Doctoral Thesis

Analytical investigation on and isolation of procyanidins from Crataegus leaves and flowers

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Analytical Investigation on and
Isolation of Procyanidins from
Crataegus Leaves and Flowers

A dissertation submitted to the
Swiss Federal Institute of Technology Zurich

for the degree of
Doctor of Natural Sciences

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Zurich 1999
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Curriculum vitae
General abbreviations

\[\alpha\]D  specific optical rotation
acetone-\textsubscript{d6}  deuterated acetone
ACN  acetonitrile
AcOH  acetic acid
APCI  atmospheric pressure chemical ionization
benzene-\textsubscript{d6}  deuterated benzene
C  carbon atom
cat  (+)-catechin
CD  circular dichroism
chloroform-\textsubscript{d1}  deuterated chloroform
CRD  chemical reaction detection
d  doublet
\delta  chemical shift
2D  two-dimensional
DAB  Deutsches Arzneibuch (German Pharmacopoeia)
DAC  Deutscher Arzneimittel Codex (German Drug Codex)
DAD  diode array detector
dd  double doublet
DEPT  distortionless enhancement by polarization transfer
DMACA  4-dimethylaminocinnamaldehyde
DMSO-\textsubscript{d6}  deuterated dimethyl sulphoxide
D\textsubscript{2}O  deuterated water
DQF-COSY  double quantum filter correlation spectroscopy
EI-MS  electron impact - mass spectrometry
epi  (-)-epicatechin
ESP  electrospray
EtOH  ethanol
FAB-MS  fast atom bombardment - mass spectrometry
H  hydrogen atom
HCl  hydrochloric acid
HClO\textsubscript{4}  perchloric acid
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tr>
<td>H₂O</td>
<td>water</td>
</tr>
<tr>
<td>HCO₂H</td>
<td>formic acid</td>
</tr>
<tr>
<td>HMBC</td>
<td>heteronuclear multiple bond correlation</td>
</tr>
<tr>
<td>HMQC</td>
<td>heteronuclear multiple quantum correlation</td>
</tr>
<tr>
<td>H₃PO₄</td>
<td>ortho-phosphoric acid</td>
</tr>
<tr>
<td>HPLC</td>
<td>high-performance liquid chromatography</td>
</tr>
<tr>
<td>Hz</td>
<td>Hertz</td>
</tr>
<tr>
<td>IR</td>
<td>infrared spectroscopy</td>
</tr>
<tr>
<td>J</td>
<td>coupling constant</td>
</tr>
<tr>
<td>K</td>
<td>Kelvin</td>
</tr>
<tr>
<td>LC-MS</td>
<td>liquid chromatography - mass spectrometry</td>
</tr>
<tr>
<td>m</td>
<td>multiplet</td>
</tr>
<tr>
<td>mAU</td>
<td>milli absorption unit</td>
</tr>
<tr>
<td>Me₂CO</td>
<td>acetone</td>
</tr>
<tr>
<td>MeOH</td>
<td>methanol</td>
</tr>
<tr>
<td>methanol-d₄</td>
<td>deuterated methanol</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>m/z</td>
<td>mass-to-charge ratio</td>
</tr>
<tr>
<td>nitrobenzene-d₅</td>
<td>deuterated nitrobenzene</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>nOe</td>
<td>nuclear Overhauser effect</td>
</tr>
<tr>
<td>n.s.</td>
<td>not specified</td>
</tr>
<tr>
<td>PC</td>
<td>paper chromatography</td>
</tr>
<tr>
<td>Ph.Helv.</td>
<td>Pharmacopoea Helvetica (Swiss Pharmacopoeia)</td>
</tr>
<tr>
<td>ppm</td>
<td>parts per million</td>
</tr>
<tr>
<td>RDA-F</td>
<td>retro-Diels-Alder fission</td>
</tr>
<tr>
<td>Rₚ</td>
<td>retention factor (TLC analysis)</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>s</td>
<td>singlet</td>
</tr>
<tr>
<td>t</td>
<td>triplet</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
</tr>
<tr>
<td>tᵣ</td>
<td>retention time (HPLC analysis)</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
</tbody>
</table>
Abbreviations for procyanidins

B1  (-)-epicatechin-(4β→8)-(+) catechin
B2  (-)-epicatechin-(4β→8)-(+) epicatechin
B3  (+) catechin-(4α→8)-(+) catechin
B4  (+) catechin-(4α→8)-(+) epicatechin
B5  (-) epicatechin-(4β→6)-(+) epicatechin
B6  (+) catechin-(4α→6)-(+) catechin
B7  (-) epicatechin-(4β→6)-(+) catechin
B8  (+) catechin-(4α→6)-(+) epicatechin
A1  (-) epicatechin-(4β→8),(2β→O→7)-(+) epicatechin
A2  (-) epicatechin-(4β→8),(2β→O→7)-(+) epicatechin
C1  (-) epicatechin-(4β→8)-(+) epicatechin-(4β→8)-(+) epicatechin
C2  (+) catechin-(4α→8)-(+) catechin-(4α→8)-(+) catechin
trimer I (-) epicatechin-(4β→6)-(+) epicatechin-(4β→8)-(+) epicatechin
trimer II (-) epicatechin-(4β→6)-(+) epicatechin-(4β→6)-(+) epicatechin
trimer III (-) epicatechin-(4β→8)-(+) epicatechin-(4β→8)-(+) catechin
trimer IV (+) catechin-(4α→8)-(+) catechin-(4α→8)-(+) epicatechin
trimer V (-) epicatechin-(4β→6)-(+) epicatechin-(4β→8)-(+) catechin
trimer VI (-) epicatechin-(4β→8)-(+) epicatechin-(4β→6)-(+) catechin
trimer VII (-) epicatechin-(4β→8)-(+) epicatechin-(4β→6)-(+) epicatechin
trimer VIII (-) epicatechin-(4β→8)-(+) epicatechin-(4α→8)-(+) catechin
tetramer I (-) epicatechin-(4β→8)-(+) epicatechin-(4β→8)-(+) epicatechin
(4β→8)-(+) epicatechin
tetramer II (-) epicatechin-(4β→6)-(+) epicatechin-(4β→8)-(+) epicatechin
(4β→6)-(+) epicatechin
tetramer III (+) catechin-(4α→6)-(+) epicatechin-(4β→8)-(+) epicatechin
(4β→8)-(+) epicatechin
Summary

The present study is dedicated to the analysis and purification of procyanidins occurring in *Crataegi folium cum flore* (hawthorn). The first part includes an extended literature overview on the analysis of procyanidins. The actual knowledge on the qualitative and quantitative analysis of procyanidins in different matrices is reviewed. Suitable sample preservation and extraction conditions are discussed followed by an overview on conventional methods (proanthocyanidin, vanillin and dimethylaminocinnamaldehyde assay). A profound overview on sample clean up procedures, chromatography and detection of procyanidins is presented in following subsections.

Based on this knowledge and results of preliminary studies a high-performance liquid chromatographic (HPLC) protocol for the qualitative and quantitative analysis of procyanidins in hawthorn leaves and flowers using UV diode array detection was developed. The sample clean up procedure was kept to a minimum and takes advantage of the solubility of procyanidins in 50 % aqueous methanol. For quantitative analysis the sample solutions were additionally filtered over a C18 cartridge to insure better repeatabilities and adequate purity of the procyanidin B2 peak. The quantitative method has been validated and allows the determination of (-)-epicatechin as well as the procyanidins B2, B4, B5 and C1. The evaluations can be based on detector responses at 280 nm or 220 nm. Best recovery rates especially for the procyanidins B4 and C1 were obtained when quantitations were based on height counts. If (-)-epicatechin is used as a standard also for the procyanidins, the evaluations have to be based on area counts at 280 nm.

The protocol for the quantitative analysis of procyanidins in hawthorn leaves and flowers was adapted to evaluate contents in fruits and flowers, too. Procyanidin B2 was the principal procyanidin in all plant tissues. The procyanidins B4, B5 and C1 could also be detected in all samples as well as (-)-epicatechin. The concentrations of the identified compounds were significantly lower in ripe fruits compared to leaves and flowers or flowers. The additionally performed LC-MS (liquid chromatography – mass spectrometry) analysis of the extracts of the different plant tissues revealed analogous procyanidin patterns up to the tetrameric level.

In preliminary studies other possible sample clean up procedures for the analysis of procyanidins in hawthorn leaves and flowers were evaluated. Ethyl acetate was found to
extract procyanidins not quantitatively from aqueous extract solutions. Applying solid phase extraction, adsorption was predominating with size exclusion sorbents. A poor loadability for the extract solutions combined with a low retention for procyanidins was the major drawback of the reversed-phase sorbents that were tested. The addition of an antioxidant to the extraction solvent generally led to lower extraction efficiencies.

Special attention was given to possible alternatives to the UV detection. Single electrode electrochemical detection was found to be more sensitive but less selective than UV diode array detection. The analysis of the calibration graphs of (-)-epicatechin and the procyanidins revealed that a quantitation in terms of monomer equivalents leads to an underestimation of the contents of procyanidin dimers. Extensive qualitative studies were performed using mass spectrometrical detection (LC-MS). Molecular ions of procyanidins could be generated using the electrospray interface (ESP) in the negative ionization mode, while considerable fragmentation occurred with atmospheric pressure chemical ionization (APCI).

The second part of this investigation starts with theoretical sections covering the distribution in the plant kingdom and general features of proanthocyanidins and the procyanidins in particular. Overviews follow in later subsections on procyanidins occurring in *Crataegus* species and on structure elucidation. The experimental sections deal with the isolation and structure elucidation of procyanidins from *Crataegus* leaves and flowers.

The purification of the aqueous acetone extracts prepared from *Crataegus* leaves and flowers includes several liquid-liquid partitionings and repeated column chromatography over Sephadex-LH 20 followed by preparative reversed-phase HPLC. The structure elucidation was based on MS (mass spectrometry) as well as on $^1$H-NMR (nuclear magnetic resonance) spectroscopy of the native and peracetylated compounds. The experimental values were compared to published data. $^{13}$C-NMR and two-dimensional techniques were also used to confirm the identities in some cases. The absolute configurations at the interflavanoid linkage of the dimers was deduced from circular dichroism (CD) measurements. By the combination of all these spectroscopic methods the identities of the isolated (-)-epicatechin as well as the procyanidins B2, B4, B5, C1, trimer I [(-)-epicatechin-(4β→6)-(-)-epicatechin-(4β→8)-(-)-epicatechin] and tetramer I [(-)-epicatechin-(4β→8)-(-)-epicatechin-(4β→8)-(-)-epicatechin-(4β→8)-]
(-)-epicatechin] could unequivocally be established. Trimer I is described for the first time in *Crataegus* species. The identity of another trimer II [(-)-epicatechin-(4ß→6)-(4ß→6)-(-)-epicatechin] could only tentatively be established based on the $^1$H-NMR data of the native compound and the MS spectrum. The NMR spectra of the peracetylated derivative were not interpretable due to extensive rotational isomerism.

Evidences were found for the presence of additional flavan-3-ols in *Crataegus* leaves and flowers. Three compounds were isolated in very low quantities which show typical procyanidin UV spectra and form dyes with dimethylaminocinnamaldehyde. LC-MS analysis revealed that the molecular weights of these compounds follow a specific algorithm. The presence of procyanidin glycosides or alternatively the formation of procyanidin degradation products is postulated. The structure of an isolated artifact I (bis-8-8'-epicatechinylmethane) could be established by spectroscopic methods.

The last part is dealing with the stability of procyanidins. In a first section a literature overview is given on possible degradation reactions procyanidins may undergo. The experimental section describes the degradation of the procyanidins B2, B5 and C1 in aqueous methanolic solutions under different temperature and light conditions followed by HPLC UV / DAD analysis. All solutions were stable at -80 °C in darkness over a time period of one year. The procyanidins were degraded under all other storage conditions. The trimeric procyanidin C1 was less stable than the dimeric procyanidins B2 and B5. The principal degradation products formed under the different conditions were largely the same for one particular procyanidin as was judged from the retention features. The identification of the degradation products based on UV characteristics and retention times was found to be speculative. The LC-MS analysis of a degraded procyanidin C1 solution revealed that the procyanidin B2 co-eluted with another degradation product which presumably is a trimeric A-type procyanidin.
Zusammenfassung


Massenspektrometrie) Analyse der Extrakte der verschiedenen Pflanzenteile ergab ein analoges Procyanidin-Muster bis zu den Tetrameren.


Die Aufreinigung der wässrigen Acetonauszüge, die aus den blühenden Zweigspitzen von Crataegus erhalten wurden, beinhaltet verschiedene flüssig-flüssig Verteilungen und wiederholte Säulenchromatographie über Sephadex LH-20, gefolgt von präparativer Umkehrphasen-HPLC. Die Strukturaufklärung basiert auf der MS (Massenspektrometrie) und 1H-NMR (kernmagnetische Resonanz) Spektroskopie der

Es wurden Hinweise für das Vorhandensein weiterer Flavan-3-ole gefunden. Drei Verbindungen, die in sehr geringen Mengen isoliert wurden, wiesen typische Procyanidin UV Spektren auf und bildeten Farbstoffe mit Dimethylaminozimtaldehyd. Die LC-MS Analyse zeigte auf, dass die Molekülmassen dieser Verbindungen einem bestimmten Algorithmus folgen. Das Vorhandensein von Procyanidin Glykosiden oder alternativ, die Bildung von Procyanidin Abbauprodukten wird postuliert. Die Struktur eines isolierten Artefaktes I (bis-8-8'-Epicatechinylmethan) konnte mittels spektroskopischer Methoden ermittelt werden.

Objectives

Hawthorn has been used as a medicinal plant for hundreds of years. The activity of the flavonoid fraction as well as the oligomeric procyanidin fraction in cases of declining heart function could be demonstrated in numerous pharmacological studies over the past years. A standardization of preparations of hawthorn either on flavonoids or procyanidins therefore seems reasonable. The quantitative analysis of the principal flavonoid aglycones after acid hydrolysis is well established. In contrast, there does not yet exist a validated protocol for the quantitative determination of the oligomeric procyanidins. The scope of the present investigation was therefore to develop a high-performance liquid chromatographic method for the quantitative analysis of oligomeric procyanidins in hawthorn. The necessary procyanidin standards were to be isolated and their structure elucidated by modern techniques. In addition, an evaluation of the preparative as well as quantitative methods that are described in the literature was intended.
Part I: Analytical part

1 Analysis of procyanidins (publication 1)

The present knowledge on the qualitative and quantitative analysis of procyanidins is reviewed. Procyanidins belong to the class of natural products known as proanthocyanidins or condensed polyphenols. The instability of procyanidins should be considered throughout sample collection, storage and clean up procedures. Extractions are preferentially conducted using aqueous acetone. Addition of an antioxidant is recommended as long as it does not interfere with the analytical procedure. Conventional methods are mainly based on color reactions, of which only functional group assays exhibit specificity for proanthocyanidins. The dimethylamino-cinnamaldehyde assay is gaining importance compared to the most widely used vanillin assay, because it is more specific, more sensitive and less subject to interferences. Individual procyanidins can only be assessed by chromatography. Reversed-phase high-performance liquid chromatography on C18 stationary phases using acidic aqueous methanol or acetonitrile as eluents is the procedure of choice. Oligomeric procyanidins do not elute according to their degree of polymerization. Polymers can not be chromatographed and hamper the most commonly used UV detection at 280 nm. Sample clean up procedures are inevitable because of these polymeric compounds and phenolic acids as well as flavonoids, which tend to co-elute with procyanidins. Liquid liquid extraction using ethyl acetate is not quantitative. Solid phase extraction over C18, polyamide or Sephadex LH-20 are described in the literature, but none of these procedures is completely validated. More selective detection modes like electrochemical detection or mass spectrometry as well as derivatization procedures are discussed as possible alternatives to extensive sample clean up procedures.

1.1 Introduction

Procyanidins are the most widely distributed members of the class of polyphenolic compounds known as proanthocyanidins (synonyms: oligoflavonoids, condensed polyphenols, condensed tannins). Proanthocyanidins are colorless compounds, occurring predominantly in woody or herbaceous plants. They got their name from the
characteristic hydrolyzation reaction they undergo in acidic medium which yields colored anthocyanidins. Proanthocyanidins consist of flavonoid precursors, which are commonly linked by carbon-carbon bonds at C4→C8 or C4→C6. A variety of different classes are known, depending on the substitution pattern of the monomer units (see Figure 1.1). More details on structures, distribution and general features of this class of compounds can be found in numerous reviews [1-6].

Figure 1.1 Substitution pattern for the most common proanthocyanidins [1] and numbering system for monomer units.

![flavanol skeleton]

<table>
<thead>
<tr>
<th>Class</th>
<th>Monomer unit</th>
<th>Substitution pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(2R, 3S stereochemistry)</td>
<td>5</td>
</tr>
<tr>
<td>Procyanidin</td>
<td>Catechin</td>
<td>OH OH OH OH H</td>
</tr>
<tr>
<td>Prodelphinidin</td>
<td>Gallocatechin</td>
<td>OH OH OH OH OH</td>
</tr>
<tr>
<td>Propelargonidin</td>
<td>Afzelechin</td>
<td>OH OH H OH H</td>
</tr>
<tr>
<td>Profisetinidin</td>
<td>Fisetinidol</td>
<td>H OH OH OH H</td>
</tr>
</tbody>
</table>

Procyanidins consist of the diastereoisomers (-)-epicatechin and (+)-catechin [1,7] (see Figures 1.2 and 1.3). Procyanidins of low molecular weight are also known as pycnogenols [8]. The nomenclature of procyanidins is similar to the one of oligo- and polysaccharides [9]. The basic structural units with a 2R configuration are named in terms of the familiar flavans (i.e. epicatechin, catechin). Units with a 2S-configuration are distinguished by the enatio-prefix. The interflavanoid linkages are indicated the
Figure 1.2 Structures of procyanidin monomer units and nomenclature of procyanidins. Trivial names according to [10-11], official nomenclature according to [9].

![Diagram of (-)-epicatechin and (+)-catechin]

<table>
<thead>
<tr>
<th>Trivial names</th>
<th>Official nomenclature</th>
</tr>
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<tr>
<td>B1</td>
<td>epicatechin-(4β→8)-catechin</td>
</tr>
<tr>
<td>B2</td>
<td>epicatechin-(4β→8)-epicatechin</td>
</tr>
<tr>
<td>B3</td>
<td>catechin-(4α→8)-catechin</td>
</tr>
<tr>
<td>B4</td>
<td>catechin-(4α→8)-epicatechin</td>
</tr>
<tr>
<td>B5</td>
<td>epicatechin-(4β→6)-epicatechin</td>
</tr>
<tr>
<td>B6</td>
<td>catechin-(4α→6)-catechin</td>
</tr>
<tr>
<td>B7</td>
<td>epicatechin-(4β→6)-catechin</td>
</tr>
<tr>
<td>B8</td>
<td>catechin-(4α→6)-epicatechin</td>
</tr>
<tr>
<td>C1</td>
<td>epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin</td>
</tr>
<tr>
<td>C2</td>
<td>catechin-(4α→8)-catechin-(4α→8)-catechin</td>
</tr>
<tr>
<td>A1</td>
<td>epicatechin-(4β→8),(2β→O→7)-catechin</td>
</tr>
<tr>
<td>A2</td>
<td>epicatechin-(4β→8),(2β→O→7)-epicatechin</td>
</tr>
</tbody>
</table>
same way as in carbohydrates, the bond and its direction are contained in brackets. The configuration of the interflavanoid bond at C4 is specified by the I.U.P.A.C.'s $\alpha,\beta$-nomenclature. In this review we will use the more convenient trivial nomenclature for the most common procyanidins which was first introduced by Weinges et al. [10] and later was extended by Thompson et al. [11]. Figure 1.2 gives an overview on the nomenclature.

**Figure 1.3** Examples of procyanidins.
The structural diversity of procyanidins is based on the two possible monomer units (+)-catechin and (-)-epicatechin, on the different types of interflavanoid bonds and on the different lengths of chains which are possible. Besides the most common C4→C8 and C4→C6 linkages doubly linked flavan-3-ol structures exist, too. In addition to a C4→C8 bond they are linked by an ether bond between O7→C2 [1] (see Figure 1.3). Cyclic structures have been proposed for procyanidins from kaki (*Diospyros kaki*) [12] and cherry (*Prunus avium*) [13]. Higher molecular weight procyanidins are usually of moderate size (up to 3'000 daltons) [14], but also polymers with very high molecular weights (20'000 to 150'000 daltons) have been reported [15]. Although procyanidins mainly occur free and unglycosylated there are exceptions to this rule: in green tea (*Camellia sinensis*) and grapes (*Vitis vinifera*) esters of gallic acid occur, and procyanidin O-glycosides have lately been isolated [16-18].

Procyanidins are quite reactive and are therefore considered as some of the most unstable natural phenolic compounds [19-20]. They are subject to enzymatic oxidation by polyphenol oxidases as well as to spontaneous oxidation [21]. Coupled oxidation reactions involving o-quinones of phenolic acids have been reported [22-24]. Procyanidins are thermally labile [25] and can easily undergo molecular rearrangements in acidic or basic media [26]. In model solutions interflavanoid bonds of procyanidins were found to be unstable, but also new carbon-carbon bonds were formed [27]. Epimerization occurs as well and mainly concerns (-)-epicatechin [28].

Some procyanidin reactions are of a very broad significance. Their ability to form complexes with proteins, alkoloids and carbohydrates is thought to be responsible for most interactions with biological systems. For example, the traditional use of proanthocyanidin extracts from barks as tanning agents is based on their ability to form complexes with proteins [20]. Sensory properties (mainly bitterness and astringency) as well as nutritional properties of fresh and processed plant food are closely associated with procyanidins and other phenolic compounds [29-30]. In food technology the formation of hazes and precipitates in beers, wines and juices is important [31-32]. Oxidations catalyzed by polyphenol oxidases can lead to undesirable color and flavor of food products [29,33]. A number of pharmacological effects have been demonstrated for procyanidins. Among them are antiviral, antimicrobial, anti-HIV (human immuondeficiency virus), radical scavenging, antioxidative, anti-complementary and
anti-tumorpromoting properties, as well as anti-hypertensive and cardiotonic activities [34-40]. The presence of procyanidins in forage may be regarded as desirable, for their protection against bloat in grazing animals [41], or as undesirable, because of their adverse effect on digestion which is rather caused by a systemic effect of oligomeric procyanidins than by a direct interaction of polyphenols with dietary proteins or digestive enzymes [42]. Last but not least, procyanidins are biologically important as part of the plant's defense mechanism against predators and microorganisms [43-44].

Since procyanidins show such a broad range of different effects their qualitative and quantitative analysis has been a major concern to food chemists, agronomists, biologists as well as pharmacists. In this article we tried to condense the present knowledge on this subject. In a first part we discuss sample preservation procedures as well as extraction of procyanidins. Because most studies covering these topics were conducted using assays for proanthocyanidins in general – and the exact composition of a given matrix is not always known – we use the more appropriate term "proanthocyanidin" rather than "procyanidin". In the next part we will give an overview on group determination procedures. Again, these methods are not only specific for procyanidins and we therefore use the correcter term "proanthocyanidin" where procyanidins are not addressed exclusively. In the last part we discuss the analysis of individual procyanidins by chromatography. This section also includes sample clean up procedures and the different detection modes that are used in procyanidin analysis.

1.2 Sample preservation

The analysis of procyanidins starts at the point of sample collection. Care should be taken not to damage the plant material when collecting it. Any disruption of membrane integrity increases the risk of oxidation reactions and the formation of complexes with biomolecules such as proteins. Consequently an alteration in extraction efficiency and chemical composition of the samples is inevitable [45-46].

There is a general consensus that analysis of fresh material yields results which come closest to the natural distribution pattern in plants. Since this approach is not feasible in most cases suitable preservation procedures must be employed. Advantages and disadvantages of such procedures with respect to polyphenol analysis have been
reviewed by several authors [47-49]. The procedure keeping the chemical composition as close as possible to the natural state is lyophilization. A study of the extractability of proanthocyanidins in the bark of several pines (Pinus sp.) showed no difference between freshly collected samples and freeze-dried samples. On the other hand drying at room temperature resulted in an increase in extractability in one sample [50]. Increased extractability after drying at room temperature compared to fresh material has also been found for Sorghum seed proanthocyanidins [51]. Using the vanillin assay (see section 1.4.1.2.2), Terrill et al. [52] detected higher (+)-catechin equivalent proanthocyanidin concentrations in freeze-dried Sericea lespedeza forage crop than in fresh frozen, sun cured and oven dried samples. Investigation of procyanidin contents in hawthorn leaves (Crataegus monogyna) using the proanthocyanidin assay (see section 1.4.1.2.1) revealed higher contents in lyophilized samples than in drying conditions reaching from room temperature up to 60 °C [53]. Earlier works by Hagerman [54] demonstrated that the amount of polyphenols extracted from leaves of maple (Acer sp.) and oak (Quercus sp.) depended on the maturity of the leaves. Early in the season, more polyphenols were extracted from lyophilized leaves than from fresh leaves, but late in the season more polyphenols were extracted from fresh leaves. A major problem with freeze dried material is its hygroscopic nature. Since, upon freezing, all cell membranes have been broken, exclusion of moisture during the subsequent storage of the samples is very important in order to avoid degradation reactions [49].

Some authors recommend to freeze the samples as an alternative to lyophilization or analysis of fresh material [48-49]. However, the risk of sample alteration prior to the extraction due to thawing during storage or sample handling should not be underestimated.

Drying at elevated temperatures should be omitted since extractability is reduced due to complexation and polymerization reactions [52]. A shift to higher molecular weight compounds upon drying has also been noticed for procyanidins from coffee pulp (Coffea sp.) [55]. The same alterations occur, if dried samples are stored too long before analysis. In coffee pulp only 46 % of the original content of proanthocyanidins could be detected after one year of storage [56]. Analysis of hawthorn leaves (humidity approximately 7.5 %) after 2 and 3 years of storage at room temperature and under dark conditions revealed an increase of total and polymeric procyanidins, and a decrease of
oligomeric procyanidins. It was concluded that polymerization of oligomers to polymers and the formation of new oligomers from monomers must have occurred [57].

1.3 Extraction

The preparation of an extract basically consists of disintegrating and homogenizing the sample followed by transferring the compounds of interest to a suitable solvent. The first step is especially delicate when dealing with reactive compounds like polyphenols. Conventional milling may lead to an undesirable micro-climate of heat and moisture, which favors polymerization and complexation of procyanidins with matrix components. Reduced particle size therefore does not yield higher extraction rates in any case. In the analysis of beans (*Phaseolus vulgaris*) for example, a significant reduction in assayable polyphenols could be related to the particle size, which in turn is a measure of the degree of grinding and milling [58]. On the other hand "Ultra-Turrax"-type homogenizers will purge the extract solution with air, causing oxidations. Adding an antioxidant to the extraction solvent can be the solution to avoid this detrimental effect. Peng and Jay-Allemand [21] added different antioxidants to the extracting solvent and examined their effect on the polyphenol content in walnut plants (*Juglans regia*) determined with a BSA (bovine serum albumin) precipitation test. An increase of 30 % to 75 % in the protein-precipitating activity was obtained in samples containing ascorbic acid or sodium metabisulfite. For the latter the increase in activity correlated with the concentration of added antioxidant. Sodium metabisulfite has been used in concentrations ranging from 0.5 % to 2 % in the extraction solvents in the analysis of apple fruit (*Malus sylvestris*) [22], grape tissues [59] and barks of European trees [60]. Ascorbic acid is used more frequently than sodium metabisulfite, up to date mainly in the analysis of grapes. Concentrations applied range from 0.1 % to 5 % in the extraction solvents [52,61-67]. In the analysis of hawthorn samples, addition of thioacetamide to the extraction solvent led to an increase of the recovery rate of spiked (-)-epicatechin from 80 % to 97 %. The mechanism involved is thought to be antioxidative and a possible complexation of metal ions or improved solubilization is discussed [68].

It has to be born in mind that total phenol determination methods based on redox reactions (i.e. Folin-Ciocalteu or Prussian blue assay, see section 1.4.1) can not be used anymore after the addition of an antioxidant to the extracting solvent. Another possible
approach to protect procyanidins from oxidation is an initial high-temperature short-time treatment which inactivates polyphenol oxidases among other oxidizing enzymes. So far, this procedure has only been applied in food technology [69]. Since oxidations are radical-mediated, exclusion of light might be advantageous during extraction and subsequent sample work up, too.

Maceration, percolation and turbo-extraction at room temperature or at 4 °C are the most common extraction methods. Sonification for 30 minutes at 4 °C is described for Sorghum grain [48] and walnut [21]. High temperatures during extraction should be omitted in view of the known reactivity of procyanidins. To our knowledge there is only one recent publication in which high temperatures (105 °C) were employed for the extraction of hawthorn tissue [68]. Comparison of the extraction rates of coffee pulp and beans using acidified aqueous acetone at room temperature and under reflux conditions demonstrated that the yields were better under the less severe conditions [56]. Analogous results were obtained in the analysis of hawthorn [70]. Pasterization and sterilization of peach (Prunus persica) and apple juices resulted in a decrease of flavan-3-ol monomers and procyanidins (B2, C1 and a tetramer) to undetectable levels [25]. In apple peel softening has sometimes been induced by initially heating the sample to 30 °C for 10 minutes and then macerating it at room temperature [71-72]. Some authors perform extractions under a nitrogen [73-74] or carbon dioxide [75-76] atmosphere. Extraction times should be optimized to be minimal since gradually decreasing estimates have been reported for the analysis of beans applying extraction times longer than twenty minutes [58].

The solvents commonly used for the extraction of condensed polyphenols are reviewed in several publications [14,47,49]. Goldstein and Swain [77] concluded from the mobility of different extracts on paper chromatograms that anhydrous organic solvents release mainly monomers and low-molecular weight compounds, whereas aqueous organic solvents are also capable of extracting higher molecular weight compounds. The reduced effectivity of pure organic solvents compared to aqueous organic solvents was recently shown for extracts of barley (Hordeum vulgare) [76], forage [52], hawthorn [70] and carob fruit (Ceratonia siliqua) [78]. Kallithraka et al. [79] examined the extraction efficiency for several compounds from grape seeds. Methanol was proven to be the best solvent for the monomers. The largest amount of procyanidin B2 and C1 was extracted with 70 % acetone and 75 % ethanol, while the
highest amount of total phenols was obtained using 70% acetone. We only know of one recent publication in which higher yields are reported in the extraction of grape seeds using pure organic solvents compared to aqueous organic solvents [80].

Polar compounds such as procyanidins are vacuole-bound in living plant cells. During drying and homogenization, the vacuoles distort or disrupt, allowing the formation of strong hydrogen bonds between procyanidins on one hand and proteins, polysaccharides and nucleic acids on the other hand [52]. It is generally accepted that water in the extraction solvent helps to solubilize procyanidins [54, 77-78]. This effect is even enhanced, if the organic component in the extraction solvent is also a hydrogen-bond-breaking agent [81]. Of the commonly used organic solvents for the extraction of procyanidins - methanol, ethanol and acetone - , the latter shows the strongest such effect [47]. Therefore, it is not surprising that higher yields result using aqueous acetone instead of aqueous methanol or ethanol. Recently, this was again demonstrated for forage [52] and carob fruit [78]. Depending on the plant material, 60% (i.e. grape seeds [82]) to 90% (i.e. pomegranate peels (Punica granatum) [83]) aqueous acetone is most suitable, while 70% to 75% aqueous acetone is used by far most often. Moreover, in the analysis of forage crop 70% aqueous acetone yielded the most consistent absorbance readings in the vanillin assay compared to 30% and 50% aqueous acetone [52]. In Sorghum grain using 70% aqueous acetone, 90% of the total extractible phenolic compounds could be obtained with four subsequent extractions [48].

Although aqueous acetone has proven to be the most potent overall extraction solvent for procyanidins, there is some evidence that extraction efficiency also depends on the structure. For example, the highest amount of procyanidin B1 was extracted from grape seeds with water [84]. On the other hand another dimeric procyanidin, A2, is known to precipitate in cold water [85]. What role the specific matrix eventually plays in this context remains obscure.

The only disadvantage of acetone containing extracting solvents is that even traces of acetone will interfere with protein precipitation assays [86]. The advantage of acetone compared to methanol (or ethanol) has been pointed out since the early 1980’s [87] and has been demonstrated for a variety of different matrices in the past twenty years. Astonishingly, aqueous methanol is still used more frequently than aqueous acetone in the extraction of procyanidins.
Some researchers recommend the use of acidified methanol, or more recently acidified acetone [56] and dilute acetic acid [68] as extraction solvents. Acidification is especially recommended for procyanidins which are bound very strongly to polar fibrous matrices, because the increased polarity of the acidic solvent shifts the partition equilibrium of polar solutes towards the solvent medium [88]. Acidification is also useful for hydrolyzable polyphenols [54] and anthocyanins, which are unstable in neutral or alkaline solutions [89]. Because of the lability of the interflavanoid bond towards acids it is very likely that alterations of the compounds occur [47,90]. Increased yields using acidified extracting solvents compared to aqueous organic solvents, recently found for coffee [56], may therefore possibly be due to a partial hydrolysis of polymeric material. Moreover, studies on *Sorghum bicolor* [81], grape skins [88] as well as maple and oak leaves [54] showed no improvement of the extraction yield using acidified organic solvents instead of aqueous acetone.

### 1.3.1 Unextractable procyanidins

Despite of all efforts, procyanidins cannot be extracted exhaustively. Bate-Smith [85] discovered in samples of six species that the specific absorbance values after hydrolysis were always higher in the plant material itself than in the extracts. The same results were found in the analysis of hawthorn [70]. The amount of unextractable compounds depends on the matrix (i.e. up to 97 % of the total proanthocyanidins in the outer bark of European conifers [50]), on the extraction solvent and on the previous history of the samples. Generally, proanthocyanidins with a high molecular weight may strongly bind to cellular components, resulting in a low extractability [52,91]. It is a common knowledge that the degree of polymerization generally increases as proanthocyanidin containing tissues mature [63,92-93]. In this way procyanidin extractability also shows seasonal dependence.

Butler et al. [43] found that the extraction of protein precipitable polyphenols from *Sorghum* seeds was reduced by moistening the seeds before grinding and extraction. The effect was shown to be reversible. In forage legumes the amount of insoluble proanthocyanidins depended on the total content. If the total content was less than 3 mg/g of dry material the greater proportion of the proanthocyanidins was recovered in the insoluble fraction. Conversely, in species with a higher total proanthocyanidin
content most of the proanthocyanidins were soluble [67]. Prodelphinidins are much less extractable than procyanidins [14].

The amount of unextractable procyanidins can be roughly estimated by heating the extracted residue with butanol-hydrochloric acid (proanthocyanidin assay). Generally, red pigment is produced which indicates the presence of cyanidin and/or delphinidin originating from proanthocyanidins bound to tissue. Recently, this assay has been performed with grape skin [65], leaves of Sesbania sesban [94] and forage legumes [67]. The validity of the method has been questioned. Clifford and Ramirez-Martinez [56] found lower contents for proanthocyanidins directly determined in coffee pulp than for the extractable proanthocyanidins. It was concluded that the in-situ hydrolysis method caused significant degradation of the pigments during autoxidation of the polymer, probably due to attacks by electrophilic carbonyls formed from carbohydrates in the acidic environment. In some studies the amount of unextractable proanthocyanidins was directly measured in the residues by the vanillin assay [78] or by thiolysis [50].

Another approach was introduced for the analysis of forage legumes: two subsequent extractions using acetone-water-diethyl ether and boiling sodium dodecyl sulphate containing 2-mercaptoethanol yielded extractable and protein bound proanthocyanidins. Fibre-bound proanthocyanidins were directly determined in the residue using the proanthocyanidin assay [46]. The distinction between protein bound and fibre-bound proanthocyanidins seems to be empirical, whereas added amounts of proanthocyanidins could be accurately predicted with this method. In the analysis of plum (Prunus domestica) proanthocyanidins complexed with proteins and carbohydrates were claimed to be extractable under reflux conditions using 70 % methanol containing 0.1 % hydrochloric acid [95].

Conclusions
One of the most critical steps in the analysis of procyanidins is their extraction. Preservation procedures and storage conditions of the crude samples as well as procedures and conditions during homogenization and extraction have a tremendous impact on the amount and composition of the extractable procyanidins. In view of a good reproducibility a complete standardization of all procedures and experimental conditions from the time of sample collection to the storage of the final extract must be
emphasized. It is recommended to analyze fresh samples. If this is not possible, lyophilization is the drying procedure of choice. Samples should not be stored at all. If this can not be avoided care must be taken to exclude moisture. The best extraction solvent is aqueous acetone in concentrations of approximately 70 % (v/v). The addition of an antioxidant, either ascorbic acid or metabisulfite, is recommended. Extractions should be performed at room temperature or even at lower temperatures.

1.4 Qualitative and quantitative analysis of procyanidins

The methods used for the analysis of procyanidins can be divided into three main categories:

- Conventional methods
- Methods based on biological effects, and
- Chromatographic methods

Conventional methods (i.e. colorimetric assays) are cheap and fast, because generally no tedious sample clean up procedures are necessary. But some disadvantages have to be mentioned. They often lack specificity for individual groups of compounds, reactions rarely follow a stoichiometric order and many variables in the experimental designs make it impossible to collect commensurable data. Therefore, conventional methods represent a compromise between feasibility and accuracy. Several comparative studies have been conducted to illustrate advantages and deficiencies of such assays. Some recent studies were made with wine [96], forage crop [46,67], sainfoin leaves (*Onobrychis vicifolia*) [63] and hawthorn inflorescences [68].

Methods based on measuring biological effects focus on specific physico-chemical features which might be important for a potential biological activity. The interaction between procyanidins and proteins is thought to be responsible for most of the biological, ecological and technological effects. Therefore, it is not surprising that most methods are based on determining polyphenol-protein interactions. The interaction of procyanidins with specific proteins is still rarely studied. The inhibition of elastase has recently been reported for extracts of hawthorn [97] and an anti-complement activity
has been described for pure procyanidins and extracts from *Crataegus sinaica* [35]. But the significance of procyanidins is not only limited to the interaction with proteins. Reactions with other biopolymers such as carbohydrates are possible. Antioxidant properties and the ability to chelate metal ions may as well be involved in their mode of action [47]. There are only a few recent studies in which the radical scavenging or antioxidant properties have been investigated in specific experiments [13,97-101]. So far, these tests have only been used for pharmacological investigations. The determination of the radical scavenging properties, could possibly be used as an alternative to the commonly applied chemical or protein precipitation methods in the analysis of procyanidins.

It must be emphasized that the results obtained with conventional methods or methods based on biological effects for complex mixtures are empirical. Considering the heterogenic effects of the matrices, the structural diversity of the analyzed polyphenolic compounds and the numerous assay designs, it is more adequate to talk of polyphenol "estimates" instead of "contents" [102]. The early users of such protocols, like i.e. Burns [103] (vanillin assay) or Lewak [104] (proanthocyanidin assay) were very much aware of the limitations of these methods. However, reading more recent publications, this knowledge seems to be forgotten or neglected again. In addition, it is very essential to realize, that there does not exist any specific assay for procyanidins. This means that, depending on the specificity of the test and the purity of the sample under analysis, other compounds will influence the results to a varying extent.

Conventional methods and methods based on biological effects are not convenient to monitor changes of individual compounds for example during storage and processing of beverages or during seasonal development of plant tissue. Only chromatographic procedures are specific enough to allow the determination of individual procyanidins. Major drawbacks are the usually extensive sample clean up procedures and compared to conventional methods a higher amount of sample that is necessary. In view of the reactivity and instability of procyanidins it is indispensable that sample work up is as fast and simple as possible to keep the samples as ever possible unaltered.

In the following sections the different analytical approaches to procyanidin analysis will be discussed in detail.
1.4.1 Conventional methods

There exist numerous reviews on this topic [47-49,58,105-108]. We therefore restrict our discussion to some specific aspects. Conventional methods can be divided into three categories:

- Colorimetric assays based on redox or complexation reactions
- Functional group assays, and
- Other methods, i.e. gravimetric or volumetric methods.

Tables 1.1 - 1.3 give an overview on the methods described in the literature. Most of the procedures listed in the first two tables are methods determining total phenolics, whereas tables 1.3A - 1.3C list the functional group assays, which are specific for proanthocyanidins.

Today, the most widely used method for the estimation of total phenolics in plant extracts as well as in foods and beverages is the Folin-Ciocalteu method. Some authors prefer the Prussian blue method, because there are less interferences from non-phenolic compounds (i.e. proteins) [48,108] and calibrations can be performed using a defined standard (Prussian blue) [109]. The major advantage of gravimetric methods over colorimetric procedures is, that they do not rely on the use of a standard. In view of the difficulties in finding appropriate standards for colorimetric assays this fact may not be underestimated. Despite this special feature gravimetric methods are scarcely used nowadays.

Procyanidin contents are commonly determined using the more specific functional group assays. If purified extracts have to be analyzed, procedures determining phenolics in general may nevertheless be quite useful. A distinction between polyphenol and non-polyphenol "contents" can be made by performing a general phenolic assay before and after precipitation with protein or adsorption onto a synthetic polymer like polyamide (Nylon 66) or polyvinylpolypyrrolidion (PVPP) [31-32].

Generally, lipids and chlorophylls have to be removed from the samples by a liquid-liquid extraction step prior to performing a colorimetric assay [2].
Table 1.1  Conventional methods used for the determination of proanthocyanidins: colorimetric procedures.

<table>
<thead>
<tr>
<th>Method</th>
<th>Principle</th>
<th>Comments</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Folin-Denis method</td>
<td>Reduction of complex polymeric ions formed from phosphomolybdic and phosphotungstic heteropoly acids to complex molybdenum-tungsten blue.</td>
<td>detection wavelength: 725 - 770 nm recommended for uniformity: 765 nm complexes and reagent are unstable in alkaline solution formation of precipitates controlled sequence and timing of the addition of reagents (reproducibility!) deviation from Beer-Lambert law (high phenol contents) reaction is stoichiometrically predictable</td>
<td>105,106,110</td>
</tr>
<tr>
<td>Folin-Ciocalteu method</td>
<td>see Folin-Denis.</td>
<td>detection wavelength: 720 nm reagent commercially available alterations: longer heating, addition of HCl and Li₂SO₄ effects compared to Folin-Denis method: no precipitates absorption band is sharper and more symmetrical more sensitive slightly less color with other reductants</td>
<td>47,49,110, 111</td>
</tr>
<tr>
<td>Price-Butler method  = Prussian blue method</td>
<td>Reduction of ferric to ferrous ions followed by formation of the hexacyanoferrate-(II)-chelate (Prussian blue).</td>
<td>detection wavelength: 720 nm complexes are unstable (precipitates), cuvettes become stained timed addition of FeCl₃ (instability!) sensitivity toward flavanols: &lt; 10⁻⁴ M use of Prussian blue standard: number of reduced moles of ferric ions can be calculated</td>
<td>58,109</td>
</tr>
<tr>
<td>Jerumanis method</td>
<td>Oxidation and complexation with ammonium ferric citrate under alkaline conditions.</td>
<td>detection wavelength: 600 nm</td>
<td>106</td>
</tr>
<tr>
<td>Hagerman-Butler method</td>
<td>Complexation with ferric chloride. Original design: after precipitation of tannins with BSA and subsequent resolubilization.</td>
<td>detection wavelength: 510 nm green color with condensed tannins, blue color with hydrolyzable tannins, but color also depends on solvent complexes are unstable original design: monomers co-precipitate</td>
<td>47,116,163</td>
</tr>
<tr>
<td>Reaction with molybdate</td>
<td>Oxidation and complexation with molybdate.</td>
<td>detection wavelength: 420 nm</td>
<td>106</td>
</tr>
</tbody>
</table>
Table 1.2  Conventional methods used for the determination of proanthocyanidins: other procedures.

