compared to the ^1L_b transition (see Fig. 7). In conclusion, positive and negative Cotton effects at the ^1L_a transition seem to indicate 3*S* and 3*R* absolute configuration, respectively. Because of the increasing complexity of procyanidins with growing chain length, determining the absolute configuration of each C-atom still poses a challenge.

The authors have declared no conflict of interest.

7 References

[1] Anderson, O., Markham, K. R. (Eds.), *Flavonoids: Chemistry, Biochemistry and Applications,* CRC Press, Boca Raton 2006.

[2] Porter, L. J., Ma, Z., Chan, B. G., Cocoa procyanidins: Major flavanoids and identification of some minor metabolites. *Phytochemistry* 1991, *30*, 1657 – 1663.

[3] Haslam, E., *Polyphenols-vegetable tannins*, in: Phillipson, J. D., Ayres D. C., Baxter J. (Eds.), *Plant Polyphenols: Vegetable Tannins Revisited*, 1st edn., Cambridge University Press, Cambridge 1989, pp. 1 – 89.

[4] Mateus, N., Silva, A. M. S., Santos-Buelga, C., Rivas-Gonzalo, J. C., de Freitas, V., Identification of anthocyanin-flavanol pigments in red wines by NMR and mass spectrometry. *J. Agric. Food Chem*. 2002, *50*, 2110 – 2116.

[5] Weinges, K., Göritz, K., Nader, F., Zur Kenntnis der Proanthocyanidine. XI. Konfigurationsbestimmung von $C_{30}H_{26}O_{12}$-Procyanidinen und Strukturaufklärung eines neuen Procyanidins. *Liebigs Ann. Chem*. 1968, *175*, 164 – 171.

[6] Thompson, R. S., Jacques, D., Haslam, E., Tanner, R. J. N., Plant proanthocyanidins. I. Introduction; the isolation, structure, and distribution in nature of plant procyanidins. *Chem. Soc. Perkin Trans.* 1972, *1*, 1387 – 1399.

[7] Hemingway, R. W., Foo, L. Y., Porter, L. J., Linkage isomerism in trimeric and polymeric 2,3-*cis*-procyanidins. *J. Chem. Soc. Perkin Trans.* 1982, *1*, 1209 – 1216.

[8] Williams, V. M., Porter, L. J., Hemingway, R. W., Molecular weight profiles of proanthocyanidin polymers. *Phytochemistry* 1983, *22*, 569 – 572.

[9] Monagas, M., Hernández-Ledesma, B., Garrido, I., Martín-Álvarez, P. J. *et al.*, Quality assessment of commercial dietary antioxidant products from *Vitis vinifera L.* grape seeds. *Nutr. Cancer* 2005, *53*, 244 – 254.

[10] Lea, A. G. H., Arnold, G. M., The phenolics of ciders: Bitterness and astringency. *J. Sci. Food Agric.* 1978, *29*, 478 – 483.

[11] Nie, L., Sun, J., Relationship between content of phenolic compounds and taste of astringency and bitterness in apple fruit. *Yuanyi Xuebao* 2005, *32*, 778 – 782.

[12] Clapperton, J., Hammerstone, J. F. Jr., Romanczyk, L. J. Jr., Yow, S., *et al.*, Polyphenols and cocoa flavor. *Bull. Liaison – Groupe Polyphenols* 1992, *16*, 112 – 116.

[13] Lea, A. G. H., Tannin and color in English cider apples. *Flüssiges Obst* 1984, *51*, 356 – 361.

[14] Esquenazi, D., Wigg, M. D., Miranda, M. M. F. S., Rodrigues, H. M. *et al.*, Antimicrobial and antiviral activities of polyphenolics from *Cocos nucifera* Linn. (*Palmae*) husk fiber extract. *Res. Microbiol.* 2002, *153*, 647 – 652.

[15] Shahat, A. A., Cos, P., De Bruyne, T., Apers, S., *et al.*, Antiviral and antioxidant activity of flavonoids and proanthocyanidins from *Crataegus sinaica*. *Planta Med.* 2002, *68*, 539 – 541.

[16] Park, J. C., Ito, H., Yoshida, T., H-NMR assignment of HIV protease inhibitor, procyanidin B3 isolated from *Rosa rugosa*. *Nat. Prod. Sci.* 2003, *9*, 49 – 51.

[17] Jerez, M., Tourino, S., Sineiro, J., Torres, J. L., Nunez, M. J., Procyanidins from pine bark: Relationships between structure, composition and antiradical activity. *Food Chem.* 2007, *104*, 518 – 527.