<table>
<thead>
<tr>
<th>Method</th>
<th>Principle</th>
<th>Comments</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Volumetry</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neubauer-Loewenthal = permanganate method</td>
<td>Direct or indirect (after precipitation of tannins) oxidative titration using permanganate.</td>
<td>poor reproducibility</td>
<td>58,105,106</td>
</tr>
<tr>
<td><strong>Gravimetry</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ytterbium</td>
<td>Precipitation of phenolics with ytterbium acetate.</td>
<td></td>
<td>287</td>
</tr>
<tr>
<td>formaldehyde</td>
<td>Precipitation of proanthocyanidins with formaldehyde in acidic solution via the formation of a methylene bridge (= Stiasny reaction).</td>
<td>addition of phloroglucinol improves precipitation efficiency precipitation is not quantitative flavonoids precipitate, too</td>
<td>47,58,111</td>
</tr>
<tr>
<td>chloroform</td>
<td>Insolubility of proanthocyanidins in chloroform. (i.e. precipitation in ethyl acetate extracts)</td>
<td>poor reproducibility recovery of procyanidins: 70 - 80 %</td>
<td>72,243</td>
</tr>
<tr>
<td><strong>Coupling with diazotized aromatic amines</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-amino benzoic acid</td>
<td>Coupling with diazotized p-amino-benzoic acid.</td>
<td>not anymore used</td>
<td>288</td>
</tr>
<tr>
<td><strong>UV Spectroscopy</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Measurement of the absorbance at 280 nm.</td>
<td>poor specificity</td>
<td>58,106</td>
</tr>
</tbody>
</table>
Table 1.3A Conventional methods specific for proanthocyanidins (functional group assays).

<table>
<thead>
<tr>
<th>Proanthocyanidin assay</th>
<th>(synonym: acid butanol assay, formerly: leucoanthocyanin method)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Principle</td>
<td>Formation of anthocyanidins by autooxidative depolymerization of proanthocyanidins in mixtures of hydrochloric acid and butyl alcohol.</td>
</tr>
<tr>
<td>Features</td>
<td></td>
</tr>
<tr>
<td>In general</td>
<td>formation of anthocyanidin depends on:</td>
</tr>
<tr>
<td></td>
<td>structure</td>
</tr>
<tr>
<td></td>
<td>degree of polymerization</td>
</tr>
<tr>
<td></td>
<td>extent of side reactions</td>
</tr>
<tr>
<td>reaction is also sensitive to:</td>
<td>see text</td>
</tr>
<tr>
<td></td>
<td>presence of transition metals</td>
</tr>
<tr>
<td></td>
<td>presence of oxidants (i.e. oxygen)</td>
</tr>
<tr>
<td></td>
<td>control required!</td>
</tr>
<tr>
<td></td>
<td>optimum: 6% or 10%</td>
</tr>
<tr>
<td></td>
<td>acetone reduces yield</td>
</tr>
<tr>
<td></td>
<td>should be &lt; 20%</td>
</tr>
<tr>
<td></td>
<td>effective; no effect</td>
</tr>
<tr>
<td></td>
<td>89,117,113</td>
</tr>
<tr>
<td></td>
<td>impurities in solvents and reagents</td>
</tr>
<tr>
<td></td>
<td>see text</td>
</tr>
<tr>
<td>detection wavelength:</td>
<td>545 - 555 nm; 550 nm being most often used</td>
</tr>
<tr>
<td></td>
<td>67,107,118</td>
</tr>
<tr>
<td></td>
<td>$\lambda_{max}$ of cyanidin: 547 nm</td>
</tr>
<tr>
<td></td>
<td>85</td>
</tr>
<tr>
<td>Original method [117]</td>
<td>no linear correlation</td>
</tr>
<tr>
<td></td>
<td>reaction time: 40 - 120 [289] minutes</td>
</tr>
<tr>
<td></td>
<td>117,85,117</td>
</tr>
<tr>
<td>Porter's reagent [113]</td>
<td>addition of ferric ions: $\text{NH}_4\text{Fe(SO}_4\text{)_2}$</td>
</tr>
<tr>
<td></td>
<td>better reproducibility</td>
</tr>
<tr>
<td></td>
<td>higher yields (factor three [113])</td>
</tr>
<tr>
<td></td>
<td>linear correlation up to A 0.6 to 0.7 (for B1, trimer, polymer)</td>
</tr>
<tr>
<td></td>
<td>use of ferric ions because they are more stable than ferrous ions</td>
</tr>
<tr>
<td></td>
<td>reaction time: 40 minutes</td>
</tr>
<tr>
<td></td>
<td>113</td>
</tr>
<tr>
<td>Sealbert et al. [121]</td>
<td>addition of ferrous ions: $\text{FeSO}_4$</td>
</tr>
<tr>
<td></td>
<td>better reproducibility</td>
</tr>
<tr>
<td></td>
<td>VIS maximum is more pronounced</td>
</tr>
<tr>
<td></td>
<td>reaction time: exactly 15 minutes</td>
</tr>
<tr>
<td></td>
<td>121</td>
</tr>
<tr>
<td>DAC method [120]</td>
<td>hydrolysis in aqueous ethanol, extraction with butanol</td>
</tr>
<tr>
<td></td>
<td>quantitation of procyanidins in <em>Crataegus</em> fruit</td>
</tr>
</tbody>
</table>
Table 1.3B  Conventional methods specific for proanthocyanidins (functional group assays).

<table>
<thead>
<tr>
<th>Reaction with aldehydes: vanillin assay</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Principle</strong></td>
</tr>
<tr>
<td>Condensation of vanillin (4-hydroxy-3-methoxybenzaldehyde) with phloroglucinol- or resorcinol-type nuclei in acidic solution.</td>
</tr>
<tr>
<td><strong>Features</strong></td>
</tr>
<tr>
<td>In general</td>
</tr>
<tr>
<td>formation of colored carbonium ions depends on:</td>
</tr>
<tr>
<td>structure</td>
</tr>
<tr>
<td>degree of polymerization</td>
</tr>
<tr>
<td>reaction is also sensitive to:</td>
</tr>
<tr>
<td>temperature: rigorous control required! (equilibrate reagents!)</td>
</tr>
<tr>
<td>timing:</td>
</tr>
<tr>
<td>water: content should be the same for samples and standards</td>
</tr>
<tr>
<td>absorbance decreases with increasing water content</td>
</tr>
<tr>
<td>solvent:</td>
</tr>
<tr>
<td>concentration of vanillin (effect temperature dependent)</td>
</tr>
<tr>
<td>quality of solvents and reagents</td>
</tr>
<tr>
<td>vanillin reagent is not stable (prepare daily)</td>
</tr>
<tr>
<td>nature of acid: sulfuric acid (H₂SO₄):</td>
</tr>
<tr>
<td>linear response with catechin [117, 121]</td>
</tr>
<tr>
<td>reaction product more stable [136]</td>
</tr>
<tr>
<td>higher absorbance values</td>
</tr>
<tr>
<td>ascorbates affect sensitivity [136]</td>
</tr>
<tr>
<td>detection wavelength</td>
</tr>
<tr>
<td>500 nm (510 nm for vanillin assay in glacial acetic acid)</td>
</tr>
</tbody>
</table>

| **Vanillin-HCl assay**                   |
| extraction with methanol                |
| conducted in methanol, 0.5 % vanillin, 4 % HCl | 48, 90, 103 |
| **Modified vanillin assay**              |
| extraction with methanol + 1 % HCl      |
| otherwise like vanillin-HCl assay, poorer reproducibility | 58, 290 |
| **Broadhurst and Jones**                 |
| samples in water                        |
| 2.4 % vanillin, 30 % HCl, 60 % methanol  | 136 |
| **Makkar and Becker**                   |
| samples in aqueous acetone or methanol  |
| modified from method of Broadhurst and Jones | 143 |
| **Vanillin assay in glacial acetic acid** |
| samples in glacial acetic acid          |
| conducted in glacial acetic acid, 0.5 % vanillin, 4 % HCl | 133, see text |
| (methanol kept to a minimum)            |
| **Vanillin-H₂SO₄ assay**                 |
| samples in water (methanol kept to a minimum) |
| reagent: 1 % vanillin in 70 % sulfuric acid | 83, 117, 121 |
Table 1.3C Conventional methods specific for proanthocyanidins (functional group assays).

<table>
<thead>
<tr>
<th>Principle</th>
<th>Reaction with aldehydes: dimethylaminocinnamaldehyde assay</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Features</strong></td>
<td><strong>References</strong></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
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<tr>
<td></td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

Protocols used for:

- **Beer, barley and hops**: 0.1 % DMACA in 25 % concentrated hydrochloric acid in methanol 147,148,291
- **Wine and grape tissues**: 0.1 % DMACA in concentrated hydrochloric acid : methanol = 1 : 99 (= 0.12 N) 152
  1.0 % DMACA in 12 N hydrochloric acid : methanol = 1 : 9 149
- **Forage legumes**: 2.0 % DMACA in 6 N hydrochloric acid : methanol = 1 : 1 67
  protocol originally developed for wine [152] 63
1.4.1.1 Colorimetric assays based on redox and complexation reactions

The color yield of assays based on redox reactions depends on the redox potential of the phenolic groups [48]. For example, applying the Prussian blue method a sevenfold difference in response between quercetin and hydroquinone has been determined [109]. The reactions of phenols with the Folin-Denis and the Folin-Ciocalteu reagents have been found to be stoichiometrically predictable [110-111]. Phloroglucinol reacts as a monophenol, whereas catechol reacts as a diphenol and therefore, yields double the molar color of phloroglucinol. The molar absorbance of oligomeric polyphenols approximate the sum of their constituent monomeric components [110]. In general, vicinal triphenols (pyrogallol-type) are oxidized very easily. Ortho-diphenols (catechol-type) are as well oxidizable, whereas mono- and meta-phenols (phloroglucinol-type) and their methoxylated derivatives are less susceptible to oxidation [106].

A major drawback of redox-based assays is that any oxidizable compound in the sample solution will react, too. Ascorbic acid, sulphur dioxide (in wines), aromatic amines and carbohydrates (production of endiol reductones in alkaline solution) are the most frequently encountered reductants [47,106].

Metal complexing assays (i.e. Jerumanis method) are generally more specific than redox-based assays, because the color of the complexes depends on a specific pattern of substitution on the phenolic rings [108]. Procyanidins form chelates with metal ions via their ortho-diphenolic groups on the B-rings [112].

1.4.1.2 Functional group assays

The following functional group assays are used: proanthocyanidin assay, vanillin assay and dimethylaminocinnamaldehyde (DMACA) assay. Details to the experimental designs are listed in Tables 1.3A - 1.3C.

1.4.1.2.1 Proanthocyanidin assay

Acid degradation is widely used to identify proanthocyanidins. In solutions of mineral acids proanthocyanidins are cleaved to form carbocations from their "upper units" and flavan-3-ols from their "lower unit". The carbocations are immediately converted to anthocyanidins by autoxidation [113]. In the case of procyanidins, red-colored cyanidin
is produced (see Figure 1.4). Orange-red colors are formed with propelargonidins, while mauve, purple and blue colors are generally formed with prodelphinidins [89]. Under appropriate conditions this reaction can be intercepted by toluene-α-thiol to produce flavanyl-4-thioethers which are widely used in the structural elucidation of proanthocyanidins [11]. The monomeric flavan-3-ols may only be detected at the beginning of the reaction [11] or, if mildly acidic conditions are applied [11,114-115]. Under the conditions used in the proanthocyanidin assay the monomers form ill-defined yellow-brown products which may influence absorbance at the detection wavelength [11,116-118].

The theoretical yield for a dimer is 50 %, but for procyanidin B2, for example, only 30 % [11,104] or determined by HPLC (high-performance liquid chromatography) 13 % [118], respectively, could be obtained. Procyanidins with a higher degree of polymerization produce more anthocyanin than dimers, because more "upper units" exist which can be converted into carbocations [113]. For a procyanidin polymer with an average degree of polymerization of 9.4 a cyanidin yield of 58 % is reported [113].

Side reactions, which lead to the formation of yet unidentified polymeric material with $\lambda_{\text{max}}$ 450 nm, commonly known as phlobaphenes, are the reason for the relatively low yields [113,117]. Some attempts have been made to characterize these pigments. Analysis of the hydrolysis products of a procyanidin polymer, using gel permeation chromatography, revealed four additional peaks absorbing between 400 nm and 540 nm, besides cyanidin [119]. HPLC analysis of the degradation products of procyanidin B2 and detection at 550 nm showed that in addition to cyanidin as the major product other pigments were formed, too [118]. It was concluded that pigment destruction or transformation had taken place. Experiments on hawthorn fruit, conducted in our laboratory according to the guidelines of the German Drug Codex (DAC) [120] followed by HPLC analysis confirm this assumption1. Side reactions which are due to matrix effects are also described by Scalbert et al. [121]. Some compounds from wood extracts containing conjugated aromatic rings led to

---

1 The oligomeric procyanidin content of samples using the DAC protocol averaged 0.89 % (triplicate determinations). HPLC analyses of samples revealed only trace amounts of cyanidin although solutions were intensely red-colored (contents in the plant material calculated as cyanidin amounted to 0.0015 %). Addition of cyanidin to the extract solutions (spike levels: 2.5 and 5 times detected concentrations) and subsequent conductance of the assay led to recoveries of 48 % and 85 %, respectively. HPLC conditions: Hypersil ODS, 5 μm, 100 x 4.0 mm; 70 % methanol in 0.5 % aqueous ortho-phosphoric acid; flow rate: 1.0 ml/min; temperature: 25.0 °C; injection volume: 5 μl; detection: 540 nm and 280 nm; retention time of cyanidin: 4.2 minutes.
Figure 1.4 Reaction sequence of procyanidin B1 in the proanthocyanidin assay.
ill-defined absorbances at 550 nm, if the reaction mixtures were incubated longer than 15 minutes. In coffee pulp – but not in extracts – the in-situ hydrolysis caused a significant degradation of the pigment [56]. In white wine, Singleton and Trousdale [122] demonstrated that polymeric polyphenols complexed quickly with added anthocyanins. The linkages were proposed to be covalent.

Proanthocyanidin structure influences the yield and kinetics of the reaction. The stability of the interflavanoid bond depends on the nature of the monomer units. 5-deoxyproanthocyanidins such as *Quebracho* polyphenols give substantially less color than procyanidin polymers such as *Sorghum* polyphenols [48]. Prodelphinidins give higher yields than procyanidins [85,116]. In proanthocyanidins the ratio of prodelphinidin to procyanidin will influence the wavelength of the maximum absorbance [85]. In B-type procyanidins 4β→8 configured dimers give higher yields than 4β→6 linked dimers, probably due to different reaction kinetics [113]. The same conclusions were drawn by Hemingway and Mc Graw [123] who analyzed the kinetics of acid-catalyzed cleavage of procyanidins in the presence of toluene-α-thiol and acetic acid. Furthermore, they found that the conversion of proanthocyanidins with "upper units" having a 2,3-cis configuration is faster compared to proanthocyanidins having a 2,3-trans configuration [123]. B-type procyanidins react much faster than A-type procyanidins [11,85]. The degradation of A-type procyanidins to cyanidin seems to be a minor pathway, whereas the major product of the acid-catalyzed cleavage is an anthocyanidin pigment with λ_{max} 535 nm [124].

It has been recommended to add transition metal ions, which are known to accelerate autoxidations, to increase reproducibility and color yield of the reaction [113]. For the first time, this effect was described in the analysis of beer by Mc Farlane [125], who noticed that usually – but not always – an increase in cyanidin yields could be obtained with ferrous and copper ions added to the reaction mixture. Later, the increase in yields, using different plant extracts, was shown not to be constant [126]. In forage the addition of iron salts generally resulted in higher but much more variable contents [46]. In the analysis of plant tissues from hawthorn the addition of ferric ions did not improve results [127]. Scalbert et al. [121] compared the original proanthocyanidin method to the Porter's reagent (see Table 1.3A) which uses ferric ions and a new method which uses ferrous ions. For wood extracts no important differences in
absorbances were found. However, they found significant differences in the shapes of the spectra, the maximum absorbance around 550 nm being most pronounced with ferrous ions. The maximum absorbance was less pronounced using the conventional proanthocyanidin assay and decreased to a shoulder with ferric ions. Based on these results a new protocol with ferrous ions was proposed. Unlike the widely applied Porter's reagent, so far, there is only very little experience with the use of ferrous ions [116].

The use of blank samples, prepared by replacing the acid component by water, is generally recommended. Preparing unheated blanks is delicate, because some compounds may also react in the cold [49] and anthocyanidins are red in acidic solutions [58]. Furthermore, this approach is only an approximation for chlorophyll containing samples because chlorophyll is partially destroyed during heating and therefore, unheated blank values tend to be too high [48]. Some researchers recommend a pre-fractionation step for pigmented samples [128].

Analytical results will depend upon the standard used for the preparation of the calibration plot. If cyanidin is used as a standard, values will be underestimated because only a certain percentage of red pigment absorbing at 550 nm is produced. Purified proanthocyanidins isolated from the matrices under analysis are therefore the most suitable standards for the proanthocyanidin assay. The problem with this approach is, that defined oligomeric proanthocyanidins are not commercially available. On the other hand, the purity of polymeric proanthocyanidins which can be purchased (i.e. Quebracho polyphenols) is often unknown and a constant quality is not guaranteed. Moreover, some proanthocyanidin polymers produce a biphasic calibration curve [49] and the color yield of some others have been reported to decrease during storage [113]. Nevertheless, there are some studies described in the literature in which quantitation was performed using purified proanthocyanidins as a standard. For the analysis of coffee pulp Quebracho polyphenols were used [129] and purified sainfoin proanthocyanidins were taken to generate a calibration plot for the analysis of forage legumes [67].

Calibration factors are most often used for the quantitation of results. For the original method (see Table 1.3A) a specific absorbance A1% of 150 is proposed, if the degree of polymerization of the procyanidins under analysis is not known [130]. For the
Porter's reagent (see Table 1.3A) the use of a specific absorbance A of 270 for dimers and 470 for higher oligomers has become common practice [55,129]. These values are averages of measured absorbance data of purified procyanidin standards originally determined by Porter et al. [113]. In pharmaceutical standardization procedures "plant-specific" calibration factors are also used [120]. These factors are determined for a specified plant extract and may only be used for quantitation of samples of this same matrix [131].

Tables 1.4A and 1.4B show a comparison of absorbance data obtained with the proanthocyanidin assay. Values for purified procyanidins as well as for extracted and unextracted plant tissues containing only procyanidins are listed. The data clearly demonstrate that calibration factors not only depend on experimental variables but also on the matrix. The use of calibration factors does therefore hardly produce true estimates for contents in plant extracts.

The only correct quantitation of results from the proanthocyanidin assay is to use purified procyanidin fractions from the matrix under analysis. Calibrations should be prepared in the extracts themselves to correct for matrix effects as much as possible. A similar approach has been used by Li et al. [67] who produced a calibration curve with purified sainfoin proanthocyanidins in a lucerne (Medicago sativa) blank for the quantitation of proanthocyanidins in forage legumes.

1.4.1.2.2 Vanillin assay

The reactivity of nucleophilic sites of phloroglucinol nucleis with aldehydes has been known for a long time. Using formaldehyde a condensation product is formed which precipitates and can be determined gravimetrically [111]. Colorimetric procedures using substituted benzaldehydes are more popular. The most often used substituted benzaldehyde is vanillin (4-hydroxy-3-methoxybenzaldehyde). Protocols based on dimethylaminocinnamaldehyde have gained interest in the last few years, whereas 2,4-dimethoxybenzaldehyde [132] – although more sensitive than vanillin [133] – is not anymore used.

In strong acidic solutions aldehydes are protonated at their carbonyloxygen, thus forming electrophilic carbocations. In substituted benzaldehydes the electrophility is reduced by the delocalization of the positive charge. Therefore, reactions only occur
Table 1.4A  Absorbance data of cyanidin and procyanidins using the proanthocyanidin assay.

<table>
<thead>
<tr>
<th>Compound</th>
<th>A$^{1%}$</th>
<th>$\varepsilon$ (l mol$^{-1}$cm$^{-1}$)</th>
<th>Solvent</th>
<th>Comments</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>cyanidin chloride</td>
<td>1027</td>
<td>33160</td>
<td>BuOH/HCl</td>
<td></td>
<td>292</td>
</tr>
<tr>
<td>cyanidin</td>
<td>1053</td>
<td>30240</td>
<td>BuOH/HCl</td>
<td></td>
<td>own results$^a$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>34700</td>
<td>EtOH/0.1% HCl</td>
<td></td>
<td>292</td>
</tr>
<tr>
<td>B2</td>
<td>90 - 106</td>
<td></td>
<td>BuOH/5%HCl</td>
<td></td>
<td>130</td>
</tr>
<tr>
<td>B1, B3, B4 (mixture)</td>
<td>136</td>
<td></td>
<td>BuOH/5%HCl</td>
<td></td>
<td>85</td>
</tr>
<tr>
<td>A-type procyanidins</td>
<td>~ 600</td>
<td></td>
<td>BuOH/5%HCl</td>
<td></td>
<td>130</td>
</tr>
<tr>
<td>trimers</td>
<td>170</td>
<td></td>
<td>BuOH/5%HCl</td>
<td></td>
<td>293</td>
</tr>
<tr>
<td>tetramers</td>
<td>140 - 180</td>
<td></td>
<td>BuOH/5%HCl</td>
<td></td>
<td>293</td>
</tr>
<tr>
<td>higher oligomers</td>
<td>200</td>
<td></td>
<td>BuOH/5%HCl</td>
<td></td>
<td>130</td>
</tr>
<tr>
<td>oligomeric procyanidins</td>
<td>43</td>
<td></td>
<td>BuOH/5%HCl</td>
<td></td>
<td>127,145</td>
</tr>
<tr>
<td>polymeric procyanidins</td>
<td>172</td>
<td></td>
<td>BuOH/5%HCl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>procyanidins</td>
<td>243</td>
<td></td>
<td>n.m.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B1</td>
<td>270</td>
<td></td>
<td>BuOH/5%HCl ferric ions added</td>
<td></td>
<td>113</td>
</tr>
<tr>
<td>B3</td>
<td>280</td>
<td></td>
<td>BuOH/5%HCl ferric ions added</td>
<td></td>
<td>113</td>
</tr>
<tr>
<td>B7</td>
<td>225</td>
<td></td>
<td>BuOH/5%HCl ferric ions added</td>
<td></td>
<td>113</td>
</tr>
<tr>
<td>trimer III</td>
<td>345</td>
<td></td>
<td>BuOH/5%HCl ferric ions added</td>
<td>9.4 - 12.5 units</td>
<td>113</td>
</tr>
<tr>
<td>polymeric procyanidins</td>
<td>450 - 490</td>
<td></td>
<td>BuOH/5%HCl ferric ions added</td>
<td></td>
<td>113</td>
</tr>
</tbody>
</table>

Abbreviations: A$^{1\%}$ = specific absorbance (coefficient of absorbance for a 1 % (w/v) solution); $\varepsilon$ = molar coefficient of absorbance; HCl = hydrochloric acid; BuOH = 1-butanol; EtOH = ethanol; n.m. = not mentioned. Trivial nomenclature of dimers and trimers, see Abbreviations for procyanidins.

a) Reference standard: cyanidin chloride (Roth AG, Germany; Rotichrom® HPLC), regression plot determined using eight standard solutions in the concentration range of 0.178 to 22.8 μg/ml (solvent composition according to literature [120]); correlation coefficient: 1.0.

Table 1.4B  Absorbance data in the proanthocyanidin assay for extracts and unextracted plant tissues containing exclusively procyanidins.

<table>
<thead>
<tr>
<th>Matrix</th>
<th>A$^{1%}$ determined in extracts$^b$</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malus sylvestris</td>
<td>7.9</td>
<td>85</td>
</tr>
<tr>
<td>Aesculus hippocastanum</td>
<td>53</td>
<td>85</td>
</tr>
<tr>
<td>Vaccinium myrtillus</td>
<td>16.8</td>
<td>85</td>
</tr>
<tr>
<td>Vaccinium vitis-idaea</td>
<td>26</td>
<td>85</td>
</tr>
<tr>
<td>Persea gratissima</td>
<td>9.5</td>
<td>85</td>
</tr>
<tr>
<td>Rhododendron ponticum</td>
<td>11</td>
<td>85</td>
</tr>
</tbody>
</table>

b) Extracts were prepared in 50 % aqueous methanol.
with phenolic compounds which show a phloroglucinol- or resorcinol-type oxygenation pattern [77,134]. The substituents may be hydroxy- or alkoxy groups, whereas electron-withdrawing groups (i.e. carbonyls) considerably reduce reactivity [134]. Figure 1.5 shows the sequence of the condensation reaction for the two most commonly used aldehydes: vanillin and dimethylaminocinnamaldehyde.

Sarkar and Howarth [135] investigated the structural prerequisites for the vanillin reaction in 70 % sulphuric acid in more detail. They found that the reaction is limited to flavanoids with free meta-oriented A-ring hydroxyl groups, a single C2-C3 bond, no carbonyl function at C4 and a free hydroxy group at C7. In this way, the reaction is largely specific for a narrow range of flavanols. Quebracho polyphenols, which are frequently used as a standard in other conventional methods, only produces little color [48]. Hydrolyzable polyphenols do not give a reaction [47]. However, some electron-rich heteroaromatics (i.e. pyrrole or indole) [134], dihydrochalcones (i.e. phloretin, phloridzin) [135] and some phloroglucinol based phlorotannins [116] will give a positive reaction. Dihydrochalcones may be removed by a diethyl ether extraction, which will also reduce monomer contents [121,136]. Anthocyanins may substantially interfere with the vanillin assay and a correction must be made by subtracting a suitable blank [58,108,135-136]. Chlorophyll may also affect color-development [137].

The condensation reaction may take place at position 6 or 8. Position 6 of the flavan skeleton is favored because it is usually less sterically hindered than position 8. In contrast to the proanthocyanidin assay the condensation reaction runs without depolymerization of the proanthocyanidins [135]. Consequently monomers also react [116] and a multitude of different dyes are produced depending on the complexity of the sample. The maxima of the chromophores of the resulting dyes are not influenced by the B-ring hydroxylation pattern (i.e. procyanidin-prodelphinidin ratio) like in the proanthocyanidin assay [116]. Absorbances are however influenced by the substitution pattern on the ring at which condensation takes place. Condensation products of phloroglucinol nucleis show absorbance maxima at about 500 nm, whereas resorcinol and pyrogallol nucleis exhibit absorbance maxima around 520 nm [77].

Differences in molar absorption coefficients occur: i.e. catechins give higher ε-values than their epimers [138]. While reactions seem to run in an approximately stoichiometric manner for simple compounds [135], chromophore production is more complex with higher oligomers and polymers. Although absorbance values are
Figure 1.5  Condensation reaction of (-)-epicatechin with vanillin and $p$-dimethylaminocinnamaldehyde.

Examples of possible condensation products are shown.
generally higher for procyanidins than for the monomers [48], reactions occur neither at all flavan-3-ol subunits nor only at the terminal units. Therefore, using a conventional assay (with hydrochloric or sulphuric acid), absorbance values can not be correlated either on a weight or on a molar basis [133]. Recently, it was suggested that this non-stoichiometric behavior may be caused by a partial depolymerization of proanthocyanidins in strong acidic vanillin reagents [96]. Butler et al. [133] developed a procedure in glacial acetic acid, in which the chromophore production is proportional to the number of reacting molecules. Under the experimental conditions applied, only flavan-3-ol end groups were accessible to the condensation reaction with vanillin. Absorbance data for the condensation products of monomers and specified procyanidins using various assay designs can be found in the literature [96,116,133,135-136,138,139-142].

The reaction kinetic of the vanillin assay depends on the chemical structure. For (+)-catechin the reaction was terminated after 7 minutes, whereas absorbances of wood extracts still increased after 100 minutes of reaction time [121]. Other researchers came to the same conclusion [90]. A kinetic study with purified proanthocyanidins showed that the initial rate of reaction is fast and that afterwards the rate decreases steadily with time [83].

Conventional assays in hydrochloric acid work best, if moisture is rigorously excluded. Since procyanidins are most effectively extracted with aqueous organic solvents, this is a major drawback. For this reason Makkar and Becker [143] developed a procedure which also allows the performance of the vanillin assay in aqueous methanol or acetone. The use of aqueous acetone is delicate, since it is known that acetone reacts with acidified vanillin to produce a chromogen exhibiting a $\lambda_{max}$ at 548 nm which may interfere determinations substantially [143].

Although, during the last twenty years numerous attempts have been made to improve vanillin assays with respect to reproducibility and accuracy, resulting in a series of different protocols, adaptions are still necessary for certain matrices [144].

For convenience, (+)-catechin rather than purified procyanidins is used to standardize the assay, although this leads to a considerable overestimation. For example, the use of (+)-catechin instead of purified proanthocyanidins as a standard for the analysis of *Sericea lespedeza* forage crop approximately doubled proanthocyanidin estimations in the samples [52].
Vanillin assays are more sensitive than the proanthocyanidin assay. In extracts of hawthorn up to five times higher values were obtained [145], whereas ten to twenty times higher absorbances were measured in wood extracts [121]. In the analysis of seventeen plant extracts considerable variations were found for the two assays, too [116]. Addition of purified proanthocyanidins to forage samples revealed good agreement between actual and measured values with the proanthocyanidin assay, whereas the vanillin assay generally overestimated the contents [46]. All these results once again show that the estimates not only depend on the methods used, but also on the matrices. Unlike the proanthocyanidin assay the vanillin reaction critically depends on timing and temperature. Therefore, the conductance of the vanillin assay is technically more difficult [49,116].

1.4.1.2.3 Dimethylaminocinnamaldehyde assay

The use of dimethylaminocinnamaldehyde (DMACA) as a reagent for the colorimetric determination of flavanols was first described by Thies and Fischer [146]. The first protocol was developed by Mc Murrough and Mc Dowell [147] for purified extracts of barley and hops (Humulus lupulus). The reaction scheme is the same as for the vanillin reaction (see Figure 1.5). The reactivity of proanthocyanidins essentially again resides in the 5,7-hydroxylation of the aromatic A-ring, a single bond between C2 and C3 and the lack of a carbonyl function at C4 [107]. Unlike the classical vanillin reagent, the DMACA reagent only reacts with terminal units, since molar absorbance values for monomers, dimers and trimers are of the same magnitude [96,147]. The following coefficients of absorbance (ε) were measured: procyanidin B2 (17'000), procyanidin B3 (16'000), procyanidin C1 (20'700), procyanidin C2 (19'000) and (+)-catechin (16'000) [96]. Other researchers reported that the absorbances depend on the stereochemistry at C2 and C3 [148-149]. In contrast to the vanillin reaction, compounds with a 2,3-cis-configuration show a greater reactivity in the DMACA assay than 2,3-trans-configurated compounds. In this way (-)-epicatechin yields a higher color response than (+)-catechin. The same findings were described for dimers: procyanidin B4 and B2, respectively, showed higher absorbance values than the all-trans procyanidin dimer B3 [148, 150].
Like in the vanillin assay, dihydrochalcones (phloretin, phloridzin) [147], indol, phloroglucinol, resorcinol among other compounds [150] may also form condensation products with the DMACA reagent absorbing at the detection wavelength of 640 nm. Indole shows a similar sensitivity like (-)-epicatechin towards DMACA. All other substrates show a very low sensitivity, which is one to several orders of magnitude lower than the one of (-)-epicatechin [151]. Other researchers found an approximately four times higher sensitivity for proanthocyanidins compared to indole on a molar basis [67]. Aromatic amino acids [44] and some flavonoids having a carbonyl function at C4 (naringin, naringenin) [151] also show very week reactions. Taking all these evidences into account it seems as if the DMACA assay is more specific than the vanillin assay [67,152].

The major advantage of the DMACA assay compared to the vanillin assay is its higher sensitivity [96,150]. In the analysis of forage legumes the DMACA reagent was about five times more sensitive than the vanillin reagent [67]. Individual compounds exhibit a different response to the two aldehyde reagents as has been demonstrated for a series of monomers, dimers and trimers [150]. Another advantage of the DMACA assay is that it is easier to perform than the vanillin assay, because the experiments can be run at room temperature without the need for a rigorous temperature control. The DMACA protocols are also much less affected by interferences from pigments (i.e. anthocyanidins) [151]. In the literature contradictory results are reported for the stability of the resulting dyes. Some authors note that the condensation products with DMACA are not as sensitive to light as the ones produced by vanillin [2], whereas others claim that the degradation of reaction products is more pronounced with DMACA than with vanillin [140].

(+)-Catechin is generally used as a standard in the DMACA assay. Calibration plots are linear and therefore, calibration factors are also applied [148]. Like in all conventional methods the use of purified procyanidins as a standard will give the correctest estimates. A blank, composed of the samples without the chromogen reagent, is also recommended. In the protocol for the analysis of forage legumes Li et al. [67] suggest a proanthocyanandin-free blank prepared from lucerne leaves. The calibration plot – using the lucern blank and added amounts of purified sainfoin proanthocyanidins – showed a linear correlation between absorbances and proanthocyanidin concentrations.
Unfortunately, there exist a variety of different protocols. None of these protocols is generally accepted as an universal method. The assay of Delcour and Janssens de Varebeke [148] has been adopted by the European Brewery Convention Analysis Committee as an official method, but a new protocol has recently been published for the analysis of beer [31]. To our knowledge, one of the most carefully optimized DMACA protocols at this time is the one of Li et al. [67] for the analysis of forage legumes.

In the past few years considerable attention has also been given to the use of the DMACA reagent as a histochemical stain in the analysis of procyanidin containing cells [67,153] and as a post-column derivatization reagent in HPLC analysis of procyanidins (see section 1.4.3.2.2) [151,154].

1.4.1.2.4 Conclusions
A general problem of all colorimetric assays is their lack of reproducibility between samples, days and laboratories. Moreover, a comparison of the results is hampered by numerous assay designs, different matrices and a variety of different polyphenol structures. Therefore, standardization of procedures is very important and should not only be limited to the assay itself, but also to the sample preparation procedures (drying and extraction). To correct for variations caused by reagents and chemicals (i.e. different batches) or by different reaction conditions several replicates (i.e. four [67]) should be analyzed and a standard sample should also be included in each set of samples.

A major drawback of all functional group assays is that a satisfactory standard does not exist. For a given sample the most appropriate standard is a purified procyanidin fraction prepared from the same matrix. The isolation and characterization of such purified fractions are laborious. Added to that procyanidins undergo oxidation, complexation and self-polymerization very easily, rendering such purified fractions only reproducible to a limited degree. At least in the proanthocyanidin assay the color reaction depends not only on the polyphenols themselves, but also on the matrix. The use of specified proanthocyanidins as a standard in a suitable blank matrix is an attempt to correct for such effects [67].
For convenience, commercially available standards (i.e. (+)-catechin) are used most often in the aldehyde condensation reactions. Some variations may still occur due to differences between standards from different suppliers. Results should be expressed as standard "equivalents", since molar reactivities of the different polyphenols vary (in the DMACA assay a molar reactivity has been demonstrated up to a degree of polymerization of three).

The use of calibration factors is even more problematic than the use of self-generated calibration graphs, because differences in experimental conditions are not considered. If calibration factors are used at all, it should in any case be specified how these factors have been generated.

Several attempts have been made to obtain a more complete picture of the phenolic composition of a sample. For this purpose some researchers propose the conductance of several complementary assays [106]. More popular is the formation of ratios which are claimed to correlate with the relative degree of polymerization. Considering the complex reaction scheme of these assays, interpretations of such ratios must be performed very carefully. In our opinion, some of them more likely reflect the specific proanthocyanidin structures than actual relative degrees of polymerization. In the last few years the following ratios have been described in the literature: dimethylaminocinnamaldehyde / proanthocyanidin ratio for wine and grape tissue [149], vanillin / dimethylaminocinnamaldehyde ratio for wine and purified standards [96] and proanthocyanidin / vanillin ratio for plums [95] as well as purified proanthocyanidins from various plant sources [155].

1.4.2 Methods based on biological effects: protein precipitation

Numerous reviews describe this type of polyphenol assay [47-48,86,108]. Again, we will only focus on some general aspects and refer the reader to the review articles for more detailed information on specific test procedures.

Precipitation reactions of polyphenols with proteins depend on the concentration and structure of the compounds and proteins involved, the pH in the reaction mixture as well as on the presence of modifiers (genuine in the extracts or reagents) [47-48]. Studies on the precipitation reaction in solution showed that the affinities of proteins to polyphenols varies over several orders of magnitude. High affinity was found for
conformationally loose, proline-rich proteins which are strong hydrogen bond acceptors [156]. Bovine serum albumine (BSA) is currently the most often used protein for precipitation assays.

The binding mechanism is based on hydrogen bonding and hydrophobic interaction [157-158]. An involvement of electrostatic attraction is unlikely in the case of procyanidins because their phenolic hydroxyls ionize only at a very alkaline pH. Associated with oxidation covalent binding may occur [43,86]. The precipitation reaction is only possible, if a minimum number of hydroxyl groups per molecule are present to enable sufficient cross linkages. The affinity of procyanidins to proteins was found to be proportional to the number of ortho-diphenol groups and increased with the number of 4ß→6 linkages and the number of galloylated substituents [159]. Polymers are therefore precipitated first, whereas oligomers may remain in solution [160-161]. But, soluble polyphenol-protein complexes may also form with high molecular weight polyphenols which is evidenced by the fact that threshold concentrations of polyphenols are necessary to effect precipitation [86]. The minimal degree of polymerization to cause denaturation of proteins is claimed to be six [162]. Monomers and low molecular weight polyphenols should therefore not react, but they may be co-precipitated with higher molecular weight compounds [163]. All these results suggest that protein precipitation assays are less suitable, if oligomeric procyanidins have to be estimated.

Some attempts have been made to correlate the results of conventional chemical assays with the results of assays based on the interaction with proteins. In sainfoin leaves at different development stages similar values were obtained for the proanthocyanidin-, DMACA-assay and a BSA microtiter test [63]. Nelson et al. [155] evaluated the Prussian blue-, proanthocyanidin-, Vanillin- and BSA radial diffusion assays in predicting effects of Desmodium polyphenols on several species of ruminal bacteria. Although working with purified proanthocyanidins, the Prussian blue assay showed the best correlation with the antimicrobial activity. Surprisingly, the BSA radial diffusion assay did not correlate well with the proanthocyanidins' antimicrobial activity. This demonstrates that the interpretation of results from protein precipitation assays in terms of biological activity or ecological effectivity has to be done with great care.
1.4.3 Chromatographic methods
1.4.3.1 Sample clean up

For the qualitative and quantitative analysis of complex matrices sample clean up is necessary to produce sufficiently resolved chromatograms. In the analysis of procyanidins the primary goal of sample preparation is to separate oligomeric procyanidins from lipids and interfering phenolic compounds, but also from polymeric procyanidins which cannot be separated chromatographically and produce an unwanted underground in chromatograms (see section 1.4.3.2.2). Knowledge of the physico-chemical features of the analytes is very important to choose an appropriate sample preparation procedure.

Procyanidins are water-soluble polyphenols independent of their molecular weight [87]. Naturally occurring procyanidin polymers show highly irregular structures, with 4→8 as well as 4→6 bondings and branch points. Branched, globular rather than linear, thread-like structures therefore predominate [164]. Helical structures have only been reported for homopolymers of (+)-catechin and (-)-epicatechin [165]. In aqueous solutions procyanidins tend to form associates via hydrogen bonds but do not necessarily precipitate. It is assumed that smaller oligomers may also form such reversible complexes with themselves or with larger molecular weight forms, especially in concentrated and aged solutions [166]. Problems regarding solubility are thought to be primarily due to proteins and carbohydrates to which procyanidins can bind in aqueous solutions [87]. Recently, colloidal behavior which was attributed to hydrophobic forces was reported for (+)-catechin polymers formed in wine-like model solutions containing acetaldehyde. Colloidal intermediates were first formed, which in a next step coagulated until, at a critical aggregation concentration, precipitation began [167]. Keeping this in mind, the development of suitable sample clean up procedures for procyanidins is rather difficult: on one hand they are hydrophilic, reflected in the ability to form both inter- and intra-molecular hydrogen bonds and on the other hand they may exhibit considerable hydrophobicity. Moreover, solubility always depends on the matrix which may be involved in precipitation or co-solvatization processes (i.e. the flavonoid quercetin, which is insoluble in water and only slightly soluble in ethanol may be detected in large quantities in some wines [168]).
1.4.3.1.1 Liquid-liquid extraction

The goal of liquid-liquid extraction can be twofold: the removal of lipids and chlorophylls on the one hand and the extraction of procyanidins from a crude extract or a beverage on the other.

Removal of lipids and chlorophylls

A variety of different solvents are used to remove lipids and chlorophylls. Among these are:

- Petroleum ether for grape seeds [62,169] and apples [22]
- n-Hexane for grape seeds [64], apples [170] and barley [171]
- Diethyl ether for leaves of Eucalyptus sp. [139] and seeds of lentil (Lens culinaris) [172]
- Dichloromethane for hawthorn tissue [173], and
- Iso-octane for beer [174].

Diethyl ether is also frequently used to delipidate extracts which are subsequently analyzed by conventional methods [46,55,67,175]. It has been reported that extraction using diethyl ether allows the quantitative removal of monomers from acidified aqueous wood extracts, while dimeric proanthocyanidins remain in the aqueous phase [121]. Recovery studies of (-)-epicatechin and (+)-catechin in synthetic wine solutions only yielded 24 % and 46 %, respectively, of the added amounts. Procyanidin B2, however, could not be detected in the lipophilic layer. Analogous results were obtained using white wine and extracts of apple pulp [176]. We did not succeed either in eliminating (-)-epicatechin from extracts of hawthorn herb using diethyl ether. Moreover, in an isolation scale experiment using n-hexane and diethyl ether we detected in HPLC chromatograms in both organic fractions significant amounts of compounds showing typical procyanidin UV-spectra (unpublished data). As an alternative we tested iso-octane as delipidating solvent for aqueous acetone extracts of hawthorn herb. Triplicate determinations revealed losses of (-)-epicatechin and procyanidin C1 below 0.2 % and losses of procyanidin B2 and B5 below 0.5 % of the
There are a few methods described in the literature which, after extraction with a lipophilic solvent and evaporation or lyophilization, respectively of the aqueous layer, directly proceed to HPLC analysis. Such a procedure is reported for the analysis of leaves of *Eucalyptus sp.* [139], hawthorn tissue [173] and grape seeds [62,64].

**Liquid-liquid extraction of procyanidins**

Ethyl acetate is almost exclusively used for the extraction of procyanidins from aqueous solutions. Sometimes, crude extracts are directly partitioned using ethyl acetate, without prior elimination of lipids and pigments. This approach seems to be especially feasible in the analysis of beverages like wine [177], beer [74,178] and apple juice [179-180]. But also crude extracts of other matrices have been directly extracted using ethyl acetate i.e. extracts from callus cultures of buckwheat (*Fagopyrum esculentum*) [114], apple tissue [181-182] and leaves and fruit of *Visnea mocanera* [183].

Since the early 1980's it is already known that ethyl acetate is not a good extraction solvent for procyanidins. Using HPLC, Jende-Strid and Møller [184] found less than 50% of the proanthocyanidin contents in barely grains after extraction with ethyl acetate compared to only defatted crude extracts. They concluded that there was a specific loss of the more polar proanthocyanidins which could not be quantitatively extracted from the aqueous phase into ethyl acetate. Kirby and Wheeler [185] noticed in the analysis of beer that repetitive extractions with ethyl acetate resulted in a better recovery of the tetramers. These findings were interpreted as a problem of low partition coefficient. Lea et al. [186-187] reported for a variety of procyanidins approximations for partition

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2 Preparation of extracts: 15 g of *Crataegus* herb were percolated with 250 ml 70 % aqueous acetone (column dimensions: \( \varnothing \) 2 x 25 cm). Acetone was evaporated below 30 °C and the aqueous layer was extracted four times with 50 ml iso-octane and four times with 50 ml ethyl acetate (both solvents presaturated with water). Combined organic phases were evaporated below 30 °C, dissolved in 3.0 ml 50 % aqueous methanol, filtered over a 0.45 μm filter and analyzed by HPLC. The water phases were lyophilized, dissolved in 10.0 ml 50 % aqueous methanol, filtered over a 0.45 μl filter and analyzed by HPLC.

HPLC conditions: LiChrosorb RP-18, 5 μm, 250 x 4.0 mm; gradient elution, A = methanol, B = 0.5 % aqueous ortho-phosphoric acid, 0 min 82 % B in A, 10 min 76 % B in A, 20 min 76 % B in A, 45 min 60 % B in A, 45.01 min 0 % B, 50 min 0 % B; flow: 1.0 ml/min; temperature: 24 °C; injection volume: 5 - 20 μl; detection: UV / DAD 280 nm. Quantitation was performed using an external calibration graph of (-)-epicatechin. Total contents were calculated as the sum of the contents of the individual extracts (iso-octane, ethyl acetate, water phases). Interpretation of data for procyanidins B2 and B5 in the water phases was not possible due to masking of the relevant peaks by matrix components.
coefficients between ethyl acetate and water, derived from distribution features in counter-current chromatography. Monomers and procyanidin B5 showed values around 4.5 while other dimers exhibited values between 0.4 and 0.9 (while partition coefficients increased in the series: procyanidin B1 < B2, B3 < B4). The partition coefficients decreased further with increasing degree of polymerization up to the hexamers which only showed a value of 0.08.