[18] Oszmianski, J., Wolniak, M., Wojdylo, A., Wawer, I., Comparative study of polyphenolic content and antiradical activity of cloudy and clear apple juices. *J. Sci. Food Agric*. 2007, *87*, 573 – 579.

[19] Fan, J., Ding, X., Gu, W., Radical-scavenging proanthocyanidins from sea buckthorn seed. *Food Chem.* 2007, *102*, 168 – 177.

[20] Shahat, A. A., Hammouda, F., Ismail, S. I., Azzam, S. A., *et al.*, Anti-complementary activity of *Crataegus sinaica*. *Planta Med.* 1996, *62*, 10 – 13.

[21] Del Bas, J. M., Fernandez-Larrea, J., Blay, M., Ardevol, A., *et al.*, Grape seed procyanidins improve atherosclerotic risk index and induce liver CYP7A1 and SHP expression in healthy rats. *FASEB J.* 2005, *19*, 479 – 481.

[22] Takano, F., Takata, T., Yoshihara, A., Nakamura, Y., *et al.*, Aqueous extract of peanut skin and its main constituent procyanidin A1 suppress serum IgE and IgG1 levels in mice-immunized with ovalbumin. *Biol. Pharm. Bull*. 2007, *30*, 922 – 927.

[23] Fridrich, D., Kern, M., Fritz, J., Pahlke, G., *et al.*, The epidermal growth factor receptor and human topoisomerases represent potential cellular targets of oligomeric procyanidins. *Mol. Nutr. Food Res*. 2007, *51*, 192 – 200.

[24] Urbonaviciute, A., Jakstas, V., Kornysova, O., Janulis, V., Maruska, A., Capillary electrophoretic analysis of flavonoids in single-styled hawthorn (*Crataegus monogyna Jacq*.) ethanolic extracts. *J. Chromatogr. A* 2006, *1112*, 339 – 344.

[25] Rasmussen, S. E., Frederiksen, H., Struntze, K., Poulsen, L., Dietary proanthocyanidins: Occurrence, dietary intake, bioavailability, and protection against cardiovascular disease. *Mol. Nutr. Food Res.* 2005, *49*, 159 – 174.

[26] Zessner, H., Pan, L., Will, F., Klimo, K., *et al.*, Fractionation of polyphenol-enriched apple juice extracts to identify constituents with cancer chemopreventive potential. *Mol. Nutr. Food Res*. 2008, *52 (Suppl. 1)*, S28 – S44.

[27] Scalbert, A., Haslam, E., Polyphenols and chemical defense of the leaves of *Quercus robur*. *Phytochemistry* 1987, *26*, 3191 – 3195.

[28] Matthews, S., Mila, I., Scalbert, A., Donnelly, D. M. X., Extractable and non-extractable proanthocyanidins in barks. *Phytochemistry* 1997, *45*, 405 – 410.

[29] Kiatgrajai, P., Wellons, J. D., Gollob, L., White, J. D., Kinetics of epimerization of (+)- catechin and its rearrangement to catechinic acid. *J. Org. Chem.* 1982, *47*, 2910 – 2912.

[30] Komatsu, Y., Suematsu, S., Hisanobu, Y., Saigo, H., *et al.*, Effects of pH and temperature on reaction kinetics of catechins in green tea infusion. *Biosci. Biotechnol. Biochem.* 1993, *57*, 907 – 910.

[31] Zhu, Q. Y., Holt, R. R., Lazarus, S. A., Ensunsa, J. L., *et al.*, Stability of the Flavan-3-ols epicatechin and catechin and related dimeric procyanidins derived from Cocoa. *J. Agric. Food Chem.* 2002, *50*, 1700 – 1705.

[32] Kondo, K., Kurihara, M., Fukuhara, K., Tanaka, T., *et al.*, Conversion of procyanidin B-type (catechin dimer) to A-type: Evidence for abstraction of C-2 hydrogen in catechin during radical oxidation. *Tetrahedron Lett*. 2000, *41*, 485 – 488.

[33] Shi, J., Nawaz, H., Pohorly, J., Mittal, G., *et al.*, Extraction of polyphenolics from plant material for functional foods – Engineering and technology. *Food Rev. Int.* 2005, *21*, 139 – 166.

[34] Santos-Buelga, C., Francia-Aricha, E. M., Escribano-Bailon, M. T., Comparative flavan-3-ol composition of seeds from different grape varieties. *Food Chem.* 1995, *53*, 197 – 201.

[35] Ferreira, D., Guyot, S., Marnet, N., Delgadillo, I., *et al.*, Composition of phenolic compounds in a Portuguese pear (*Pyrus communis L. Var. S. Bartolomeu*) and changes after sun-drying. *J. Agric. Food Chem.* 2002, *50*, 4537 – 4544.

[36] Waterman, P. G., Mole, S., *Analysis of phenolic plant metabolites*. Blackwell Scientific Publications, London 1994, pp. 66 – 103.

[37] Clifford, M. N., Ramirez-Martinez, J. R., Tannins in wet-processed coffee beans and coffee pulp. *Food Chem.* 1991, *40*, 191 – 200.

[38] Peng, S., Jay-Allemand, C., Use of antioxidants in extraction of tannins from walnut plants. *J. Chem. Ecol.* 1991, *17*, 887 – 896.

[39] Amiot, M. J., Tacchini, M., Aubert, S., Nicolas, J., Phenolic composition and browning susceptibility of various apple cultivars at maturity. *J. Food. Sci.* 1992, *57*, 958 – 962.

[40] Kantz, K., Singleton, V. L., Isolation and determination of polymeric polyphenols using Sephadex LH-20 and analysis of grape tissue extracts. *Am. J. Enol. Viticult.* 1990, *41*, 223 – 228.

[41] Suarez, B., Picinelli, A., Mangas, J. J., Solid-phase extraction and high-performance liquid chromatographic determination of polyphenols in apple musts and ciders. *J. Chromatogr. A* 1996, *727*, 203 – 209.

[42] Li, Y. G., Tanner, G., Larkin, P., The DMACA-HCl protocol and the threshold proanthocyanidin content for bloat safety in forage legumes. *J. Sci. Food Agric.* 1996, *70*, 89 – 101.

[43] Goldstein, J. L., Swain, T., Changes in tannins in ripening fruits. *Phytochemistry* 1963, *2*, 371 – 383.

[44] Bate-Smith, E. C., Phytochemistry of proanthocyanidins. *Phytochemistry* 1975, *14*, 1107 – 1113.

[45] Vrhovsek, U., Rigo, A., Tonon, D., Mattivi, F., Quantitation of polyphenols in different apple varieties. *J. Agric. Food Chem.* 2004, *52*, 6532 – 6538.

[46] Mane, C., Souquet, J. M., Olle, D., Verries, C., *et al.*, Optimization of simultaneous flavanol, phenolic acid, and anthocyanin extraction from grapes using an experimental design: Application to the characterization of Champagne grape varieties. *J. Agric. Food Chem.* 2007, *55*, 7224 – 7233.

[47] Sudjaroen, Y., Haubner, R., Wuertele, G., Hull, W. E., *et al.*, Isolation and structure elucidation of phenolic antioxidants from tamarind (*Tamarindus indica L.*) seeds and pericarp. *Food Chem. Toxicol.* 2005, *43*, 1673 – 1682.

[48] Galletti, G. C., Self, Ron. The polyphenols (vegetable tannins) of grape skins and pressed fruit residues. *Ann. Chim.* 1986, *76*, 195 – 211.

[49] Meagher, L. P., Spencer, P., Lane, G. A., Sivakumaran, S., Fraser, K., What do green tea, grape seeds, and docks have in common? *Chem. N. Z.* 2005, *69*, 6 – 9.

[50] Renard, C. M. G. C., Baron, A., Guyot, S., Drilleau, J. F., Interactions between apple cell walls and native apple polyphenols: Quantification and some consequences. *Int. J. Biol. Macromol.* 2001, *29*, 115 – 125.

[51] Shoji, T., Masumoto, S., Moriichi, N., Akiyama, H., *et al.*, Apple procyanidin oligomers absorption in rats after oral administration: Analysis of procyanidins in plasma using the Porter method and high-performance liquid chromatography/tandem mass spectrometry. *J. Agric. Food Chem.* 2006, *54*, 884 – 892.

[52] Sano, A., Yamakoshi, J., Tokutake, S., Tobe, K., *et al.*, Procyanidin B1 is detected in human serum after intake of proanthocyanidin-rich grape seed extract. *Biosci. Biotechnol. Biochem.* 2003, *67*, 1140 – 1143.

[53] Koga, T., Moro, K., Nakamori, K., Yamakoshi, J., *et al.*, Increase of antioxidative potential of rat plasma by oral administration of proanthocyanidin-rich extract from grape seeds. *J. Agric. Food Chem.* 1999, *47*, 1892 – 1897.