Recovery studies for (+)-catechin and (-)-epicatechin in synthetic wine solutions performed by HPLC revealed differences between these stereoisomers: 79 % (-)-epicatechin was recovered compared to 97 % (+)-catechin [176]. We evaluated the use of ethyl acetate as extraction solvent in the analysis of extracts of hawthorn herb. Four subsequent extractions were performed and determinations were carried out in triplicate. From the total contents only 82 % (-)-epicatechin and 43 % procyanidin C1 could be recovered in the ethyl acetate extracts. The proportions of procyanidins B2 and B5 in the ethyl acetate extracts could not be determined because the peaks in the water phases were superimposed by matrix components. LC-MS (liquid chromatography – mass spectrometry) analysis of the water phases however proved the presence of procyanidins B2, B5 and C1 besides other dimeric, trimeric and tetrameric procyanidins (see Figure 1.6).3

If procyanidins are to be analyzed quantitatively, ethyl acetate extractions should be omitted because every procyanidin exhibits a specific partition coefficient. For the qualitative analysis of procyanidins one should be aware that procyanidins of lower degree of polymerization are extracted more easily into ethyl acetate than procyanidins of higher degree of polymerization. Therefore, ethyl acetate fractions have already been termed as "dimer rich", whereas ethyl acetate insoluble fractions have been referred to as "oligomer rich" [55]. In any case, the number of extractions as well as the phase volumes should be standardized in order to get reproducible results. Some researchers advocate the use of methylethylketone instead of ethyl acetate because extractability is supposed to be better [188-189].

3 Experimental conditions: see previous footnote.
HPLC conditions for LC-MS analysis: LiChrosorb ODS 2, 5 μm, 250 x 4.0 mm; gradient elution: A = 24.5 % methanol, 75 % water, 0.5 % acetic acid; B = 44.5 % methanol, 55 % water, 0.5 % acetic acid; C = 99.5 % methanol, 0.5 % acetic acid; 0 - 90 min 100 % A, 110 min 100 % B, 170 min 100 % B, 170.1 min 100 % C, 190 min 100 % C; flow: 0.2 ml/min; temperature: ambient; injection volume: 20 μl.
Detection: mass spectrometry; SSQ detector (Finnigan MAT); interface: ESI; mode: negative ion; scan rate: 15 sec; capillary: 200 °C; spray: 1.5 kV; auxiliary gas: position 10.
Figure 1.6 Extraction of procyanidins using ethyl acetate.

Top: Chromatogram of ethyl acetate extract using UV/DAD detection at 280 nm.
Middle: Chromatogram of water phase after ethyl acetate extraction using UV/DAD detection at 280 nm.
Bottom: Chromatogram of water phase after ethyl acetate extraction using LC-MS detection.

- m/z: 289 = monomers
- m/z: 577 = dimeric procyanidins
- m/z: 865 = trimeric procyanidins
- m/z: 1153 = tetrameric procyanidins

RIC = total ion current; abscissa: scans (scan rate: 15 sec).

1 = (-)-epicatechin; 2 = procyanidin B2; 3 = procyanidin B5; 4 = procyanidin Cl; 5 = epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin.

Experimental: see text.
To separate neutral phenolics from phenolic acids in wine [190] and apple juice [179-180], an extraction with ethyl acetate at fixed pH values of seven and two has been proposed. Extraction efficiencies determined with standard solutions of (+)-catechin, (-)-epicatechin and procyanidin A2 were higher than 90 %, however, recoveries of procyanidin B2, B3 and B4 amounted only to 30 to 40 % [190].

1.4.3.1.2 Solid phase extraction
Solid phase extraction is the method of choice for sample clean up in the analysis of procyanidins. Using this technology procyanidins are selectively and reversibly bound to a solid stationary phase. Because of their highly polar nature and their ability to form multiple hydrogen bonds to almost any other polar surface quantitative desorption of procyanidins can be a major problem.

Three sorbents are almost exclusively used: C18 (octadecylsilane), polyamide and Sephadex LH-20. Beverages – after removal of ethanol – and crude extracts are applied to the cartridges or columns, respectively, without prior removal of lipids and chlorophylls in a liquid-liquid extraction step.

C18
Table 1.5 gives an overview on the methods described in the literature. Sep-Pak® cartridges from Waters are almost exclusively used, although Suárez et al. [66] reported that the use of cartridges from Lida yielded better results. The loadability is a major problem. For the C18 sorbent from Lida a loadability of 8 mg of total phenolics per gram of sorbent is reported [66]. Using Sep-Pak® cartridges from Waters 0.5 ml to 5 ml wine or juices, respectively, can be applied (500 mg sorbent) [191-192]. Two cartridges in series have been frequently used in order to increase the applied sample amount [75,193-195]. Lately, larger cartridges with 900 mg (Long Body Sep-Pak Plus® by Waters) [196] and 1000 mg (Lida) [66,197] sorbent have been reported, too.

Acidic phenolics are generally eliminated by washing with water of pH 7.0. The volume seems to be critical and monomers may also partially be eluted. In the analysis of wine small proportions of chlorogenic acid [198] or gallic acid [195], respectively, always remained in the neutral fraction. When using immiscible solvents careful drying of the cartridges with nitrogen is required [61]. Some validation experiments have been
Table 1.5  Solid phase extraction using C18.

<table>
<thead>
<tr>
<th>Matrix</th>
<th>References</th>
<th>Washing solvent</th>
<th>eluted compounds</th>
<th>Elution solvent (in water)</th>
<th>eluted compounds</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>wine grape tissue</td>
<td>193,195,294</td>
<td>water pH 7.0</td>
<td>acidic phenolics</td>
<td>EtOAc water pH 2.0</td>
<td>cats, OPC</td>
<td>two cartridges in series (last three eluents not always used)</td>
</tr>
<tr>
<td></td>
<td>75,194</td>
<td>dry with nitrogen</td>
<td></td>
<td>ACN 16%, pH 2.0</td>
<td>PPC, anthocyanins</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>MeOH</td>
<td>PPC, anthocyanins</td>
<td></td>
</tr>
<tr>
<td>grape tissue wine, juices</td>
<td>61 266</td>
<td>0.01 N HCl</td>
<td></td>
<td>EtOAc MeOH / HCl</td>
<td>phenolics</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>dry with nitrogen</td>
<td></td>
<td></td>
<td>anthocyanins</td>
<td></td>
</tr>
<tr>
<td>wine white wine grape must</td>
<td>191 196,242</td>
<td>water pH 7.0</td>
<td>acidic phenolics</td>
<td>ACN 16%, pH 2.0</td>
<td>cats, PC</td>
<td>eluted wash fraction is cleaned up over a second cartridge equilibrated at pH 2.0 (additional analysis of phenolic acids) slight modifications in some protocols</td>
</tr>
<tr>
<td>grape tissue</td>
<td>267 295</td>
<td>dry with nitrogen</td>
<td></td>
<td>EtOAc MeOH</td>
<td>flavonols</td>
<td></td>
</tr>
<tr>
<td>grape juice grape juice</td>
<td>199 192</td>
<td>water pH 7.0</td>
<td>acidic phenolics</td>
<td>MeOH</td>
<td>polymers</td>
<td></td>
</tr>
<tr>
<td>apple juice / cider</td>
<td>66 197</td>
<td>water pH 7.0</td>
<td>acidic phenolics</td>
<td>MeOH</td>
<td>anthocyanins</td>
<td></td>
</tr>
<tr>
<td>apple juice / tissue</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tea brews</td>
<td>297</td>
<td></td>
<td></td>
<td>ACN 15%</td>
<td>PC</td>
<td></td>
</tr>
<tr>
<td>sainfoin leaves</td>
<td>63</td>
<td>1 % AcOH</td>
<td></td>
<td>70% MeOH</td>
<td>PPC</td>
<td>OPC removed with EtOAc (discarded!) use of Adsorbex RP-18 (Merck) analysis as phloroglucinol adducts</td>
</tr>
<tr>
<td>wine, grape juice wine, grape tissue</td>
<td>273 272</td>
<td>water 1M NH₄OH</td>
<td>sugars, organic acids phenolic acids</td>
<td>MeOH</td>
<td>phenolics, anthocyanins</td>
<td>only qualitative analysis</td>
</tr>
</tbody>
</table>

Abbreviations: EtOAc = ethyl acetate; ACN = acetonitrile; MeOH = methanol; HCl = hydrochloric acid; AcOH = acetic acid; NH₄OH = ammonium hydroxide; cats = monomers; OPC = oligomeric procyanidins; PPC = polymeric procyanidins; PC = procyanidins.
performed for five protocols [61,191,199]. The ones for apple must / cider [66] and wine [193] were conducted with a broad range of procyanidin standards.

Recently, Romani et al. [200] published a sophisticated solid phase extraction procedure for the qualitative analysis of grape skins. In a first extraction on Extrelut®20 (kieselguhr, Merck) polyphenols are separated from anthocyanins. The polyphenols are subsequently fractionated on a C18 cartridge (Bond Elut®, Varian) into phenolic acids and esters, procyanidins, flavonol glycosides and acylated anthocyanins.

Generally, it is recommended to use two different sorbents for clean up and subsequent analysis to take advantage of different selectivities. The use of C18 cartridges in sample clean up procedures followed by analytical HPLC on the same stationary phase material is therefore probably not the best choice. However, the availability of C18 in pre-packed cartridges is a major advantage compared to the other sorbents that are used. The packing of columns is usually too slow to be included in routine analysis and gives rise to additional variabilities between laboratories.

Polyamide
Polyamide was first used for the quantitative determination of procyanidins in various fruits and vegetables by Hanefeld and Herrmann [201]. Hydrogen bonds are responsible for the adsorption of procyanidins on polyamide resins. Compounds which are able to form multiple hydrogen bonds (i.e. polymers) show a strong bonding potential and may displace or exclude components with a lower bonding potential [202]. Procyanidins are adsorbed stronger to polyamide than to C18 material. This is reflected in the necessity to use strong hydrogen-bondbreaking solvents like acetone or N,N-dimethylformamide as eluents. Some phenolic compounds cannot be desorbed even with such strong solvents [59,203]. Columns (generally: Ø 2 x 25 cm) are individually packed using almost exclusively polyamide TLC6 quality from Macherey-Nagel. There exists only one study in which pre-packed cartridges (Chromabond® PA 1000 mg, Macherey-Nagel) are applied in the analysis of beer, barley and hops [203]. Using 1.2 g of polyamide (TLC6, Macherey-Nagel) 2 ml to 5 ml of wine can be applied [204]. Most studies are performed according to the publication of Ricardo da Silva et al. [204], containing a detailed description of the procedure as well as validation data for a variety of procyanidins determined in wine and grape tissue. Table 1.6 gives an overview on the conditions and methods described in the literature.
Table 1.6  Solid phase extraction using polyamide and Sephadex LH-20.

<table>
<thead>
<tr>
<th>Matrix</th>
<th>References</th>
<th>Washing solvent</th>
<th>eluted compounds</th>
<th>Elution solvent (in water)</th>
<th>eluted compounds</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Polyamide</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>wine, grape tissue</td>
<td>204,238,244, 268,298</td>
<td>water pH 7.0</td>
<td>phenolic acids</td>
<td>ACN 30 %</td>
<td>monomers PC</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>acetone 75 %</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>299</td>
<td>water pH 2.5</td>
<td>pigments</td>
<td>MeOH 30 % pH 2.5</td>
<td>phenolics</td>
<td></td>
</tr>
<tr>
<td>beer, barley, hops wine</td>
<td>32,189</td>
<td>water</td>
<td>phenolic acids</td>
<td>acetone 75 %</td>
<td>phenolics</td>
<td></td>
</tr>
<tr>
<td></td>
<td>243</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>column dimensions: Ø 1 x 12 cm [32]</td>
</tr>
<tr>
<td></td>
<td>245</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>use of Nylon 66 (Imperial Chemicals) [32]</td>
</tr>
<tr>
<td>beer, barley, hops</td>
<td>203,269</td>
<td>1 % AcOH</td>
<td></td>
<td>MeOH DMF</td>
<td>phenolics</td>
<td></td>
</tr>
<tr>
<td>white wine</td>
<td>32</td>
<td>water</td>
<td>phenolic acids</td>
<td>MeOH</td>
<td>phenolics</td>
<td>60 % of total phenolics eluted</td>
</tr>
<tr>
<td></td>
<td>49</td>
<td></td>
<td></td>
<td>MeOH +2 % AcOH DMF</td>
<td>monomers PA</td>
<td>85 % of total phenolics eluted</td>
</tr>
<tr>
<td>green tea</td>
<td>282</td>
<td>-</td>
<td></td>
<td>MeOH</td>
<td>chlorogenic acid, PA</td>
<td>only qualitative analysis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>MeOH</td>
<td></td>
<td>column dimensions: Ø 2 x 15 cm</td>
</tr>
<tr>
<td><strong>Sephadex LH-20</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>grape juice</td>
<td>209</td>
<td>MeOH 20 %</td>
<td>sugars, phenolic acids, some monomers</td>
<td>MeOH</td>
<td>PC</td>
<td>column dimensions: Ø 0.8 x 4 cm 10 ml juice applied</td>
</tr>
<tr>
<td>apple juice</td>
<td>60</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>grape tissue</td>
<td>59</td>
<td>-</td>
<td></td>
<td>MeOH 60 %</td>
<td>non-polymeric PP</td>
<td>column dimensions: Ø 0.5 x 15 cm samples should be dissolved in water (max. 10 % MeOH)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+ 0.2 % AcOH acetone 50 % + 0.2 % AcOH</td>
<td>polymeric PP</td>
<td>only qualitative analysis</td>
</tr>
<tr>
<td>hawthorn tissue</td>
<td>227</td>
<td>water</td>
<td></td>
<td>MeOH: acetone = 1:1</td>
<td>PC</td>
<td>column dimensions: Ø 2 cm</td>
</tr>
</tbody>
</table>

Abbreviations: ACN = acetonitrile; MeOH = methanol; DMF = N,N-dimethylformamide; AcOH = acetic acid; EtOH = ethanol; PC = procyanidins; PA = proanthocyanidins; PP = polyphenols; LLE = liquid-liquid extraction; SPE = solid phase extraction.
Considering the fact that polyamide has been completely replaced by gel chromatography sorbents like Sephadex LH-20 in isolation procedures for procyanidins, it is more than likely that this development will also take place in the field of sample clean up.

**Sephadex LH-20**

Sephadex LH-20 (Pharmacia) is a sorbent for gel chromatography and is widely used in isolation protocols for proanthocyanidins. It is composed of dextran molecules which are three-dimensionally cross-linked with hydroxypropyl groups [205]. Proanthocyanidins are separated by filtration (molecule sieve effect of the gel) and adsorption (hydrogen bonding). Retention increases primarily with increasing (!) molecular weight and C4→C6 linked procyanidins are more retarded than their C4→C8 linked counterparts [206-207]. Sephadex LH-20 has first been used for sample clean up in the analysis of apple ciders by Lea and Timberlake [208]. So far, no pre-packed columns exist on the market. All methods described in the literature work with different column dimensions (see Table 1.6). Standardization would help to make results more comparable. Some validation procedures have been performed for two protocols [185,209].

**1.4.3.1.3 Other sample preparation techniques**

In some qualitative analyses the insolubility of polyphenols in chloroform is used to precipitate them from crude [189] and ethyl acetate extracts [210] or to separate chloroform soluble compounds from dry extracts [71-72,211]. There is some evidence that this is not a quantitative procedure because oligomeric procyanidins have been detected and analyzed after chloroform extraction in cacao beans (Theobroma cacao) and grape seeds [82].

Separation of the acetone and water phase in crude extracts has sometimes been achieved by saturation of the aqueous layer with sodium chloride [82,210,212]. Only qualitative analyses have been performed using this technique. In our opinion, there exists a certain risk to precipitate procyanidins by dehydration.
1.4.3.1.4 Conclusions

The main criterion for a successful extraction is that the procedure is exhaustive, or, if this is not possible, that it is perfectly reproducible [168]. It seems as if this prerequisite is not fully accomplished with neither of the two main sample preparation procedures. Liquid-liquid extraction using ethyl acetate works very well with flavan-3-ol monomers [190,197]. This might be the reason why this sample clean up procedure is still widely used in procyanidin analysis. However, present knowledge suggests that solid phase extraction yields better results than liquid-liquid extraction in sample clean up procedures. In the analysis of apple musts and ciders Picinelli et al. [197] compared ethyl acetate extraction (water phase at pH 7.0) and solid phase extraction using C18 (washing: water pH 7.0, elution: methanol). In the ethyl acetate extracts they could only detect 49 % procyanidin B1, 70 % procyanidin B2, 75 % procyanidin B5, 34 % procyanidin C1 and 21 % of a tetrameric procyanidin compared to the amounts obtained by solid phase extraction. Furthermore, repeatability was better when solid phase extraction was used. On the other hand Escribano-Bailon et al. [65] did not succeed in separating flavan-3-ols from matrix components of grape tissue using either polyamide, Sephadex LH-20 or C18 cartridges alone. They finally chose a combination of C18 and Sephadex LH-20, but they registered losses of flavan-3-ols between 7 % and 20 % during purification. The degree of loss seemed to be random.

Validation data, which are mostly unsatisfactory and / or incomplete, seem to confirm these findings. Table 1.7 gives an overview on the validation experiments performed for various protocols. In our opinion the method which is most completely validated and also yields good results is the one of Ricardo da Silva et al. [204]. The performance of this protocol is surely not trivial since columns have to be packed individually and loading as well as washing of the columns must be done with great care. Reproducibility and ruggedness may therefore be a problem. The best approach is probably still to keep sample clean up procedures to a minimum.
Table 1.7  Validation of methods.

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Ref.</th>
<th>Sample Prep.</th>
<th>Repeatability coef. of variation</th>
<th>Accuracy percent recovery</th>
<th># levels</th>
<th>height</th>
<th>Comments</th>
<th>Compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td>wine</td>
<td>204</td>
<td>SPE polyamide</td>
<td>2.5 - 5.2 % (10 rep)</td>
<td>94.0 - 101.0 % (4 rep)</td>
<td>1</td>
<td>1 - 7</td>
<td>detection threshold determined, too</td>
<td>B1, B2, B3, B4</td>
</tr>
<tr>
<td>wine</td>
<td>193</td>
<td>SPE C18</td>
<td>“good“ (10 rep)</td>
<td>92.0 - 100.6 % (n.m.)</td>
<td>n.m.</td>
<td>1</td>
<td>(synthetic wine sol.)</td>
<td>B2, B3, B4, C1, trimers III</td>
</tr>
<tr>
<td>wine</td>
<td>190</td>
<td>LLE EtOAc</td>
<td>11 - 23 % (10 rep)</td>
<td>78 - 121 % (n.m.)</td>
<td>3</td>
<td>0.5 - 7</td>
<td>recovery of model sol. determined, too</td>
<td>B2, B3, B4, A2</td>
</tr>
<tr>
<td>wine</td>
<td>191</td>
<td>SPE C18</td>
<td>n.d.</td>
<td>85 % (synthetic wine sol.)</td>
<td>n.m.</td>
<td>n.m.</td>
<td></td>
<td>B3</td>
</tr>
<tr>
<td>grape juice</td>
<td>209</td>
<td>SPE Sephadex</td>
<td>2.7 - 6.9 % (3-4 rep)</td>
<td>n.d.</td>
<td></td>
<td></td>
<td>only standard solution</td>
<td>B1, B2, B3, B4</td>
</tr>
<tr>
<td>grape tissue</td>
<td>61</td>
<td>SPE C18</td>
<td>2.0 - 2.7 % (5 rep)</td>
<td>86.2 - 90.7 % (5 rep)</td>
<td>2</td>
<td>0.5, 1</td>
<td></td>
<td>B3</td>
</tr>
<tr>
<td>grape tissue</td>
<td>199</td>
<td>SPE C18</td>
<td>n.d.</td>
<td>104.2 - 118.0 % (n.m.)</td>
<td>1</td>
<td>1.5 - 2</td>
<td></td>
<td>B2, B3</td>
</tr>
<tr>
<td>apple cider, must</td>
<td>66</td>
<td>SPE C18</td>
<td>&lt; 5 % (2 rep)</td>
<td>84 - 110 % (2 rep)</td>
<td>n.m.</td>
<td>n.m.</td>
<td></td>
<td>n.m.</td>
</tr>
<tr>
<td>apple juice</td>
<td>180</td>
<td>LLE EtOAc</td>
<td>n.d.</td>
<td>93 - 106 % (3 rep)</td>
<td>3</td>
<td>n.m.</td>
<td>only monomers ?</td>
<td>n.m.</td>
</tr>
<tr>
<td>beer</td>
<td>73</td>
<td>none</td>
<td>3.3 % (3 rep)</td>
<td>n.d.</td>
<td></td>
<td></td>
<td></td>
<td>B3</td>
</tr>
<tr>
<td>beer</td>
<td>32</td>
<td>SPE polyamide</td>
<td>n.d.</td>
<td>78 % (3 rep)</td>
<td>n.m.</td>
<td>n.m.</td>
<td>only standard solution</td>
<td>B3</td>
</tr>
<tr>
<td>beer, barley, hops</td>
<td>189</td>
<td>SPE polyamide</td>
<td>5 - 6 % (2 rep)</td>
<td>n.d.</td>
<td></td>
<td></td>
<td>CHCl₃ precipitation prior to SPE</td>
<td>B3, C2</td>
</tr>
</tbody>
</table>

Procyanidin derivatives (i.e. galloylated procyanidins) and flavan-3-ol monomers are not mentioned. Trivial nomenclature of dimers and trimers, see list of Abbreviations for procyanidins. Abbreviations: SPE = solid phase extraction; LLE = liquid-liquid extraction; C18 = octadecylsilane; EtOAc = ethyl acetate; CHCl₃ = chloroform; rep = replicates; n.m. = not mentioned; n.d. = not determined; height = height of spike level in relation to detected sample concentrations; sol. = solution.
The crucial question for the final chromatographic analysis is, which procyanidins are to be expected in a given matrix. Naturally occurring procyanidins predominantly consist of either (-)-epicatechin or (+)-catechin. In the (-)-epicatechin series procyanidins B2, B5, C1 and an (-)-epicatechin tetramer co-occur (i.e. in *Crataegus* sp.), while principal components in the (+)-catechin series are procyanidins B3, B6, C2 and a (+)-catechin tetramer (i.e. in *Salix* sp.). Predominance of procyanidins B1 (i.e. in *Rubus* sp.) or B4 (i.e. in *Sorghum* sp.) is relatively seldom in the plant kingdom. The main procyanidins are always accompanied by the respective monomers and various minor oligomers [1]. Enantiomeric procyanidins are relatively rare but are described, too [213-217].

There is no final consensus on whether procyanidin biosynthesis is controlled thermodynamically or enzymatically. In either case proanthocyanidins are synthesized through sequential addition of flavan-3,4-diol units (in their reactive forms as carbocations or quinone methides) to a flavan-3-ol monomer [218]. Based on the latest findings there is some evidence that different condensation enzymes might exist which are specific for each type of flavan-3,4-diol [64] and that polymer synthesis would be subject to a very complex regulatory mechanism [63]. But so far, no enzyme synthetase systems have been isolated and enzymatic conversion of flavanols to proanthocyanidins could not be demonstrated in vitro [219]. If biosynthesis was thermodynamically controlled, the variation in proanthocyanidin composition could be explained by synthesis at different times or in different compartments [64]. The hypothesis of a thermodynamically controlled biosynthesis is based on the fact that naturally and chemically synthesized procyanidin dimers occur as a mixture of 4->8 and 4->6 linked isomers in approximate ratios of 3-4 : 1 [220]. Porter [164] found analogous ratios of 4->8 and 4->6 linkages in proanthocyanidin polymers.

Whether biosynthesis is enzymatically or thermodynamically driven, naturally occurring procyanidins in either case display a multitude of structural diversity of various degrees of polymerization, which is simply beyond the separating capacity of any chromatographic system. Nevertheless, all classical chromatographic procedures have been applied to procyanidin analysis. Some reviews on the chromatography of procyanidins are also available [26,107,221-222].
1.4.3.2.1 Gas chromatography and thin layer chromatography

Gas chromatography has been successfully applied only to the analysis of dimers in beer [223]. In order to improve volatilities derivatization is inevitable; trimethylsilyl [223] and trifluoroacetyl [224] derivatives are described in the literature. Drawbacks of gas chromatography are that higher molecular weight oligomers are less volatile, that the resolution in chromatograms is not improved compared to HPLC chromatograms using detection at 280 nm and that the derivatization in complex matrices may not be quantitative or reproducible [202,225].

For qualitative analysis Lea's thin layer chromatographic method [186,226] on silica using the TAF-solvent (toluene / acetone / formic acid) is well established. It is possible to separate monomers and procyanidins according to their molecular weights up to the hexameric [186] or heptameric [80] level, respectively. Stereoisomers are scarcely distinguishable [226].

To our knowledge only two densitometric protocols for the analysis of procyanidins exist. Vanhaelen and Vanhaelen-Fastre [227] developed purified extracts from hawthorn on silica and detected procyanidins using vanillin reagent. The coefficient of variation for repeatability experiments was reported to be smaller than 6 %. Procyanidins were quantified as (-)-epicatechin and for this monomer a linear correlation was obtained in the range of 1 to 14 μg. However, in view of the complex reaction mechanism of the vanillin reaction the accuracy of quantitation in terms of monomer equivalents should be demonstrated. Furthermore, higher molecular weight procyanidins overlap [227] and a considerable background may hamper detection [68]. We also tried this method in our laboratory and made the same experiences. The thin layer chromatograms could only be analyzed with great difficulties because of extensive streaking of higher molecular weight compounds on the silica gel (unpublished data).

Kappenberg [68] derivatized sample solutions of inflorescenses of hawthorn and lime (Tilia sp.), among other species, with dansylchloride (5-dimethylamino-1-naphthalinsulfonyl chloride) prior to thin layer chromatography on silica. Detection is based on the measurement of fluorescence at 365 nm. Under the applied experimental conditions phenolic acids and flavonoid glycosides remained at the start, whereas derivatives of B-type procyanidin dimers were developed as a single spot. The sensitivity was reported to be ten times higher compared to the vanillin condensation.
products. Drawbacks of this method are that it is sensitive to light and that so far, no studies on reaction kinetics of individual procyanidins have been performed.

1.4.3.2.2 High-performance liquid chromatography
HPLC is most commonly used in the analysis of procyanidins. Normal-phase HPLC allows the separation of procyanidins according to their degree of polymerization. It is only used for qualitative analysis. Silica is predominantly chosen as stationary phase [28,82,92]. The procyanidins are eluted in order of increasing molecular weight. Generally, a considerable baseline drift occurs. Resolution up to the pentamers in cacao [82] and heptamers in apple juice (using a cyano-column) [228] has been reported. Under the chromatographic conditions applied by Rigaud et al. [82] flavan-3-ol monomers and some dimers are resolved as discrete peaks.

Reversed-phase HPLC
Individual procyanidins can be separated on reversed-phase HPLC. So far, resolution is only possible up to the tetrameric level [64,66,180,193,209]. The reasons why higher molecular weight procyanidins can not be detected are manifold: the peak capacity of stationary phases is exploited in complex samples, the concentrations of individual compounds decrease because the number of possible isomers increases with increasing degree of polymerization (provided that biosynthesis is mainly thermodynamically controlled) and the sensitivity of the detection is not sufficient. Unresolved humps or applying long linear gradients at least raises or a drift of the baseline are therefore typical of chromatograms of procyanidin samples. Chromatograms of ethyl acetate extracts generally look nicer, because solubility of higher oligomers and polymers is relatively low in this solvent [79].

Several researchers have investigated the nature and features of these characteristic humps in chromatograms of procyanidin samples. Guyot et al. [229] showed that they disappeared after thiolysis, which indicates that they consist of polymeric proanthocyanidins. By incorporating a sharp change in a non-linear gradient, Kantz and Singleton [59] eluted polymers of grape tissue extracts as an envelope peak. From the peak area of this envelope peak they estimated the percentage of polymeric polyphenols in the sample. The values obtained correlated well with the ones measured with the
Folin-Ciocalteu assay for total phenols. Lea [230] demonstrated a similar chromatographic behavior for oxidized procyanidins. He concluded that oxidized procyanidins did not elute with defined $k'$ values (capacity factor) under isocratic or continuous linear gradient conditions but were spread out over the whole area of the chromatogram. Putman and Butler [160] noticed that the elution profile of oligomeric procyanidins changed from a broad band to a series of discrete peaks after switching the linear gradient to a step gradient. In analogy to the chromatographic behavior of proteins they proposed that a fast increase in concentration of organic solvent in the mobile phase causes a conformational change or an aggregation of procyanidins which leads to a rapid desorption. Figure 1.7 gives an example to the chromatographic behavior of higher molecular weight procyanidins.

Low molecular weight procyanidins, which can be chromatographed as individual peaks on reversed-phase material, do not elute according to their degree of polymerization. Factors which influence retention time are: stereochemistry, substitution on ring B, molecular weight and the overall polarity. Pyrogallol-type proanthocyanidins elute faster than catechol-type proanthocyanidins due to their increased polarity [231]. (-)-Epicatechin and its oligomers are retarded more than (+)-catechin derivatives [154,166,231-232]. Procyanidins with $4\rightarrow 6$ linkages as well as doubly linked procyanidins show longer retention times compared to the $4\rightarrow 8$ linked counterparts [64,154]. Dimers and trimers with a terminal (+)-catechin unit are eluted earlier than analogously linked procyanidins with a terminal (-)-epicatechin unit [8,64]. The elution order of the dimeric and the most widespread trimeric procyanidins (C1, C2) is surprisingly constant. It is largely unchanged by the type of eluent, acid and stationary phase that are normally used. The retention times of procyanidins B1, B4 and C1 are slightly more affected by changes in the chromatographic system than the ones of the other common procyanidins.

Several researchers analyzed the chromatographic behavior of individual procyanidins. Stafford and Lester [166] generated plots of log $k'$ values of monomers up to trimers vs. increasing amounts of methanol in an acetic acid containing aqueous eluent. They noticed that the slopes (known as "S-value" [233]) were parallel to each other in the (+)-catechin series and in mixed dimers, whereas the lines of the monomer, dimer and trimer in the $4\beta\rightarrow 8$ linked (-)-epicatechin series became
Figure 1.7  Chromatographic behavior of higher molecular weight procyanidins.

Example: Fraction of an ethyl acetate extract of Crataegus herb, twice cleaned up over Sephadex LH-20 (gradient of methanol – water – aqueous acetone and ethanol isocratic, respectively).

A: Elution as a sharp peak (t_R = 42.1 min) upon a fast change in solvent strength.
HPLC conditions: LiChrosorb RP-18, 5 μm, 250 x 4.0 mm; gradient elution, A = methanol, B = 0.5 % aqueous ortho-phosphoric acid, 0 min 80 % B in A, 35 min 65 % B in A, 40 min 65 % B in A, 40.01 min 10 % B in A, 45 min 10 % B in A; flow: 1.0 ml/min; temperature: 24 °C; injection volume: 6 μl; concentration: 4.86 mg/ml.

B: UV spectrum of peak at 42 min.

C: Elution as a broad peak using a linear gradient.
HPLC conditions: see A; gradient elution, 0 min 65 % B in A, 30 min 0 % B in A.
increasingly steeper. This can be interpreted in the way that the distance between members of the (-)-epicatechin series in the chromatograms increases with decreasing amounts of organic phase in the eluent. Lea [230] found a slightly concave relationship in the semi-log plots of \(k'\) vs. percent methanol for oligomeric procyanidins, which is indicative for an unusual chromatographic behavior. Furthermore, S-values of procyanidins were found to be relatively high. Normally, S-values of small compounds range from three [230] to five [234], whereas the ones of procyanidin oligomers show an average value of eight on C8 and C6 sorbents [230]. For procyanidin B2 an S-value of 7.4 is reported on a C18 stationary phase [234]. Compounds exhibiting high S-values are far more affected by small changes of chromatographic parameters than compounds showing low S-values. Therefore, the use of shallow gradients is recommended for the analysis of compounds with high S-values [234].

The chromatographic behavior of procyanidins is also affected by the concentration of injected sample solutions. When increasing amounts of a procyanidin fraction were added to a C18 column, the procyanidin peaks in the elution profile shifted towards lower retention times. This concentration effect was reversible. It was suggested that polar complexes of smaller oligomers with themselves or with larger molecular weight compounds might have been formed [166]. Putman and Butler [160] reported the occurrence of on-column reactions which were thought to be caused by trace metal impurities. They recommend the addition of a chelator to the eluent. Phytic acid was found to be more effective than ethylenediamine tetraacetic acid (EDTA). We found solvent dependent peak anomalies (see Figure 1.8). These effects could be suppressed by dissolving the samples in a solvent of lower solvent strength.

Procyanidins are often accompanied by a large number of phenolic compounds like hydroxycinnamic acid derivatives and flavonoids which show a similar chromatographic behavior [66,151,168,235]. Chlorogenic acid, for example, tends to co-elute with procyanidin B2 applying methanol [176,236], acetonitrile [170,181] as well as tetrahydrofuran gradients [237]. To cope with this problem, Lea [236] removed phenolic acids at pH 7.0 at the beginning of a chromatographic run, then he reverted to pH 2.5 and eluted the procyanidins without any interferences.

Procyanidin samples are commonly chromatographed on C18 sorbents. In the analysis of wine and grape tissue some authors prefer C8 columns, because retention times are shorter [204,238]. C6 columns have been used in the analysis of apple tissue.
Figure 1.8  Solvent dependent peak anomalies.

Example: Fraction of an ethyl acetate extract of Crataegus herb, cleaned up over Sephadex LH-20 (gradient of methanol – water – aqueous acetone).

A: Chromatogram and UV spectra. Injection volume: 6 µl; concentration: 0.547 mg/ml (in methanol). 1 = procyanidin B2; 2 = (-)-epicatechin.

B: Injection volume: 12.5 µl; concentration: 0.547 mg/ml (in methanol).

C: Injection volume: 25 µl; concentration: 0.274 mg/ml (in methanol).

HPLC conditions: Lichrosorb RP-18, 5 µm, 250 x 4.0 mm; gradient elution, A = methanol, B = 0.5 % aqueous ortho-phosphoric acid, 0 min 80 % B in A, 35 min 65 % B in A, 40 min 65 % B in A, 40.01 min 10 % B in A, 45 min 10 % B in A; flow: 1.0 ml/min; temperature: 24 °C.
juices and ciders [66,197,239]. The eluent should include an acid to suppress ionization of phenolic acids. Gradients are applied starting at a high percentage of aqueous phase (usually 80 % to 100 %). As organic component of the eluents are used: methanol (i.e. [79,154,179-180,194,209,240]), acetonitrile (i.e. [64,207,241-242]) and acetic acid (i.e. [243-245]). Only one method for the analysis of hawthorn procyanidins makes use of tetrahydrofuran [237]. Acetic acid gradients in water are reported to exhibit a large selectivity [168], but the UV cut-off at 240 nm is a major drawback. The positioning of an internal standard is quite difficult because the chromatograms are very crowded. Very few methods therefore include an internal standard at all. Protocatechuic acid (5 mg/l) in the analysis of beer, barley and hops using electrochemical detection [73-74], α-naphthol (3.2 mg/l) in the analysis of apple juice applying UV detection at 220 nm [179], peracetylated epigallocatechin-3-gallate in the analysis of peracetylated procyanidins in apple tissue using UV detection at 280 nm [72] and carbutamide in the analysis of fractions from Saint Johns wort (Hypericum perforatum) using UV detection at 280 nm [246] have been reported.

**Derivatization for HPLC analysis**

On principle, pre- and post-column derivatization can be performed. Derivatization of complex samples prior to chromatographic separation is more problematic because matrix effects may alter reactions. Therefore, pre-column derivatization procedures are less suitable for quantitative analysis. Nevertheless quantitative procedures are described in the literature. Piretti et al. [71-72,247] analyzed peracetylated flavan-3-ol monomers and procyanidin dimers among other compounds in apple tissue after acetylation on a nitrile stationary phase under normal-phase conditions. Tarnai et al. [13] used the same approach in the analysis of procyanidins from cherry tissue. Incomplete acetylation was never observed [71], but so far validation data are not available.

Tetreuter [151,154] analyzed procyanidin condensation products after post-column derivatization with dimethylaminocinnamaldehyde. Intensive studies on reaction kinetics [154], linearity [154], sensitivity [235] as well as on cross reactivity [151] have been performed. The method has been used to assay procyanidins in apple tissue [93], cherry tissue [248] and wine [235]; other matrices have been analyzed qualitatively
The main disadvantage of this method is the requirement of special equipment (knitted reaction coil, acid-resistant pump, easy-to-clean flow cell).

Some derivatization reactions which are frequently used for the structural elucidation of procyanidins have been adopted for HPLC analysis. Koupai-Abyazani et al. [231] developed a qualitative HPLC procedure to separate flavan-3-ol monomers and phloroglucinol adducts. The reaction is based on the acid degradation of procyanidins in the presence of phloroglucinol. The procedure has been used for quantitation of polymeric proanthocyanidins from sainfoin leaves [63]. HPLC analysis of benzylthioethers after acid degradation of procyanidins in the presence of toluene-α-thiol has so far only been used for qualitative analysis [250-251].

Conclusions
Reversed-phase HPLC is the method of choice for the quantitative analysis of procyanidins. In view of the known instability of procyanidins and the problem of developing suitable sample clean up procedures direct analysis of crude extracts is probably the best approach for quantitation. However, the separation capacity of HPLC in combination with the most commonly used UV detection at 280 nm is generally insufficient to generate useful quantitative results. Direct chromatographic determination of procyanidins in qualitative analysis has been frequently performed for example in the analysis of wine [168,252], beer [32], grape seeds [28], rhizomes of tormentil (Potentilla tormentilla) [253], Sesbania sesban leaves [94] and leaves of witch hazel (Hamamelis virginiana) [254]. Quantitation has so far only been described for extracts of apple peel [255] and callus cultures of hawthorn [256]. Direct chromatographic analysis of extracts and beverages can reasonably only be accomplished by using a more selective detection mode. Post-column derivatization, described for wine [235] and extracts of apples [93] as well as dual-electrode electrochemical detection, described for beer [31,73,76] are possible solutions.

Micellar electrokinetic capillary chromatography (MECC) has recently been evaluated for the analysis of procyanidins from hawthorn [257] and flavan-3-ol monomers from green tea [258]. In view of the relatively insensitive detection methods (see section 1.4.3.3) it is very doubtful that this technology will replace HPLC in the near future, because only very small sample amounts can be analyzed.
1.4.3.3 Detection

Procyanidins can usually not be completely separated from each other, from phenolic acids and from accompanying flavonoids by sample clean up and/or chromatographic procedures. The differentiation between these individual compounds can therefore only by accomplished by the selectivity of a suitable detection method. UV / DAD (ultra violet diode array detection), electrochemical and mass spectrometry detection have been used for the identification and quantitative determination of procyanidins.

1.4.3.3.1 UV and diode array detection

Absorption spectra of phenolic compounds consist of two bands which show maxima at 230 - 240 nm and at 270 nm [259-260]. A third band between these two maxima may occur, if substituents are conjugated to the aromatic ring (i.e. gallic acid). The position of the maximum at the longer wavelength depends on the number, position and type of additional substituents on the ring. Ortho- and meta- substitution, for example, result in a bathochromic shift [259]. Procyanidins, whose A- and B-rings behave as a set of uncoupled oscillators, exhibit only one pronounced maximum at 280 nm and a much less pronounced shoulder around 240 nm [141]. The shape of the maximum at 280 nm depends on the composition of proanthocyanidin polymers: homogeneous procyanidin polymers show a symmetrical maximum at 280 nm, whereas symmetry is disturbed in mixed procyanidin-prodelphinidin polymers [261]. Galloylation also leads to a hypsochromic shift, in addition to a band broadening which can be diagnostically used to distinguish acylated from non-acylated compounds [80].

Since most other plant phenolics show two or more absorption maxima between 250 and 600 nm, procyanidins can easily be identified in chromatograms with the help of an UV / DAD. However, chromatograms are usually dominated by other compounds, because molar absorption coefficients of procyanidins at the detection wavelength of 280 nm are relatively low compared to phenolic acids and flavonoids (see Figure 1.9). A possible solution to low sensitivities at 280 nm is the quantitation of procyanidins at 220 nm. This approach has been used in the analysis of apple juice [179].

The B-ring substitution pattern influences the absorbance. Pure prodelphinidin polymers show much lower specific absorption coefficients (E₅% = 62) than pure procyanidin polymers (E₅% = 130) [141]. The molar absorption coefficients of dimeric
Figure 1.9  UV spectra and absorbance features of flavan-3-ol monomer, phenolic acids and flavonoids.

Spectra:  UV / DAD spectra of A = (+)-catechin, B = caffeic acid, C = quercetin, D = hyperoside, E = vitexin.

Table:  Absorbances for 1 mM solutions of reference compounds at 280 nm, calculated from HPLC data (linear correlation between absorbances and concentrations is assumed).

HPLC conditions: Spherisorb ODS 2, 5 μm, 250 x 4.0 mm; gradient elution according to [151] using methanol and 0.5 % aqueous ortho-phosphoric acid; flow: 1.0 ml/min; temperature: 25.0 °C; injection volume: 25 μl; concentration range of injected reference compounds: 0.2 - 1.3 mg/ml; detection: 280 nm.
and higher oligomeric procyanidins are similar (see Table 1.8). The overall UV absorbance of an oligomer approximately amounts to the sum of the absorbances of the monomer units [226,254]. However, UV detection of individual procyanidins after chromatographic separation implies that—in this case—molar absorbances depend on structure. In the series (+)-catechin, procyanidin B2, C1 and a tetramer molar absorbances decreased [262]. Treutter et al. [154] found lower molar absorbances for dimers with a 2,3-cis stereochemistry (procyanidin B2 and B5) compared to (-)-epicatechin and (+)-catechin which nearly showed identical molar calibration plots. The molar absorbance of the doubly-linked procyanidin A2 was much higher than the ones of the monomers.

The major drawback 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 (see Figure 1.10). Despite this fact, numerous attempts have been made to gain additional structural information from the UV spectra. One possibility is the formation of absorbance ratios. A quotient is calculated from the absorbances at a master (= denominator) wavelength and an operating (= numerator) wavelength [263]. Reproducibility is best for wavelengths close to absorbance maxima [264]. Absorbance ratios are characteristic for a given compound regardless of concentration, column efficiency, lamp energy, peak shape, flow rate and retention time [263]. On the other hand, these ratios are influenced by the eluent, impurities and co-eluting compounds absorbing at the same wavelengths. For the identification of structurally closely related compounds the calculation of at least two or three ratios is recommended. Distinction between compounds with similar chromophores remains difficult [263-264].