[54] Déprez, S., Brezillon, C., Rabot, S., Philippe, C., *et al.*, Polymeric proanthocyanidins are catabolized by human colonic microflora into low-molecular-weight phenolic acids. *J. Nutr.* 2000, *130*, 2733 – 2738.

[55] Osborne, N. J. T., McNeill, D. M., Characterisation of Leucaena condensed tannins by size and protein precipitation capacity. *J. Sci. Food Agric.* 2001, *81*, 1113 – 1119.

[56] Naczk, M., Amarowicz, R., Zadernowski, R., Shahidi, F., Protein precipitating capacity of condensed tannins of beach pea, canola hulls, evening primrose and faba bean. *Food Chem.* 2001, *73*, 467 – 471.

[57] Naczk, M., Grant, S., Zadernowski, R., Barre, E., Protein precipitating capacity of phenolics of wild blueberry leaves and fruits. *Food Chem.* 2006, *96*, 640 – 647.

[58] Hagerman, A. E., Butler, L. G., The specificity of proanthocyanidin-protein interactions. *J. Biol. Chem.* 1981, *256*, 4494 – 4497.

[59] *Pharmacopoea Europaea*, 4th edn, Deutscher Apotheker Verlag, Stuttgart 2002.

[60] Harbertson, J. F., Picciotto, E. A., Adams, D. O., Measurement of polymeric pigments in grape berry extract and wines using a protein precipitation assay combined with bisulfite bleaching. *Am. J. Enol. Vitic*. 2003, *54*, 301 – 306.

[61] Montedoro, G., Fantozzi, P., Determination of the tannin phenolics in musts and wines by methyl cellulose (MC) and evaluation of other phenolic fractions. *Lebensm. Wiss. Tech*. 1974, *7*, 155 – 161.

[62] Sarneckis, C. J., Dambergs, R. G., Jones, P., Mercurio, M., *et al.*, Quantification of condensed tannins by precipitation with methyl cellulose: Development and validation of an optimized tool for grape and wine analysis. *Aust. J. Grape Wine Res.* 2006, *12*, 39 – 49.

[63] Porter, L. J., Hrstich, L. N., Chan, B. G., The conversion of procyanidins and prodelphinidins to cyanidin and delphinidin. *Phytochemistry* 1986, *25*, 223 – 230.

[64] Del Pino, M. C. A., Hervas, G., Mantecon, A. R., Giraldez, F. J., Frutos, P., Comparison of biological and chemical methods, and internal and external standards, for assaying tannins in Spanish shrub species. *J. Sci. Food Agric.* 2005, *85*, 583 – 590.

[65] Terrill, T. H., Rowan, A. M., Douglas, G. B., Barry, T. N., Determination of extractable and bound condensed tannin concentrations in forage plants, protein concentrate meals and cereal grains. *J. Sci. Food Agric.* 1992, *58*, 321 – 329.

[66] Geiger, H., in: Van Sumere, C. F., Lea, P. (Eds.), *The biochemistry of plant phenolics, Annual Proceedings of the Phytochemical Society of Europe*, Vol. 25, Clarendon Press, Oxford 1985, pp. 45 – 46.

[67] McMurrough, I, McDowell, J., Chromatographic separation and automated analysis of flavanols. *Anal. Biochem.* 1978, *91*, 92 – 100.

[68] Oszmianski, J., Bourzeix, M., Comparison of methods for determining the content and polymerization of proanthocyanidins and catechins. *Pol. J. Food Nutr. Sci.* 1996, *5*, 43 – 50.

[69] Treutter, D., Chemical reaction detection of catechins and proanthocyanidins with 4-dimethylaminocinnamaldehyde. *J. Chromatogr. A* 1989, *467*, 185 – 193.

[70] Lang, F., Wilhelm, E., Quantitative determination of proanthocyanidins in *Ginkgo biloba* special extracts. *Pharmazie* 1996, *51*, 734 – 737.

[71] Oelschlaeger, C., Milde, J., Schempp, H., Treutter, D., Polyphenols and antioxidant capacity of *Sorbus domestica* L. fruits. *J. Appl. Botany Food Qual.* 2004, *78*, 112 – 116.

[72] De Pascual-Teresa, S., Santos-Buelga, C., Rivas-Gonzalo, J. C., Quantitative analysis of flavan-3-ols in Spanish foodstuffs and beverages. *J. Agric. Food Chem.* 2000, *48*, 5331 – 5337.

[73] Sun, B., Leandro, M. C., de Freitas, V., Spranger, M. I., Fractionation of red wine polyphenols by solid-phase extraction and liquid chromatography. *J. Chromatogr. A* 2006, *1128*, 27 – 38.

[74] Walton, M. F., Haskins, F. A., Gorz, H. J., False positive results in the vanillin-hydrochloric acid assay of tannins in sorghum forage. *Crop Sci.* 1983, *23*, 197 – 200.

[75] Goldstein, J. L., Swain, T., Methods for determining the degree of polymerization of flavans. *Nature* 1963, *198*, 587 – 588.

[76] Hagerman, A. E., Butler, L. G., Assay of condensed tannins or flavonoid oligomers and related flavonoids in plants. *Methods Enzymol.* 1994, *234*, 429 – 437.

[77] Butler, L. G., Price, M. L., Brotherton, J. E., Vanillin assay for proanthocyanidins (condensed tannins): Modification of the solvent for estimation of the degree of polymerization. *J. Agric. Food Chem.* 1982, *30*, 1087 – 1089.

[78] Mole, S., Waterman, P. G., A critical analysis of techniques for measuring tannins in ecological studies. *Oecologia* 1987, *72*, 137 – 147.

[79] Prior, R. L., Gu, L., Occurrence and biological significance of proanthocyanidins in the American diet. *Phytochemistry* 2005, *66*, 2264 – 2280.

[80] Fuezfai, Z., Molnar-Perl, I., Gas chromatographic-mass spectrometric fragmentation study of flavonoids as their trimethylsilyl derivatives: Analysis of flavonoids, sugars, carboxylic and amino acids in model systems and in citrus fruits. *J. Chromatogr. A* 2007, *1149*, 88 – 101.

[81] Balaban, M., Identification of the main phenolic compounds in wood of *Ceratonia siliqua* by GC-MS. *Phytochem. Anal.* 2004, *15*, 385 – 388.

[82] Ohara, S., Yasuta, Y., Ohi, H., Structure elucidation of condensed tannins from barks by pyrolysis /gas chromatography. *Holzforschung* 2003, *57*, 145 – 149.

[83] Matsuo, T., Ito, S., On mechanisms of removing astringency in persimmon fruits by carbon dioxide treatment. Part II. The chemical structure of kaki-tannin from immature fruit of the persimmon (*Diopyros kaki L.*). *Agric. Biol. Chem.* 1978, *42*, 1637 – 1643.

[84] Karonen, M., Leikas, A., Loponen, J., Sinkkonen, J., *et al.*, Reversed-phase HPLC-ESI/MS analysis of birch leaf proanthocyanidins after their acidic degradation in the presence of nucleophiles. *Phytochem. Anal.* 2007, *18*, 378 – 386.

[85] Fu, C., Loo, A. E. K., Chia, F. P. P., Huang, D., Oligomeric proanthocyanidins from mangosteen pericarps. *J. Agric. Food Chem.* 2007, *55*, 7689 – 7694.

[86] Chirinos, R., Campos, D., Costa, N., Arbizu, C., *et al.*, Phenolic profiles of Andean mashua (*Tropaeolum tuberosum* Ruiz & Pavon) tubers: Identification by HPLC-DAD and evaluation of their antioxidant activity. *Food Chem.* 2008, *106*, 1285 – 1298.

[87] Sueiro, L., Yousef, G. G., Seigler, D., de Mejia, E. G., *et al.*, Chemopreventive potential of flavonoid extracts from plantation-bred and wild *Aronia melanocarpa* (black chokeberry) fruits. *J. Food Sci.* 2006, *71*, C480 – C488.

[88] Yanagida, A., Shoji, T., Shibusawa, Y., Separation of proanthocyanidins by degree of polymerization by means of size-exclusion chromatography and related techniques. *J. Biochem. Biophys. Methods*. 2003, *56*, 311 – 322.

[89] Lea, A. G. H., The phenolics of ciders: Oligomeric and polymeric procyanidins. *J. Sci. Food Agric.* 1978, *29*, 471 – 477.

[90] Rigaud, J., Escribano-Bailon, M. T., Prieur, C., Souquet, J.-M., Cheynier, V., Normal-phase high-performance liquid chromatographic separation of procyanidins from cocoa beans and grape seeds. *J. Chromatogr. A* 1993, *654*, 255 – 260.