Because of the simplicity of UV spectra of procyanidins, it is reasonable to only form one absorbance ratio. For the characterization of procyanidins the ratios from absorbances at 220 nm / 275 nm [179] and 260 nm / 290 nm [263] (only monomers) are reported. The diastereoisomers (-)-epicatechin and (+)-catechin could not be distinguished properly [263]. The value of absorbance ratios is dramatically diminished in the presence of matrix, which has been demonstrated in the analysis of apple juices [179]. We calculated absorbance ratios of procyanidins in extracts and purified fractions of hawthorn leaves and flowers and came to the same conclusion (unpublished data).
Table 1.8  Absorbance data for monomers and individual procyanidins at 280 nm.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$A^{1%}$ conf. interval (%)</th>
<th>Corrected $A^{1%}$</th>
<th>$\varepsilon$ (l mol$^{-1}$ cm$^{-1}$)</th>
<th>$r$</th>
<th>Solvent</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>$(+)\text{-catechin}$</td>
<td>150</td>
<td></td>
<td>3800</td>
<td></td>
<td>water</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td>132</td>
<td></td>
<td>3890</td>
<td></td>
<td>water</td>
<td>131</td>
</tr>
<tr>
<td></td>
<td>142</td>
<td></td>
<td>3800</td>
<td></td>
<td>MeOH</td>
<td>246</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3800</td>
<td></td>
<td></td>
<td>water</td>
<td>131</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3000</td>
<td></td>
<td></td>
<td>MeOH</td>
<td>301</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5130</td>
<td></td>
<td></td>
<td>MeOH</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1910</td>
<td></td>
<td></td>
<td>MeOH</td>
<td>301</td>
</tr>
<tr>
<td>$(-)\text{-epicatechin}$</td>
<td>130</td>
<td>80</td>
<td>3780</td>
<td>0.994</td>
<td>MeOH</td>
<td>a)</td>
</tr>
<tr>
<td></td>
<td>138</td>
<td>137</td>
<td></td>
<td></td>
<td>MeOH</td>
<td>246</td>
</tr>
<tr>
<td>$B1$</td>
<td>132</td>
<td></td>
<td></td>
<td></td>
<td>MeOH</td>
<td>113</td>
</tr>
<tr>
<td>$B2$</td>
<td>136</td>
<td>98</td>
<td>7850</td>
<td>0.999</td>
<td>MeOH</td>
<td>a)</td>
</tr>
<tr>
<td></td>
<td>139</td>
<td>140</td>
<td></td>
<td></td>
<td>MeOH</td>
<td>246</td>
</tr>
<tr>
<td></td>
<td>146</td>
<td></td>
<td></td>
<td></td>
<td>water</td>
<td>88</td>
</tr>
<tr>
<td>$B3$</td>
<td>137</td>
<td></td>
<td>7100</td>
<td></td>
<td>MeOH</td>
<td>96</td>
</tr>
<tr>
<td>$B4$</td>
<td>141</td>
<td>90</td>
<td>8170</td>
<td>0.999</td>
<td>MeOH</td>
<td>a)</td>
</tr>
<tr>
<td>$B5$</td>
<td>135</td>
<td>99</td>
<td>7810</td>
<td>0.999</td>
<td>MeOH</td>
<td>a)</td>
</tr>
<tr>
<td>$B7$</td>
<td>140</td>
<td></td>
<td></td>
<td></td>
<td>MeOH</td>
<td>113</td>
</tr>
<tr>
<td>$C1$</td>
<td>136</td>
<td>90</td>
<td>11800</td>
<td>0.999</td>
<td>MeOH</td>
<td>a)</td>
</tr>
<tr>
<td>$C2$</td>
<td>132</td>
<td>141</td>
<td></td>
<td></td>
<td>MeOH</td>
<td>96</td>
</tr>
<tr>
<td>trimer III</td>
<td>130</td>
<td></td>
<td>10600</td>
<td>0.999</td>
<td>MeOH</td>
<td>113</td>
</tr>
<tr>
<td>trimer</td>
<td>140</td>
<td></td>
<td>9800</td>
<td></td>
<td>MeOH</td>
<td>113</td>
</tr>
<tr>
<td>tetramer</td>
<td>134 - 138</td>
<td></td>
<td></td>
<td></td>
<td>water</td>
<td>88</td>
</tr>
<tr>
<td>polymer II</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>MeOH</td>
<td>113</td>
</tr>
</tbody>
</table>

Abbreviations: $A^{1\%}$ = specific absorbance (coefficient of absorbance for a 1 % (w/v) solution); conf. interval = confidence interval of intercept; corrected $A^{1\%}$ = transformation of regression plots through origin; $\varepsilon$ = molar coefficient of absorbance; $r$ = correlation coefficient; n.m. = not mentioned; MeOH = methanol. Trivial nomenclature of dimers and trimers, see Abbreviations for procyanidins. polymer II = procyanidin polymer from *Chaenomeles* sp., average chain length: 9.4 - 12.5.

a) Standards: $(-)\text{-epicatechin}$ (Roth AG, Germany; Rotichrom® TLC), procyanidins isolated from *Crataegus* herb; regression plots determined using seven standard solutions; concentration ranges: $(-)\text{-epicatechin}$ (0.021 - 0.131 mg/ml), B2 (0.006 - 0.107 mg/ml), B5 (0.006 - 0.108 mg/ml), B4 (0.016 - 0.105 mg/ml), C1 (0.018 - 0.111 mg/ml).
Figure 1.10 Examples of UV/DAD spectra of procyanidins and an artifact.

1 = (-)-epicatechin; 2 = procyanidin B4; 3 = procyanidin B2; 4 = procyanidin B5; 5 = procyanidin C1; 6 = epicatechin-(4β→6)-epicatechin-(4β→8)-epicatechin; 7 = epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin; 8 = artifact of a procyanidin in aqueous methanolic solution (dimeric procyanidin linked by a methylene bridge). HPLC conditions: Lichrosorb RP-18, 5 μm, 250 x 4.0 mm; gradient elution, A = methanol, B = 0.5 % aqueous ortho-phosphoric acid, 0 min 80 % B in A, 35 min 65 % B in A, 40 min 65 % B in A, 40.01 min 10 % B in A, 45 min 10 % B in A; flow: 1.0 ml/min; 24 °C.
The only valuable application of absorbance ratios is described by Treutter et al. [154], who developed a post-column derivatization procedure using dimethylamino-cinnamaldehyde. The identification and characterization of individual procyanidins is based on retention time and absorbance ratios. The same approach has already successfully been applied to the analysis of flavonoids after post-column derivatization with shift-reagents [265]. In the method of Treutter the ratios are calculated from the absorbances at 640 nm after derivatization and absorbances at 280 nm before derivatization. Under fixed conditions the ratios were found to be structurally related: 2,3-cis-configurated procyanidins exhibited higher ratios than 2,3-trans-configurated compounds and 4β→6-linked procyanidins showed higher ratios than 4β→8-linked procyanidins. The ratios generally decreased with increasing degree of polymerization and doubly-linked procyanidins (i.e. A2) exhibited very low values. In oligomeric procyanidins the "upper units" mainly influence the magnitude of the ratios. These variations in the UV absorbance ratios could be explained by different reaction kinetics.

Another attempt to get structural information from UV spectra is described by Bartolomé et al. [232]. They invented the so-called "min-max distance", which is the distance in nanometers between the maximum and minimum absorbances between 220 nm and 280 nm. The minimum absorbance is derived from the second derivative of the original spectrum. It was found that this "min-max distance" decreased by 1.3 nm upon each additional monomer unit in the molecule. This regularity was demonstrated up to the tetrameric level. In routine analysis, the value of this spectral parameter is questionable because spectra must be recorded with a high resolution and blank spectra must be subtracted. So far, it has only been applied to the analysis of procyanidins from *Visnea mocanera* [183].

**Quantitation using UV absorbance**

The fact that molar absorbances of procyanidin oligomers are roughly the sum of the absorbances of the monomer units has generally been taken as a legitimation for the quantitation using only monomeric reference standards (i.e. (+)-catechin) [185,226]. Such an approach is not wrong, but it has to be considered, that obvious differences in absorptivity of individual procyanidins after chromatographic separation occur [154,189,243,262]. Because of its easy feasibility, quantitation in terms of monomer equivalents has been extensively used, for example in the analysis of apple tissue.
[22,207], apple juice [69,179-181], white wine [196,242], red wine [266], grape juice [209,266], musts [267], grape tissue [79,268], beer and its primary materials [185,203,269] as well as in the analysis of leaves of witch hazel [254].

The use of calibration factors is an attempt to correct for differences in molar absorbances. This approach has been chosen for the quantitation of procyanidins in grape tissue using (+)-catechin [194,270] and in red wine using procyanidin B2 (and B2-3'O-gallate) [244]. The disadvantage of such quantitation procedures is, that the experimental (i.e. chromatographic) conditions must be kept unchanged, which sometimes is difficult to accomplish.

Individual procyanidin standards are not used very often, considering the number of quantitative chromatographic procedures described in the literature. This is mainly due to the fact that procyanidins are not commercially available. On the other hand, isolation or synthesis require laborious procedures. Table 1.9 summarizes the studies which use individual procyanidin standards for quantitation. This approach still remains the only way to quantify procyanidins correctly. In view of the demand for validated protocols on one hand and the countless quantifiable procyanidins in the chromatograms on the other hand, it is more than tempting (and surely reasonable, too) to develop standardized methods using merely one commercially available monomeric reference standard.

Conclusions

Since procyanidins show low absorption coefficients, samples have to be concentrated and relatively high amounts of reference compounds are necessary to generate calibration plots. If phenolic acids and flavonoids are not separated by suitable sample clean up procedures, the chromatograms are dominated by these compounds. The selectivity of UV detection at a single wavelength is insufficient, since many compounds show a similar chromatographic behavior as procyanidins and absorb at the detection wavelength of 280 nm as well. The use of UV / DAD enables the distinction between procyanidins and other eluting compounds (i.e. flavonoids, phenolic acids). Furthermore, peak purity can be estimated. Structural information may be obtained in pure samples (standards) using absorbance ratios or additional spectral parameters. In "real" samples these approaches are of little value. Post-column derivatization using dimethylaminocinnamaldehyde enhances selectivity drastically and offers the
<table>
<thead>
<tr>
<th>Matrix</th>
<th>Procyanidin standards</th>
<th>Determination of</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Linearity</td>
<td>Detection limit</td>
</tr>
<tr>
<td>wine</td>
<td>B2, B3, B4, A2</td>
<td>yes</td>
<td>n.d.</td>
</tr>
<tr>
<td>wine</td>
<td>B1, B2, B3, B5, B6, B7, C1 b)</td>
<td>n.d.</td>
<td>yes</td>
</tr>
<tr>
<td>wine, grape tissue</td>
<td>B1, B2, B3, B4, trimer, tetramer</td>
<td>yes</td>
<td>n.d.</td>
</tr>
<tr>
<td>wine, grape tissue</td>
<td>B1, B2, B3, B4, C1, trimer II</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>beer, barley, hops</td>
<td>B3, C2</td>
<td>yes</td>
<td>n.d.</td>
</tr>
<tr>
<td>apple tissue, green tea</td>
<td>B1, B2, B3, B4 a)</td>
<td>yes</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>B2, B5, A2 b)</td>
<td>yes</td>
<td>n.d.</td>
</tr>
<tr>
<td>wine</td>
<td>B2, B3, B4, A2</td>
<td>no doc.</td>
<td>n.d.</td>
</tr>
<tr>
<td>wine</td>
<td>B1, B2, B3, B4, trimer, tetramer</td>
<td>no doc.</td>
<td>n.d.</td>
</tr>
<tr>
<td>white wine</td>
<td>B1, B2, B3, B4, trimer, tetramer</td>
<td>no doc.</td>
<td>n.d.</td>
</tr>
<tr>
<td>grape tissue</td>
<td>B1, B2, B3, B4, C1</td>
<td>no doc.</td>
<td>n.d.</td>
</tr>
<tr>
<td>barley tissue</td>
<td>B1, B2, B3, B4</td>
<td>no doc.</td>
<td>n.d.</td>
</tr>
<tr>
<td>apple tissue</td>
<td>B2</td>
<td>no doc.</td>
<td>n.d.</td>
</tr>
<tr>
<td>apple tissue</td>
<td>B1, B2, B5, C1, trimer II b)</td>
<td>no doc.</td>
<td>n.d.</td>
</tr>
<tr>
<td>hawthorn tissue</td>
<td>B2</td>
<td>no doc.</td>
<td>n.d.</td>
</tr>
<tr>
<td>buckwheat callus culture</td>
<td>B2</td>
<td>no doc.</td>
<td>n.d.</td>
</tr>
<tr>
<td>cherry tissue</td>
<td>B1, B2 a)</td>
<td>no doc.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

Procyanidin derivatives (i.e. galloylated procyanidins) and flavan-3-ol monomers are not mentioned. Trivial nomenclature of dimers and trimers, see Abbreviations for procyanidins. Abbreviations: no doc. = no documentation in publication; n.d. = not determined.

a) Detection of peracetylated compounds.
b) Detection at 640 nm after derivatization with DMACA (dimethylaminocinnamaldehyde).
opportunity to obtain additional structural information via the calculation of absorbance ratios.

1.4.3.3.2 Electrochemical detection
For electrochemical detection molecules are oxidized or reduced by an electrical potential built up between two electrodes. The electrons that are exchanged on the surface of the working electrode produce a measurable current, which is proportional to the number of reacting molecules [271]. The electrochemical behavior of compounds can be studied with the help of voltammograms, which relate the current response to the applied potential. Depending on the experimental setup, hydrodynamic or cyclic voltammograms can be distinguished. In hydrodynamic voltammograms the potential is changed stepwise and actual chromatograms are recorded. Hydrodynamic voltammograms are especially suitable for impure compounds or for compounds which are only available in small quantities. Effects of the mobile phase (i.e. gradient elution) influence the acquired data, too [74,272]. Figure 1.11 shows examples of hydrodynamic voltammograms of procyanidins, some flavonoids and a phenolic acid.

In cyclic voltammograms the detector cell is continuously floated with a solution of the analyte, while the potential is gradually changed. This approach is less time-consuming, but does not reflect influences from chromatography [272-273]. The use of dual-mode electrochemical detection gives the opportunity to access reversibility of redox reactions. The shape of the resulting curves depends on the electrochemical properties of the analyte, but also on the cell geometry, the condition of the electrodes, the flow rate and some other factors [274-275].

As already outlined in the section which covers colorimetric assays based on redox reactions (section 1.4.1.1), ortho-diphenols are more easily oxidized to the respective quinones than meta-diphenols. This has been confirmed by recording cyclic voltammograms of (-)-epicatechin. Two separate oxidation reactions occurred, the first one being responsible for the oxidation of the B-ring ortho-diphenols and the second one – at higher potential – representing oxidation of the A-ring meta-diphenols. The oxidation of the B-ring proved to be reversible while A-ring oxidation was irreversible. Slightly different potential values for the completion of the oxidations and reductions are reported: B-ring oxidation: +0.49 V to +0.6 V; A-ring oxidation: +0.85 V to +0.8 V;
Figure 1.11 Hydrodynamic voltammograms.

Responses for 1 μM solutions of reference compounds, calculated from HPLC data (linear correlation between electrochemical response and concentrations is assumed).

HPLC conditions: Spherisorb ODS 2, 5 μm, 100 x 4.0 mm; isocratic elution, 25 % methanol in 0.5 % aqueous ortho-phosphoric acid (procyanidin B2, C1, (+)-catechin, caffeic acid), 30 % methanol in 0.5 % aqueous ortho-phosphoric acid (quercetin, hyperoside); flow: 1.0 ml/min; temperature: 25.0 °C; injection volume: 5 μl; concentration range: 0.11 - 3.9 μg/ml.

Detection: electrochemical; amperometric detector LC-4B (Bioanalytical Systems Inc.); mode: single electrode; cell geometry: thin layer, 2 μm; working electrode: glassy carbon; reference electrode: silver / silver chloride; range: 10 nA.
B-ring quinone reduction: +0.46 V to +0.3 V [272,275]. Hydrodynamic voltammograms revealed two plateau regions for (+)-catechin starting at around +0.85 V and +0.65 V, respectively [262,275]. The hydrodynamic voltammograms of procyanidin B2 does not anymore show pronounced plateau regions. In the series from dimeric to tetrameric procyanidins only the slopes of the voltammograms change, which is indicative of the ease with which the molecules can be oxidized. In electrochemical detection, responses of monomers, which are most easily oxidized, are therefore always higher than the responses of procyanidins [31,262,272].

The selectivity of electrochemical detection can be improved by the use of two electrodes (dual-mode detection). Basically, two different combinations are used: two amperometric cells and the combination of a coulometric cell with an amperometric cell. The difference between these two cell types is that in amperometric cells only a fraction of the eluting analytes react, whereas in coulometric cells analytes (and all other eluting compounds!) may be quantitatively converted depending on the working potential. Using these two combinations a variety of different experimental setups are possible [74,271].

In the analysis of procyanidins the goal of dual-mode electrochemical detection is the assessment of parameters which are diagnostic for the shapes (i.e. slopes) of the respective voltammograms. In this way procyanidins can be distinguished from other eluting compounds and in addition, some structural information can be obtained [74,272-273]. Lunte defined two parameters: the ratio of $E_{1/2} / E_{\text{max}}$, which gives information on the degree of polymerization of procyanidins [272], and the collection efficiency $N_0$, which gives information on the hydroxylation pattern [273]. The ratio of $E_{1/2} / E_{\text{max}}$ defines the quotient of the detector responses at the plateau potential and at the half-response level. It is a measure for the slope of the voltammograms. The potential at the half-response level seems to depend on the matrix, since different settings were chosen for grape seeds (+0.55 V) and wine (+0.70 V) [272,274]. The collection efficiency $N_0$, is a measure for the reversibility of the electrochemical oxidation. It is determined at potentials of +1.0 V (upstream) and 0.0 V (downstream) and is expressed as a percent value. Collection efficiencies of procyanidins are around 10 % independent of their degree of polymerization. Flavonoid aglycones with ortho-diphenol moieties show similar values, whereas glycosides exhibit values around 30 %. The collection efficiencies of mono- and tri-phenols are smaller than 10 % [273].
Sensitivities in electrochemical detection are much improved compared to UV detection [26,31,274], although some authors claim it to be of the same order [272]. This may again reflect the dependence of detector responses on the condition and type of electrodes on one hand and the type of detector cell on the other. For (−)-epicatechin a detection limit in the pico-molar range has been reported in the dual-mode detection [272]. Other researchers found detection limits of 0.1 mg/l for (+)-catechin, (−)-epicatechin and procyanidin B3 [74]. Mc Murrough and Baert [31], who analyzed beer by direct injection HPLC, noticed a 40% decrease of responses using dual-mode electrochemical detection compared to single-mode detection. Because of improved stability, reliability and accuracy they advocate the use of dual-mode detection after all.

Table 1.10 gives an overview on the methods described in the literature which make use of electrochemical detection. So far, quantitative determination is limited to the analysis of beer, hops and barley. A linear correlation has been reported in the range of 0.1 to 6 mg/l for (+)-catechin, (−)-epicatechin and procyanidin B3 [31, 73-74]. In a broader concentration range of 0.1 to 1000 mg/l a linear correlation for (−)-epicatechin could only be obtained by generating a double-logarithmic plot [275]. Chiavari et al. [262] made calibration plots using (+)-catechin and procyanidins B2, C1 and a tetramer. The responses of the procyanidins were in the same order and slightly depended on the degree of polymerization. (+)-Catechin produced a much steeper calibration graph than the procyanidins. The calibration plots imply that quantitation of procyanidins in terms of monomer units cause larger mistakes in electrochemical detection than in UV detection. Quantitation based on monomer equivalents using electrochemical detection has so far only been described once [76].

Conclusions
Electrochemical detection is critically dependent on many influencing factors, which makes it by far not an "easy-to-use" technology. Only relatively shallow gradients can be used because the eluent produces a background current which leads to high baseline drifts in steep gradients. In the analysis of procyanidins high potentials must be used which lead to high noise levels. Since phenolic groups of procyanidins react gradually there are no pronounced plateau region in hydrodynamic voltammograms. Measurements are therefore not very rugged because already small changes in the applied potential have a great impact on the response. The sensitivity of the working
Table 1.10  Procyanidin analysis using electrochemical detection.

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Mode</th>
<th>Electrodes</th>
<th>Potential</th>
<th>Compounds</th>
<th>Comments</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>wine, grape juice</td>
<td>dual amperometric</td>
<td>Ag/AgCl (ref) glassy carbon</td>
<td>+ 1.0 V; 0.0 V</td>
<td>B1, B2, B4, C1</td>
<td>qualitative analysis in series with UV / DAD</td>
<td>273</td>
</tr>
<tr>
<td>wine, grape tissue</td>
<td>dual amperometric</td>
<td>Ag/AgCl (ref) glassy carbon</td>
<td>+ 1.1 V; 0.55 (0.70) V</td>
<td>B2, B4, C1</td>
<td>qualitative analysis</td>
<td>272</td>
</tr>
<tr>
<td>beer, barley, hops</td>
<td>single amperometric</td>
<td>Ag/AgCl (ref) n.m.</td>
<td>+ 0.85 V</td>
<td>B3</td>
<td>quantitative analysis</td>
<td>73, 32</td>
</tr>
<tr>
<td>beer, barley</td>
<td>dual coulometric amperometric</td>
<td>palladium (ref) porous graphite glassy carbon</td>
<td>+ 0.35 V - 0.65 V</td>
<td>B3, C2</td>
<td>quantitative analysis</td>
<td>74, 178, 76</td>
</tr>
<tr>
<td>beer</td>
<td>dual coulometric amperometric</td>
<td>n.m. porous graphite glassy carbon</td>
<td>+ 0.01 V + 0.35 V</td>
<td>B3</td>
<td>quantitative analysis in series with UV</td>
<td>31</td>
</tr>
<tr>
<td>maize silage</td>
<td>single amperometric</td>
<td>n.m. glassy carbon</td>
<td>+ 1.1 V</td>
<td>B2, C1, tetramer</td>
<td>qualitative analysis in series with UV</td>
<td>262</td>
</tr>
</tbody>
</table>

Procyanidin derivatives (i.e. galloylated procyanidins) and flavan-3-ol monomers are not mentioned. Trivial nomenclature of dimers and trimers, see Abbreviations for procyanidins. Abbreviations: ref = reference electrode; Ag/AgCl = silver / silver chloride electrode; UV / DAD = UV-diode array detection; UV = ultra violet detection; n.m. = not mentioned in publication.
electrode gradually decreases by adsorption of matrix components, which is known as electrode-fouling. Since higher molecular weight procyanidins show tanning properties this is a major problem in procyanidin analysis. Too frequent polishing of the working electrode of thin-layer cells on the other hand will degrade it faster [274-275].

The advantage of electrochemical detection over UV detection is its much greater sensitivity. Its major drawback is that it is much less selective than UV / DAD. Flavonoids and phenolic acids are also easily oxidized at high working potentials (around +1.0 V) used in the analysis of procyanidin samples. The discrimination between procyanidins and non-procyanidins in single-mode electrochemical detection is merely based on different retention times. Application of dual mode detection enhances selectivity, but flavonoid aglycones with ortho-diphenol moieties show a similar electrochemical behavior as procyanidins. Although voltammograms are diagnostic for specific procyanidins [272], the recording of only two response values at two different potentials in the dual-mode does not result in sufficient information to be successfully applied in the analysis of complex matrices. The use of electrochemical array detectors, now commercially available, might give a more complete picture of voltammograms, which in turn would make electrochemical detection more tempting in the analysis of procyanidins.

1.4.3.3.3 Liquid chromatography – mass spectrometry

In liquid chromatography – mass spectrometry (LC-MS) ions are generated from the eluting molecules of an HPLC device. They are subsequently separated by a mass-selective detector according to their mass-to-charge (m/z) ratio. Numerous different interfaces are on the market to connect the HPLC with the mass detection device and to produce the ion species. Currently, heated nebulizers (enabling APCI = atmospheric pressure chemical ionization) and electrospray or ionspray (ESP, ISP) are the most widely used interfaces. Besides various experimental variables (i.e. composition of the eluent, positive or negative ionization mode) the type of interface has a tremendous influence on the ionization of the eluting compounds. Mass detection is mostly performed using a quadrupole mass spectrometer. By connecting three quadrupoles in series additional fragment ions of one ion species can be produced (LC-MS-MS experiment). The use of ion traps enables the recording of multiple MS experiments of
selected ions (LC-MS\(^n\)). More details on this relatively new technology can be found elsewhere [276-278].

Table 1.11 gives an overview on the LC-MS methods described in the literature. The vast majority of studies has been performed with the thermospray interface in the positive ionization mode. Thermospray detection is not very sensitive [279]. For procyanidins a detection limit of 1 \(\mu g\) has been reported [269]. No data are available on the detection limits of procyanidins using other types of interfaces. So far, LC-MS has not yet been applied to the quantitative analysis of procyanidins.

**Ionization behavior of procyanidins**

Using thermospray interfaces in the positive ionization mode protonated molecular ions are generated in small amounts and have so far only been reported up to the trimeric level [269]. Fragmentation predominantly occurs by cleavage of the interflavanoid bonds. Thus, sequence ions are produced, of which the protonated monomer (m/z = 291) usually constitutes the base peak [269]. Information on the molecular weight are more easily accessible in the negative ionization mode. Oligomeric and monomeric fragments may however occur in small amounts, signaling that the interflavanoid bond is labile in this mode, too [276,280]. Molecular ions up to the heptameric level are reported in the negative mode using off-line FAB-MS (fast atom bombardment) [281]. Guyot et al. [229] reported a complete series of polymeric procyanidins from a degree of polymerization of four up to seventeen using an off-line electrospray ionization mass spectrometry (ESI) technique in the negative mode. Some of the ion species were doubly-charged. Cluster ions may frequently be detected in on-line measurements in the positive ionization mode. Sodium adducts [282], acetic acid adducts [280] or – using buffer ionization – ammonium adducts [283] have been reported. Experiments in our laboratory revealed cluster ions of procyanidins in negative ionization mode, too (unpublished data).

Kiehne et al. [282] who analyzed green tea using a thermospray interface in the positive ionization mode could distinguish fragments originating from upper (T) and lower (B) units of dimeric procyanidins. Cleavage of the interflavanoid bond resulted in a carbenium-ion (T-1) and a neutral fragment from the lower unit which could be protonated under the experimental conditions to form a pseudo molecular ion (B + H\(^+\)).
Table 1.11  Procyanidin analysis using mass spectrometry detection.

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Interface</th>
<th>Ionization Type</th>
<th>Mode</th>
<th>Compounds</th>
<th>Comments</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>tea leaves</td>
<td>TSP</td>
<td>no buffer</td>
<td>positive</td>
<td>flavan-3-ol-gallates</td>
<td>CID experiment</td>
<td>284</td>
</tr>
<tr>
<td>green tea</td>
<td>TSP</td>
<td>buffer</td>
<td>positive</td>
<td>flavan-3-ol-gallates, Cl</td>
<td></td>
<td>279, 282, 282</td>
</tr>
<tr>
<td>beer, malt, hops</td>
<td>TSP</td>
<td>buffer</td>
<td>positive</td>
<td>B3, trimer</td>
<td>in series with UV</td>
<td>269</td>
</tr>
<tr>
<td>barley</td>
<td>HN</td>
<td>APCI</td>
<td>positive</td>
<td>dimer</td>
<td>CID experiment</td>
<td>171</td>
</tr>
<tr>
<td>litchi (Litchi chinensis)</td>
<td>n.m.</td>
<td>n.m.</td>
<td>negative</td>
<td>A- and B-type dimers, trimers, tetramer</td>
<td></td>
<td>304</td>
</tr>
<tr>
<td>grape tissue</td>
<td>ESP</td>
<td>ESI</td>
<td>negative</td>
<td>benzylthioethers of monomers</td>
<td>analysis after thiolysis</td>
<td>251</td>
</tr>
<tr>
<td>none</td>
<td>TSP</td>
<td>buffer</td>
<td>positive</td>
<td>dimer</td>
<td>analysis of standard</td>
<td>280</td>
</tr>
</tbody>
</table>

Flavan-3-ol monomers are not mentioned. Trivial nomenclature of dimers and trimers, see Abbreviations for procyanidins. Abbreviations: TSP = thermospray; HN = heated nebulizer; ESP = electrospray; CI = chemical ionization; APCI = atmospheric pressure chemical ionization; ESI = electrospray ionization; CID = collision induced dissociation; n.m. = not mentioned in publication.
Lin et al. [284] studied heterocyclic ring cleavage of procyanidins and flavonoids in collision induced dissociation experiments using the thermospray interface. Loss of the mass fragment 138 u (u = mass unit) was found to be diagnostic for the identification of procyanidins in complex matrices. Loss of the mass fragment 152 u was not only observed with procyanidins but also with flavonoids. The loss of this fragment results from Retro-Diels-Alder fission of the heterocyclic ring system, which is known as a typical reaction of procyanidins in both ionization modes from off-line FAB-MS experiments [239,285-286]. Loss of mass fragment 123 u was observed with procyanidin monomers and flavonols having an oxygen functionality at C3 position, whereas loss of the mass fragment 165 u could only be detected with flavan-3-ols and flavanons but not with flavons and flavonols [284].

Conclusions
The advantage of LC-MS detection over UV or electrochemical detection is its tremendously improved selectivity. Flavonoids, phenolic acids but also certain oxidation and degradation products of procyanidins can be suppressed by monitoring specific ion traces. Sample clean up procedures can therefore be minimized. Figure 1.12 illustrates the benefit of LC-MS detection in the analysis of complex matrices. A major drawback of this technology in the analysis of procyanidins is its low sensitivity, even compared to UV detection [282].

LC-MS analysis gives unequivocal information on the degree of polymerization of the eluting procyanidins. Furthermore, mass spectra are much more characteristic than UV spectra. Galloylated procyanidins, for example, can easily be detected by the occurrence of the mass fragment of gallic acid (170 u) [282-283]. It should even be possible to distinguish A- and B-type procyanidins, which exhibit mass differences of only two mass units. Although this differentiation is probably not easy to accomplish in complex matrices in a first order mass spectrum. Detection should be performed in the negative ionization mode, because pseudo molecular ions are generally more stable in this mode than in the positive ionization mode. Since specific ion traces can be selected in LC-MS analyses co-eluting procyanidins of higher degrees of polymerization are not displayed. In this way, LC-MS chromatograms do not show the characteristic "humps" typical of chromatograms of procyanidin extracts using UV or electrochemical detection. However, a chromatographic separation of oligomeric procyanidins is
Figure 1.12  Potential of LC-MS in procyanidin analysis.

Top: LC-MS chromatogram of an ethyl acetate extract of *Crataegus* herb.  
- m/z = 280 - 1700: total ion current; m/z = 291: monomers; m/z = 579: dimeric procyanidins; m/z = 867: trimeric procyanidins; m/z = 1155: tetrameric procyanidins.

Bottom: Mass spectra.  
- A = (-)-epicatechin, tR 15.21 min;  
- B = procyanidin B2, tR 14.10 min;  
- C = procyanidin C1, tR 17.12 min;  
- D = epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin, tR 17.91 min.

HPLC conditions: C8 column, 250 x 4.6 mm; gradient elution, A = methanol, B = 0.5% aqueous acetic acid, 0 min 80% B in A, 35 min 65% B in A, 40 min 65% B in A, 40.01 10% B in A, 45 min 10% B in A; flow: 1.0 ml/min; temperature: 24 °C; injection volume: 1 μl; concentration: 23.44 mg/ml.

Detection: mass spectrometry; LCQ detector (Finnigan MAT); interface: ESI; mode: positive ionization.
necessary, because under the currently employed experimental conditions interflavanoid bonds are not stable and cluster ions may be formed. In both cases secondary ion species are produced. These ions show masses which may be very similar to the masses of genuine procyanidins, rendering a qualitative or quantitative interpretation of results difficult or even impossible.

There are some limitations to MS detection. On one hand, it is impossible to differentiate between the stereoisomers (+)-catechin and (-)-epicatechin and on the other hand the configuration of the interflavanoid bond can not be determined [171,269]. However, results from off-line FAB-MS suggest that additional structural information, which up to now is not accessible with any other detection mode, might be obtained. Karchesy et al. [285-286] could distinguish between linear and branched trimers from the different ion species that were produced. Furthermore, slight differences in abundance of characteristic ions for 4β→8-linked and 4β→6-linked procyanidins B1 and B7 were observed. Such additional information can only be exploited in LC-MS-MS or multiple MS experiments, since the matrices may considerably hamper interpretation of the first order MS spectrum. Performance of additional MS experiments on selected ions might as well be useful in the confirmation of identities, since loss of characteristic mass fragments (i.e. 152 u or 138 u) could be induced.

**Epilogue**

Although numerous chromatographic methods have been published for the quantitative analysis of procyanidins, there does not exist any procedure which could be addressed as a "standard procedure". The reasons are manifold and they are to a large extent due to the complexity and uncommon physico-chemical features of this class of natural products. Added to that the separation of procyanidins from accompanying matrix components is a challenge for itself. There still remains a lot of research to do in this field. Nevertheless, we hope that this review article will serve the reader engaged in procyanidin analysis as a guidance in finding appropriate solutions to his/her analytical problem.
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2 Analysis of procyanidins in *Crataegus*

2.1 Quantitative reversed-phase high-performance liquid chromatography of procyanidins in *Crataegus* leaves and flowers (submitted version of publication 2)

A quantitative reversed-phase high-performance liquid chromatographic method using UV diode array detection has been developed for the determination of (-)-epicatechin and procyanidins B2, B5, C1 and B4 in *Crataegus* leaves and flowers. The chromatographic method has been optimized with respect to stationary phase, eluent composition, temperature, flow rate and injection volume. A sample preparation procedure including a filtration step over C18 cartridges is proposed for quantitative determinations, while a less laborious procedure is useful as a fingerprint method for qualitative analysis. The final method has been validated.

2.1.1 Introduction

*Crataegus* (hawthorn) has already been used by the Romans and is considered as one of the oldest medicinal plants of the western world [1]. Nowadays preparations of *Crataegus* are given in cases of declining cardiac performance equivalent to stages I and II of the NYHA (New York Heart Association) classification [2]. The pharmacological effects are ascribed to the flavonoid fraction (hyperoside, vitexin, vitexin-2′′O-rhamnoside) as well as to the procyanidin fraction ((-)-epicatechin, procyanidins B2, B5, C1 and oligomers) [3,4]. Procyanidins occurring in *Crataegus* are primarily composed of (-)-epicatechin units while the diastereoisomeric (+)-catechin is only represented as a minor component. Further structural diversity is based on the different types of interflavanoid bonds (C4 → C8 or C4 → C6) and on the different degrees of polymerization (see Figure 2.1).

The quantitative analysis of the flavonoids in *Crataegus* is well established [5]. For the analysis of oligomeric procyanidins however there still does not exist a validated analytical method. For quantitative analysis of total procyanidin contents colorimetric
Figure 2.1 Examples of procyanidins found in Crataegus sp.

determination of cyanidin after acid hydrolysis has been widely used [6-9]. Hiermann et al. [10] colorimetrically analyzed an oligomeric and a polymeric fraction obtained from chromatography over Sephadex G25. This method has also been applied in studies on seasonal changes of procyanidin contents [11,12] as well as in a study on cell cultures of Crataegus [13]. The major drawbacks of colorimetric assays are that the reactions are not quantitative and that they often lack reproducibility [14]. For the determination
of individual procyanidins a variety of different qualitative HPLC (high-performance liquid chromatography) methods have been described [4,7,15,16], but resolution is generally too bad for a quantitative determination. Two densitometric protocols have been published. The method of Vanhaelen and Vanhaelen-Fastre [17] is based on thin layer chromatography and detection with vanillin reagent after sample clean up over Sephadex LH-20. The quantitation is hampered by considerable background absorbance [14]. Kappenberg [18] derivatized sample solutions of inflorescences of Crataegus with dansylchloride prior to thin layer chromatography on silica. Drawbacks of this method are that it is sensitive to light and that so far, no studies on reaction kinetics of procyanidins have been performed [14].

The analysis of procyanidins is not only hampered by their structural diversity, but also by the instability of this class of polyphenolic compounds. Procyanidins are subject to enzymatic and spontaneous oxidation reactions [19,20], they are thermally labile [21,22] and may undergo molecular rearrangements (i.e. epimerization) [23-25]. With numerous compounds like proteins and carbohydrates they can form reversible as well as irreversible complexes [26].

The scope of the present work was to develop a validated HPLC protocol using UV diode array detection for the determination of the major low molecular weight procyanidins in Crataegus leaves and flowers.

2.1.2 Experimental

Materials
Crataegus leaves and flowers (Crataegi folium cum flore Ph.Helv.VII / DAB10) was obtained from Dixa (CH-St. Gallen). (-) Epicatechin (E; HPLC grade) was purchased from Extrasynthese (F-Genay). Chlorogenic acid was of CHR grade and was obtained from Roth (CH-Reinach). Procyanidin dimers B2 [(-)-epicatechin-(4ß→8)-(-)-epicatechin], B5 [(-)-epicatechin-(4β→6)-(−)-epicatechin], B4 [(+)-catechin-(4α→8)-(−)-epicatechin] and trimer C1 [(-)-epicatechin-(4β→8)-(−)-epicatechin-(4β→8)-(−)-epicatechin] were isolated from Crataegus leaves and flowers as described elsewhere [27]. Methanol, acetonitrile, tetrahydrofuran and acetone were of HPLC grade (Romil Chemicals, UK-Shepshed). Ortho-phosphoric acid (Ph.Helv.VI / Eur.1)
was purchased from Hänseier (CH-Herisau), formic acid (p.a.) and acetic acid (p.a.) from Merck (CH-Dietikon). Water was obtained using a NANOpure cartridge system (Skan, CH-Basel). Celite 535 was purchased from Fluka (CH-Buchs). Sep-Pak® tC18 solid phase extraction columns (3ml, 500 mg) were bought from Waters (USA-Milford, MA). Nylon filters (Spartan 30/B, 0.45 μm) were obtained from Spartan (D-Dassel).

**Instrumentation**

All HPLC analyses were performed using a Hewlett-Packard instrument (Model 79994A analytical workstation, model 1090 liquid chromatograph, model 1040 diode-array detector). A Knauer (D-Berlin) prepacked cartridge column (250 x 4 mm I.D., 5 μm) filled with LiChrosorb RP-18 (for validation experiments: batch L233233646, series MD 186) and a guard column (10 x 4 mm I.D.) of the same material was used for the final chromatographic separation. For chromatographic method development additional columns of the same dimensions were evaluated: Spherisorb ODS II (5 μm, Knauer, D-Berlin), Nucleosil 50 C18 (5 μm, Macherey-Nagel, CH-Oensingen) and Spherisorb C6 (5 μm, Knauer, D-Berlin). For lyophilization an alpha 1-4 model of Christ (D-Osterode am Harz) was used. Sonification was performed with a model TOC-300 (35 kHz) instrument of Merck (CH-Dietikon).

**Chromatographic conditions**

The chromatographic method was optimized with respect to the type of stationary phase (see 2.2 Instrumentation), the composition of the eluent (organic component, acid), the column temperature (24, 30, 35 and 42 °C), the flow rate (0.6 to 1.0 ml/min) and the injection volume (5 to 20 μl).

The various stationary phases were evaluated using methanol - 0.5 % aqueous ortho-phosphoric acid gradients at 24 °C and applying flow rates of 1.0 ml/min. Different gradients of acetonitrile and methanol in 0.5 % aqueous ortho-phosphoric acid, as well as effects of tetrahydrofuran as modifier in methanol (2.5 % (v/v)) were tested on the LiChrosorb RP-18 column. The influence of different acids (ortho-phosphoric acid, formic acid, acetic acid) and concentrations (0.1 % to 5 %) were evaluated at 24 °C at a flow rate of 1.0 ml/min on the same stationary phase. The following methanol gradient was used for these studies: 0 - 35 min from 20 % to 35 % methanol in acidic aqueous
phase, 35 - 40 min 35 % methanol, 40.01 - 45 min 90 % methanol, 45.01 - 50 min 100 % methanol, 50.01 - 60 min 20 % methanol.

The mobile phase of the optimized chromatographic method consisted of solvent A (methanol) and solvent B (0.5 % (v/v) ortho-phosphoric acid in water). The elution profile was: 0 min 18 % A in B, 0-10 min 18 % to 24 % A in B, 10 - 20 min 24 % A in B, 20 - 45 min 24 % to 40 % A in B, 45.01 - 50 min 100 % A (wash-out), 50.01 - 60 min 18 % A in B (re-conditioning). All gradients were chosen to be linear. The flow rate was set to 1.0 ml/min, the column temperature was fixed at 24.0 °C and the injection volume was chosen to be 5 μl. The detection wavelengths were set at 280 nm and 220 nm, spectra were recorded in the range 190 nm to 600 nm. Peak purity was checked using the software of the photodiode array detector. Upslope, apex and downslope spectra of the peak under consideration were normalized and matches were calculated. Perfect matches are assigned values of 1000; values below 990 are considered to be indicative of co-eluting impurities. The peak detection threshold was set to 0.5 mAU for validation experiments of B5 and to 1.0 mAU for the remaining experiments. Peak identification was based on retention times, UV / DAD spectra and spiking of authentic reference compounds to sample solutions.

2.1.2.1 Sample preparation

Sample preparation procedure I

15.00 g of dried and pulverized (mesh 1 mm) plant material were macerated with 50 ml of aqueous acetone 70 % (v/v) for approximately 15 minutes. The suspension was transferred to a column (dimensions: Ø 20 x 250 mm) and was percolated at an approximate speed of one drop per second. 250 ml of extract were collected (requiring 2.5 to 3 hours). 25.0 ml of the extract (= extract solution A) were transferred to a tared flask. The extract solution was evaporated to approximately 5 ml applying temperatures below 30 °C. The flask was again weighed and a sufficient volume of methanol was added to produce a 50 % (v/w) aqueous solution. The suspension was poured over a sintered glass filter (3G1, dimensions: Ø 40 x 40 mm) containing an approximately 5 mm layer of Celite 535. The flask and filter was washed in portions using another 30 ml of 50 % (v/v) aqueous methanol. If necessary, the flask was held in an ultrasonic bath for a short time. The methanol was evaporated below 30 °C and the aqueous
extract solution was immediately frozen and lyophilized. The brown residue was dissolved in 50 % (v/v) aqueous methanol and was transferred to a 5.0 ml volumetric flask (= sample solution B). Wash solutions were again sonificated for a short time to solubilize residues sticking to the glass wall. All sample solutions were filtered over a 0.45 μm nylon filter before HPLC analysis.

Sample preparation procedure II
Sep-Pak® tC18 cartridges were activated with 10 ml of methanol and pre-conditioned using 5 ml 50 % (v/v) aqueous methanol. 2.0 ml of sample solution B were applied and the filtrate was collected in a 5 ml volumetric flask. The cartridge was further eluted with 2.3 ml of 50 % (v/v) aqueous methanol using a 5.0 ml Pipetman® of Gilson (Villiers-le-Bel, France). During the whole filtration the vacuum was adjusted to approximately -400 mbar which resulted in a speed of filtration of one drop per second. The volumetric flask was filled up to the mark with 50 % (v/v) aqueous methanol (= sample solution C) and was directly used for HPLC analysis.

Validation experiments
Validation samples to determine recovery rates were prepared by adding weighed amounts of reference compounds to the extract solutions A. Spiking levels were set based on preliminary tests in which concentrations were calculated in (-)-epicatechin equivalents. The levels were chosen to approximate 0.5 to 2 times the levels detected in unspiked extract solutions A. To each validation set three unspiked samples were worked up, the average of which were used to calculate the recovery rates. The results of the recovery experiments are given in Tables 2.1 and 2.2.

Quantitation was carried out by the external standard method on the basis of area and height counts at 280 nm as well as 220 nm. All procyanidins were also quantified in terms of (-)-epicatechin equivalents using calibration curves of (-)-epicatechin. Calibration graphs were generated using six to eight calibration solutions. Calibration solutions of procyanidins were filled into micro-vials to reduce gas-phase and consequently to prevent oxidation reactions as much as possible. These micro-vials were only used for one analysis (the remaining calibration solutions were stored at -20 °C). All graphs were linear. Correlation coefficients for graphs of procyanidins were better than 0.9993, while correlation coefficients for (-)-epicatechin graphs were
Table 2.1 Results of recovery experiments for sample preparation procedure I. Quantitation using individual reference compound calibration graphs.

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<th>280 nm height counts</th>
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$^a$ Calculated concentrations of spikes of reference compounds in sample solution B.
Table 2.2  Results of recovery experiments for sample preparation procedure II. Quantitation using individual reference compound calibration graphs.

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<sup>a</sup> Calculated concentrations of spikes of reference compounds in sample solution C.
better than 0.9980 (exceptions: quantitation of (-)-epicatechin at 220 nm area counts: 0.9869; quantitation of procyanidin B2 in (-)-epicatechin equivalents at 280 nm and 220 nm area counts: 0.9969 and 0.9974, respectively). Detection limits (amount of analyte that produces a signal to noise ratio of two to five) are given in Table 2.3 and were determined using additional calibration solutions.

**Table 2.3** Limits of detection (ng).

<table>
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The repeatability of the method was determined using 14 (procedure I) and 13 (procedure II) replicates. The calculation of the relative standard deviation was based on detector responses or detected concentrations expressed in (-)-epicatechin equivalents, respectively. Results are given in Table 2.4.

### 2.1.3 Results and discussion

#### Extraction

70 % aqueous acetone is the extraction solvent of choice in the analysis of procyanidins [14]. This extraction solvent was also proven to be superior over methanol and 80 % aqueous methanol in the analysis of hawthorn leaves and flowers determined by acid hydrolysis [28].