[91] Karonen, M., Ossipov, V., Sinkkonen, J., Loponen, J., *et al.*, Quantitative analysis of polymeric proanthocyanidins in birch leaves with normal-phase HPLC. *Phytochem. Anal.* 2006, *17*, 149 – 156.

[92] Shoji, T., Masumoto, S., Moriichi, N., Kanda, T., Ohtake, Y., Apple (*Malus pumila*) procyanidins fractionated according to the degree of polymerization using normal-phase chromatography and characterized by HPLC-ESI/MS and MALDI-TOF/MS. *J. Chromatogr. A* 2006, *1102*, 206 – 213.

[93] Kelm, M. A., Johnson, J. C., Robbins, R. J., Hammerstone, J. F., Schmitz, H. H., High-performance liquid chromatography separation and purification of cacao (*Theobroma cacao* L.) procyanidins according to degree of polymerization using a diol stationary phase. *J. Agric. Food Chem.* 2006, *54*, 1571 – 1576.

[94] Kallithraka, S., Garcua-Vuguera, C., Bridle, P., Bakker, J., Survey of solvents for the extraction of grape seed phenolics. *Phytochem. Anal.* 1995, *6*, 265 – 267.

[95] Koupai-Abyazani, M. R., McCallum, J., Bohm, B. A., Identification of the constituent flavanoid units in sainfoin proanthocyanidins by reversed-phase high-performance liquid chromatography. *J. Chromatogr.* 1992, *594*, 117 – 123.

[96] Kahle, K., Kraus, M., Richling, E., Polyphenol profiles of apple juices. *Mol. Nutr. Food Res.* 2005, *49*, 797 – 806.

[97] Rohr, G. E., Meier, B., Sticher, O., in: Rahman, A.-U. (Ed.), *Studies in Natural Products Chemistry*, Vol. 21, Elsevier Science, Amsterdam 2000, pp. 497 – 570.

[98] Putman, L. J., Butler, L. G., Separation of high molecular weight sorghum procyanidins by high-performance liquid chromatography. *J. Agric. Food Chem.* 1989, *37*, 943 – 946.

[99] Dallas, C., Ricardo-da-Silva, J. M., Laureano, O., Degradation of oligomeric procyanidins and anthocyanins in a Tinta Roriz red wine during maturation. *Vitis* 1995, *34*, 51 – 56.

[100] Gu, L., Kelm, M., Hammerstone, J. F., Beecher, G. *et al.*, Fractionation of polymeric procyanidins from lowbush blueberry and quantification of procyanidins in selected foods with an optimized normal-phase HPLC-MS fluorescent detection method. *J. Agric. Food. Chem.* 2002, *50*, 4852 – 4860.

[101] Huemmer, W., Dietrich, H., Will, F., Schreier, P., Richling, E., Content and mean polymerization degree of procyanidins in extracts obtained from clear and cloudy apple juices. *Biotechnol. J.* 2008, *3*, 234 – 243.

[102] Treutter, D., Santos-Buelga, C., Gutmann, M., Kolodziej, H., Identification of flavan-3-ols and procyanidins by high-performance liquid chromatography and chemical reaction detection. *J. Chromatogr. A* 1994, *667*, 290 – 297.

[103] Feucht, W., Treutter, D., Santos-Buelga, C., Christ, E., Catechin as a radical scavenger in paraquat-treated *Prunus avium*. *Angew. Bot.* 1996, *70*, 119 – 123.

[104] Rigaud, J., Perez-Ilzarbe, J., Ricardo da Silva, J. M., Cheynier, V., Micro method for the identification of proanthocyanidin using thiolysis monitored by high-performance liquid chromatography. *J. Chromatogr.* 1991, *540*, 401 – 405.

[105] Guyot, S., Marnet, N., Drilleau, J., Thiolysis-HPLC characterization of apple procyanidins covering a large range of polymerization states. *J. Agric. Food Chem.* 2001, *49*, 14 – 20.

[106] Koehler, N., Wray, V., Winterhalter, P., Preparative isolation of procyanidins from grape seed extracts by high-speed counter-current chromatography. *J. Chromatogr. A* 2008, *1177*, 114 – 125.

[107] Ricardo da Silva, J. M., Rigaud, J., Cheynier, V., Cheminat, A., Moutounet, M., Procyanidin dimers and trimers from grape seeds. *Phytochemistry* 1991, *30*, 1259 – 1264.