Preliminary tests revealed that collection of 250 ml of extract solution is exhaustive. Subsequent extraction of the same plant material using another 250 ml of extracting solvent showed that more than 99 % of the detected amounts of (-)-epicatechin and procyanidins B2, B5, C1 and procyanidin B4 could be recovered in the first extraction as long as the extraction time exceeded 2.5 hours.
Table 2.4  Determination of repeatability. Relative standard deviation (%) of 14 (procedure I) and 13 (procedure II) replicates.

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a  Relative standard deviations determined on the basis of detector responses.
b  Determination based on detected concentrations expressed in (-)-epicatechin equivalents.
c  One value was considered as an outlier (values beyond +/- 2s).
d  Two values were considered as outliers (values beyond +/- 2s).

Sample preparation

Liquid-liquid extraction procedures are generally hampered by the amphiphilic nature of procyanidins while their complexing ability limits the use of solid supports in sample preparation procedures [14]. In view of these facts and the known instability of procyanidins it was attempted to minimize sample preparation procedures as much as possible.

Chlorophylls and lipids may be eliminated by extraction of the aqueous extracts with iso-octane. Losses of (-)-epicatechin and procyanidins B2, B5 and C1 were shown to be below 0.5 % [14]. The method presented here is less laborious and makes use of the insolubility of chlorophylls and lipids in 50 % (w/v) aqueous methanol. It is necessary
to weigh the remaining amount of aqueous extract A after evaporation of the acetone, since an exceeding amount of methanol is capable of dissolving significant amounts of more lipophilic matrix. On the other hand, if the methanol content is too low, precipitates are extremely sticky, clinging to the glass wall of the flasks and tending to clog in the following filtration step.

Attempts to develop a solid phase extraction procedure specific for low molecular weight procyanidins using commercially available solid phase extraction cartridges were unsuccessful because of the limited loadability of the cartridges [27]. This is a well-known problem in procyanidin analysis and the reason why validated protocols up to date merely exist for fruit juices and wine which seem to be less complex matrices than herbal plant materials [14]. Sample preparation procedure II only represents a filtration step over C18 material. Higher molecular weight procyanidins are retained and analytical chromatography is therefore assumed to be less affected by these compounds which do not elute as discrete peaks but tend to be spread over the whole chromatogram [14].

Recoveries of calibration solutions in the filtration step over C18 cartridges were determined in preliminary tests and were found to be good for (-)-epicatechin (100.5 %), B2 (100.8 %), B4 (96.4 %) as well as C1 (98.9 %). The recovery of procyanidin B5 (95.3 %), which is the latest eluting procyanidin on C18, is still acceptable.

**Chromatography**

The chromatographic method for the analysis of procyanidins in Crateagus leaves and flowers was developed empirically. The merit of optimization programs is very limited because of interfering minor matrix peaks and due to the fact that peak tracking is delicate since UV spectra of procyanidins do not show any obvious differences [14]. As a starting point the method was limited to 60 minutes. Furthermore, the proportion of organic phase in the eluent should at least amount to 5 %.

The LiChrosorb RP-18 stationary phase yielded slightly sharper peaks than the Spherisorb ODS II material under the same conditions. The pore size of Nucleosil 50 C18 columns is 50 Â, which leads to a 30 % increase in specific surface area compared to the one of common C18 columns showing pore sizes of 100 Â (i.e. LiChrosorb or Spherisorb columns). However, this increased surface area of the Nucleosil 50 column
did not improve the separating capacity of the stationary phase in the present investigation.

C6 columns have already been used in the analysis of procyanidins in apple tissues, juices and ciders [29-31]. In the analysis of *Crataegus* extracts flavan-3-ols elute earlier than on the RP-18 supports applying the same experimental conditions. If the methanol gradient is adjusted in the way that procyanidin B2 elutes approximately at the same time like on LiChrosorb RP-18 using the final chromatographic method \((t_R \text{ approximately } 13 \text{ min}), (-)-epicatechin elutes approximately 5 \text{ minutes earlier than on the RP-18 support. Procyanidin C1 is masked by matrix components under these conditions. By increasing the column temperature to } 35 \text{ °C the elution order is changed (i.e. } (-)-epicatechin → B2 → C1 → B5) \text{ compared to separations achieved using C18 supports or analyses at } 24 \text{ °C (i.e. B2 → C1 → (-)-epicatechin → B5). Best separations were obtained at } 42 \text{ °C starting the gradient at } 5 \% \text{ methanol.}

Increasing the column temperature also led to sharper peaks and better separations with the LiChrosorb RP18 column. However, it was not possible to establish a rugged method using higher column temperatures, because temperature control in the air-heated oven was not sufficient for this delicate separation and retention times therefore were not stable enough. Furthermore, repetitive injections at a column temperature of 42 °C over 9 hours showed constantly decreasing retention times which proved that the column was not yet equilibrated after this time interval. This is a well-known problem of air-heated devices and can only be solved by the use of block-heaters in combination with pre-heated eluents [32]. It was therefore decided to optimize the method at 24 °C for LiChrosorb C18, which was the most promising stationary phase at this temperature.

Acetonitrile was excluded as organic component in the eluent, because initial concentrations of at least 5 % in the eluent did not allow satisfactory separation of the flavan-3-ols. Tetrahydrofuran, which has already been tried as a possible eluent in the analysis of hawthorn [16], did not affect the selectivity of the methanol gradient and merely shifted retention times to lower values which yielded worse separation. Because it shows an even stronger elution power than acetonitrile, it could not be added alone as organic component to the eluent in reasonable initial concentrations. Methanol was therefore considered the organic component of choice for the present separation problem.
In a next step the influence of different types and concentrations of acids in the eluent was evaluated. The addition of an acid is necessary to suppress tailing of hydroxycinnamic acid derivatives, which show a similar retention behavior like procyanidins [14]. The type of acid can influence the selectivity of the chromatographic system by interaction with the stationary phase. In RP-chromatography such an interaction is especially expected with organic acids. The concentration affects the ionic strength of the eluent and may alter retention times as well. An increase in ionic strength led to longer retention times of flavan-3-ols in the analysis of apple juice using a methanol – aqueous ortho-phosphoric acid gradient [33]. We could not see such an effect applying 0.1 % to 5.0 % aqueous ortho-phosphoric acid in methanol in the analysis of extracts of Crataegus leaves and flowers. Retention times as well as resolution was practically unchanged. An increase of acetic acid concentrations from 0.5 % to 5 % in the aqueous methanol gradient caused a dramatic reduction of retention times with considerable loss of resolution. Low concentration of acetic acid produce chromatograms with similar resolution like eluents containing ortho-phosphoric acid. The UV cut-off at 240 nm is however a major drawback. The use of formic acid at a concentration of 0.5 % did not affect retention times significantly compared to the other acids at the same concentration levels, but resolution was worse. Based on these results it was decided to use 0.5 % ortho-phosphoric acid as aqueous component in the methanolic eluent.

The methanol gradient was further optimized. Separation of procyanidin B2 from accompanying matrix peaks was found to ask for a slight gradient while separation of procyanidin C1 and (-)-epicatechin is best achieved by isocratic elution. The resolution in the latter region of the chromatogram is not absolutely satisfying. LC-MS (liquid chromatography – mass spectrometry) analysis revealed the presence of higher molecular weight procyanidins up to the pentameric level. The (4β→8)-linked tetramer of (-)-epicatechin was isolated and was shown to elute in this region, too [27]. Reduction of the flow rate increased peak width slightly, but did not improve resolution. Variation of the injection volume revealed that best separations could be achieved using 5 µl. An increase of the injection volume resulted in a worse separation of procyanidin C1 and (-)-epicatechin.
With the final chromatographic method described in section 2.3 peak purities of
(-)-epicatechin, and procyanidins B5, B4 and C1 were better than 990 for both
procedures, which indicates the absence of impurities originating from non-
procyanidins. Peak purities of procyanidin B2 were between 985 and 990 for six
replicates of the repeatability samples using sample preparation procedure I. All
replicates of procedure II showed peak purities above 990 counts. Comparison of
different batches of LiChrosorb RP-18 columns indicated that the method is quite
rugged. The simultaneous resolution of procyanidin C1 and (-)-epicatechin on one hand
and the separation of procyanidin B2 from matrix components on the other hand,
however remain critical. Care should be taken to keep chromatographic parameters
constant, since already small changes (i.e. temperature) may affect the separation. This
behavior is typical for compounds showing unusual chromatographic features. For
some lower molecular weight procyanidins evidence for such irregularities have been
described [14]. An example of a typical chromatogram of an unspiked sample solution
C is given in Figure 2.2.

Validation
Using the sample preparation procedure I recoveries are higher than: 83 % for
(-)-epicatechin, 87 % for B2 and 81 % for B5, regardless of the calculation mode (see
Table 2.1). There is a slight tendency of reduced recovery rates with increasing spike
levels. This tendency is even more pronounced with procyanidins B4 and C1. If only
the lowest two spike levels are considered (which lie closest to the detected
concentrations), recovery rates are higher than: 92 % for (-)-epicatechin, 91 % for B2,
96 % for B5, 83 % for B4 and 80 % for C1. Best recovery rates for all five compounds
are obtained with quantitations based on area counts, although for example
(-)-epicatechin shows best recovery rates, if height counts are used for the quantitation.

Using the sample preparation procedure II recoveries are higher than: 89 % for
(-)-epicatechin, 81 % for B2, 89 % for B5, 72 % for B4 and 63 % for C1, regardless of
the calculation mode (see Table 2.2). There is not anymore an obvious tendency of
reduced recovery rates with increasing spike levels. Since this phenomenon is only
pronounced with sample preparation procedure I, it is assumed that complexation
reactions with matrix components might be responsible for these effects rather than
oxidation reactions. If only the lowest two spike levels are considered recovery rates are
Figure 2.2  Typical chromatogram of unspiked sample solution C recorded at 280 nm.

Retention times (min): procyanidin B4: 9.7, procyanidin B2: 12.8, chlorogenic acid: 14.5, procyanidin C1: 17.6, (-)-epicatechin: 18.3 and procyanidin B5: 38.4. Different integration modes for the quantitation of procyanidin C1 and (-)-epicatechin were evaluated. Integration in terms of "baseline-all-valleys" yielded the best results. For conditions, see Experimental.
higher than: 95 % for (-)-epicatechin, 85 % for B2, 94 % for B5, 80 % for B4 and 75 % for C1. Best recovery rates for all five compounds are obtained with quantitation based on height counts.

LC-MS analysis revealed the presence of procyanidins of higher degree of polymerization which elute closely to procyanidins B4 and C1 [27]. If the lower recovery rates of procyanidin B4 and C1 are due to interferences from these almost co-eluting minor procyanidins (i.e. complexation reactions) is not clear. The trimeric procyanidin C1 is much less stable than (-)-epicatechin or procyanidins B2 and B5, which might be an explanation for lower recovery rates, too [27].

The recovery rates obtained in this study are comparable to the ones described in the literature for other matrices. In grape tissue recoveries of procyanidin B3 were 86 to 91 % (5 replicates) [34], in wine recoveries for procyanidins B1 and B3 were 88 to 96 % [35], while validation experiments in apple cider and must yielded recoveries of 84 to 110 % (2 replicates, nature of spiked procyanidins is not specified) [30]. Better recoveries were only obtained with synthetic wine solutions (92 to 101 % for dimers, trimers and tetramers) [36] and by using individually packed polyamide columns in the analysis of wine (94 to 101 % for dimers and trimers) [37]. A more detailed list of methods for the analysis of procyanidins which at least have been partially validated is given in a review article on procyanidin analysis [14].

Comparison of the recovery rates for sample preparation procedure I and II reveals that none of the two procedures is superior over the other for all tested compounds. Results of the repeatability tests indicate that procedure II produces slightly lower relative standard deviations. This tendency is even more pronounced, if procyanidins are quantified in (-)-epicatechin equivalents (see Table 2.4).

Although quantitation of procyanidins is usually carried out at a detection wavelength of 280 nm the results presented clearly demonstrate that quantitation can as well be performed at 220 nm. So far this approach has only been applied to the quantitative analysis of apple juice [38]. The major advantage of the detection at 220 nm is its increased sensitivity (see Table 2.3). Although all procyanidins possess the same chromophore, differences in the limits of detection arise primarily from different elution times. Furthermore, higher molecular weight procyanidins tend to elute
as broader peaks, which can be seen from the relatively high limit of detection that was obtained for procyanidin C1.

Because procyanidins are not commercially available the quantitation of procyanidins in terms of (-)-epicatechin equivalents is very widespread [14]. All recovery experiments were also conducted using (-)-epicatechin calibration graphs. The recovery rates for the determinations based on 280 nm area counts of both sample preparation procedures corresponded very well with the respective values of the experiments run with individual procyanidin calibration graphs. The use of the other calculation modes generally led to significant overestimations and should therefore not be applied (experimental details are published elsewhere [27]).

Using sample preparation procedure II, the following contents in Crataegus leaves and flowers were detected: (-)-epicatechin: 0.120 %, B2: 0.106 %, C1: 0.060 %, B5: 0.052 % and B4: 0.051 %. Literature values for procyanidin contents in Crataegus leaves and flowers are between 1.9 % and 4 % [7,10,11,17,39]. However, all these results are based on colorimetric detection methods which are known to be not quantitative [14]. Furthermore, all methods (exception: [17]) do not quantify individual procyanidins but the total of oligomeric and/or polymeric procyanidins.

Conclusions

Although chromatograms of procedure I and II look almost the same, procedure II should be favored, if quantitative analyses are to be performed. The repeatability is slightly better, there are no obvious matrix effects and peak purity data for procyanidin B2 are more favorable. As long as sensitivity is no problem, quantitation should be based on 280 nm height counts, because possible interferences are reduced at this higher wavelength. If the lowest two spike levels are considered (which lie closest to the detected concentrations), recoveries were higher than 94 % for (-)-epicatechin, procyanidins B4 and B5. Recoveries of procyanidin B2 were higher than 87 % and recoveries for procyanidin C1 were higher than 81 %. Interpretation of results of procyanidins B4 and C1 should be done with great care, since other procyanidins of higher degree of polymerization co-elute.

Quantitation in terms of (-)-epicatechin equivalents should be based on area counts at 280 nm. Overestimations result, if responses at 220 nm or height counts at 280 nm
are used for quantitation. Sample procedure I, which is less laborious, is very suitable as a fingerprint method.

It is postulated that the recovery rates could be further improved by the performance of the filtration over solid phase cartridges under a nitrogen atmosphere and the use of a cooled sample tray for the vials during the HPLC analysis. This special equipment was however not available for the present study.

2.1.4 References

2.2 Additional results to publication 2

Figure 2.3 gives an overview on the sample preparation procedure described in section 2.1.2.1.

Tables 2.5 and 2.6 summarize the results of recovery experiments for the two sample preparation procedures using quantitation in (-)-epicatechin equivalents. The recovery rates for the determinations based on 280 nm area counts of both sample preparation procedures correspond well with the respective values of the experiments run with individual procyanidin calibration graphs. Significant overestimations result, if calculations are based on height counts or area counts at 220 nm. For experimental details, see section 2.1.2.

Figures 2.4 and 2.5 show typical chromatograms of the two sample preparation procedures recorded at 280 and 220 nm. Underground absorbances are higher at 220 nm than at 280 nm. Therefore, determinations preferentially should be performed at 280 nm. Sample preparation procedure II should be favored, if quantitative analyses have to be made. The repeatability is slightly better, there are no obvious matrix effects and peak purity data for procyanidin B2 are better using this sample preparation procedure. For experimental details, see section 2.1.2.
Crataegus leaves and flowers

Percolation with acetone 70 %

Evaporation to a small volume

Weighing
Addition of the same volume of methanol

Filtration over Celite 535

Evaporation of organic phase

Lyophilization of aqueous phase

Dissolution in 50 % methanol

HPLC for fingerprint analysis

Filtration over C18 cartridge

HPLC for quantitative analysis

Figure 2.3 Scheme of the sample preparation procedure for the qualitative and quantitative HPLC analysis of proanthocyanidins in Crataegus leaves and flowers.
Table 2.5  Results of recovery experiments for sample preparation procedure I. Quantitation in (-)-epicatechin equivalents.

<table>
<thead>
<tr>
<th>Reference compound</th>
<th>Spikea (µg/ml)</th>
<th>Recoveries (%), calculation based on</th>
</tr>
</thead>
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<tr>
<td></td>
<td>280 nm</td>
<td>280 nm</td>
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<tr>
<td></td>
<td>area counts</td>
<td>height counts</td>
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<tr>
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<tr>
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a Calculated concentrations of spikes of reference compounds in sample solution B.
Table 2.6   Results of recovery experiments for sample preparation procedure II. Quantitation in (-)-epicatechin equivalents.

<table>
<thead>
<tr>
<th>Reference compound</th>
<th>Spike&lt;sup&gt;a&lt;/sup&gt; (μg/ml)</th>
<th>Recoveries (%), calculation based on 280 nm area counts</th>
<th>280 nm height counts</th>
<th>220 nm area counts</th>
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<sup>a</sup> Calculated concentrations of spikes of reference compounds in sample solution C.
Figure 2.4 Typical chromatograms of unspiked sample solution B.
Sample preparation procedure I.

Top: Recorded at 280 nm.
Bottom: Recorded at 220 nm.
Figure 2.5  Typical chromatograms of unspiked sample solution C.
Sample preparation procedure II.

Top: Recorded at 280 nm.
Bottom: Recorded at 220 nm.
2.3 Analysis of *Crataegus* flowers and fruits

The flowers as well as the fruits of *Crataegus* are commercially available besides the most commonly used leaves and flowers. Official monographs exist of all three plant tissues: leaves and flowers (Ph. Helv. 8; DAB 1998; Ph. Franç. X), fruits (Ph. Eur. 3, suppl. 1998; DAC (German Drug Codex) 1986, suppl. 4, 1992) and flowers (Ph. Franç. X; DAC 1986, suppl. 2, 1990).

There have been made a number of colorimetric surveys in which the contents of the different plant parts have been compared. Higher procyanidin levels were found in the leaves and flowers than in the fruits (leaves and flowers: 1.9 - 3.2 %, fruits: 0.1 - 1 % [1,2]; leaves and flowers: 3.8 %, fruits 1.4 % [3]). In the fruits the pulp is the richest source of procyanidins followed by the skin and the stalks [2]. Flowers are generally reported to show higher contents than the leaves (determined colorimetrically [2,4]; determined by densitometry [5]; shown for monomers quantified by HPLC [6]). Lower procyanidin levels in the flowers compared to the leaves have been reported for *Crataegus monogyna* samples from a homogeneous climatic area [7] and in a study on procyanidin levels in different *Crataegus* species [8]. Quantitation in both studies was based on colorimetric assays. The barks seem to be a rich source for flavan-3-ols, too. Oszmianski and Bourzeix [9] described a procedure to obtain monomer and procyanidin standards from the bark of *Crataegus azarolus*. They succeeded in isolating 450 mg of flavan-3-ols from 100 g of fresh bark.

The two most commonly encountered species, *Crataegus monogyna* and *Crataegus laevigata*, have been reported to not show any obvious differences with respect to the total contents or qualitative composition of procyanidins [1,10]. The red-blooming variety of *Crataegus monogyna* has been reported to show tendentiously higher levels of total procyanidins than the white-blooming variety [7]. Hecker-Niediek [8] analyzed the leaves of twelve species and subspecies of *Crataegus* by a combination of preparative TLC and a colorimetric assay. In contrast to the results cited above, large differences in contents were found (1.5 to 8.6 %). The procyanidin pattern of the leaves however remained unchanged over the whole season in all tested species and was the same as determined for the fruits and flowers.

Several studies focus on the seasonal development of procyanidin levels in the different plant organs. All these studies make use of colorimetric quantitation...
techniques. The results are again sometimes contradictory. In the leaves highest contents were measured during the blooming period in some studies [7,11], while in other studies the levels increased from the beginning of the development of the leaves until the ripening of the fruits in early fall and only subsequently decreased in the period before the falling of the leaves [8,12]. The buds show lower flavan-3-ol contents than the flowers [6,7].

In the fruits, highest procyanidin contents are measured in the unripe states. The levels are reported to decrease until the end of summer (i.e. from 6.9 % to 0.9 % [8]) [7,11]. If the procyanidin concentrations are calculated per fruit, the contents remain nearly unchanged over the whole ripening period [8]. This behavior is typical of procyanidin containing fruits in general (i.e. it has also been reported for apple fruits [13]). Based on this feature the hypothesis has been established that after an initial burst of procyanidin synthesis a steady state concentration is maintained [14,15]. As the tissues mature further the average degree of polymerization increases in a lot of species (i.e. Vaccinium myrtillus [16], Vitis vinifera [17,18]), while the average molecular weight remains nearly unchanged in other species (i.e. Hordeum vulgare [19]) [20].

The very few studies on seasonal changes of selected procyanidins in plant matrices other than Crataegus imply that the different low molecular weight compounds exhibit individual concentration profiles [13]. The simplified view that tissue aging is generally accompanied by an increase of compounds of higher molecular weight has also been defeated by the study of Koupai-Abyazani et al. [21] on the developmental changes of the composition of proanthocyanidins in sainfoin leaves. Evidences were found for a very complex regulatory mechanism which might also include catabolism of polymeric compounds.

The re-evaluation of the concentration profiles of individual procyanidins over a whole vegetation period in different plant tissues of Crataegus sp. would therefore be very informative and would surely help to clarify the somewhat contradictory results on contents of procyanidin fractions which are reported in the literature.

The scope of this present investigation was to analyze the qualitative composition of procyanidins in Crataegus flowers and ripe fruits using the adapted method originally developed for the leaves and flowers and to give estimates for the contents of individual compounds.
2.3.1 Experimental

*Crataegus* flowers (lot 48264L) and fruits (DAC, lot 44175L) were obtained from Dixa AG (CH-St. Gallen). All other reference standards, reagents and instruments were the same as described in section 2.1.2 (HPLC / DAD analyses) and section 5.2 (LC-MS analyses), respectively.

Sample preparation
15.00 g of dried and pulverized (mesh 1 mm) plant material were macerated with 50 ml of aqueous acetone 70 % (v/v) for approximately 15 minutes. The suspension was transferred to a column (dimensions: Ø 20 x 250 mm) and was percolated at an approximate speed of one drop per second (requiring 2.5 to 3 hours). Glass beads were placed on top of the pulverized fruits during the percolation to prevent floating of the plant material. 250 ml of extract (= extract solution A) was collected and the organic phase was evaporated applying temperatures below 30 °C. The aqueous solution was immediately frozen and lyophilized. The brown residue was dissolved in 50 % (v/v) aqueous methanol and was transferred to 50.0 ml (samples of flowers) or 25.0 ml (samples of fruits) volumetric flasks, respectively. Wash solutions were again sonificated for a short time to solubilize residues sticking to the glass wall. The volumetric flask was filled up to the mark (= sample solution B). Aliquots of these solutions were analyzed by HPLC after filtration over 0.45 μm nylon filters.

Sep-Pak® tC18 cartridges were activated with 10 ml of methanol and preconditioned using 5 ml 50 % (v/v) aqueous methanol. 2.0 ml of the sample solutions B were applied and the filtrates were collected in 5.0 ml volumetric flasks. The cartridges were further eluted with 2.3 ml of 50 % (v/v) aqueous methanol using a 5.0 ml Pipetman® of Gilson (F-Villiers-le-Bel). The volumetric flasks were filled up to the mark with 50 % (v/v) aqueous methanol and were directly used for HPLC analysis (= sample solutions C). The samples were prepared in triplicate.

The chromatographic conditions were the same as described in section 2.1.2. The injection volumes were chosen to be 5 μl for samples of flowers and 20 μl for samples of fruits. Results are expressed in (-)-epicatechin equivalents. Seven calibration solutions of (-)-epicatechin were prepared in the concentration range 1140 to 18 μg/ml (correlation coefficient: 0.9979).
LC-MS analyses

Concentrated and lyophilized extract solutions A were dissolved in 50 % (v/v) aqueous methanol (concentration of the solutions: flower extract: 216.7 mg/ml, fruit extract: 225.2 mg/ml). The extracts were filtered over a 0.45 μm nylon filter prior to injection. The chromatographic conditions were the same as described in section 5.2.

2.3.2 Results and discussion

Preliminary tests revealed that the collection of 250 ml of extract is exhaustive for both types of samples (more than 99 % of the total extractable amounts of procyanidins B4, B2, B5 and (-)-epicatechin and more than 98 % of the total extractable amounts of procyanidin C1 could be recovered in the first 250 ml of extract).

The final filtration step over C18 cartridges should be performed, if quantitative estimations are intended. The results from the triplicate determinations indicate that the relative standard deviations are much lower after the filtration step over C18 cartridges than before. The mean relative standard deviations of sample solutions B amounted to: 10.5 % (fruits) and 4.0 % (flowers) while the mean relative standard deviations of sample solutions C were calculated to be: 7.3 % (fruits, without considering procyanidin C1: 2.5 %) and 0.9 % (flowers), respectively. The peak purity data indicate no impurities originating from non-procyanidins in sample solutions B and C for the samples prepared from flowers (values above 990 counts). The peak purity data of the samples from fruits indicate the presence of impurities in sample solutions B for the peak of procyanidin B2 (one sample showing a value of 964 counts) and the broad peak of (-)-epicatechin / procyanidin C1 (values between 958 and 983 counts). The peak purity data of the sample solutions C from fruits were again above 990 counts for all compounds. Typical chromatograms of sample solutions B and C are given in Figures 2.6 and 2.7.

The chromatograms of Crataegus fruits are characterized by a huge unresolved underground which is postulated to be due to oligomeric procyanidins which can not be separated chromatographically. This view is supported by the fact that the UV spectra of the column effluent after about 20 minutes of the run indicates the prevailing presence of flavan-3-ols. Petri et al. [2] also describe a higher average degree of polymerization of procyanidins in fruits than in leaves and flowers. An additional
procyanidin peak is detectable at around 11 minutes which may be attributed to trimer I and an unidentified tetramer from the LC-MS data (see below).

Figure 2.6 Chromatogram of *Crataegus* flowers recorded at 280 nm.
Top: Sample solution B (before filtration over C18 cartridge).
Bottom: Sample solution C (after filtration over C18 cartridge).
Peak numbering: 1 = procyanidin B4; 2 = procyanidin B2; 3 = procyanidin C1; 4 = (-)-epicatechin; 5 = procyanidin B5. Injection volume: 5 µl.
Figure 2.7 Chromatogram of *Crataegus* fruits recorded at 280 nm.

Top: Sample solution B (before filtration over C18 cartridge).
Bottom: Sample solution C (after filtration over C18 cartridge).
Peak numbering: 1 = procyanidin B4; 2 = procyanidin B2; 3 = procyanidin C1; 4 = (-)-epicatechin;
5 = procyanidin B5. Injection volume: 20 µl.
The separation of (-)-epicatechin and procyanidin C1 in the samples from fruits is very delicate. These two compounds are not separated in the chromatograms of sample solutions B and appear as a broad peak (see Figure 2.7). In the chromatograms of sample solutions C procyanidin C1 can be detected as a shoulder. The removal of some impurities combined with the injection of a less concentrated solution is thought to be responsible for this better resolution. The quantitative estimation of the two compounds however remains critical due to the difficulties in drawing suitable baselines for the integration of the respective peaks.

The chromatograms of Crataegus flowers look very similar to the ones of Crataegus leaves and flowers (see Figures 2.4, 2.5 and 2.6). In the range of t\textsubscript{R} 27 to 34 min some peaks occur which show UV spectra similar to procyanidins. These peaks are also present in samples of Crataegus leaves and flowers. At around 11 minutes a procyanidin peak occurs which may again be attributed to trimer I and an unidentified tetramer from the LC-MS data (see below). The peak purity data (mean value: 982 counts) however indicate some co-eluting impurities which forbid a quantitation.

The results of the quantitative evaluations are given in Table 2.7. The values obtained for the flowers and fruits can only serve as estimates, because the methods have not been validated. Some qualitative information can be taken from the data after all: The contents of individual procyanidin dimers and trimers are significantly lower in the fruits compared to the flowers (and also leaves and flowers). (-)-Epicatechin can be detected in significant amounts in all samples. Procyanidins B5, B4 and C1 are also present in all samples, but in much lower concentrations than the principal procyanidin B2.

The LC-MS chromatograms show identical procyanidin patterns up to the tetrameric level for all plant tissues (see Figures 2.8, 2.9 and section 5 Figure 5.7A). Procyanidin B5 could not be detected in the samples of fruits and flowers due to instrumental problems (loss of sensitivity after about 500 scans). The pentameric (m/z 1441) and hexameric (m/z 1729) ion traces of the flower extracts resemble the ones of the extracts of leaves and flowers very much. In contrast, no individual pentameric or hexameric peaks of significant abundance could be detected in the fruit sample. However, the extended hump in the chromatograms of the fruit samples recorded using UV detection at 280 nm suggests that higher oligomers might be present in this sample as well.
Presumably the concentrations of individual oligomeric procyanidins are too low to be detectable in LC-MS analysis.

**Table 2.7** Results of the analysis of *Crataegus* flowers and fruits.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Fruits(^a) (%)</th>
<th>Flowers(^a) (%)</th>
<th>Leaves and flowers(^b) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(-)-epicatechin</td>
<td>0.0349(^c)</td>
<td>0.146</td>
<td>0.120</td>
</tr>
<tr>
<td>procyanidin B2</td>
<td>0.0286</td>
<td>0.189</td>
<td>0.106</td>
</tr>
<tr>
<td>procyanidin B5</td>
<td>0.0063</td>
<td>0.080</td>
<td>0.052</td>
</tr>
<tr>
<td>procyanidin B4</td>
<td>0.0075</td>
<td>0.040</td>
<td>0.051</td>
</tr>
<tr>
<td>procyanidin C1</td>
<td>0.0081(^c)</td>
<td>0.071</td>
<td>0.060</td>
</tr>
</tbody>
</table>

\(^a\) Quantitation in (-)-epicatechin equivalents.  
\(^b\) Quantitation using individual flavan-3-ol calibration graphs (see section 2.1).  
\(^c\) Estimates tentatively too low due to the application of the integration mode „baseline- all valleys“ resulting in very steep baselines.

**Conclusions**

The principal procyanidin in all tested plant tissues is procyanidin B2. The procyanidins B5, B4 and C1 can be detected as well, besides significant amounts of (-)-epicatechin. The concentrations of all individual procyanidins are significantly lower in ripe fruits compared to flowers or leaves and flowers. This result is in agreement with reported findings which are based on colorimetric assays.

LC-MS chromatograms revealed analogous procyanidin patterns for all tested plant tissues up to the tetrameric level. No individual higher oligomers of significant abundance were detectable in the fruit sample. In the extract of the flowers some peaks which might be attributable to individual pentamers and hexamers were detectable. Analogous peaks were also present in the samples prepared from leaves and flowers.
Figure 2.8  LC-MS chromatogram of *Crataegus* flowers.

Ion traces correspond to monomers (m/z 289), dimers (m/z 577), trimers (m/z 865), tetramers (m/z 1153), pentamers (m/z 1441) and hexamers (m/z 1729). RIC = total ion current; ordinate: relative abundance; abscissa: time (scans; rate: 15 sec); injection volume: 20 μl; concentration: 216.7 mg/ml. ESP interface, negative ionization mode.
Figure 2.9 LC-MS chromatogram of *Crataegus* fruits.

Ion traces correspond to monomers (m/z 289), dimers (m/z 577), trimers (m/z 865), tetramers (m/z 1153), pentamers (m/z 1441) and hexamers (m/z 1729). RIC = total ion current; ordinate: relative abundance; abscissa: time (scans; rate: 15 sec); injection volume: 20 µl; concentration: 225.2 mg/ml. ESP interface, negative ionization mode.
2.3.3 References

3 Solid phase extraction and addition of antioxidants to the extraction solvent in the quantitative analysis of procyanidins in *Crataegus*

3.1 Solid phase extraction of procyanidins

Since procyanidins are accompanied by a lot of compounds which show similar retention and/or absorbance behavior (i.e. flavonoids and phenolic acids) sample clean up is inevitable. In view of the instability of procyanidins liquid-liquid extraction should be omitted, because shaking leads to a thorough mixing of the two-phase system with the gas phase and consequently promotes oxidation reactions. Furthermore, liquid-liquid extraction is quite laborious. As an alternative the possible use of solid phase extraction as a sample clean up procedure for extracts of *Crataegus* leaves and flowers has been evaluated.

The scope of the investigation was to find suitable conditions to be able to separate low molecular weight flavan-3-ols (monomers up to tetramers) from matrix components (flavonoids, phenolic acids) on one hand and to be able to possibly separate (-)-epicatechin from procyanidins on the other hand.

3.1.1 Experimental

Materials and instrumentation

In order to ensure reproducibility only commercially available solid phase extraction cartridges were evaluated. The following size exclusion sorbents were used: Oasis® HLB (based on divinylbenzene-co-N-vinylpyrrolidone, 30 mg, 1 ml, Waters, USA-Milford, MA), Insolute ENV+® (200 mg, 3 ml, ict, CH-Basel) and Chromabond® HP-P (200 mg, 3 ml, Macherey-Nagel, D-Düren). The latter two phases are based on polystyrene-divinylbenzene copolymers. The following mainly reversed-phase sorbents were evaluated: Sep-Pak® tC18 (500 mg, 3 ml, Waters) as well as the following Chromabond® cartridges (100 mg, 1 ml) from Macherey-Nagel: C8, phenyl, diol, nitrile, amino, dimethylamino and SB (anion exchange material).
The ethyl acetate extract was prepared from *Crataegus* leaves and flowers (Ph.Helv.VII / DAB10, Dixa, CH-St. Gallen) as described in section 8.3. Solutions of approximately 20 mg/ml were prepared in 5 % and 30 % aqueous methanol. Only the solution in 30 % aqueous methanol showed no turbidity at room temperature.

(-) Epicatechin (HPLC grade) was purchased from Extrasynthese (F-Genay), (+)-catechin (Rotichrom® CHR) and chlorogenic acid (CHR grade) were from Roth (CH-Reinach). All procyanidins were isolated from *Crataegus* leaves and flowers (see sections 8 and 10). Acetone, acetonitrile, ethyl acetate, methanol and tetrahydrofuran were of HPLC grade (Romil Chemicals, UK-Shepshed). Water was obtained using a NANOpure cartridge system (Skan, CH-Basel). pH adjustments were made with diluted solutions of ammonia (32 %, puriss., Merck, CH-Dietikon) and hydrochloric acid (Ph.Eur., Hänseler, CH-Herisau). Formic acid (p.a.) was obtained from Merck; sulphuric acid (puriss.) and ortho-phosphoric acid (Ph.Helv.VI / Eur.) were purchased from Hänseler. 4-Dimethylaminocinnamaldehyde (DMACA, purum) was obtained from Fluka (CH-Buchs).

**Chromatographic conditions**

TLC analyses were developed on silica gel 60F254 (Merck) over a distance of approximately 8 cm. The eluent was composed of ethyl acetate – formic acid – H2O (90:5:5, v/v). Detection was performed at UV 254 nm, UV 365 nm and using a 1 % (w/v) solution of DMACA in methanolic sulphuric acid (8 ml H2SO4 ad 100 ml methanol). Procyanidins turn green to blue; upon heating spots become brown but turn back to the original colors after a while.

HPLC analyses were performed using a Hewlett-Packard instrument (Model 79994A analytical workstation, model 1090 liquid chromatograph, model 1040 diode-array detector). A Knauer (D-Berlin) prepacked cartridge column (250 x 4 mm I.D., 5 μm) filled with LiChrosorb RP-18 and a guard column (10 x 4 mm I.D.) of the same material was used for chromatographic separations. The mobile phase consisted of solvent A (methanol) and solvent B (0.5 % (v/v) ortho-phosphoric acid in water). The elution profile was: 0 min 20 % A in B, 0 - 35 min from 20 % to 35 % A in B, 35 - 40 min 35 % A in B, 40.01 - 45 min 90 % A in B, 45.01 - 50 min 100 % A (wash-out), 50.01 - 60 min 20 % A in B (re-conditioning). All gradients were chosen to be linear. The flow rate was set to 1.0 ml/min, the column temperature was fixed at 24.0 °C and
the injection volume was chosen to be 20 μl. Chromatograms were recorded at 280 nm, spectra were measured in the range 190 nm to 600 nm. Peak identification was based on comparison of retention times with authentic reference compounds and on UV / DAD spectra. Peak purities were checked using the software of the photodiode array detector.

**Procedure**

The 3 ml cartridges were conditioned using 10 ml of methanol followed by 5 ml of H₂O pH 7; the 1 ml cartridges were conditioned using 5 ml of methanol followed by 2 ml of H₂O pH 7. If cartridges were washed with acidic or alkaline H₂O, they were conditioned with the same solutions. The analyses were conducted applying 0.2 to 2 ml aqueous methanolic solutions. Loadability tests were performed with the 5 % aqueous solutions.

The cartridges were washed with H₂O pH 7 (1 to 5 ml in two portions depending on the amount of sorbent). Elution from size exclusion sorbents was achieved using methanol (5 to 6 ml in portions), followed by aqueous acetone 70 % (5 to 10 ml in portions). Elution from the other sorbents was performed using aqueous acetonitrile 16 % pH 2 (4 to 10 ml in portions) and methanol (4 to 10 ml in portions). If not all compounds could be eluted using this procedure, subsequent elution with aqueous acetone 70 %, ethyl acetate, acetonitrile and tetrahydrofuran was tried (amino and dimethylamino sorbents). The effect of the pH of the wash fraction (H₂O pH 7, pH 2 and pH 9) was evaluated using the size exclusion sorbents and the tC18 phase.

All eluates were investigated by TLC. Eluates, which showed some promising separation were evaporated below 30 °C, dissolved in 0.5 ml 50 % aqueous methanol and additionally analyzed by HPLC.

**3.1.2 Results and discussion**

Solid phase extraction of procyanidins is mainly performed using aqueous methanol, acetonitrile and acetone as eluents [1]. The most commonly used acetonitrile eluent (16 % in H₂O, pH 2) [2-6] was shown to be weaker than methanol or aqueous acetone in preliminary tests. The elution order was therefore chosen as described above.
Size exclusion sorbents

None of the tested sorbents worked on the basis of gel filtration, that is to say polymeric procyanidins (remaining at the start on TLC chromatograms) could not be separated from low molecular weight procyanidins (monomers up to tetramers). Adsorption effects did not cause a separation of matrix components absorbing at 254 nm or 365 nm from low molecular weight procyanidins, either.

The loadability of the Chromabond® HP-P sorbent (< 4 mg extract per 200 mg sorbent) is significantly lower than the one of the other two cartridges (approximately 35 to 40 mg extract per 200 mg sorbent). Only (-)-epicatechin is retained on Chromabond® HP-P, all procyanidins are already eluted with the wash fraction. In contrary, all procyanidins are retained on Oasis® HLB and Insolute ENV+® sorbents during the washing of the cartridges. Methanol is capable of eluting all procyanidins and interfering matrix components. Changing the pH of the wash solutions to alkaline and acidic values does not allow the separation of these interfering components from the procyanidins. If the cartridges are overloaded, (-)-epicatechin elutes before the procyanidins on Oasis® HLB cartridges, while procyanidins B2 and C1 first elute from Insolute ENV+® cartridges.

Reversed-phase and other sorbents

Best separations are achieved using the amino sorbent. (-)-Epicatechin is eluted to a great extent in the wash fraction together with trace amounts of (+)-catechin and two unidentified compounds (see Figure 3.1). TLC and HPLC chromatograms of the acetonitrile fractions indicate that polymeric procyanidins and matrix components can greatly be separated from the low molecular weight procyanidins (see Figure 3.1). Peak purity for all identified procyanidins and (-)-epicatechin were better than 990. Four unknown peaks showing procyanidin spectra or spectra similar to procyanidins and peak purities over 990 counts eluted between minutes 20 and 27, too. Methanol does not elute further low molecular weight procyanidins. Brown-colored among other matrix components (i.e. chlorogenic acid) can not be eluted from the cartridge even applying very strong eluents. Flavan-3-ols behave similarly on the dimethylamino sorbent with the exception that the greater proportion of (-)-epicatechin elutes together with the procyanidins in the aqueous acetonitrile fraction. The loadability for procyanidins for both cartridges is about 10 mg extract per 100 mg sorbent.
Figure 3.1  Solid phase extraction of an ethyl acetate extract of *Crataegus* leaves and flowers using a Chromabond® amino cartridge.

Top:  Chromatogram of the wash fraction.
Bottom:  Chromatogram of the aqueous acidic acetonitrile eluate.
Only peaks exhibiting typical UV spectra are labeled; peak identification, see Figure 3.2.
For experimental details, see text.
Using the anion exchange sorbent a lot of flavan-3-ols, but only one spot showing activity at 365 nm, are eluted with the wash fraction (see Figure 3.2). However, compared to the amino sorbent, less individual procyanidins can be identified and peak purity data for procyanidin B4 and (-)-epicatechin indicate the presence of some impurities. No peak purity data were available for procyanidin C1. Aqueous acetonitrile and acetone elute more polymeric material (at the start on TLC chromatograms), chlorogenic acid and remaining compounds showing activity at 365 nm.

Figure 3.2  Solid phase extraction of an ethyl acetate extract of Crataegus leaves and flowers using a Chromabond® SB cartridge. Chromatogram of the wash fraction.

Peak identification:

1. Procyanidin B4
2. Trimer I
3. (+)-Catechin
4. Procyanidin B2
5. Chlorogenic acid
6. Procyanidin C1
7. Tetramer I
8. (-)-Epicatechin
9,11 Unknown compounds showing typical procyanidin spectra
10,12,13,15 Unknown compounds showing spectra similar to procyanidins
14. Procyanidin B5

Only peaks exhibiting typical UV spectra are labeled. For experimental details, see text.
The tC18 cartridge is capable of retaining more (-)-epicatechin and procyanidin dimers than the C8 cartridge, which is reflected in lower amounts of these flavan-3-ols in the wash fractions of the tC18 sorbent. The loadability for procyanidins for tC18 was about 30 mg extract per 500 mg solid support. The vast majority of procyanidins (including some polymeric procyanidins) are eluted with aqueous acetonitrile. The peak purity data of procyanidin B2 indicate no co-eluting impurities, which is an improvement to the data obtained with the ethyl acetate extract (see Figure 3.3). However, procyanidin C1 is masked by matrix peaks and no peak purity data were available for procyanidin B4. The purity data for (-)-epicatechin indicate some impurities, too. Methanol elutes remaining amounts of low molecular weight procyanidins and more polymeric procyanidins. Chlorogenic acid is eluted in wash and in aqueous acetonitrile fractions. Adjusting the pH of wash fractions to pH 9 makes it possible to remove chlorogenic acid together with two other components absorbing at 365 nm in the wash step. However, some flavan-3-ols still elute with the wash fraction.

The diol sorbent is not suitable for solid phase extraction of procyanidins at all, because all flavan-3-ols partially already elute in the wash fraction. The same is true for the phenyl and nitrile sorbents, which however adsorb flavan-3-ols stronger than the diol sorbent. (-)-Epicatechin is washed from the cartridges before the procyanidin dimers, followed by the trimers.

**Conclusions**

Size exclusion sorbents are not suitable to separate oligomeric from polymeric procyanidins because adsorption effects predominate. Diol, phenyl and nitrile cartridges did not yield satisfactory separation. The results of C8 and dimethylamino sorbents were worse than the ones obtained with tC18 and amino cartridges, respectively.

The use of tC18 cartridges for solid phase extraction of oligomeric procyanidins seems to only be applicable to less complex matrices like fruit juices, wines and extracts from grapes [1]. In the case of more complex matrices like extracts from leaves (Onobrychis sp. [7]) or aerial parts of plants (Cistus sp. [8]) merely filtration over the sorbent has been performed (i.e. flavan-3-ols are eluted right after application to the cartridges). The results of the present investigation supports such an approach for Crataegus leaves and flowers, too.
Figure 3.3  Solid phase extraction of an ethyl acetate extract of *Crataegus* leaves and flowers using a Sep-Pak<sup>®</sup> tC18 cartridge and chromatogram of the ethyl acetate extract before solid phase extraction.

Top: Solid phase extraction of an ethyl acetate extract of *Crataegus* leaves and flowers using a Sep-Pak<sup>®</sup> tC18 cartridge. Chromatogram of the aqueous acidic acetonitrile eluate.

Bottom: Chromatogram of an ethyl acetate extract of *Crataegus* leaves and flowers in 50% aqueous methanol (23.44 mg/ml, injection volume: 5 µl).

Only peaks exhibiting typical UV spectra are labeled; peak identification, see Figure 3.2.

For experimental details, see text.
With the amino sorbent separation of procyanidins from the majority of (-)-epicatechin as well as separation from matrix components can be achieved. The use of an anion exchange sorbent like Chromabond® SB to separate compounds exhibiting UV 365 nm activity from flavan-3-ols by filtration is promising. For both sorbents experimental conditions should be further optimized and quantitative recovery of low molecular weight procyanidins and (-)-epicatechin should be demonstrated. Since ethyl acetate does not extract low molecular weight procyanidins quantitatively [1], these two sorbents would have to be tested with crude extracts, too. The loadability for procyanidins is expected to be even lower than with ethyl acetate extracts. This problem can be overcome by connecting cartridges in series, which already has been described in the literature for C18 cartridges [1,9] and combinations of Extrelut® and Bond Elut® cartridges [8,10]. Some researchers also make use of cartridges with higher amounts of sorbents [1].

The solubility of the extract in reasonable concentrations for subsequent UV / DAD detection (ideally 20 mg/ml) causes the main problem in solid phase extraction of procyanidins. Ethyl acetate extracts of *Crataegus* leaves and flowers are only completely dissolved in 30 % aqueous methanol. This solvent, however, is already such a strong eluent that procyanidins are not anymore retained on the sorbents. In order to study elution features suspensions in 5 % aqueous methanol had to be applied to the cartridges. Separation of the precipitate and subsequent TLC analysis revealed that also low molecular weight procyanidins (i.e. dimers and trimers) were not completely dissolved in 5 % aqueous methanol. The application of suspensions to solid phase extraction cartridges is however not a good laboratory practice, if quantitative determinations are intended, because reproducibility may suffer substantially.

3.2 Addition of antioxidants to the extraction solvent

The addition of antioxidants to the extracting solvent (i.e. [11]) or directly to the analyzed fruit juices (i.e. [12]) is quite common in the analysis of procyanidins [1]. Favorable effects on polyphenol contents determined by the protein precipitating activity could be demonstrated in *Juglans* sp. [13], but there are no data published concerning the effects of antioxidants on low molecular weight procyanidins. The scope
of this investigation was to evaluate the use of ascorbic acid and sodium metabisulfite as antioxidants in the extracting solvent in the analysis of *Crataegus* leaves and flowers.

**Experimental**
Sodium metabisulfite (purum) was purchased from Siegfried (CH-Zofingen). Ascorbic acid (Ph.Eur.) was obtained from Hänseler (CH-Herisau). All other materials, instruments and chromatographic conditions were the same as described in section 2.1.2.

Three replicates were made using ascorbic acid (1 g/l) in the extracting solvent, four replicates were made using sodium metabisulfite (10 g/l) in the extracting solvent and two replicates were worked up without the addition of any antioxidant. The concentrations of the antioxidants were chosen according to the literature [1]. Extractions were performed as described in section 2.1.2. The samples were extracted a second time with another 250 ml of the same extracting solvent. All extract solutions (250 ml) were worked up analogously as described in section 2.1.2. Comparison of individual procyanidins and (-)-epicatechin levels were based on 280 nm area counts.

**Results and discussion**
The peak areas of procyanidins B4 and B2 of the samples prepared with sodium metabisulfite varied strongly, but were significantly lower (17 % and 18 %, respectively) than the average peak areas obtained with aqueous acetone. (-)-Epicatechin produced an average peak area which was 17 % higher than the one obtained with aqueous acetone. Peak areas of procyanidins C1 and B5 were comparable to the reference values (± 5 %). The second extraction of the samples did not yield significant additional amounts of procyanidins. Interpretation of these findings is difficult. It is assumed that the relatively high concentration of sodium metabisulfite in the aqueous acetone, which is close to the saturation point, is responsible for the greater variations of peak areas compared to the other samples containing ascorbic acid or no antioxidant. The high salt content of the extracting solvent could also favor the formation of complexes or aggregates which could be an explanation for low concentrations of individual procyanidins. The high concentration of (-)-epicatechin can
only be explained by depolymerization reactions, because (-)-epicatechin is quite stable at room temperature compared to the procyanidins.

Average peak areas of all procyanidins and (-)-epicatechin in the samples prepared with ascorbic acid were lower (8% to 12%) than the ones of the samples prepared without the addition of an antioxidant. Again, extraction of the same plant materials did not yield any significant additional amounts of (-)-epicatechin or procyanidins. Possible explanations for these findings could be that ascorbic acid forms complexes with procyanidins, or that the alteration of the pH of the extracting solvent may lead to hydrolysis of procyanidins.

Conclusions
The addition of antioxidants alters the amounts of detectable flavan-3-ols significantly. Under the experimental conditions applied, concentrations of procyanidins were generally lower than the ones obtained with extracting solvents containing no antioxidant. An alteration of extraction efficiency could be excluded, since consecutive extraction of the same plant materials did not yield more procyanidins compared to the reference samples. Although protection of the procyanidins from oxidation during extraction and sample work up is reasonable, a suitable procedure has still to be developed. More studies would be necessary to be able to explain the behavior of low molecular weight procyanidins during extraction in the presence of matrix and antioxidants.

3.3 References


Diode array and single electrode electrochemical detection were evaluated for their use in the analysis of (-)-epicatechin and oligomeric procyanidins in reference solutions and extracts of Crataegus leaves and flowers. Linearity of regression curves was good for both detection modes. Diode array detection was found to be more selective but less sensitive than electrochemical detection. The absorbance ratio 220 nm / 280 nm gives information on the degree of polymerization in reference solutions. Quantitation in terms of monomer equivalents using electrochemical detection leads to an underestimation of contents for procyanidin dimers, but it is applicable using detection at 280 nm. In view of the better selectivity and the easier handling, diode array detection is recommended for the analysis of procyanidins in complex matrices.

4.1 Introduction

Procyanidins, which occur widely in woody or herbaceous plants, consist of 3', 4', 5, 7-tetrahydroxyflavan-3-ol units, which are linked by C4 -> C8 or C4 -> C6 bonds. Monomer units are the diastereomers (-)-epicatechin and (+)-catechin, which show different stereochemistry at C2 and C3 (Haslam, 1989; Hemingway, 1989; Porter, 1994). Studies of over twenty-nine plant species revealed that one of the diastereoisomers generally predominates in a specific species (Thompson et al., 1972). Procyanidins in Crataegus leaves and flowers almost exclusively consist of (-)-epicatechin units, while procyanidin B2 [(-)-epicatechin-(4ß→8)-(4)-epicatechin], procyanidin B5 [(-)-epicatechin-(4β→6)-(4)-epicatechin], procyanidin B4 [(+)-catechin-(4α→8)-(4)-epicatechin] and procyanidin C1 [(-)-epicatechin-(4β→8)-(4)-epicatechin-(4β→8)-(4)-epicatechin] are the principal compounds among the procyanidin oligomers.
Crataegus (hawthorn) is widely used as medicinal plant to improve the heart function. Procyanidins as well as flavonoids are responsible for the beneficial effects in cases of declining cardiac performance, deficiency of coronary blood supply and mild forms of arrhythmia (Ammon and Kaul, 1994a, b; Kaul, 1998; Sticher and Meier, 1998). The flowering tops are mainly harvested from Crataegus monogyna JACQ. and Crataegus laevigata (POIR.) DC. (syn.: C. oxyacantha L.).

The quantitative analysis of procyanidins in different matrices has already kept the scientific community busy for a long time — so far without a real breakthrough. Analysis of procyanidins is especially hampered by their tremendous structural diversity and their chemical reactivity, which include oxidation reactions and complexation i.e. to proteins and carbohydrates (Porter, 1992). The scope of this study was to evaluate different modes of detection for the analysis of procyanidins in Crataegus leaves and flowers.

4.2 Experimental

Materials

Crataegus leaves and flowers (Crataegi folium cum flore Ph.Helv.VII / DAB10) were obtained from Dixa (CH-St. Gallen). (-)-Epicatechin (HPLC grade) was purchased from Extrasynthese (F-Genay). Chlorogenic acid and (+)-catechin were of CHR grade and were obtained from Roth (CH-Reinach). Procyanidin dimers B2 [(-)-epicatechin-(4β→8)(-)-epicatechin], B5 [(-)-epicatechin-(4β→6)(-)-epicatechin], B4 [(+)-catechin-(4α→8)(-)-epicatechin]; procyanidin trimers C1 [(-)-epicatechin-(4β→8)(-)-epicatechin-(4β→8)(-)-epicatechin], trimer I [(-)-epicatechin-(4β→6)(-)-epicatechin-(4β→8)(-)-epicatechin], trimer II [(-)-epicatechin-(4β→6)(-)-epicatechin-(4β→6)(-)-epicatechin (tentatively identified)], the procyanidin tetramer [(-)-epicatechin-(4β→8)(-)-epicatechin-(4β→8)(-)-epicatechin-(4β→8)(-)-epicatechin] as well as a dimeric artifact (bis-epicatechinylmethane) were isolated from Crataegus leaves and flowers as described elsewhere (Rohr, 1999).

Acetone, ethyl acetate and methanol were of HPLC grade (Romil Chemicals, UK-Shepshed). Iso-octane (puriss. p.a.) was obtained from Fluka (CH-Buchs) and orthophosphoric acid (Ph.Helv.VI / Ph. Eur.) was purchased from Hänseler (CH-Herisau).
De-ionized water was obtained using a NANOpure cartridge system (Skan, CH-Basel). Celite 535 was purchased from Fluka (CH-Buchs). Sep-Pak® tC18 solid phase extraction columns (3 ml, 500 mg) were bought from Waters (USA-Milford, MA) and nylon filters (Spartan 30/B, 0.45 μm) were obtained from Spartan (D-Dassel).

Instrumentation
All HPLC analyses were performed using a Hewlett-Packard model 1090 instrument with low pressure pumps and a model 1040 diode array detector (= DAD) coupled to a Hewlett-Packard ChemStation (model 79994A). The detection wavelengths of the DAD were set at 280 nm, 220 nm, 260 nm and 290 nm. Spectra were taken at the leading edge, the apex and the tailing edge of the peaks to monitor for purity. Perfect matches are assigned values of 1000; values below 990 are considered to be indicative of co-eluting impurities.

The electrochemical detector (= ECD) model LC-4B (Bioanalytical Systems, UK-Stockport) was connected in series to the DAD and detector signals were fed into the ChemStation via an analog digital converter (Hewlett-Packard, Interface 35900). The detector cell was a 2 μm amperometric thin layer cell containing the glassy carbon working electrode (reference electrode: silver / silver chloride). The potential was set to 1.0 V, the range to 500 nA and the 2s filter was used. The working electrode was polished frequently. The HPLC system was passivated approximately once a month (using 6 M nitric acid followed by 0.02 M phosphate buffer pH 8 containing 20 mM EDTA). In order to prevent air spikes, eluents were continuously purged with helium and the backpressure was raised after the ECD by squeezing the tubing.

Off-line absorbance spectra were recorded on an Uvikon 930 instrument (Kontron Instruments, CH-Zürich). The data interval was set to 1.0 nm (0.2 nm for the determination of derivatives of spectra for the calculation of "min-max distances"), the scan speed was 200 nm/min (50 nm/min for derivatives) and the sensitivity was set to 0.1 absorbance units. For lyophilization an alpha 1-4 model of Christ (CH-Osterode am Harz) was used.

Chromatographic conditions
For chromatographic separations a Knauer (D-Berlin) prepacked cartridge column (250 x 4 mm I.D., 5 μm) filled with LiChrosorb RP-18 and a guard column (10 x 4 mm I.D.)
of the same material was employed. The mobile phase consisted of solvent A (methanol) and solvent B (0.5 % (v/v) ortho-phosphoric acid in water). The elution profile was: 0 min 18 % A in B, 0-10 min 18 % to 24 % A in B, 10 - 20 min 24 % A in B, 20 - 45 min 24 % to 40 % A in B, 45.01 - 50 min 100 % A (wash-out), 50.01 - 60 min 18 % A in B (re-conditioning). All gradient steps were linear. The flow rate was set to 1.0 ml/min, the column temperature was fixed at 24.0 °C and the injection volume was chosen to be 5 µl. Peak identification was based on retention times, UV / DAD spectra and spiking of authentic reference compounds to sample solutions.

Procedures

Crataegus leaves and flowers were extracted with 70 % (v/v) aqueous acetone. The organic phase was evaporated and sufficient methanol was added to produce a 50 % (w/v) aqueous methanolic solution. The suspension was filtered over Celite 535, was concentrated and lyophilized. Samples were redissolved in 50 % (v/v) aqueous methanol and filtered over a Sep-Pak® tC18 cartridge before HPLC analysis (Rohr et al., 1999).

Preparation of ethyl acetate extract. 15 g of Crataegus leaves and flowers were percolated with 250 ml of 70 % (v/v) aqueous acetone (column dimensions: Ø 2 x 25 cm). The organic phase was evaporated below 30 °C and the resulting aqueous suspension was subsequently extracted four times with 50 ml iso-octane and four times with 50 ml ethyl acetate (both solvents were saturated with water before use). The ethyl acetate fractions were combined, evaporated below 30 °C and redissolved in 3.0 ml 50 % (v/v) aqueous methanol. Samples were filtered over a nylon filter before HPLC analysis.

Reference solutions. For the determination of the linearity eight to twelve solutions of reference compounds in methanol were prepared. The concentrations were between approximately 1 µg/ml (ECD determination of (-)-epicatechin: 0.2 µg/ml) and 110 µg/ml (determinations of (-)-epicatechin: 130 µg/ml). The calibration graphs were generated by a least squares regression method. All calculations were based on area counts. Additional solutions were prepared to determine the detection limits (amount of analyte that produces a signal to noise ratio of two to five).
Collection of off-line absorbance data. Linear regression plots were generated using seven standard solutions in methanol. Concentration ranges: (-)-epicatechin (0.021 - 0.131 mg/ml), procyanidin B2 (0.006 - 0.107 mg/ml), procyanidin B5 (0.006 - 0.108 mg/ml), procyanidin B4 (0.016 - 0.103 mg/ml) and procyanidin C1 (0.018 - 0.111 mg/ml).

Recording of voltammograms. Hydrodynamic voltammograms were recorded by injecting reference solutions in triplicate. Chromatography was conducted on a Knauer (Berlin, Germany) Spherisorb ODS 2 (100 x 4 mm I.D., 5 μm) column. The following conditions were applied: isocratic elution using 25 % (v/v) methanol in 0.5 % (v/v) aqueous ortho-phosphoric acid, flow rate: 1.0 ml/min, column temperature: 25 °C, injection volume: 5 μl. The potential of the ECD was successively changed from 0.1 to 1.3 V.

4.3 Results and discussion

4.3.1 Diode array detection
Procyanidins can easily be identified in chromatograms from their DAD spectra which show a maximum at 280 nm and a shoulder at around 220 nm. The maximum at 280 nm is relatively weak compared to other phenolics. This is the reason why phenolic compounds showing similar retention behavior like procyanidins (i.e. cinnamic acids) usually dominate the chromatograms to such an extent, that procyanidins almost disappear in the baseline (see Figure 4.1). Detection at 220 nm is a possible solution, but peak purities have to be watched carefully since interferences by matrix components are more likely at this shorter detection wavelength. So far, quantitation of procyanidins at 220 nm has only been described for apple juices (Delage et al., 1991) and extracts from Crataegus (Rohr et al., 1999).

The limits of detection for most flavan-3-ols at 220 nm are roughly half the ones obtained at 280 nm (see Table 4.1). In cases where sensitivity is a problem quantitation should therefore rather be based on detection at 220 nm. The relatively high detection limit of procyanidin C1 is consistent with the tendency of procyanidins of higher molecular weight to elute as broader peaks.
Figure 4.1  Typical chromatograms of samples of *Crataegus* leaves and flowers.

Top:  Recorded at 280 nm.
Bottom:  Recorded at 220 nm.

Peak identification:

1  Procyanidin B4  
2  Procyanidin B2  
3  Chlorogenic acid  
4  Procyanidin C1  
5  (-)-Epicatechin  
6  Procyanidin B5.

For conditions, see Experimental.
Table 4.1  Limits of detection (ng).

<table>
<thead>
<tr>
<th>Reference compound</th>
<th>UV 280 nm</th>
<th>UV 220 nm</th>
<th>Electrochemical detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>(-)-epicatechin</td>
<td>20.9</td>
<td>10.5</td>
<td>1.05</td>
</tr>
<tr>
<td>procyanidin B2</td>
<td>5.5</td>
<td>2.8</td>
<td>0.17</td>
</tr>
<tr>
<td>procyanidin B5</td>
<td>14.0</td>
<td>5.6</td>
<td>0.35</td>
</tr>
<tr>
<td>procyanidin B4</td>
<td>8.2</td>
<td>3.3</td>
<td>0.41</td>
</tr>
<tr>
<td>procyanidin C1</td>
<td>44.2</td>
<td>17.7</td>
<td>1.11</td>
</tr>
</tbody>
</table>

For details, see Experimental.

Table 4.2 summarizes the data obtained for the determination of linearity. Correlation coefficients were superior to 0.999 for both detection wavelengths and for all tested compounds (exception: (-)-epicatechin at 280 nm: 0.998). Quantitation in terms of monomer equivalents has been extensively used in the analysis of procyanidins. A prerequisite for such an approach is that molar absorbances of procyanidin oligomers are the sum of the composing monomer units. Table 4.3 illustrates that this is roughly the case. Considering molar coefficients of absorbance, dimers and trimers seem to be slightly overestimated, if they are quantified using an (-)-epicatechin calibration graph. Such differences in linear regression plots have also been reported by other authors (Chiavari et al., 1987; Bourzeix et al., 1986; Jerumanis, 1985; Treutter et al., 1994). If a chromatographic separation is performed additional effects have to be considered: higher molecular weight procyanidins can not be chromatographed and tend to be spread over the whole chromatogram (i.e. Wall et al., 1996; Rohr et al., in press), added to that, unusual chromatographic behavior has been described for some procyanidins (Lea, 1980; Stafford and Lester, 1980). The validity of quantitation in terms of monomer equivalents has therefore to be demonstrated by recovery experiments for every matrix. Analysis of procyanidins in *Crataegus* leaves and flowers using calibration graphs of (-)-epicatechin yielded similar recovery rates like using calibration graphs of individual procyanidins, if quantitation was based on 280 nm. Calculations based on detector responses at 220 nm resulted in an
overestimation (Rohr, 1999). This overestimation of procyanidin contents by using (-)-epicatechin calibration graphs can also be seen from the linearity data in Table 4.2.

Table 4.2 Determination of linearity.

<table>
<thead>
<tr>
<th>Reference compound</th>
<th>Detection mode</th>
<th>Regression curve</th>
<th>Corr. coeff. (r)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(-)-epicatechin</td>
<td>280 nm</td>
<td>2006.632x - 0.509</td>
<td>0.998</td>
</tr>
<tr>
<td></td>
<td>220 nm</td>
<td>11483.12x + 20.75</td>
<td>0.999</td>
</tr>
<tr>
<td></td>
<td>ECD</td>
<td>52354.54x + 230.2</td>
<td>0.996</td>
</tr>
<tr>
<td>procyanidin B2</td>
<td>280 nm</td>
<td>2229.906x - 0.630</td>
<td>1.000</td>
</tr>
<tr>
<td></td>
<td>220 nm</td>
<td>16347.01x + 3.920</td>
<td>1.000</td>
</tr>
<tr>
<td></td>
<td>ECD</td>
<td>18335.36x + 5.699</td>
<td>1.000</td>
</tr>
<tr>
<td>procyanidin B5</td>
<td>280 nm</td>
<td>2256.873x + 0.546</td>
<td>1.000</td>
</tr>
<tr>
<td></td>
<td>220 nm</td>
<td>16954.46x - 3.955</td>
<td>1.000</td>
</tr>
<tr>
<td></td>
<td>ECD</td>
<td>13938.49x + 2.667</td>
<td>1.000</td>
</tr>
<tr>
<td>procyanidin B4</td>
<td>280 nm</td>
<td>2247.006x - 4.126</td>
<td>0.999</td>
</tr>
<tr>
<td></td>
<td>220 nm</td>
<td>14859.91x + 12.75</td>
<td>1.000</td>
</tr>
<tr>
<td></td>
<td>ECD</td>
<td>6436.455x - 0.701</td>
<td>0.998</td>
</tr>
<tr>
<td>procyanidin C1</td>
<td>280 nm</td>
<td>2357.848x - 5.834</td>
<td>0.999</td>
</tr>
<tr>
<td></td>
<td>220 nm</td>
<td>18663.04x - 34.28</td>
<td>0.999</td>
</tr>
<tr>
<td></td>
<td>ECD</td>
<td>151808.3x + 63.06</td>
<td>1.000</td>
</tr>
</tbody>
</table>

For details, see Experimental.
Abbreviations: corr. coeff. = correlation coefficient, ECD = electrochemical detection
Table 4.3  Absorbance data for flavan-3-ols in methanol at 280 nm.

<table>
<thead>
<tr>
<th>Reference compound</th>
<th>A(^{1%a})</th>
<th>A(^{1%}) corrected(^b)</th>
<th>(\varepsilon^c) (1 mol(^{-1}) cm(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>(-)-epicatechin</td>
<td>130</td>
<td>137</td>
<td>3780</td>
</tr>
<tr>
<td>procyanidin B2</td>
<td>136</td>
<td>140</td>
<td>7850</td>
</tr>
<tr>
<td>procyanidin B5</td>
<td>135</td>
<td>139</td>
<td>7810</td>
</tr>
<tr>
<td>procyanidin B4</td>
<td>141</td>
<td>146</td>
<td>8170</td>
</tr>
<tr>
<td>procyanidin C1</td>
<td>136</td>
<td>141</td>
<td>11800</td>
</tr>
</tbody>
</table>

For details, see Experimental.

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

\(^b\) A\(^{1\%}\) corrected = transformation of regression plots through origin.

\(^c\) \(\varepsilon\) = molar coefficient of absorbance.

The features of the UV spectra of some procyanidins in the presence of matrix were also examined. In order to eliminate effects of polymeric procyanidins an ethyl acetate extract was prepared (see Figures 4.2A and 4.2B). Peak purity data for (-)-epicatechin and procyanidins B2, B4, B5 and C1 were superior to 996 (match index) which indicates no interferences from non-procyanidins. The UV spectrum of procyanidin C1 shows a small tail after the maximum at 280 nm, which is absent in spectra of pure reference solutions. Attempts to separate this co-eluting impurity failed. Higher molecular weight procyanidins (Cho, 1990; Putman and Butler, 1989), galloylated procyanidins (Escribano-Bailón et al., 1992) or \(p\)-coumaroylated procyanidins (Bartolomé et al., 1997) are known to show such tails in the spectra. However, we could not find any further evidence for the presence of galloylated or coumaroylated species in *Crataegus* nor are there any reports in the literature. Furthermore, polymeric compounds are scarcely extracted into ethyl acetate (Rohr et al., in press). It was concluded that these anomalies in the spectrum of procyanidin C1 are probably due to an underground composed of oxidation products which do not elute with a defined k'-value (Lea, 1980). Such oxidized flavan-3-ols are known to exhibit UV absorbance between 300 nm and 400 nm (Guyot et al., 1996; Rouet-Mayer et al., 1990; Singleton,
Figure 4.2A Chromatogram of an ethyl acetate extract of Crataegus leaves and flowers recorded at 280 nm.
For conditions, see Experimental. Peak identification, see Figure 4.2B.
Figure 4.2B UV / DAD apex spectra of selected peaks.

1  Procyanidin B4
2  Procyanidin B2
3  Chlorogenic acid
4  Procyanidin C1
5  (-)-Epicatechin
6  Procyanidin B5
Similar tailing effects of (+)-catechin and procyanidin B2 in the presence of matrix also occurred in the analysis of grape seed extracts (Revilla et al., 1991).

At first sight, UV spectra of procyanidins and monomeric flavan-3-ols do not show any obvious differences (see Figure 4.2B). Nevertheless, several attempts have been made to gain additional structural information from the spectra to be able to identify individual compounds. One possibility is the formation of absorbance ratios. The use of the following absorbance ratios were evaluated in this study: 220 nm / 280 nm, 280 nm / 260 nm, 280 nm / 290 nm and 260 nm / 290 nm. The ratio 220 nm / 280 nm shows the largest differences between individual compounds (see Table 4.4). The other ratios tested did not reveal any obvious differences between individual flavan-3-ols and relative standard deviations were higher (up to 5 %) using pure reference solutions. The higher dispersions are due to measurements near the absorbance minimum (260 nm) or the selection of too close wavelengths (280 nm / 290 nm), respectively.

Table 4.4 Determination of 220 nm / 280 nm peak height ratios of reference compounds.

<table>
<thead>
<tr>
<th>Reference compound</th>
<th>220nm / 280 nm height ratio (mean values)</th>
<th>Standard deviation</th>
<th>Relative standard deviation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(-)-epicatechin</td>
<td>5.68&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.017</td>
<td>0.3</td>
</tr>
<tr>
<td>(+)-catechin</td>
<td>5.63&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.011</td>
<td>0.2</td>
</tr>
<tr>
<td>procyanidin B2</td>
<td>7.30&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.030</td>
<td>0.4</td>
</tr>
<tr>
<td>procyanidin B5</td>
<td>7.35&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.085</td>
<td>1.2</td>
</tr>
<tr>
<td>procyanidin B4</td>
<td>6.96&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.091</td>
<td>1.3</td>
</tr>
<tr>
<td>procyanidin C1 trimer I</td>
<td>7.94&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.045</td>
<td>0.6</td>
</tr>
<tr>
<td>trimer II</td>
<td>7.69&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.040</td>
<td>0.5</td>
</tr>
<tr>
<td>tetramer I</td>
<td>8.14&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.020</td>
<td>0.3</td>
</tr>
<tr>
<td>dimeric artifact</td>
<td>8.18&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.037</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>6.87&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.025</td>
<td>0.4</td>
</tr>
</tbody>
</table>

For details, see Experimental.

<sup>a</sup> More than 6 determinations on different days.

<sup>b</sup> Between 4 and 5 determinations on different days.
Applying the ratio 220 nm / 280 nm, a discrimination between procyanidins of different degree of polymerization is possible, if pure references are used. Considering the mean values ± one standard deviation the following intervals can be distinguished: monomers 5.6 - 5.7, dimers 6.8 - 7.4 and trimers 7.6 - 8.2. The data of the tetrameric procyanidin indicates that absorbance ratios increase further with increasing degree of polymerization. Similar tendencies have been reported for apple procyanidins using 220 nm / 275 nm absorbance ratios (Delage et al., 1991).

Distinction among structural isomers (procyanidins B2 and B5) or diastereomers ((-)-epicatechin and (+)-catechin) is not possible. The ratios of degradation products of procyanidins B2 and C1 were also analyzed. The references were dissolved in aqueous methanol (50 %, v/v) and were stored at different temperatures. Aliquots of the solutions were sampled and analyzed over a time period of several months. The ratios of three out of five degradation products of procyanidin B2 showed values below 5.6, while the remaining two compounds were in the same intervals like procyanidins. The ratios of three out of six degradation products of procyanidin C1 were outside the intervals of procyanidins, of which only one showed a value below 5.6 (experimental data are not included). A dimeric artifact produced a ratio in the same interval like procyanidin dimers, too (see Table 4.4). All these results show, that absorbance ratios are only of limited value in the discrimination of degradation products of procyanidins.

Measurements of absorbance ratios of procyanidins in purified fractions and in extracts of *Crataegus* leaves and flowers revealed values in the same order of magnitude, but relative standard deviations were very high (2 % up to 12 %). The results indicate that measurements are strongly influenced by the matrix. Again, it is postulated that these effects are due to oxidized or higher molecular weight procyanidins which can chromatographically not be separated from the compounds under investigation. As a conclusion, absorbance ratios might be useful for the characterization of pure compounds, but they can not be beneficially interpreted in the presence of matrix.

Bartolomé et al. (1996) reported that the distance in nanometers between the maximum and the minimum absorbance between 220 nm and 280 nm ("min-max distance") decreased by 1.3 nm upon each additional monomer unit in the molecule. They used this parameter to determine the degree of polymerization of eluting procyanidins in extracts of grape seeds (Bartolomé et al., 1996) and lentils (Bartolomé et al., 1997). We
tried to duplicate these results in an off-line experiment using reference solutions in methanol. We tendentiously could confirm the findings, but only by applying data intervals of 0.2 nm. Differences between monomers and dimers ranged from 1.6 nm ((-)-epicatechin - B4) to 2.6 nm ((-)-epicatechin - B5). The "min-max distance" for the two dimeric procyanidins B4 and B5 differed by 1.0 nm. The differences between dimers and procyanidin C1 amounted to 1.0 nm (B5) to 2.0 nm (B4). Our results indicate that the use of the "min-max distance" is very delicate and seems to only be applicable by exactly duplicating the experimental (i.e. chromatographic) conditions used by Bartolomé et al.

4.3.2 Electrochemical detection

The electrochemical behavior of procyanidins can be studied with voltammograms, which relate the current response to the applied potential. Figure 4.3 shows hydrodynamic voltammograms of (-)-epicatechin, procyanidins B2, C1 and chlorogenic acid. The first plateau region in the voltammogram of (-)-epicatechin is due to the oxidation of the B-ring ortho-diphenols, while the second plateau, starting at approximately 1.2 V, represents the oxidation of the A-ring meta-diphenols (Chiavari et al., 1987; Hayes et al., 1987a). The voltammograms of procyanidin B2 and C1 do not anymore show pronounced plateau regions. This results from the increased number of phenolic groups that are gradually oxidized in oligomers compared to monomers. For the analysis of procyanidins, relatively high working potentials are necessary which lead to high noise levels, too. At the normally applied potential of 1.0 V, phenolic acids (see Figure 4.3) as well as common flavonoids are also oxidized. Since measurements can not be performed in a plateau region, determinations are expected not to be very rugged.

ECD is about 20 to 40 times more sensitive than UV detection at 280 nm (see Table 4.1). The linearity of regression curves is good (correlation coefficients superior to 0.996, see Table 4.2) as long as low concentrations are used and the calibration solutions are injected in series. If extract samples are injected in between, the sensitivity of the working electrode is dramatically decreased by adsorption of matrix components which makes quantitative analysis impossible. This is a well known problem in the analysis of polyphenols and can be solved by the application of pulsed amperometric detection (Chiavari et al., 1987; Hayes et al., 1987b).
Figure 4.3  Hydrodynamic voltammograms of reference compounds.
For conditions, see Experimental.

Chromatograms of *Crataegus* extracts using ECD look similar to the ones applying DAD (see Figure 4.4). This again illustrates that, at the relatively high working potential applied, matrix components like flavonoids and phenolic acids respond, too. However, some differences between DAD and ECD can be found: Procyanidin B2 almost co-elutes with an electrochemically active, but UV inactive compound. Furthermore, (-)-epicatechin shows higher relative response than procyanidin dimers (and also cinnamic acid derivatives like chlorogenic acid) in ECD compared to DAD. This can be seen from the linearity data, too (see Table 4.2). Chiavari et al. (1987) also calculated much steeper calibration plots for monomers than for procyanidins.
Figure 4.4  Chromatogram of an ethyl acetate extract of *Crataegus* leaves and flowers using electrochemical detection.

Range: 20 nA; other conditions, see Experimental. Peak identification, see Figure 4.1.
If quantitation is based on monomer equivalents, these differences in responses would lead to incorrect results.

Using single electrode ECD peak identification is only based on retention times, which is a major drawback compared to DAD. The selectivity of ECD might be enhanced by using dual electrode or electrochemical array detectors, which both would give more information on the shape of the voltammograms of the eluting compounds (Lunte et al., 1988; Madigan et al., 1994).

4.4 Conclusions

Using DAD, characterization of flavan-3-ols with respect to their degree of polymerization is possible in reference solutions via the formation of the absorbance ratio 220 nm / 280 nm. In the presence of matrix the value of these absorbance ratios is questionable because of high standard deviations. Information on other structural features of procyanidins can not be drawn from UV spectra.

Quantitation in terms of monomer equivalents is applicable in DAD using a detection wavelength of 280 nm, but accuracy should be demonstrated in the presence of matrix, because a chromatographic underground may influence detection. In ECD, monomers are more easily oxidized than procyanidin dimers and quantitation in terms of monomer equivalents leads to an underestimation of contents.

ECD is more sensitive but less selective than DAD. It is by far not an easy-to-use technology and its poor selectivity in single electrode mode as well as its poor repeatability does not justify its use in the analysis of procyanidins in complex matrices at the moment. The major drawback of DAD is, that oligomeric procyanidins and monomeric flavan-3-ols can not be distinguished from co-eluting procyanidins or degradation products of procyanidins which in part show similar chromophors.
4.5 References


5 Evaluation of different detection modes for the analysis of procyanidins in *Crataegus* leaves and flowers. Part II. Liquid chromatography – mass spectrometry (submitted version of publication 4)

Liquid chromatography – mass spectrometry using electrospray as well as atmospheric pressure chemical ionization in the negative and positive ionization mode have been evaluated for the qualitative analysis of (-)-epicatechin and oligomeric procyanidins in reference solutions and extracts of *Crataegus* leaves and flowers. The use of the electrospray interface in the negative ionization mode yields molecular ions as principal signals with all tested compounds. In the positive ionization mode and using the interface for atmospheric pressure chemical ionization considerable fragmentation due to cleavage of the interflavanoid bonds and *retro*-Diels-Alder fission of the heterocyclic rings occur.

5.1 Introduction

Preparations of *Crataegus* (hawthorn) have been used as a heart remedy in folk medicine in the western world for a long time. Many pharmacological studies have scientifically proven their actions on cardiac performance and blood pressure. The effects could be ascribed to the procyanidin and flavonoid fractions (Kaul, 1998; Sticher and Meier, 1998; Tauchert et al., 1994). Procyanidins in food stuffs (i.e. contained in grapes and tea) have gained considerable attention in the past few years because of their protective effects against chronic deseases like atherosclerosis and coronary heart disease (Maffei Facinó et al., 1996; Ricardo da Silva et al., 1991; Teissedre et al., 1996). Especially their antioxidative and radical scavenging activities are discussed as possible modes of action. Food technologists are also interested in procyanidins because they influence taste of food products and are known as precursors of undesirable hazes or discolorations in plant derived food stuffs (Beveridge et al., 1998; Lee, 1992).
Thus, there is a broad interest in suitable techniques for the analysis of procyanidins in plant matrices. Common detection modes like UV diode-array and electrochemical detection lack specificity for individual procyanidins (Rohr et al., submitted). Investigations using off-line fluorescence detection revealed a decrease in the intensities of the emission bands with increasing degree of polymerization, but polymeric compounds still showed activity (Cho, 1990). In reversed-phase chromatography, higher oligomeric procyanidins tend to be spread over the whole chromatogram which may hamper detection of individual compounds. Moreover, phenolic acids and flavonoids which show similar retention behavior in reversed-phase chromatography like procyanidins also respond in UV and electrochemical detection. Thus, the use of a more selective detection method like mass spectrometry is very promising.

The most suitable LC-MS (liquid chromatography – mass spectrometry) coupling for the reversed-phase chromatographic analysis of polar compounds like procyanidins are the heated nebulizer (for APCI = atmospheric pressure chemical ionization) and electrospray (ESP = electrospray) interfaces (Niessen et al., 1992). The thermospray interface, which has been extensively used in the analysis of polar natural products in the past few years (Wolfender et al., 1994), is generally more complicated with respect to method development. A lot of interdependent experimental parameters must be optimized, i.e. amount and type of buffer, temperature of vaporizer and ion source, position and potential of electrodes (Niessen et al., 1992). These obvious drawbacks are the main reason why the thermospray interface is not anymore used very often at present. In this study the possible use of the heated nebulizer and electrospray interfaces for the qualitative analysis of procyanidins in Crataegus leaves and flowers has been evaluated.

In the APCI interface the column effluent is nebulized in a heated nebulizer. The heated solvent and analyte vapour mixture is then introduced in the atmospheric pressure ion source. Chemical ionization is initiated by a corona discharge electrode. The ions are subsequently transferred to the mass spectrometer by a special skimmer system. The advantage of the heated nebulizer interface over the electrospray interface is that even pure aqueous mobile phases at high flow rates (i.e. 1 - 2 ml/min) can be used (Niessen et al., 1992). This is a very important feature for the analysis of procyanidins, since gradients generally start with an aqueous proportion of 80 % to
100 % (Rohr et al., in press). To our knowledge there is only one method described in
the literature which makes use of this interface in the analysis of procyanidin dimers
from barley (Maillard et al., 1996).

In the ESP interface the eluent enters a spray chamber through a needle, which is
kept at ground potential. The surrounding cylindrical electrodes are set at a positive or
negative potential, which causes the liquid to be electrosprayed from the tip of the
needle. The generated positively or negatively charged droplets are further evaporated
and the analyte ions thus formed are introduced into the mass selective detector
(Niessen et al., 1992). The specific ionization mode in the electrospray probe may lead
to different ion species compared to the heated nebulizer interface. ESP ionization of
peptides i.e. often results in the formation of multiply-charged ions (Mann et al., 1989).
The ESP interface has already been used in the analysis of procyanidins from cider
apples, litchi pericarp as well as grape seeds in the negative ionization mode (Cheynier
et al., 1997) and in the analysis of procyanidins from unripe sweet almond fruits in the
positive ionization mode (de Pascual-Teresa et al., 1998).

5.2 Experimental

Materials

Crataegus leaves and flowers (Crataegi folium cum flore Ph.Helv.VII / DAB 10) were
obtained from Dixa (CH-St. Gallen). (-)-Epicatechin (HPLC grade) was purchased from
Extrasynthese (F-Genay). Procyanidin dimers B2 [(-)-epicatechin-(4ß→8)-
(-)-epicatechin], B5 [(-)-epicatechin-(4ß→6)(-)-epicatechin], B4 [(+)-catechin-
(4α→8)(-)-epicatechin]; procyanidin trimers C1 [(-)-epicatechin-(4ß→8)(-)-epi-
catechin-(4β→8)(-)-epicatechin], trimer I [(-)-epicatechin-(4β→6)(-)-epicatechin-
(4β→8)(-)-epicatechin], trimer II [(-)-epicatechin-(4β→6)(-)-epicatechin-(4β→6)-
(-)-epicatechin (tentatively identified)]; tetramer I [(-)-epicatechin-(4β→8)(-)-epi-
catechin-(4β→8)(-)-epicatechin-(4β→8)(-)-epicatechin] as well as a dimeric artifact
(bis-epicatechinylmethane) were isolated from Crataegus leaves and flowers as
described elsewhere (Rohr, 1999).

Materials for sample work up were the same as described in a previous paper (Rohr
et al., submitted). For the LC-MS analyses the following reagents were used: methanol
(HPLC grade; Scharlau, EGT Chemie, CH-Tägerig) and acetic acid (p.a.; Merck, CH-Dietikon).

**Instrumentation**

All LC-MS analyses were performed with a Finnigan MAT model SSQ 710 instrument (Finnigan MAT, USA-San Jose, CA). A model 600 MS pump and a model 717 autosampler by Waters (USA-Milford, MA) were used for solvent delivery and sample injection, respectively. In the final method the ESP interface in the negative ionization mode was used. The following conditions were employed: potential of spray: 1.5 kV, temperature of capillary: 200 °C, sheath gas: 4.5 bar, auxiliary gas: position 10, scan range: 200 - 2000 and scan rate: 15 seconds. The compounds and fragments are characterized in terms of m/z (mass to charge ratio) or are given in terms of u (mass units), if the species do not carry any charge.

An UV detector (model 486 MS; Waters, USA-Milford, MA) was connected before the mass selective detector for the analysis of extracts. The detection wavelength was set to 278 nm (in the spectra defined as user trace).

**Chromatographic conditions**

For chromatographic separations a Knauer (D-Berlin) prepacked cartridge column (250 x 4 mm I.D., 5 μm) filled with LiChrosorb-100 RP-18 and a guard column (10 x 4 mm I.D.) of the same material was employed. The mobile phase consisted of solvent A: methanol - water - acetic acid (24.5 : 75.0 : 0.5, v/v), solvent B: methanol - water - acetic acid (44.5 : 55.0 : 0.5, v/v) and solvent C: methanol - acetic acid (99.5 : 0.5, v/v). The elution profile was: 0 - 90 min 100 % A, 90 - 110 min from 100 % A to 100 % B, 110 - 170 min 100 % B, 170.1 - 190 min 100 % C (wash-out), 190.1 - 340 min 100 % A (re-conditioning). The flow rate was set to 0.2 ml/min, the column temperature was ambient and the injection volume was chosen to be 5 to 20 μl. The conditions for the mass detection with the ESP interface were optimized with respect to the potential of the spray (0.4 - 5.0 kV). Peak identification was based on mass spectra and comparison of retention times with authentic reference compounds.
Procedures

Preparation of extracts and reference solutions. The extracts were prepared as described in a previous paper (Rohr et al., submitted). Reference compounds were diluted in 50 % (v/v) aqueous methanol at concentrations of 1 to 5 mg/ml (procyanidin C1 also 55 mg/ml).

Flow injection analyses. The APCI interface was tested in the positive and negative ionization mode using different corona currents (0.3 - 5 μA) and vaporizer temperatures (300 °C and 450 °C). The temperature of the capillary was fixed at 200 °C. Using the ESP interface, the potential of the spray was set to 4 kV and the temperature of the capillary to 200 °C. Both ionization modes were evaluated. The scan range was 200 to 900 (APCI) and 200 to 1500 (ESP) at a rate of 3 seconds. The sheath gas was set to 4.5 bar and the auxiliary gas was turned off.

1 to 5 μl of solutions of reference compounds were injected with a Rheodyne rotary valve (model 7725, Ismatec SA, CH-Zürich) fitted with a 5 μl loop. The mobile phase consisted of 63 % solvent A (methanol) and 37 % solvent B (water containing 0.5 % acetic acid (v/v)). The flow rate was set to 0.1 ml/min.

5.3 Results and discussion

5.3.1 Flow injection analysis using the APCI interface

Positive ionization

In the positive ionization mode the abundance of the molecular ion of (-)-epicatechin (m/z 291) amounts to 20 % (corona current: 3.5 to 5 μA) up to 40 % (corona current: 0.5 to 1 μA). The principal signals are ions m/z 139 and m/z 123. The fragment ion m/z 139 results from retro-Diels-Alder fission (RDA-F) of the heterocyclic ring system (see Figure 5.1). This type of cleavage is well-known and characteristic for flavonoids and flavan-3-ols (Gracey and Barker, 1976; Lin et al., 1993). The fragment ion m/z 123 results from cleavage between O/C2 and C2/C3 of the pyran ring (see Figure 5.2). Other ions (m/z 207, 165 and 147) showing abundances below 30 % have also been detected in collision induced dissociation experiments (Lin et al., 1993). Fragment m/z 273 (30 %) results from loss of water (u 18) at position C3.
Figure 5.1 Retro-Diels-Alder fission of the pyran ring of (-)-epicatechin.
Example: APCI interface, positive ionization mode.

Figure 5.2 Heterocyclic ring cleavage of (-)-epicatechin.
Example: APCI interface, positive ionization mode.
The molecular ion of procyanidin B2 (m/z 579) counts for less than 5% applying a corona current of 1.5 μA (which results in a reasonable signal to noise ratio). Principal signals are ions m/z 271 (fragment of RDA-F: m/z 409 - u 138) and m/z 291 (monomer fragment). Other typical fragments of RDA-F are: m/z 427 (m/z 579 - u 152; 20%) and m/z 289 (m/z 427 - u 138; 60%). This latter fragment might as well be a product of the cleavage of the interflavanoid bond following the quinone-methide mechanism, which has been first postulated by Karchesy et al. (1989; see Figure 5.3, in the positive ionization mode the monomer fragments are detectable at m/z 289 and m/z 291, respectively). m/z 409 (60%) results from loss of water preferably at position C3 of m/z 427. All these major ions have also been detected in off-line experiments with procyanidins B1 and B7 using FAB-MS (fast atom bombardment – mass spectrometry; Karchesy et al., 1986).

![Figure 5.3](image)

Figure 5.3 Cleavage of the interflavanoid linkage following the quinone-methide mechanism postulated by Karchesy et al. (1989).