[108] Scharbert, S., Holzmann, N., Hofmann, T., Identification of the astringent taste compounds in black tea infusions by combining instrumental analysis and human bioresponse. *J. Agric. Food Chem*. 2004, *52*, 3498 – 3508.

[109] Bartolome, B., Bengoechea, M. L., Galvez, M. C., Perez-Ilzarbe, F. J., *et al.*, Photodiode array detection for elucidation of the structure of phenolic compounds. *J. Chromatogr.* 1993, *655*, 119 – 125.

[110] Vennat, B., Pourrat, A., Texier, O., Pourrat, H., Gaillard, J., Proanthocyanidins from the roots of *Fragaria vesca*. *Phytochemistry* 1986, *26*, 261 – 263.

[111] Adamson, G. E., Lazarus, S. A., Mitchell, A. E., Prior, R. L., *et al.*, HPLC method for the quantification of procyanidins in cocoa and chocolate samples and correlation to total antioxidant capacity. *J. Agric. Food Chem.* 1999, *47*, 4184 – 4188.

[112] Lazarus, S. A., Adamson, G. E., Hammerstone, J. F., Schmitz, Harold H., High-performance liquid chromatography/ mass spectrometry analysis of proanthocyanidins in foods and beverages. *J. Agric. Food Chem.* 1999, *47*, 3693 – 3701.

[113] Mitchell, A. E., Hong, Y.-J., May, J. C., Wright, C. A., Bamforth, C. W., A comparison of polyvinylpolypyrrolidone (PVPP), silica xerogel and a polyvinylpyrrolidone (PVP) – silica co-product for their ability to remove polyphenols from beer. *J. Inst. Brewing* 2005, *111*, 20 – 25.

[114] Rohr, G. E., Giggio, G., Meier, B., Sticher, O., Evaluation of different detection modes for the analysis of procyanidins in leaves and flowers of *Crataegus* spp. Part I. Diode array and electrochemical detection. *Phytochem. Anal*. 2000, *11*, 106 – 112.

[115] Donovan, J. D., Luthria, L. D., Stremple, P., Waterhouse, A. L., Analysis of (+)-catechin, (–)-epicatechin and their 3′- and 4′-*O*-methylated analogs – A comparison of sensitive methods. *J. Chromatogr. B* 1999, *726*, 277 – 283.

[116] Hammerstone, J. F., Lazarus, S. A., Mitchell, A. E., Rucker, R., Schmitz, H. H., Identification of procyanidins in cocoa (*Theobroma cacao*) and chocolate using high-performance liquid chromatography/mass spectrometry. *J. Agric. Food Chem*. 1999, *47*, 490 – 496.

[117] Wu, Q., Wang, M., Simon, J. E., Determination of proanthocyanidins in fresh grapes and grape products using liquid chromatography with mass spectrometric detection. *Rapid Commun. Mass Spectrom.* 2005, *19*, 2062 – 2068.

[118] Flamini, R., Mass spectrometry in grape and wine chemistry. Part I: Polyphenols. *Mass Spectrom. Rev.* 2003, *22*, 218 – 250.

[119] Cheynier, A., Fulcrand, H., in: Santos-Buelga, C., Williamson, G. (Eds.), *Methods in polyphenol analysis*, The Royal Society of Chemistry, Cambridge 2003, pp. 284 – 313.

[120] Guyot, S., Doco, T., Souquet, J. M., Moutounet, M., Drilleau, J. F., Characterization of highly polymerized procyanidins in cider apple (*Malus sylvestris var. kermerrien*) skin and pulp. *Phytochemistry* 1997, *44*, 351 – 357.

[121] Karonen, M., Loponen, J., Ossipov, V., Pihlaja, K., Analysis of procyanidins in pine bark with reversed-phase and normal-phase high-performance liquid chromatography-electrospray ionization mass spectrometry. *Anal. Chim. Acta* 2004, *522*, 105 – 112.

[122] Friedrich, W., Eberhardt, A., Galensa, R., Investigation of proanthocyanidins by HPLC with electrospray ionization mass spectrometry. *Eur. Food Res. Technol.* 2000, *211*, 56 – 64.

[123] De Pascual-Teresa, S., Rivas-Gonzalo, J. C., Santos-Buelga, C., Prodelphinidins and related flavanols in wine. *Int. J. Food Sci. Technol*. 2000, *35*, 33 – 40.

[124] Ohnishi-Kameyama, M., Yanagida, A., Kanda, T., Nagata, T., Identification of catechin oligomers from apple (*Malus pumila cv. Fuji*) in matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and fast-atom bombardment mass spectrometry. *Rapid Comm. Mass Spectrom.* 1997, *11*, 31 – 36.