No molecular ions can be detected after injection of procyanidin C1 regardless of the applied corona current. However, typical fragments of the cleavage of the heterocyclic ring are present in the spectra: m/z 409 (RDA-F and loss of water in a dimeric fragment: m/z 579 - u 152 - u 18; 20%), m/z 271 (RDA-F: m/z 409 - u 138; 80%) and
m/z 257 (RDA-F: m/z 409 - u 152; 40 %). m/z 287 constitutes the principal signal at a corona current of 1.5 μA. Monomeric fragments would be expected to exhibit m/z 291 or m/z 289, respectively. The mass fragment m/z 287 is known to be characteristic for A-type procyanidins which show an additional ether bond between the two monomeric units (Karchesky et al., 1989; Karchesky et al., 1986). A possible explanation for the occurrence of this ion as a result of fragmentation of procyanidin C1 is, that an oxidation reaction produces another quinoide structure in ring B of the fragment m/z 289. Such oxidation reactions of flavan-3-ols are well-known (Hayes et al., 1987).

Negative ionization

In the negative ionization mode abundance of the molecular ion of (-)-epicatechin (m/z 289) constitutes the principal signal up to a corona current of 5 μA. Other typical fragments are: m/z 271 (loss of water: m/z 289 - u 18; 50 %) and m/z 151 (RDA-F: m/z 289 - u 138; 30 %).

At a corona current of 5 μA no molecular ions are produced with procyanidins B2 and C1. At a corona current of 1.5 μA molecular ions of procyanidin B2 (m/z 577.0) amount to 20 % (see Figure 5.4 and 5.5). Principal signals of the spectrum of procyanidin B2 are: m/z 406.9 (RDA-F and loss of water: m/z 577 - u 152 - u 18) and slightly less abundant m/z 289.3 (monomer fragment). Other typical fragments are: m/z 425.5 (m/z 577 - u 152; 30 %) and m/z 268.5 (m/z 407 - u 138; 70 %).

Lowering the vaporizer temperature from 450 °C to 300 °C (corona current 1.5 μA) leads to significantly lower fragmentation, but the intensity of the molecular ion of procyanidin B2 decreases from 20 % to about 2 %. The monomer fragment m/z 289 is the principal signal.

Applying a corona current of 1.5 μA only traces of the molecular ion of procyanidin C1 (m/z 865) are produced, while m/z 287 (quinoide monomer fragment) constitutes the principal signal (see Figure 5.3, in the negative ionization mode the monomer fragments are detectable at m/z 287 and m/z 289, respectively). The fragment ion m/z 289 is also present, but in lower concentrations. If the corona current is raised to 2 - 5 μA this latter fragment ion m/z 289 is more abundant than the m/z 287 ion. An increase of the sample concentration to 55 mg/ml does not yield higher abundance of the molecular ion applying a corona current of 1.5 μA. The dimeric fragment (m/z 577)
Figure 5.4  Flow injection analysis of procyanidin B2 using the APCI interface in the negative ionization mode.

Corona current: 1.5 μA; vaporizer temperature: 450 °C; capillary temperature: 180 °C; concentration: 4.7 mg/ml; injection volume: 3 μl. For conditions, see Experimental.
Figure 5.5  Fragmentation scheme of procyanidin B2 using the APCI interface in the negative ionization mode.
is present in an abundance of about 20 % among other fragments of the RDA-F (m/z 425, m/z 407 and m/z 269).

In the positive as well as in the negative ionization mode considerable fragmentation of the heterocyclic ring system as well as cleavage of the interflavanoid linkage in dimers and trimers take place. Although different types of cleavages of the pyran ring occur in (-)-epicatechin, only fragments of the RDA-F of the heterocyclic ring resulting in the loss of u 152 and u 138, respectively, are detected with procyanidins. This predominant cleavage between O/C2 and C3/C4 can be attributed to the formation of stable aryl carbonium ions (Lin et al., 1993). The preponderance of fragment ions of the heterocyclic ring compared to molecular ions or sequence ions resulting from cleavage of the interflavanoid bonds demonstrates, that the interflavanoid bonds are less stable than the pyran rings under our experimental conditions.

In principle, the same fragment ions are generated in the negative and positive ionization mode. However, fragmentation seems to occur more extensively in the positive ionization mode, because the abundance of molecular ions in the negative ionization mode is more pronounced. These results are in agreement with the ones obtained from off-line FAB-MS experiments using positive and negative ionization modes (de Bruyne et al., 1996).

Lowering the corona current or the vaporizer temperature does not increase the abundance of molecular ions significantly, but it leads to lower sensitivities and higher noise levels, because the total ion current is reduced. We did not succeed in generating molecular ions of procyanidins as principal signals using the APCI interface under our experimental conditions. Therefore, the ESP ionization, which is extensively used in the analysis of proteins and is known to also be a very mild ionization technique, was evaluated.

5.3.2 Flow injection analysis using the ESP interface

The abundance of the molecular ion of procyanidin B2 (m/z 579) accounts for less than 10 % in the positive ionization mode, setting the spray to 4.0 kV and the capillary temperature to 200 °C. No fragment ions from cleavage of the heterocyclic ring nor sequence ions from cleavage of the interflavanoid linkage can be detected. We did not succeed in rationalizing the principal signal m/z 242.
In the negative ionization mode the molecular ion of procyanidin B2 (m/z 577) represents the principal signal (see Figure 5.6). Additional ions occur at m/z 637 (cluster with acetic acid: m/z 577 + u 60; 25 %) and m/z 1155 (cluster with another analyte molecule: m/z 577 + u 578; 40 %). Fulcrand et al. (1996) who analyzed acetaldehyde induced polymerization of flavan-3-ols using an ion spray interface, which is very similar to the electrospray interface, also found better responses in the negative than in the positive ionization mode. They also detected cluster ions with ethanol in the negative mode with monomers and procyanidins.

The abundance of the cluster ions are dependent on the vacuum produced by the skimmer pump (measurements mentioned above were performed at 880 millitorrs). Lower abundances of cluster ions are measured, if the vacuum is higher (810 millitorrs: m/z 1155: 30 %, m/z 637: 10 %). The detection limit for procyanidin B2 (amount of analyte which produces a signal to noise ratio of 3) is 150 ng.

The same ions are generated with the other dimeric procyanidins, but the abundances vary. The principal signal of procyanidin B4 is the molecular ion m/z 577, while the clusters amount to 20 % (m/z 1154) and 10 % (m/z 637), respectively. The principal signal of procyanidin B5 is the cluster with acetic acid m/z 637, while the molecular ion (m/z 577) and the cluster with itself (m/z 1155) amount to approximately 30 % (pressure of the skimmer pump: 870 - 890 millitorrs). These results imply that the ionization behavior of the 4 \rightarrow 6 linked procyanidin B5 is different from the one of the 4 \rightarrow 8 linked procyanidins B2 and B4 under our experimental conditions.

The molecular ion of (-)-epicatechin (m/z 289) only amounts to 15 %, while the cluster with another (-)-epicatechin molecule (m/z 579) is the principal signal. The cluster with acetic acid (m/z 349) shows an abundance of 50 %. The principal signals of procyanidin C1 and trimers I and II are the molecular ions m/z 865. Dimeric fragments (m/z 577) can be detected in low abundances, too. The dimeric artifact (bis-epicatechinylmethane) isolated from Crataegus extracts, shows a principal signal at m/z 591 which clearly characterizes this compound as a non-procyanidin. The UV spectrum of this artifact, however, cannot be distinguished from the one of a procyanidin, because the chromophore is exactly the same.
Figure 5.6: Flow injection analysis of procyanidin B2 using the ESP interface in the negative ionization mode.

Potential of spray: 4.0 kV; capillary temperature: 200 °C; concentration: 4.7 mg/mL; injection volume: 1 μL. For other conditions, see Experimental.
If MS spectra are interpreted of higher molecular weight species, the natural abundance of heavier isotopes has to be considered. Taking into account the isotops $^{13}$C (1.112 %), $^2$H (0.015 %), $^{17}$O (0.038 %) and $^{18}$O (0.200 %) the following average molecular weights can be calculated (Pretsch et al., 1990): monomers: 290.3, dimers: 578.5, trimers: 866.8, tetramers: 1155.0, pentamers: 1443.3 and hexamers: 1731.5. Molecular weights determined without considering the naturally occurring isotopes are about 0.2 mass units lower for each monomeric unit in the molecule. These effects can already be seen in the analysis of the tetramer I which shows a more intense molecular ion at m/z 1154 than at m/z 1153. The dimeric fragment (m/z 577) constitutes the principal signal, while the molecular ion only amounts to 20 %.

Compared to the APCI interface, spectra obtained with the ESP interface show very few ions. Molecular ions generally constitute the principal signals, if the negative ionization mode is used. Only ions resulting from cleavage of the interflavanoid bond can be detected, while the heterocyclic ring systems are stable. The mild ionization conditions lead to the formation of cluster ions, generally composed of two analyte molecules and more seldom of an analyte molecule and acetic acid.

The problem which arises in the LC-ESP-MS analysis of oligomeric procyanidins is that ions of similar masses can be produced by different molecular species. Molecular ions have the general formula $[M-1]^{+}$, while cluster ions with another analyte molecule have the formula $[2M-1]^{+}$. Cheynier et al. (1997) detected doubly charged ($[M-2H]^{2+}$) and triply charged ions ($[M-3H]^{3+}$) of procyanidins. Furthermore, procyanidins showing an additional ether linkage between monomeric units (A-type procyanidins) lack two mass units compared to the molecules only exhibiting the most often encountered C4 → C8/6 interflavanoid linkage. Looking at the mass region of i.e. a tetrameric procyanidin the following ions might therefore be detected in the negative ionization mode: m/z 1153 (molecular ion of a tetramer), m/z 1155 (cluster ion of a dimer), m/z 1152 (doubly charged molecular ion of an octamer), m/z 1151.7 (triply charged molecular ion of a dodecamer) and m/z 1151 (molecular ion of a tetramer with one A-type linkage in the molecule; i.e. isolated from *Pavetta owariensis* (Baldé et al., 1995)). If the natural abundance of heavier isotops (0.2 mass units for each monomer unit) and the accuracy of the mass detector (app. ± 0.5 - 1 mass unit) are considered, it is obvious that a chromatographic separation is inevitable and that the retention times of the principle procyanidins should be established with pure reference standards.
The presence of multiply charged ions can be determined on the basis of the mass difference to the adjacent isotopic peak(s) in the mass spectra (i.e. 0.5 mass units in doubly charged ions). We could not detect such multiply charged ions under our experimental conditions. Interpretation of the mass spectra should in any case be based on spectra which have not been corrected for the background, because actually measured masses are displayed that way.

5.3.3 Chromatographic analysis using the ESP interface

For the chromatographic separation of the procyanidins in extracts of *Crataegus* leaves and flowers a method previously developed for the detection with UV was adapted (Rohr et al., 1999). In order to apply flow rates of 0.2 ml/min the auxiliary gas has to be turned on. The attempts to use higher flow rates (i.e. by raising the temperature of the capillary) were unsuccessful. If the potential of the spray was kept at 5 kV, like in the flow injection analyses, no procyanidin signals could be detected in the negative ionization mode. A possible explanation is that the nitrogen molecules (of the auxiliary gas) are also ionized at this high potential and cause such a huge background that analyte signals can not be detected anymore by the spectrometer. The potential of the spray was optimized to a value of 1.5 kV. Potentials higher than 3.0 kV result in an almost complete loss of sensitivity for procyanidins.

Chromatographic analysis of reference solutions reveal that the molecular ions are the principal signals for all procyanidins. The abundance of the cluster ions of dimers amount to 20 % up to 80 % (procyanidin B5). A high abundance of the acetic acid cluster (m/z 637; 40 %) can be found with procyanidin B5 like in the flow injection analysis. This cluster ion can not be detected with the other dimers. Cluster ions of trimeric procyanidins (m/z 1731) are only detected with procyanidin C1 and trimer II in low abundances. The principal signal in the spectrum of (-)-epicatechin was the molecular ion (m/z 289), while the cluster with itself (m/z 579) and with acetic acid (m/z 349) can also be detected.

Figure 5.7A shows a chromatogram of an extract of *Crataegus* leaves and flowers and Figure 5.7B shows some examples of spectra of eluting procyanidins. The MS spectra of the known procyanidins show the same ions like the ones obtained with reference solutions. The abundances of individual ions vary slightly. Trimer II, which
Figure 5.7A  LC-MS chromatogram of an extract of *Crataegus* leaves and flowers using the ESP interface in the negative ionization mode.

Ion traces correspond to monomers (m/z 289), dimers (m/z 577), trimers (m/z 865), tetramers (m/z 1153), pentamers (m/z 1441) and hexamers (m/z 1729). User trace = UV detection at 278 nm, RIC = total ion current. Ordinate: relative abundance (UV trace: absorbance units); abscissa: time (scans; rate 15 sec). Injection volume: 20 µl, concentration: 214 mg/ml. For other conditions, see Experimental.

Peak identification: 1 = procyanidin B4, 2 = trimer I, 3 = tetramer A, 4 = procyanidin B2 (and its cluster in the tetrameric ion trace), 5 = pentamer B, 6 = tetramer C, 7 = procyanidin C1, 8 = tetramer I, 9 = (-)-epicatechin, 10 = pentamer D, 11 = hexamer E, 12 = procyanidin B5. Identity of procyanidins labeled with letters has not been verified by isolation.
Figure 5.7B  MS spectra of selected peaks.
Top: Procyanidin CI molecular ion: m/z 864.8
Middle: (-)-Epicatechin molecular ion: m/z 288.6, cluster ion: m/z 578.7
Tetramer I molecular ion: m/z 1153.3
Bottom: Procyanidin B5 molecular ion: m/z 577.5, cluster ion: m/z 1154.6
cluster ion with acetic acid: m/z 637.2
Ordinate: relative abundance, abscissa: mass to charge ratio (m/z).
elutes after procyanidin B5, could not be detected in any of the recorded chromatograms of extracts. However, it was noticed in a lot of chromatograms that the total ion current dropped towards the end of the chromatographic run which was attributed to an insufficient evaporation of the effluent. A possible explanation for the lack of trimer II in the chromatograms is that the sensitivity becomes too low for the detection of the low amounts of this trimer that are present in the extracts.

Additional procyanidins can be detected in the chromatograms of extracts of *Crataegus* leaves and flowers (see Figure 5.7A): A tetramer A (peak number 3) elutes after procyanidin B4 and trimer I. The molecular ion (m/z 1153.8) confirms its identity, while the hump eluting just before represents the cluster ion of procyanidin B4. Another tetramer C (m/z 1153.4, peak number 6) elutes at around 290 scans. The spacing of the isotopic signals of both tetramers is approximately 1 mass unit, which indicates that the ions are singly charged. Two pentameric procyanidins B and D (m/z 1441.3, peak number 5 and m/z 1441.4, peak number 10) can be detected as singly charged ions. The ions at m/z 1442, which would be expected as principal signals taking into account the natural abundance of heavier isotops, show intensities of 60 % (B) and 90 % (D) compared to the signal at m/z 1441. A hexameric singly charged procyanidin E (m/z 1730.0, peak number 11) elutes at about 500 scans. The identities of these procyanidins would have to be proven irrefutably by MS-MS detection. More severe ionization conditions can produce characteristic sequence ions or fragment ions originating from RDA-F as has been demonstrated by the use of the APCI interface in this study. De Pascual-Teresa et al. (1998), using an ESP interface in the positive ionization mode and an ion trap as mass detector, successfully identified procyanidins from purified fractions of unripe sweet almond fruit with this approach.

The same extract was also analyzed in the positive ionization mode at a spray voltage of 5.0 kV. The chromatogram looked the same like using the negative ionization mode, but signal intensities roughly amounted to one tenth. This is in agreement with the results obtained from flow injection analysis.
5.4 Conclusions

The ESP interface in the negative ionization mode is recommended as interface for the LC-MS detection of procyanidins. It has been proven to yield molecular weight information for procyanidins up to a degree of polymerization of four. The use of the positive ionization mode or the use of the APCI interface results in considerable fragmentation due to cleavage of the interflavanoid bonds and RDA-F of the heterocyclic rings.

ESP-MS is a highly selective detector for the determination of procyanidins in complex matrices. Compounds which tend to co-elute with procyanidins, like i.e. phenolic acid derivatives and oxidized or higher molecular weight procyanidins can easily be separated by the selection of specific ion traces. This feature is an enormous advantage compared to diode array or electrochemical detection, for which complete chromatographic separation of all these compounds is mandatory for an unequivocal identification and quantitation.

The interpretation of MS spectra becomes increasingly complicated as the molecular weight of the procyanidins increases. It is therefore inevitable to establish ionization features for a defined set of experimental conditions with reference compounds. Higher molecular weight procyanidins which can not be isolated in sufficient quantities should be identified not only by single MS, but by additional fragmentation experiments using MS-MS.

The method presented here would need to be optimized further, if it shall be used as a quantitative method. Possibilities to improve sensitivity are: use of oxygen instead of nitrogen as the sheath gas, use of a sheath liquid (i.e. 2-methoxyethanol) added coaxially around the needle of the ESP probe and optimization of the pH of the eluent. The application of higher flow rates is already possible with the newest generation of interfaces (Rohr et al., in press). However, the main question in the analysis of procyanidins is, on which ion species quantitative determination should be based. Procyanidins show different ionization features under the same experimental conditions as could be seen with the dimeric procyanidins. It is therefore unlikely to be able to develop conditions under which all procyanidins can be detected merely as molecular ions (no fragmentation and no clustering occurring). On the other hand, complete fragmentation to the monomeric units yields two fragment ions which differ by two mass units (see Figure 5.3). Co-eluting compounds are likely to influence ionization as
well. Thus, there still remains a lot of basic research to do until LC-MS technology can also be applied successfully to the quantitative analysis of procyanidins in complex matrices.

5.5 References


Part II: Purification and structure elucidation

6 Distribution and general features of proanthocyanidins

Procyanidins belong to the group of natural products commonly termed as polyphenols or (vegetable) tannins. This group is further divided into the proanthocyanidins (synonym: condensed polyphenols; based on flavonoid precursors connected by carbon-carbon bonds) and the hydrolyzable polyphenols (polyesters of gallic and hexahydroxydiphenic acid with glucose or other polyols) [1]. The procyanidins represent the most frequently encountered subgroup of the proanthocyanidins.

In contrast to the hydrolyzable polyphenols, which only occur in some orders of the Dicotyledons [2], proanthocyanidins are extremely widespread in Dicotyledons, but have also been found in numerous species of Monocotyledons and in some species of Gymnosperms, Pteridophyta and Bryophyta. They are typically present in plants which show a woody or tree-like habit of growth [3,4]. Proanthocyanidins do not occur in prokaryotes, fungi or animals [1]. Hydrolyzable and condensed polyphenols have been reported to co-occur in some species, i.e. in Fragaria sp. or Rubus sp. [5].

The structural diversity among the proanthocyanidins results from the variable hydroxylation pattern on the A- and B-rings (see section 1, Figure 1.1) [6]. The great majority of naturally occurring proanthocyanidins are procyanidins (3,3',4',5,7-pentahydroxy flavans) and to a lesser extent prodelphinidins (3,3',4',5',5,7-hexahydroxy flavans) [3,7,8]. Polymers are often composed of both, procyanidin and prodelphinidin units [5]. Polymers exclusively based on procyanidin units are quite rare, but seem to be common in the Rosaceae [9,10]. Procyanidin-prodelphinidin oligomers have been described [11] and are well-known to occur in Camellia sinensis (Theaceae [12]). Other examples have also been isolated more recently from Vaccinium vitis-idaea (Ericaceae [13]), Croton lechleri (Euphorbiaceae [14]), Onobrychis spp. (Fabaceae [15,16]), Quercus petraea (Fagaceae [17]), Hamamelis virginiana (Hamamelidaceae [18]) and Lotus pedunculatus (Leguminosae [19]). Mixed procyanidin-propelargonidins (3,4',5,7-tetrahydroxy flavans) are seldom [11]. Some
Oligomers have more recently been reported from *Prunus spinosa* [20] and *Prunus prostata* [21] (Rosaceae), *Krameria triandra* (Krameriaceae [22]) and *Pavetta owariensis* (Rubiaceae [23]).

Williams et al. [24] found five classes of molecular weight distribution patterns for proanthocyanidins, based on gel permeation chromatography of the peracetate derivatives of 32 samples of different species and organs. The molecular range was mainly dependent on the species and to a lesser extent on the plant organ or tissue maturity. *Hordeum vulgare* (Gramineae) for example contained virtually only dimers and trimers, while proanthocyanidins of other species covered a very broad range of molecular weights. Very high degrees of polymerization are unusual (common are up to 15 [25] and 40 [24] units). Based on results from photophysical studies (fluorescence, circular dichroism) and molecular modeling, polymers have been found to display conformational versatility ranging from disordered compact random coils to highly extended rod-like chains with a locally helical structure [26]. Hemingway et al. [27] and Porter [28] postulated that polymers, which mainly show C4 → C8 linkages (i.e. in *Aesculus* and *Theobroma* sp.) are linear or threadlike, while polymers showing a lot of C4 → C6 linkages (i.e. in *Photinia* and *Vicia* sp.) are highly branched and therefore rather show globular structures. Evidence for cyclic structures has been found for proanthocyanidins from *Diospyros kaki* (Ebenaceae [29]) and *Prunus avium* (Rosaceae [10]).

The highest concentrations of proanthocyanidins are generally found in non-living or lignified tissues (i.e. wood, bark, stems, seeds) and in diseased tissues [1,30]. Because of these distribution features at the periphery, proanthocyanidins have been recognized as part of the plants’ natural defence strategy against pathogenic microorganisms [31] and animal predators [1,32]. Quinoids, which are formed upon disruption of the tissue by oxidation of the polyphenols, are considered to be the active principle. These intermediates are highly reactive and lead to a chemical modification or denaturation of biopolymers thereby damaging the exposed organism [33]. In fallen leaves, such reactions may affect the rate of metabolism by soil organisms, too. It has been postulated that the release of nitrogen, phosphorus and sugars from dead leaves is thus slowed down sufficiently to ensure a better supply of these essential nutrients for plant growth the following spring [1].
6.1 Structural features of procyanidins

The structural diversity among procyanidins is mainly based on the two different diastereoisomeric monomer units (+)-catechin and (-)-epicatechin, different chain lengths and different types of interflavan linkages (C4 → C8 or C4 → C6). Generally one of the flavan-3-ol monomers predominates in a species; occasionally to the apparent exclusion of the other isomer [8]. Plants which are rich in (-)-epicatechin generally contain procyanidin B2 as major dimer, while procyanidin B3 is usually the principal dimer in plants rich in (+)-catechin [34]. C4 → C8 linkages are most common [6,35]. The C8 position is sterically favored because the alkoxy oxygen is tied back as part of the pyran ring system. In contrast, C6 shows hydroxyl groups on each side, that rotate free around the C–O-bonds and thus partially block this position [36].

The occurrence of A-type procyanidins, which exhibit an additional ether bond, is limited to a restricted number of families (Rosaceae [21,37], Lauraceae [38], Hippocastanaceae [39], Ericaceae [13], Sterculiaceae [40], Fabaceae [41], Rubiaceae [42]). Their presence is therefore interesting from a systematic point of view [7].

The occurrence of procyanidins containing enantiomeric monomer units (i.e. (+)-epicatechin or (-)-catechin) has first been described in the Palmae [43]. Singly-bonded enantiomeric procyanidins have been reported from: Chamaerops humilis [43] and Metroxylon sagus [44] (Palmae), Cassia sp. (Leguminosae [45]) and Byrsonima crassifolia (Malpighiaceae [46]). Enantiomeric, doubly-bonded (A-type) procyanidins have been reported from: Prunus spinosa [47,48], Pavetta owariensis [23,42] and Theobroma cacao (Sterculiaceae [40]).

Procyanidins overwhelmingly occur in their free phenolic forms. However, some derivatives of procyanidins are described in the literature. Well-known are esters of gallic acid in Vitis vinifera (Vitaceae [49,50]) and Rheum sp. (Polygonaceae [51,52]). Although galloylation preferably takes place at the 3-O of the heterocycle, also gallates at the 3'-O position of the B-ring are present in grapes and rhubarb. 3-O gallic acid esters have also been isolated from Sanguisorba officinalis (Rosaceae [53]), Hamamelis virginiana [18] and Ximenia americana (Olacaceae, only monomers [54]). From Byrsonima crassifolia dimers and trimers have been isolated which contain 3-O-gallates not only at the terminal units, but also at the upper monomeric units [46].
Glycosides of procyanidins are rarely found in nature. They have been described in *Potentilla viscosa* (Rosaceae [11]), *Quercus* sp. (Fagaceae [11,55]), *Rheum* spp. (Polygonaceae [11]), *Erythroxylum novogranatense* (Erythoxylaceae [56]) and *Theobroma cacao* [40]. Glycosides of monomers additionally have been isolated from *Cinnamomum cassia* (Lauraceae [38]), *Betula* spp. (Betulaceae [57]), *Picea abies* (Pinaceae [58]) and *Rhaphiolepis umbellata* (Rosaceae [59]). The presence of a monomeric di-glucoside was furthermore evidenced by FAB-MS determinations in *Krameria triandra* [60]. Mainly O-glycosides occur which preferentially are positioned at 3-O and rarely at 7-O [57,59]. C-glycosides have been reported at positions C6 and C8 [11,38]. As sugar moiety predominantly glucose occurs; rhamnose [11,56], arabinose [40], xylose [57] and rutinose [55] have also been reported.

Glycosides of higher oligomers and polymers have been detected on the basis of spectroscopic and chemical degradation studies in: *Crataegus* sp. (Rosaceae, procyanidin hexamer [61]), *Prunus avium* (oligomeric procyanidin [10]), *Pistacia lentiscus* (Anacardiaceae, polymeric procyanidin [62]) and *Lotus corniculatus* (Leguminosae, procyanidin-prodelphinidin polymer [63] and procyanidin octamer [19]). These results have also been interpreted in terms of a possible covalent linkage of proanthocyanidins to a polysaccharide matrix within the plants [61].

Other derivatives of procyanidins that are described in the literature are 3'-methoxy derivatives of monomers in *Pinus sylvestris* [64] and *Picea abies* [58] (Pinaceae), a 7-methoxy monomer in *Prunus prostrata* (Rosaceae [21]) and a 3-O-(4-hydroxy)-benzoate of procyanidin B1 in *Hamamelis virginiana* [18]. At the C4-position, substitution of (-)-epicatechin with a phloroglucinol nucleus in *Netia meyeri* (Mesembryanthemaceae [65]) and carboxymethyl derivatives of (-)-epicatechin and procyanidin B2 in *Davallia divaricata* (Davalliaceae [66]) have been reported. A methyl-pyran derivative of (-)-epicatechin, linked at position C7 and C8, has been isolated from *Lupinus angustifolius* (Leguminosae [67]).

Phenylpropanoid derivatives, linked at position C7 and C8 of the A-ring, have been found in several species, i.e. in *Cinchona succirubra* (Rubiaceae, referred to as cinchonains [68,69]) and in *Kandelia candel* (Rhizophoraceae, referred to as kandelins [70]). FAB-MS measurements also revealed their presence in *Krameria triandra* [60].
It has been postulated that these derivatives could arise from a Michael addition of a nucleophilic A-ring onto caffeic acid followed by esterification [36].
7 Procyanidins in *Crataegus*

There are many publications to be found in the literature which are dealing with the qualitative composition of the flavan-3-ols in *Crataegus* sp. However, there are only very few contributions in which the identification or structure elucidation of the procyanidins has been performed unequivocally (see Table 7.1, data are listed chronologically). It is therefore justified to reinvestigate this species with respect to its procyanidin composition.

From the literature data it can be concluded that the main flavan-3-ol monomer in *Crataegus* sp. is (-)-epicatechin. It is accompanied by small amounts of (+)-catechin. The principal dimer is procyanidin B2, while the principal trimer is procyanidin C1. Minor procyanidins are the dimeric procyanidin B5 and a tetrameric procyanidin (all-trans-(-)-epicatechin). Procyanidin B4 has been identified by means of degradation studies [71] and in the early 70ies by paper chromatography [8]. At least one other trimer besides procyanidin C1 seems to be present, too [72]. The presence of procyanidin B3 is questionable, since it has only been reported once, based on speculations from the *R*<sub>f</sub>-value on TLC chromatograms without reference substance [73].

Whether procyanidin B1 is present in *Crataegus* sp. in significant amounts can not be concluded definitely from the literature data. In more recent studies, its identification is merely based on *R*<sub>f</sub>-values without any reference compounds [74], or additionally on mass spectrometry [75]. Since mass spectra of the eight possible isomeric B-type procyanidin dimers do not exhibit any differences, and *R*<sub>f</sub>-values on silica TLC chromatograms are also very similar, identification based on only these methods are not unambiguous. Much of this confusion goes back to the widely cited, excellent publication of Thompson et al. [8] on isolation, structure elucidation and distribution of procyanidins in twenty-nine plant species. In a table therein, procyanidin B2 and B1 are reported to have been isolated from *Crataegus monogyna*. However, the isolation of procyanidin B1 is reported with respect to another literature of Weinges et al. [76] on procyanidins isolated from different fruits. In this latter publication the isolation and structure elucidation of B-type procyanidin dimers (B1, B2, B3, B4) from *Cola acuminata, Vaccinium vitis idaea* and *Gleditschia triacanthos* is described. Samples from other fruits are stated to have been isolated analogously, but a figure on schematic
Table 7.1 Identification of flavan-3-ol monomers and procyanidins from *Crataegus* sp.

<table>
<thead>
<tr>
<th>Species</th>
<th>Organ</th>
<th>Procedure</th>
<th>Method</th>
<th>Identified flavan-3-ols</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. sinaica</em></td>
<td>fruits</td>
<td>isolation</td>
<td>¹H-NMR, ¹³C-NMR, FAB-MS</td>
<td>epi, B2, B5, A2, C1</td>
<td>154</td>
</tr>
<tr>
<td><em>C. monogyna</em></td>
<td>leaves</td>
<td>isolation</td>
<td>not specified</td>
<td>B2</td>
<td>155</td>
</tr>
<tr>
<td><em>C. azarolus</em></td>
<td>bark</td>
<td>isolation</td>
<td>thiolysis + HPLC&lt;sup&gt;a&lt;/sup&gt;</td>
<td>epi, B2, B4, B5, C1</td>
<td>71</td>
</tr>
<tr>
<td><em>Crataegus sp.</em></td>
<td>flowers</td>
<td>qual. analysis</td>
<td>TLC (densitometry, dansylchloride)</td>
<td>epi, cat, B-type dimers, trimers</td>
<td>20</td>
</tr>
<tr>
<td><em>Crataegus sp.</em></td>
<td>(Polish Pharm.IV)</td>
<td>flowers</td>
<td>qual. analysis</td>
<td>HPLC&lt;sup&gt;a&lt;/sup&gt;</td>
<td>156</td>
</tr>
<tr>
<td><em>C. laevigata</em></td>
<td>leaves, flowers, buds</td>
<td>qual. analysis</td>
<td>HPLC&lt;sup&gt;a&lt;/sup&gt;, TLC (IR diffuse reflectance spectr.)</td>
<td>epi, cat</td>
<td>157</td>
</tr>
<tr>
<td><em>C. monogyna / C. laevigata</em></td>
<td>leaves and flowers</td>
<td>isolation: qual. analysis</td>
<td>FAB-MS, TLC (dansylchloride)</td>
<td>epi, B2, C1, tetramer, trimers (?)</td>
<td>72</td>
</tr>
<tr>
<td><em>C. monogyna</em></td>
<td>leaves and flowers</td>
<td>fract. + qual. analysis</td>
<td>TLC (dansylchloride, vanillin)</td>
<td>monomers, B1, B2 (UV)</td>
<td>74</td>
</tr>
<tr>
<td><em>C. monogyna</em></td>
<td>fruits</td>
<td>isolation</td>
<td>TLC&lt;sup&gt;a&lt;/sup&gt;, ¹H-NMR, CD of derivatives</td>
<td>B2, B5, C1, tetra 1</td>
<td>158</td>
</tr>
<tr>
<td><em>Crataegus sp.</em></td>
<td>leaves</td>
<td>isolation: fract. + qual. analysis</td>
<td>EI-MS, TLC (acetate) (acetate)</td>
<td>B1 (UV)</td>
<td>75</td>
</tr>
<tr>
<td><em>C. monogyna / C. laevigata</em></td>
<td>leaves</td>
<td>fract. + qual. analysis</td>
<td>TLC&lt;sup&gt;a&lt;/sup&gt; (vanillin)</td>
<td>B2 (UV)</td>
<td>73</td>
</tr>
<tr>
<td><em>C. monogyna</em></td>
<td>leaves, fruits</td>
<td>qual. analysis</td>
<td>2D-PC&lt;sup&gt;a&lt;/sup&gt; (Gibbs, Prussian blue reagents)</td>
<td>epi, cat, B1, B2, B4, B5, C1, tetramer</td>
<td>8</td>
</tr>
<tr>
<td><em>C. laevigata</em></td>
<td>fruits</td>
<td>isolation</td>
<td>TLC&lt;sup&gt;a&lt;/sup&gt;, [α], ¹H-NMR, MS of derivatives</td>
<td>B2</td>
<td>76, 159</td>
</tr>
<tr>
<td><em>C. laevigata</em></td>
<td>fruits</td>
<td>isolation</td>
<td>hydrolysis, derivatives (elem. anal., [α], IR)</td>
<td>dimer (= B1 ?)</td>
<td>77</td>
</tr>
<tr>
<td><em>C. laevigata</em></td>
<td>leaves</td>
<td>qual. analysis</td>
<td>2D-TLC</td>
<td>3 procyianidin spots</td>
<td>160</td>
</tr>
<tr>
<td><em>C. monogyna</em></td>
<td>fruits</td>
<td>isolation</td>
<td>hydrolysis, derivatives (elem. anal.)</td>
<td>epi, dimer (= B2)</td>
<td>161</td>
</tr>
<tr>
<td><em>C. laevigata</em></td>
<td>fruits</td>
<td>isolation</td>
<td>hydrolysis, [α], derivatives (elem. anal.)</td>
<td>dimer (= B2)</td>
<td>162</td>
</tr>
<tr>
<td><em>Crataegus sp.</em></td>
<td>fruits, leaves</td>
<td>isolation: qual. analysis</td>
<td>not specified</td>
<td>epi, cat</td>
<td>163</td>
</tr>
</tbody>
</table>

<sup>a</sup> Identification was made with respect to reference compounds.
TLC chromatograms of peracetylated procyanidins in different fruits shows that only procyanidin B2 has been isolated from *Crataegus*.

Some evidence for the possible presence of procyanidin B1 in *Crataegus* sp. comes from the experimental data of Lewak and Radominska [77], who isolated a procyanidin dimer from *Crataegus laevigata* fruits, of which the acetate derivative showed a melting point of 232 °C, which is identical to the one reported by Weinges et al. [76] for procyanidin B1 isolated from *Vaccinium vitis-idaea*. However, the data for the optical rotation differ significantly: for procyanidin B1 $[\alpha]_{378}$-values of $+110^\circ$ (in chloroform, $c = 1.21$ [8]) and $+107.4^\circ$ (in acetone, $c = 2$ [76]) have been reported, while Lewak and Radominska [77] found a value of $+13^\circ$ (in acetone, $c = 1$), which is much closer to values determined for procyanidin B2. All these data show, that a phytochemical re-investigation of *Crataegus* sp. with respect to the presence of procyanidin B1 with modern analytical tools would be necessary.

Studies on procyanidin contents in *Crataegus monogyna* by fractionation of extracts of flowers, leaves and fruits into an oligomeric and a polymeric group and subsequent colorimetric determination showed that the oligomers were more abundant than the polymers [78]. The same results were obtained when samples were harvested at different times of the season [79]. Oligomeric procyanidins generally include compounds up to a degree of polymerization of five [80] to six [81].

Studies on the molecular weight distribution of polymeric procyanidins of unripe berries of *Crataegus laevigata* by gel permeation chromatography of the peracetates yielded an average degree of polymerization of seven. The molecular weight profile was found to roughly obey a normal distribution [24]. Measurements of the average molecular weight of polymers of unripe fruits of *Crataegus laevigata* in two successive seasons by vapour pressure osmometry also revealed an average degree of polymerization of seven [28]. All these results corroborate the view that procyanidins of relatively low degree of polymerization are predominating in *Crataegus*.

A variety of colorimetric studies on procyanidin contents in leaves and flowers compared to fruits revealed that procyanidin contents are higher in herb [75,82-84]. The isolation of reference compounds in the present study was therefore performed with *Crataegus* leaves and flowers.
7.1 Pharmacological activities of procyanidins from *Crataegus*

The procyanidin pattern of the lower molecular weight compounds is not only typical for *Crataegus*. Similar procyanidin distribution patterns can be found in a wide variety of species from a lot of different families as can be seen from Table 7.2 (Criteria for the inclusion were: procyanidin B2 is the major dimer and is accompanied by at least procyanidin B5, procyanidin B3 and (+)-catechin are absent or minor compounds and no esters of gallic acid or prodelphinidins are described in that particular species).

The occurrence of similar distribution patterns in other species is of considerable significance in view of the ascribed pharmacological activity of procyanidins of *Crataegus*. None of the species listed in Table 7.2 is reported to be used in cases of declining heart function. The only procyanidin containing plant species which shows a related use in traditional medicine to the one of *Crataegus* is the Chinese herb *Melastoma candidum* (Melastomataceae). Procyanidin B2 among other more active compounds (castalagin, helichrysonside) have been isolated from this plant and all these compounds are reported to exert antihypertensive effects through an inhibition of the sympathetic activity and a reduction of the vascular tone [85].

Results from other pharmacological tests with individual procyanidins show that this class of compounds has distinct activities which may explain a supporting effect in cases of declining heart function in extracts of *Crataegus*: An inhibition of *in vitro* human LDL (low density lipoprotein) oxidation has been reported for low molecular weight procyanidins. Procyanidins B2 and C1 have been shown to be more active than procyanidins B3, B4 and C2 [86]. Studies on the radical scavenger activity revealed that procyanidins of higher degree of polymerization (hexamers / pentamers > tetramers > dimers / trimers) [87,88] and galloylated procyanidins [89] are most active. Procyanidins from *Crataegus sinaica* showed a stronger anti-complementary activity than the isolated flavonoids, while procyanidins C1 and A2 were the most active compounds tested [90]. All the activities listed above might prevent or reduce degenerative processes (i.e. atherosclerosis) which are known to play an important role in the development of the coronary heart disease.
Table 7.2  Species which exhibit similar procyanidin patterns like *Crataegus* sp.

<table>
<thead>
<tr>
<th>Species</th>
<th>Family</th>
<th>Organ</th>
<th>Isolated procyanidins</th>
<th>Use / active principle</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Malus sylvestris</em></td>
<td>Rosaceae</td>
<td>fruits, juice</td>
<td>epi, cat, B2, B5, B1, A2, tetra I</td>
<td>foodstuff</td>
<td>112,142, 164</td>
</tr>
<tr>
<td></td>
<td></td>
<td>fruits&lt;sup&gt;a&lt;/sup&gt;</td>
<td>tri II</td>
<td></td>
<td>31</td>
</tr>
<tr>
<td><em>Guzuma ulmifolia</em></td>
<td>Sterculiaceae</td>
<td>bark</td>
<td>C1, tri I, tri III</td>
<td></td>
<td>165</td>
</tr>
<tr>
<td><em>Nelvia meyeri</em></td>
<td>Mesembryanthemaceae</td>
<td>leaves&lt;sup&gt;f&lt;/sup&gt;</td>
<td>epi, B2, B5, C1, tri I, tri VII, tetra I</td>
<td>diarrhoea</td>
<td>101</td>
</tr>
<tr>
<td><em>Theobroma cacao</em></td>
<td>Sterculiaceae</td>
<td>fruits&lt;sup&gt;d,e&lt;/sup&gt;</td>
<td>epi, cat, B2, B5, C1</td>
<td></td>
<td>40</td>
</tr>
<tr>
<td><em>Albizia lebbek</em></td>
<td>Leguminosae</td>
<td>bark</td>
<td>epi, cat, B2, B5, tetra I</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td><em>Davallia mariesii</em></td>
<td>Davalliaceae</td>
<td>rhizomes</td>
<td>B2, B5, tri VII, tetra II</td>
<td>medicinal, n.s.</td>
<td>114,166</td>
</tr>
<tr>
<td><em>Rhaphiolepis umbellata</em></td>
<td>Rosaceae</td>
<td>bark&lt;sup&gt;g&lt;/sup&gt;</td>
<td>epi, B2, B5, B1, A2, C1</td>
<td>dying agent</td>
<td>59</td>
</tr>
<tr>
<td><em>Cinnamomum cassia</em></td>
<td>Lauraceae</td>
<td>bark&lt;sup&gt;h&lt;/sup&gt;</td>
<td>epi, B2, B5, C1, tetra&lt;sup&gt;h&lt;/sup&gt;, penta&lt;sup&gt;b&lt;/sup&gt;, hexa&lt;sup&gt;h&lt;/sup&gt;</td>
<td>medicinal, n.s.</td>
<td>38</td>
</tr>
<tr>
<td><em>Dioscorea cirrhosa</em></td>
<td>Dioscoreaceae</td>
<td>tuber</td>
<td>epi, cat, B2, B5, B1, C1, tri VIII, tri IX, tetra I, tetra III</td>
<td>foodstuff (yam)</td>
<td>115</td>
</tr>
<tr>
<td><em>Cinchona succirubra</em></td>
<td>Rubiaceae</td>
<td>bark&lt;sup&gt;g&lt;/sup&gt;</td>
<td>epi, B2, B5, C1, A2</td>
<td>quinine, quinidine</td>
<td>68</td>
</tr>
</tbody>
</table>

Abbreviations: trivial nomenclature of dimers and trimers, see also Abbreviations for procyanidins; epi = (-)-epicatechin; cat = (+)-catechin; tri I = epi-(4β→6)-epi-(4β→8)-epi; tri II = epi-(4β→6)-epi-(4β→8)-epi; tri III = epi-(4β→8)-epi-(4β→6)-epi; tri VII = epi-(4β→8)-epi-(4β→6)-epi; tri VIII = epi-(4β→8)-epi-(4α→8)-cat; tri IX = epi-(4β→8)-cat-(4α→8)-epi; tetra I = epi-(4β→8)-epi-(4β→8)-epi-(4β→8)-epi; tetra II = epi-(4β→6)-epi-(4β→8)-epi-(4β→8)-epi; tetra III = cat-(4α→6)-epi-(4β→8)-epi-(4β→8)-epi.

<sup>a</sup> Detected in HPLC analysis using chemical reaction detection; <sup>b</sup> detected as phloroglucinol derivatives by HPLC; <sup>c</sup> unfermented beans; <sup>d</sup> isolation of procyanidins with enantiomeric monomer units; <sup>e</sup> isolation of procyanidin glycosides; <sup>f</sup> isolation of phloroglucinol derivative; <sup>g</sup> isolation of phenylpropanoid derivatives; <sup>h</sup> tetramer, pentamer and hexamer (all-(4β→8)-epi) referred to as cinnamtannins.
Procyanidin containing fractions from *Crataegus* sp. have been shown to increase the coronary blood supply, to improve contractility and to affect the heart frequency (both, an increase and a decrease of the heart rate has been reported) [91]. A direct action on the heart performance has lately again been reported of a high molecular weight fraction of procyanidins from *Vitis vinifera* seeds. This fraction reduced the ventricular contracture during ischemia and improved cardiac mechanical performance upon reperfusion in the Langendorff rabbit heart model [92]. Effects of procyanidin containing fractions from *Crataegus* sp. also show beneficial effects on the peripheral circulation and on blood pressure [91]. Recently, inhibitory effects of individual procyanidins on ACE (angiotensin converting enzyme) have been demonstrated. Galloylated procyanidin B2 was the most active compound tested, but also non-galloylated procyanidins showed activity [93].