[125] Es-Safi, N. E., Guyot, S., Ducrot, P. H., NMR, ESI/MS, and MALDI-TOF/MS Analysis of pear juice polymeric proanthocyanidins with potent free radical scavenging activity. *J. Agric. Food Chem.* 2006, *54*, 6969 – 6977.

[126] Yang, Y., Chien, M., Characterization of grape procyanidins using high-performance liquid chromatography/mass spectrometry and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *J. Agric. Food Chem.* 2000, *48*, 3990 – 3996.

[127] Neto, C. C., Krueger, C. G., Lamoureaux, T. L., Kondo, M., *et al.*, MALDI-TOF MS characterization of proanthocyanidins from cranberry fruit (*Vaccinium macrocarpon*) that inhibit tumor cell growth and matrix metalloproteinase expression *in vitro*. *J. Sci. Food Agric.* 2006, *86*, 18 – 25.

[128] Reed J. D, Krueger C. G, Vestling M., M. MALDI-TOF mass spectrometry of oligomeric food polyphenols. *Phytochemistry* 2005, *66*, 2248 – 2263.

[129] Ku, C. S., Mun, S. P., Characterization of proanthocyanidin in hot water extract isolated from *Pinus radiata* bark. *Wood Sci. Technol.* 2007, *41*, 235 – 247.

[130] Northen, T. R., Yanes, O., Northen, M. T., Marrinucci, D., *et al.*, Clathrate nanostructures for mass spectrometry. *Nature* 2007, *449*, 1033 – 1036.

[131] Friebolin, H., *Basic one and two dimensional NMR spectroscopy*, 3rd Edn., Wiley-VCH, New York 2004.

[132] Tarascou, I., Barathieu, K., André, Y., Pianet, I., *et al.*, An improved synthesis of procyanidin dimers: Regio- and stereocontrol of the interflavan bond. *Eur. J. Org. Chem.* 2006, *23*, 5367 – 5377.

[133] Steynberg, P. J., Steynberg, J. P., Bezuidenhoudt, B. C. B., Ferreira, D., Oligomeric flavonoids. Part 19. Reductive cleavage of the interflavanyl bond in proanthocyanidins. *J. Chem. Soc. Perkin Trans.* 1995, *1,* 3005 – 3012.

[134] Foo, L. Y., Karchesy, J. J., Procyanidin tetramers and pentamers from Douglas fir bark. *Phytochemistry* 1991, *30*, 667 – 670.

[135] Nonaka, G., Hsu, F., Nishioka, I., Structures of dimeric, trimeric, and tetrameric procyanidins from *Areca catechu L*. *J. Chem. Soc. Chem. Commun.* 1981, 781 – 783.

[136] Vdovin, A. D., Kuliev, Z. A., Abdullaev, N. D., ^{1}H and ^{13}C NMR spectroscopy in the study of flavan-3-ols, proanthocyanidins, and their derivatives. II. Proton magnetic resonance spectroscopy of proanthocyanidins. *Chem. Nat. Comp*. 1997, *33*, 101 – 112.

[137] Shoji, T., Mutsuga, M., Nakamura, T., Kanda, T., *et al.*, Isolation and structural elucidation of some procyanidins from apple by low-temperature nuclear magnetic resonance. *J. Agric. Food Chem*. 2003, *51*, 3806 – 3813.

[138] Lightner, D. A., Gurst, J. E., *Organic conformational analysis and stereochemistry from circular dichroism spectroscopy.* Wiley-VCH, New York 2000.

[139] Van Rensburg, H., Steynberg, P. J., Burger, J. F. W., Van Heerden, P. S., Ferreira, D., Circular dichroic properties of flavan-3-ols. *J. Chem. Res*. 1999, *7*, 450 – 451.

[140] Bate-Smith, E. C., Haemanalysis of tannins. The concept of relative astringency. *Phytochemistry* 1973, *12*, 907 – 912.

[141] Foo, L. Y., Polymeric proanthocyanidins of *Photinia glabrescens*; Modification of molecular weight and nature of products from hydrogenolysis. *Phytochemistry* 1982, *21*, 1741 – 1746.

[142] Bolde, A. M., Pieters, L. A., Gergely, A., Kolodziej, H., *et al.*, A-type proanthacyanidins from stem-bark of *Pavetta owariensis*. *Phytochemistry* 1991, *30*, 337 – 342.