Although none of the plant species which show a similar procyanidin pattern like *Crataegus* is used in cases of declining heart function, it can be concluded from the pharmacological studies on individual procyanidins and procyanidin containing fractions from *Crataegus* sp. that these compounds contribute to the overall activity of extracts of *Crataegus.*
8 Purification of procyanidins from *Crataegus*

8.1 Introduction

Procyanidins are not commercially available. Their procurement can be done either by purification from plant material or by synthesis. Since purification procedures are laborious synthesis is quite tempting. The biomimetic condensation of (+)-taxifolin and flavan-3-ol monomers to produce oligomeric procyanidins with (+)-catechin as upper unit is well established. The synthesis of oligomeric procyanidins with (-)-epicatechin as upper unit by an analogous procedure is not possible due to the lack of a commercially available (or easily purifiable) flavanonol showing a 2,3-cis stereochemistry. At the time there does not exist a procedure which allows the synthesis of oligomeric procyanidins with (-)-epicatechin as upper unit in reasonable yields. The common way to obtain such procyanidins is therefore still the purification from plant material.

8.1.1 Purification of procyanidins from plant matrices

The crucial question in any isolation procedure is, how accurately the composition of compounds isolated from a plant tissue reflects the genuine distribution. This question is of significant importance when dealing with procyanidins, because compounds exhibiting ortho-diphenol groups are susceptible to oxidation reactions catalized by various enzymes [33,94], of which the polyphenol oxidases are the best-known ones [95]. In the wine making process, for example, even the addition of sulphur dioxide cannot prevent partial degradation of phenolic compounds by such enzymes during crushing and pressing of the grapes [96]. Moreover, procyanidins themselves are relatively unstable molecules and may disproportionate at the natural pH of their aqueous solutions [25]. The occurrence of small quantities of either (-)-epicatechin or (+)-catechin has already been claimed to be possibly due to isomerization processes during extraction [34]. Even the presence of small quantities of doubly-linked (A-type) procyanidins may be due to the conversion of B-type procyanidins [25,97]. Therefore, methods must be developed that minimize such secondary modifications of procyanidins during isolation and purification procedures. Possibilities are the addition
of antioxidants or the conductance of the isolation at low temperatures and the
reduction or even exclusion of oxygen. If procyanidins are not needed in their native
phenolic forms (i.e. as reference standards), peracetylation of the extracts and
subsequent isolation of the derivatives is a possible solution, too [98,99].

The extraction of the plant material is commonly performed with aqueous acetone
(i.e. [100]), ethanol (i.e. [46,101]) or methanol (i.e. [102,103]). For the extraction of
low-molecular weight procyanidins the use of aqueous ethyl acetate has lately been
recommended, too [104]. This first extract, after evaporation of the organic phase, is
most commonly extracted with an apolar solvent to remove chlorophylls and lipids. A
subsequent liquid-liquid partitioning using ethyl acetate and / or n-butanol extracts the
procyanidins together with flavonoids and phenolic acids. For a more detailed
description on these procedures, see section 1.4.3.1.1.

The procyanidin extracts are further fractionated by repeated column
chromatography. Sephadex LH-20 (Pharmacia Biotech) is most often employed. It is a
dextran gel, which is three-dimensionally cross-linked by hydroxypropylene groups
[105]. The separation mechanism is primarily based on adsorption and to a lesser extent
on gel filtration and distribution [106]. Fractionations roughly follow the molecular
weight, while lower molecular weight procyanidins elute ahead of higher molecular
weight ones. The number of phenolic groups in the molecules is therefore mainly
responsible for the adsorption effects. The first fractionation of (+)-catechin among
many other flavonoid compounds on Sephadex LH-20 has been described by Johnston
et al. [107]. They noticed that band spreading using this gel is greater than using
polyamide. Nevertheless polyamide has only been employed until the early 1980s for
the isolation of procyanidins (i.e. [108]). For sample work up in quantitative and
qualitative analysis it has been used until the early 1990s [109,110].

As mobile phases aqueous methanol and ethanol are used for the fractionation of
monomers up to tetramers. Higher oligomers can mostly be eluted with aqueous
acetone. Foo [111] recommend fractionations on Sephadex LH-20 with a change of
eluents: ethanol is stated to elute only monomers up to trimers, while methanol elutes
tetramers and 70 % aqueous acetone is capable of removing pentamers and hexamers.

One fractionation over Sephadex LH-20 rarely yields a satisfying separation
[103,112,113]. More common is a repeated chromatography (i.e. [14,55,57,114]). A
change of the stationary phase is often advantageous. The following phases have been employed: MCI-gel CHP 20 P (Mitsubishi Chemical Industries; styrene-divinylbenzene copolymer carrying macropores; i.e. [13,22,38,46,70,101,102,115]), Diaion HP-20 (Mitsubishi Chemical Industries; highly porous polystyrene gel; i.e. [59,100]) and Fractogel TSK HW-40 (S) (Merck; hydroxylated polyvinyl gel; i.e. [116-118]). The use of even more than two different gels has also been reported [40,100]. The separation on all these gels is again based on molecular sieving and affinity effects. Mostly a final clean-up of the procyanidin containing fractions by reversed-phase preparative HPLC is necessary.

A crude separation of plant polyphenols has also been performed using counter-current chromatography [72,119-123]. The main advantages of this procedure are that no adsorption to the stationary phase occurs and that it is fast. Disadvantages are that emulsification problems may occur [124] and that there are relatively few solvent mixtures that provide a suitable selectivity [125]. The solvents used often show high boiling points (i.e. butanol and propanol) or a salt is included which might be difficult to remove completely in the course of the isolation procedure. A combination with other preparative techniques like column chromatography over Sephadex LH-20 or preparative HPLC is mandatory to obtain pure compounds.

8.2 Summary

Based on the knowledge from the literature study the strategy for the isolation of procyanidins from Crataegus leaves and flowers was established. The extraction using aqueous acetone was conducted at 4 °C in order to minimize oxidation reactions. Because counter-current chromatography did not yield satisfactory results in preliminary tests the crude extract was manually partitioned between different solvents. For the purification procedure Sephadex LH-20 chromatography and reversed-phase preparative HPLC were chosen.

The presence of (-)-epicatechin, procyanidins B2, B4, B5, C1, trimer I and tetramer I in Crataegus leaves and flowers could be demonstrated. Trimer I is described for the first time in Crataegus sp. (+)-Catechin was identified in ethyl acetate extracts and purified fractions. The formation only via epimerization of (-)-epicatechin is very
unlikely, because considerable concentrations of procyanidin B4 can be detected. Therefore, (+)-catechin is also considered as a genuine component, which could not be detected though in freshly prepared samples by LC-MS, because concentrations were below the detection limit. Trimer II has never been detected in freshly prepared samples by LC-MS. It might be present in concentrations below the LC-MS detection limit in leaves and flowers, but it might as well be a degradation product of higher molecular weight procyanidins. Procyanidin B1 and A2, which have been described to be present in some species of *Crataegus* (see section 7, Table 7.1) have not been detected in the present sample.

Analysis of isolated compounds as well as investigations on purified fractions and freshly prepared samples demonstrate that procyanidins are susceptible to degradation reactions. Precautions during isolation work are therefore mandatory although they are rarely ever mentioned in publications on procyanidins. One degradation product was isolated and could be identified as a methylene bridged flavan-3-ol (artifact I, see section 10.4.9). A variety of compounds show molecular weights which seem to follow a specific algorithm. Speculations on the nature of these compounds have been made, but their validity would have to be demonstrated in additional phytochemical investigations.

The application of different methods for the characterization like Rf-values, retention times, UV-ratios or reaction with dyes specific for flavan-3-ols show that only LC-MS is specific enough to be able to discriminate between common procyanidins and structurally closely related flavan-3-ols.

For the isolation of reference standards *Crataegus* bark seems to be more suitable than leaves and flowers, because procyandins are more abundant in this woody tissue [71].

### 8.3 Experimental section

#### 8.3.1 Materials and instrumentation

*Crataegus* leaves and flowers (*Crataegi folium cum flore Ph.Helv.VII / DAB 10; lot: 49125L) was obtained from Dixa (CH-St. Gallen). Cyanidin chloride (HPLC grade) and (+)-catechin (CHR grade) were from Roth (CH-Reinch) and (-)-epicatechin (HPLC
grade) was obtained from Extrasynthese (F-Genay). Acetone, diethyl ether, ethanol, ethyl acetate and methanol were of HPLC quality (Romil Chemicals, GB-Shepshed). n-Butanol (HPLC quality) was purchased from Fluka (CH-Buchs) and toluene (p.a.) from Scharlau (EGT Chemie, CH-Tägerig). De-ionized water was obtained using a NANO-pure cartridge system (Skan, CH-Basel). Formic acid (p.a.) and acetic acid (100 %, p.a.) were from Merck (CH-Dietikon); sulphuric acid (puriss.), hydrochloric acid (Ph.Eur.) and ortho-phosphoric acid (Ph.Helv.VI / Ph. Eur.) were purchased from Hänseler (CH-Herisau). 4-Dimethylaminocinnamaldehyde (DMACA, purum) was obtained from Fluka (CH-Buchs).

Sephadex LH-20 (lots: RE 14413 and 231531) was obtained from Pharmacia Biotech (S-Uppsala) and dextran blue (Leuconostoc ssp., molecular weight: ~2’000’000) was from Fluka (CH-Buchs). For preparative chromatography a Knauer (D-Berlin) prepacked cartridge column (250 x 16 mm I.D., 5 μm) filled with Spherisorb S5 ODS II was used. The guard column (25 x 4.0 mm I.D., 10 μm) was filled with LiChrosorb RP-18 (Merck, D-Darmstadt). For analytical chromatography a Knauer (D-Berlin) prepacked cartridge column (250 x 4 mm I.D., 5 μm) filled with LiChrosorb RP-18 and a guard column (5 x 4 mm I.D.) of the same material was used.

Nylon filters (0.45 μm, 25 mm) were from Scientific Resources (Infochroma, CH-Zug). Celite 535 was purchased from Fluka (CH-Buchs). The plastic foil for light protection was bought at the Plastic-Haus AG (CH-Basel).

Instrumentation

Mill: Centrifugal mill model ZM1
(Retsch, Schieritz and Hauenstein AG, CH-Arlesheim);
sieve size: 1.0 mm.

Pump for forced flow extraction: model 681 (Büchi, CH-Flawil);
gradient former: model 687 (Büchi, CH-Flawil).

Extraction column: model B-685 (Büchi, CH-Flawil), Ø 7 x 100 cm.

Centrifuge: MSE, model Mistral 2000 (Zivy, CH-Oberwil).

Lyophilizator: Lyolab C (LSL Secfroid, CH-Aclens-Lausanne);
pump: model Pascal VDE 0530.
Modulyo (Edwards, GB-West Sussex);
pump: model E2M5.
FPLC system for medium pressure chromatography: Pharmacia Biotech (S-Uppsala).
- pump: model P-500; liquid chromatography controller: model LCC-500;
- motor valves: model MV-7 and MV-8; 10 ml superloop; fraction collector: model FRAC-100; UV detector: monitor UV-M, 254 nm.

Columns for medium pressure chromatography: model B-685 (Büchi, CH-Flawil).
- column chromatography I: \( \varnothing \) 4.8 x 80 cm.
- column chromatography II: \( \varnothing \) 1.5 x 46 cm (\( \varnothing \) 2.6 x 46 cm for F339-403)

HPLC for preparative chromatography: Merck-Hitachi (D-Darmstadt).
- Pump: model L-6200; UV-detector: model L-4000, 280 nm;
- integrator: model D-2500; fraction collector: model L-7650.

HPLC for analytical chromatography: Hewlett-Packard (D-Waldbronn).
- Models 1090 and 1090 Series II, equipped with a DAD.

LC-MS equipement: see section 5.2.

### 8.3.2 Procedures for the characterization of extracts and procyanidins

**TLC analyses**

*Procyanidin TLC.* Analyses were performed on silica gel 60F\(_{254}\) (Merck, CH-Dietikon) over a distance of approximately 8 cm. The eluent was composed of ethyl acetate – formic acid – \( \text{H}_2\text{O} \) (90:5:5, v/v). For detection, the chromatograms were sprayed with a 1 % (w/v) solution of DMACA in methanolic sulphuric acid (8 ml H\(_2\)SO\(_4\) ad 100 ml methanol). Procyanidins turn green to blue; upon heating, spots become brown but turn back to the original colors after a while.

*TLC according to Lea et al. [124].* Analyses were performed on silica gel 60F\(_{254}\) (Merck, CH-Dietikon) over a distance of approximately 14 cm. The eluent was composed of toluene – acetone – formic acid (30:30:10, v/v). Detection was performed using a 1 % (w/v) solution of DMACA in methanolic sulphuric acid (8 ml H\(_2\)SO\(_4\) ad 100 ml methanol).
Analytical HPLC analyses

Fingerprint HPLC. The mobile phase consisted of solvent A (methanol) and solvent B (0.5 % (v/v) ortho-phosphoric acid in water). The elution profile was: 0 min 20 % A in B, 0 - 35 min from 20 % to 35 % A in B, 35 - 40 min 35 % A in B, 40.01 - 45 min 90 % A in B, 45.01 - 50 min 100 % A (wash-out), 50.01 - 60 min 20 % A in B (re-conditioning). All gradients were linear. The flow rate was set to 1.0 ml/min and the column temperature was fixed at 24.0 °C. Chromatograms were recorded at 280 nm, spectra were measured in the range 190 nm to 600 nm.

HPLC for quantitative analysis. See section 2.1.2.

HPLC for the analysis of wash-out peaks. The mobile phase consisted of solvent A (methanol) and solvent B (0.5 % (v/v) ortho-phosphoric acid in water). The elution profile was: 0 min 25 % A in B, 0 - 60 min from 25 % to 80 % A in B, 60.01 - 70 min 25 % A (re-conditioning). All gradients were linear. The flow rate was set to 1.0 ml/min and the column temperature was fixed at 24.0 °C. Chromatograms were recorded at 280 nm, spectra were measured in the range 190 nm to 600 nm.

LC-MS analysis. See section 5.2.

HPLC-CRD analysis. The HPLC-CRD (chemical reaction detection) analyses using DMACAC were conducted according to Treutter [126-127] in the research group of Prof. Dr. W. Feucht and Dr. D. Treutter at the Institute for Agriculture and Horticulture, Technical University of Munich (D-85350 Freising-Weihenstephan).

Complete acid hydrolysis of extracts

The complete acid hydrolysis was performed according to the procedure described in [128]. Samples were dissolved in 1 ml concentrated hydrochloric acid – methanol (1:5, v/v). The flasks were sealed and kept in a boiling water bath for 10 min. The solutions were analyzed by TLC on a cellulose F254 stationary phase (Merck, CH-Dietikon). The mobile phase consisted of acetic acid – concentrated hydrochloric acid – water (30:3:10, v/v). Detection was performed in daylight. The Rf-values were compared to the one of authentic cyanidin standard (Rf 0.5).
8.3.3 Extraction

The principle of the extraction and purification procedures is summarized in Figure 8.1. The plant material was extracted with aqueous acetone. The organic phase was evaporated and the water phase was subsequently lyophilized. Portions of the extract were redissolved in water and this water phase was successively extracted with diethyl ether, ethyl acetate and n-butanol. The remaining water phase was again lyophilized. The flavan-3-ols contained in the ethyl acetate fraction were purified by chromatography over Sephadex LH-20 and preparative HPLC (see section 8.3.4).

*Crataegus* leaves and flowers (mesh 1.0 mm) were combined with 5 % (w/w) Celite 535 and were macerated with degased 70 % (v/v) aqueous acetone at 5 °C for one hour. The mixture was transferred to the extraction column and was subsequently extracted exhaustively under forced-flow conditions at 5 °C (rate: approximately one drop/sec; pressure: 6 bars raising to 12 bars). The organic phase was evaporated at room temperature. The remaining water phase was frozen in a -80 °C ice bath (acetone and solid carbon dioxide) and lyophilized. The residue contained 7-11 % water (determined at 100 - 105 °C) and was stored under nitrogen at -20 °C.

Portions of the crude extract were re-suspended in water and were exhaustively extracted with diethyl ether (saturated with water). An undissolved residue was filtered over cotton wool after four extractions and was extracted with boiling water. This extract was again added to the water phase for subsequent extractions with diethyl ether (*procyanidin A2 is soluble in hot, but not in cold water according to Bate-Smith [7]). The ether fractions were back-extracted with water and evaporated below 30 °C to a small volume. Remaining diethyl ether was removed in a stream of nitrogen. The wet residue was frozen at -20 °C, lyophilized under exclusion of light and stored under nitrogen at -20 °C.

The water phase was exhaustively extracted with ethyl acetate (saturated with water). A sticky precipitate was removed by filtration over cotton wool after six extractions. In order to improve phase separation, phases were centrifuged (2000 rpm, 5 min) where necessary. The ethyl acetate fractions were back-extracted with water, evaporated below 30 °C, frozen at -20 °C and lyophilized under exclusion of light. The extracts were stored under nitrogen at -20 °C.

The water phase was subsequently extracted with n-butanol (saturated with water). Again, phases were centrifuged (2000 rpm, 5 min) for better phase separation where
Crataegus leaves and flowers (990 g)

Forced-flow extraction
70% aqueous acetone at 5 °C

Evaporation of acetone

Lyophilization
Storage under N₂ at -20 °C

Dissolution in water (in three portions)

1. Liquid-liquid extraction diethyl ether (10.8 g)

2. Liquid-liquid extraction ethyl acetate (14.2 g)

Column chromatography I
Sephadex LH-20
methanol / water / acetone

Column chromatography II
Sephadex LH-20
ethanol

Preparative HPLC
C18 methanol / water

4. Lyophilization of water phase (162.1 g)

3. Liquid-liquid extraction n-butanol (53.7 g)

Figure 8.1 Overview on the isolation procedure.
necessary. The undissolved residue of the ethyl acetate extraction was suspended in n-butanol and these extracts were added to the n-butanol fractions. The combined n-butanol fractions were back-extracted with water and evaporated below 40 °C by forming an azeotrope with water. The residue was frozen at -20 °C, was lyophilized under exclusion of light and was stored under nitrogen at -20 °C.

The remaining water phase was frozen in a -80 °C ice bath, was lyophilized under exclusion of light and was stored under nitrogen at -20 °C, too.

In preliminary tests diethyl ether, n-hexane and dichloromethane were tested as possible solvents for the removal of lipids. In the dichloromethane extracts cyanidin could be detected after acid hydrolysis. Diethyl ether was favored over n-hexane because removal of chlorophylls was shown to be more effective with this first solvent. Moreover, the presence of cyanidin after acid hydrolysis of n-hexane extracts could not be excluded definitely. If diethyl ether extraction was performed first, subsequent aqueous acetone extracts were still green-colored, indicating the presence of chlorophylls. Therefore, extraction with diethyl ether was performed after the extraction of the plant material with aqueous acetone.

The extraction was conducted at 5 °C and under forced-flow conditions using degased aqueous acetone to minimize oxidation reactions. Subsequent liquid-liquid extractions were carried out at room temperature. No additional precautions against oxidations were taken, because liquid-liquid extraction was relatively fast compared to the forced-flow extraction procedure. Additional protection against oxidation could be achieved by using degased solvents and by exchanging the air in the separatory funnels by nitrogen.

TLC analysis of the ethyl acetate extract showed five spots which turned green to blue with DMACA reagent, indicating the presence of flavan-3-ols. The n-butanol extract exhibited a main spot at the start and some weaker spots with higher Rf-values. No flavan-3-ols were detected in the range of the two spots which showed the highest Rf-values in the sample of the ethyl acetate extract. These results indicate that ethyl acetate extracts flavan-3-ols of lower molecular weights (showing higher Rf-values), whereas n-butanol mainly extracts procyanidin of higher molecular weights (showing lower Rf-values or remaining at the start). Moreover, HPLC chromatograms of the n-butanol extract showed a more pronounced unresolved hump than the chromatograms of the ethyl acetate extract which supports this interpretation, too.
Monomers are known to be soluble in diethyl ether [129]. In TLC chromatograms of the diethyl ether fraction (-)-epicatechin could therefore be detected. However, (-)-epicatechin was also present in the ethyl acetate fraction as could be demonstrated by spiking experiments and subsequent HPLC analysis. TLC analysis of the water phase revealed the presence of polymeric procyanidins which remained at the start.

The sticky precipitates which formed during the extraction with diethyl ether and ethyl acetate were not soluble in ethyl acetate or n-butanol. TLC and HPLC analysis showed the presence of mainly polymeric procyanidins, which remained at the start or eluted as a broad peak exhibiting (-)-epicatechin spectrum, respectively.

8.3.4 Fractionation of the ethyl acetate extract and isolation of pure compounds

The ethyl acetate extract was further fractionated by column chromatography over Sephadex LH-20. A first fractionation was performed with a gradient from aqueous methanol over methanol to aqueous acetone. Selected fractions were again chromatographed over Sephadex LH-20 using ethanol as an eluent. The final purification was performed by preparative HPLC. An overview on the whole fractionation procedure is given in Figures 8.2A and 8.2B.

The Sephadex LH-20 was swollen in the solvent of the first eluent and was then filled as slurry into the columns. The quality of the column packing for column chromatography I was checked by measuring the exclusion volume with a solution of dextran blue in 50 % aqueous methanol. The other column packings were checked visually. The conditions applied are summarized in Table 8.1.

The extracts were dissolved in 50 % aqueous methanol and were filtered prior to application to the columns. The collected fractions were monitored by TLC. Similar fractions were combined and were evaporated below 30 °C under exclusion of light.

Remaining water was removed by lyophilization (column chromatography I). The combined fractions were again checked by TLC and HPLC and were stored under nitrogen at -20 °C.
Figure 8.2A Fractionation of the ethyl acetate extract and isolation of pure compounds. Part I.
Figure 8.2B  Fractionation of the ethyl acetate extract and isolation of pure compounds. Part II.
Table 8.1  Conditions for column chromatography over Sephadex LH-20.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Column chromatography I</th>
<th>Column chromatography II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elution program</td>
<td>0 - 24 h 70 % MeOH 24 - 36 h 70 % MeOH 36 - 38 h 100 % MeOH 38 - 44 h 70 % Me$_2$CO 44 - 60 h 70 % Me$_2$CO</td>
<td>100 % EtOH</td>
</tr>
<tr>
<td>Flow rate</td>
<td>2 ml/min</td>
<td>2 ml/min</td>
</tr>
<tr>
<td>Temperature</td>
<td>ambient</td>
<td>ambient</td>
</tr>
<tr>
<td>Fraction size</td>
<td>F 1 - 476 15 ml F 477 - 490 20 ml</td>
<td>F 1 ~ 70 10 - 15 ml F ~70 - end 15 - 20 ml</td>
</tr>
</tbody>
</table>

For preparative HPLC, fractions were dissolved in 50 % aqueous methanol and filtered over a 0.45 µm filter. All elutions were performed isocratically at a flow rate of 5 ml/min. After each run the column was washed with 100 % methanol and was then re-equilibrated. Collection of fractions from preparative HPLC was done manually for F50-59 ((-)‐epicatechin), F85-96 (procyanidin B2), F72-84 (Pc B) and F99-124 (procyanidin C1). The flasks for collection of the fractions were wrapped with black plastic foil and were set in an ice-bath to prevent oxidation reactions. All other preparative HPLC experiments were conducted using an automatic fraction collector. Peaks were collected in a previously defined time-window. The flasks for collection of the fractions were wrapped with black plastic foil and a stream of nitrogen was continuously applied to each flask. The isolated fractions were evaporated below 30 °C, were frozen and lyophilized under exclusion of light. They were analyzed by TLC and HPLC prior to storage under nitrogen at -20 °C (impure fractions) or -80 °C (procyanidins), respectively.
The first chromatography over Sephadex LH-20 separates a lot of matrix components (i.e. flavonoids and phenolic acids; 1.66 g in fractions F1-192) from procyanidins. HPLC chromatograms of the procyanidin fractions F223-423 (3.27 g) are given in Figures 8.3A to 8.3D. Besides very few exceptions, all peaks eluting during the gradient exhibit an (-)-epicatechin spectrum. Fractions F223-403 contain all the principal procyanidins, while only minor procyanidin peaks can be detected in fractions F404-423. The enormous number of minor peaks illustrates the structural diversity of this class of compounds. The occurrence of countless minor procyanidin peaks in HPLC chromatograms is common in the analysis of procyanidins (i.e. [30,130]).

The wash-out peaks of fractions F323-423 also show typical (-)-epicatechin spectra. These fractions were additionally analyzed with an HPLC method which enables the spreading of this late section in the chromatogram. No principal procyanidin peaks could be detected. The chromatograms were characterized by unresolved humps which showed minor spikes of individually resolved peaks on top of them.

(-)-Epicatechin (F193-222) elutes before the procyanidins which is in compliance with other published data [13,45,70,115]. If merely gel filtration occurred, compounds with higher molecular weights would elute ahead of compounds with lower molecular weights. Since the monomeric flavan-3-ol, (-)-epicatechin, elutes before the procyanidins adsorption and/or distribution effects predominate over the size exclusion effects, which generally are characteristic for the chromatography over Sephadex LH-20 [105]. This behavior of procyanidins is however well-known and is mainly attributable to their ability to form multiple hydrogen bonds.

The elution order of the procyanidins is as follows: Pc A → B2 → B4 → C1 / Pc B → artifact I → Pc C → trimer I → tetramer I → B5 → trimer II. The elution order of 4→8 linked procyanidins follows the degree of polymerization. Procyanidins of higher molecular weight are retained longer, which again demonstrates stronger adsorption to the Sephadex LH-20 with increasing number of hydroxyl groups in the molecules. Procyanidins which exhibit 4→6 linkages elute later than their 4→8 counterparts (e.g. trimer I and II elute after procyanidin C1 and procyanidin B5 after procyanidin B2). Bergmann et al. [131] demonstrated by time-resolved fluorescence measurements of model compounds and molecular mechanics calculations (MM2) that 4→6 linked procyanidins tend to be more compact than their 4→8 linked counterparts. The elution
order found here implies therefore, that in the elution of procyanidins with 4\(\rightarrow\)6 linkages additional size exclusion effects occur besides the adsorption effects described above.

Similar elution orders are described in the literature for the chromatography of procyanidins over Sepadex LH-20 using methanol-water gradients: Morimoto et al. [13] fractionated an extract from the whole body of *Vaccinium vitis-idaea* L. (B1 \(\rightarrow\) B3 \(\rightarrow\) B7 \(\rightarrow\) A1 \(\rightarrow\) A2 \(\rightarrow\) trimers \(\rightarrow\) tetramers) and Hsu et al. [115] fractionated an extract from tubers of *Dioscorea cirrhosa* LOUR. (B1 \(\rightarrow\) B2 \(\rightarrow\) trimer \(\rightarrow\) B5 \(\rightarrow\) C1 \(\rightarrow\) trimers \(\rightarrow\) tetramers). The rule that procyanidins elute according to their degree of polymerization and, that procyanidins showing 4\(\rightarrow\)6 interflavanoid linkages generally elute later than isomeric procyanidins with 4\(\rightarrow\)8 linkages also holds for elutions with ethanol [8,57].

The procyanidin containing fractions could be separated further with the second column chromatography over Sephadex LH-20 using ethanol as eluent, but no pure procyanidin fraction could be obtained. Again, numerous minor procyanidin peaks were present in the chromatograms. The analysis of the wash-out peaks showing (-)-epicatechin spectra revealed that no major procyanidins, which can be chromatographed, were present. Spike experiments with (-)-epicatechin revealed the presence of this monomer in some early eluting fractions. This indicates that degradation reactions of procyanidins occurred. Since the fraction collector of the FPLC apparatus can not be cooled, a possible solution would be to conduct the entire chromatographies over Sephadex LH-20 in a cold room at 5 °C.

Preparative reversed-phase chromatography of procyanidins is hampered by the relatively low loadability of the sorbents for procyanidins. This feature is likely to be attributable to an abnormous chromatographic behavior which has been reported for some procyanidins (see section 1.4.3.2.2). The injection of relatively pure fractions and automation is therefore a reasonable strategy in the isolation of procyanidins.

Protection from oxidation is especially important in the final purification step. The use of nitrogen to prevent oxidations in the collected pure fractions is very favorable, because the volatilization of the methanol containing eluent withdraws energy from the solutions which results in a considerable cooling of the fractions.
Figure 8.3A  HPLC fingerprint chromatograms of fractions of the first chromatography over Sephadex LH-20. Part I.

Top:  Ethyl acetate extract (approximately 20 mg/ml)
Bottom: Fraction 223 - 243  (0.547 mg/ml)

Peak identification:
1  (-)-Epicatechin
2  (+)-Catechin
3  Pc A
4  Procyanidin B2
5  Compound X
6  Procyanidin B4
7  Procyanidin C1
8  Pc B
9  Compound Y
10 Artifact I  bis-epicatechinyl-methane
11 Pc C
12 Trimer I  (-)-epicatechin-(4β→6)(-)-epicatechin-(4β→8)(-)-epicatechin
13 Tetramer I  (-)-epicatechin-(4β→8)(-)-epicatechin-(4β→8)(-)-epicatechin-(4β→8)(-)-epicatechin
14 Procyanidin B5
15 Trimer II  (-)-epicatechin-(4β→6)(-)-epicatechin-(4β→6)(-)-epicatechin
16 Chlorogenic acid
Figure 8.3B  HPLC fingerprint chromatograms of fractions of the first chromatography over Sephadex LH-20. Part II.

Top:  Fraction 244 - 272  (0.193 mg/ml)
Middle: Fraction 289 - 305  (14.07 mg/ml)
Bottom: Fraction 306 - 322  (12.71 mg/ml)

Peak identification: see Figure 8.3A.
Figure 8.3C  HPLC fingerprint chromatograms of fractions of the first chromatography over Sephadex LH-20. Part III.

Top:  Fraction 323 - 338  (8.72 mg/ml)
Middle: Fraction 339 - 355  (8.96 mg/ml)
Bottom: Fraction 356 - 368  (8.98 mg/ml)

Peak identification: see Figure 8.3A.
Figure 8.3D HPLC fingerprint chromatograms of fractions of the first chromatography over Sephadex LH-20. Part IV.

Top: Fraction 369 - 386 (8.86 mg/ml)
Middle: Fraction 387 - 403 (11.32 mg/ml)
Bottom: Fraction 404 - 423 (11.24 mg/ml)

Peak identification: see Figure 8.3A.
8.3.5 Characterization of purified procyanidins

The compounds which were detectable in the procyanidin containing fractions of the first column chromatographic separation over Sepahdex LH-20 were characterized by TLC analyses and HPLC analyses using different detection modes (UV, MS, CRD). Table 8.2 gives an overview on the results of these investigations. Structural formulae of the identified compounds are given in Figures 10.1A to 10.1C (see section 10); details on the structure elucidation are given in section 10.

Although pure procyanidins are expected to be colorless from their UV spectra, compounds with a degree of polymerization above two are generally beige-colored [132]. Thompson et al. [8] reported that procyanidins exposed to light become darker. These discolorations, which finally result in red to brown colored compounds, occur as a result of oxidation to quinones followed by polymerization reactions [25,133-136].

8.3.5.1 HPLC analyses and elution order on C18

The two HPLC methods for fingerprint and quantitative analysis (see Table 8.2) only differ in the applied gradients. The procedure for quantitative analysis allows a better separation of procyanidin C1 from (-)-epicatechin. Over a period of three years, using different column batches and HPLC equipments, a variation of retention times up to one minute was noticed.

Procyanidins elute according to their degree of polymerization on silica [49,137]. However, the resolution of individual procyanidins using normal phase HPLC is difficult and has only been described for flavan-3-ol monomers and some dimers [138]. The analysis of individual procyanidins is therefore conducted using reversed-phase sorbents. Flavan-3-ols do not anymore elute according to their degree of polymerization from these sorbents. Table 8.3 gives an overview on the elution order of procyanidins from C18 columns. As can be seen, the elution order is largely unaffected by the choice of the organic or acid components. The most common elution order for the dimers and the most widespread trimers (C1 and C2) and tetramer (tetramer I) is: C2 → [B3 → B1 → (+)-catechin → B4] → B6 / B2 → B8 → [(-)-epicatechin → B7 → C1 → tetramer I] → A2 → B5. There are two areas (see parentheses), which are characterized by the elution of compounds exhibiting very similar elution features. The order of
Table 8.2  Characterization of compounds of the procyanidin containing fractions from *Crataegus* leaves and flowers.

<table>
<thead>
<tr>
<th>No.</th>
<th>Identity</th>
<th>t&lt;sub&gt;R&lt;/sub&gt; HPLC fingerprint</th>
<th>t&lt;sub&gt;R&lt;/sub&gt; HPLC quant.</th>
<th>Ratio&lt;sup&gt;c&lt;/sup&gt;</th>
<th>R&lt;sub&gt;f&lt;/sub&gt;-value TLC procyanidins&lt;sup&gt;b&lt;/sup&gt;</th>
<th>R&lt;sub&gt;f&lt;/sub&gt;-value TLC Lea&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Purity&lt;sup&gt;d&lt;/sup&gt; (%)</th>
<th>MW LC-MS&lt;sup&gt;b&lt;/sup&gt; (mass units)</th>
<th>HPLC-CRD Treutter&lt;sup&gt;e,f,i&lt;/sup&gt;</th>
<th>color</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>epicatechin catechin</td>
<td>18.1</td>
<td>19.1&lt;sup&gt;g&lt;/sup&gt;</td>
<td>5.7&lt;sup&gt;g&lt;/sup&gt;</td>
<td>0.87</td>
<td>0.71&lt;sup&gt;g&lt;/sup&gt;</td>
<td>n.d.</td>
<td>100</td>
<td>290</td>
<td>confirmed</td>
</tr>
<tr>
<td>2</td>
<td>catechin</td>
<td>10.3</td>
<td>10.5&lt;sup&gt;g&lt;/sup&gt;</td>
<td>5.6&lt;sup&gt;g&lt;/sup&gt;</td>
<td>n.d.</td>
<td>n.d.</td>
<td>-</td>
<td>290&lt;sup&gt;f&lt;/sup&gt;</td>
<td>confirmed</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>B2</td>
<td>12.6</td>
<td>12.9</td>
<td>7.3</td>
<td>0.64</td>
<td>0.46</td>
<td>97.8&lt;sup&gt;k&lt;/sup&gt;-99.5</td>
<td>578</td>
<td>confirmed</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>B4</td>
<td>9.2</td>
<td>9.6</td>
<td>7.0</td>
<td>0.65</td>
<td>0.46</td>
<td>98.8&lt;sup&gt;l&lt;/sup&gt;</td>
<td>578</td>
<td>positive</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>B5</td>
<td>34.5</td>
<td>39.0</td>
<td>7.4</td>
<td>0.74</td>
<td>0.54</td>
<td>98.2</td>
<td>578</td>
<td>positive</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>C1</td>
<td>17.3</td>
<td>17.4</td>
<td>8.0</td>
<td>0.43</td>
<td>0.32</td>
<td>98.5&lt;sup&gt;k&lt;/sup&gt;-100&lt;sup&gt;k&lt;/sup&gt;</td>
<td>866</td>
<td>confirmed</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>trimer I</td>
<td>9.9</td>
<td>10.2</td>
<td>7.7</td>
<td>0.47</td>
<td>0.32</td>
<td>76.7</td>
<td>866</td>
<td>positive</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>trimer II</td>
<td>39.7</td>
<td>42.7</td>
<td>8.1</td>
<td>0.51</td>
<td>0.35</td>
<td>85.1</td>
<td>866</td>
<td>positive</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>tetramer I</td>
<td>17.7</td>
<td>18.3</td>
<td>8.2</td>
<td>0.26</td>
<td>0.23</td>
<td>100</td>
<td>1154-1155</td>
<td>positive</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>artifact I</td>
<td>38.1</td>
<td>41.5</td>
<td>6.9</td>
<td>0.77</td>
<td>0.55</td>
<td>94.9</td>
<td>592</td>
<td>positive</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Pc A</td>
<td>18.2</td>
<td>19.5</td>
<td>6.0</td>
<td>0.67</td>
<td>not det.</td>
<td>65.0</td>
<td>not det.</td>
<td>n.d.</td>
<td>positive</td>
</tr>
<tr>
<td>8</td>
<td>Pc B</td>
<td>20.7</td>
<td>22.4</td>
<td>6.1</td>
<td>0.64</td>
<td>0.40</td>
<td>63.4-79.8</td>
<td>740-741</td>
<td>positive</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Pc C</td>
<td>23.9</td>
<td>28.5</td>
<td>7.2</td>
<td>0.43</td>
<td>0.29</td>
<td>42.3</td>
<td>1028-1029</td>
<td>negative</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>X&lt;sup&gt;f&lt;/sup&gt;</td>
<td>25.4</td>
<td>28.8</td>
<td>5.4</td>
<td>0.84</td>
<td>0.65</td>
<td>-</td>
<td>452-453</td>
<td>positive</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Y&lt;sup&gt;f&lt;/sup&gt;</td>
<td>27.0</td>
<td>30.1</td>
<td>n.d.</td>
<td>0.50-0.69</td>
<td>n.d.</td>
<td>-</td>
<td>740-741</td>
<td>positive</td>
<td></td>
</tr>
</tbody>
</table>

* Numbering according to elution order from Sephadex LH-20; <sup>b</sup> see section 8.3.2; <sup>c</sup> see section 4; <sup>d</sup> determined at 280 nm (area counts) using the HPLC fingerprint method, concentrations: 1-5 mg/ml; <sup>e</sup> chemical reaction detection using DMACA: confirmed = based on t<sub>R</sub> and ratio 640 nm / 280 nm, positive = no identification, but response at 640 nm, negative = no response at 640 nm; <sup>f</sup> measurements performed with purified fractions or extracts; <sup>i</sup> measurements performed with commercially available reference standards; <sup>k</sup> isolated by manual collection; <sup>l</sup> isolated from F85-98: 92.0 %; <sup>m</sup> isolated from F85-98: 93.9 %; <sup>o</sup> trivial nomenclature of procyanidins, see Abbreviations for procyanidins; n.d. = not determined; not det. = not detectable.
elution may therefore vary slightly. In the first group around (+)-catechin, procyanidin B1 may elute before B3 or after (+)-catechin. Procyanidin B4 sometimes is reported to elute before (+)-catechin. There is only one reference in which (+)-catechin elutes before procyanidin B1 [139], or procyanidin C2 elutes after procyanidins B3 and B1 [113], respectively. In the second group around (-)-epicatechin, C1 and the tetramer I may elute before the monomer, but the trimeric procyanidin always elutes before the tetramer. There is only one reference, in which procyanidin B7 is reported to elute after procyanidin C1 [118].

In the dimer series some general elution rules are obvious:

- Procyanidins composed of (+)-catechin units elute before procyanidins of the (-)-epicatechin series (B3 → B2; B6 → B5).
- Procyanidins exhibiting a terminal (+)-catechin unit elute earlier than procyanidins showing (-)-epicatechin terminal units (B1 → B2; B7 → B5).
- Procyanidins with 4→8 interflavanoid linkages elute earlier than procyanidins with 4→6 linkages (B1 → B7; B2 → B5; B3 → B6; B4 → B8).
- The elution features of A-type procyanidins are similar to the one of the corresponding 4→6 linked procyanidins (A2/B5).

The establishment of elution rules for trimers is more difficult, because there does not yet exist many data. But, analogous tendencies can be seen: procyanidins composed of (+)-catechin units elute before procyanidins composed of (-)-epicatechin units (C2→C1). In mixed procyanidins, compounds with a terminal (+)-catechin unit elute before corresponding compounds with a terminal (-)-epicatechin unit. The elution order of corresponding procyanidins with respect to the interflavanoid linkages is: 4→8 — 4→8 before 4→6 — 4→8 before 4→8 — 4→6 before 4→6 — 4→6 [113,140-141].

The elution order of the procyanidins determined with the HPLC methods for quantitative or fingerprint analysis is in agreement with the literature data. In the methods presented, procyanidin B4 elutes before (+)-catechin, while procyanidin C1 as well as tetramer I elute before (-)-epicatechin.
<table>
<thead>
<tr>
<th>Eluent components</th>
<th>Elution order</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>MeOH / H2O / H3PO4</td>
<td>B3→B1→B4→cat→B2→trimer(^{a})→tetramer(^{a})→epi</td>
<td>130, 167, 168</td>
</tr>
<tr>
<td>MeOH / H2O / H3PO4</td>
<td>B1→cat→B2→C1→tetramer(^{a})→epi</td>
<td>169</td>
</tr>
<tr>
<td>MeOH / H2O / H3PO4</td>
<td>cat→B1→B2→epi</td>
<td>139</td>
</tr>
<tr>
<td>MeOH / H2O / HCO3H(^{b})</td>
<td>B3→B1→C2→B4→triV→B2→triVI→B7→C1→tetral→triI→B5→triII</td>
<td>113</td>
</tr>
<tr>
<td>MeOH / H2O / HCO3H</td>
<td>B3/C2→B1→cat→B2→epi→B7→C1→A2→B5→triII</td>
<td>126</td>
</tr>
<tr>
<td>MeOH / H2O / HCl</td>
<td>C2→B3→B1→B4→cat→B2→epi→A2→B5</td>
<td>170</td>
</tr>
<tr>
<td>MeOH / H2O / HClO4</td>
<td>cat→B2→trimer(^{a})→epi→C1→tetramer(^{a})</td>
<td>171</td>
</tr>
<tr>
<td>MeOH / H2O / HClO4</td>
<td>cat→B1→B2→epi→C1</td>
<td>172</td>
</tr>
<tr>
<td>MeOH / H2O / AcOH</td>
<td>B3→cat→B4→B2→epi→A2</td>
<td>173</td>
</tr>
<tr>
<td>MeOH / H2O / AcOH</td>
<td>B3→B1→cat→B4→B2→epi</td>
<td>174</td>
</tr>
<tr>
<td>ACN / H2O / H3PO4</td>
<td>B1→B3→cat→B4→B2→epi→C1</td>
<td>175</td>
</tr>
<tr>
<td>ACN / H2O / H3PO4(^{b})</td>
<td>B3→cat→B4→epi</td>
<td>176</td>
</tr>
<tr>
<td>ACN / H2O / HCO3H</td>
<td>C2→B3→B1→cat→triIII→B4→triIV→triV→B6→triI→B2→epi→triVI→C1→B7→triVII→B5</td>
<td>140, 141</td>
</tr>
<tr>
<td>ACN / H2O / HCO3H</td>
<td>B1→B3→B4→B2→B6→B8→B7→B5→A2</td>
<td>177</td>
</tr>
<tr>
<td>ACN / H2O / AcOH</td>
<td>B3→cat→B1→triIII→B8→B4→triV→B6→triI→B2→epi→triVI→C1→B7→triVII→B5</td>
<td>118</td>
</tr>
<tr>
<td>ACN / H2O / AcOH</td>
<td>B3→B1→triIII→cat→triI→B2→C1→tetramer(^{a})→B5</td>
<td>178</td>
</tr>
<tr>
<td>ACN / H2O / AcOH</td>
<td>B1→B3→B4→B2→B6→B8→C1→B7→B5</td>
<td>179</td>
</tr>
<tr>
<td>ACN / H2O / AcOH</td>
<td>B1→B3→cat→B4→B2→epi</td>
<td>180</td>
</tr>
<tr>
<td>ACN / H2O / AcOH</td>
<td>B3→B1→cat→B2→epi</td>
<td>181</td>
</tr>
<tr>
<td>ACN / H2O / AcOH</td>
<td>B3→B1→cat→B2→epi</td>
<td>182</td>
</tr>
<tr>
<td>ACN / H2O / AcOH</td>
<td>cat→B2→epi→B5</td>
<td>183</td>
</tr>
<tr>
<td>ACN / H2O</td>
<td>B1→cat→trimer(^{a})→trimer(^{a})→B2→epi→C1→B5</td>
<td>165</td>
</tr>
<tr>
<td>ACN / H2O</td>
<td>B1→cat→B4→B2→epi</td>
<td>184</td>
</tr>
<tr>
<td>ACN / H2O</td>
<td>B3→cat→B2→epi→C1</td>
<td>185, 186</td>
</tr>
<tr>
<td>H2O / AcOH</td>
<td>B3→cat→B1→B4→B2→epi→trimer(^{a})→tetramer(^{a})</td>
<td>187-189</td>
</tr>
<tr>
<td>H2O / AcOH</td>
<td>B3→B1→cat→B4→B2→epi→C1</td>
<td>104</td>
</tr>
<tr>
<td>H2O / AcOH</td>
<td>B3→B1→trimer(^{a})→B4→B2→C1</td>
<td>190, 191</td>
</tr>
</tbody>
</table>

Galloylated and enantiomeric flavan-3-ols are not included. Nomenclature for procyanidins: dimers and trimers: see Abbreviations for procyanidins, epi = (-)-epicatechin; cat = (+)-catechin; triI = epi-(4β→6)-epi-(4β→6)-epi; triII = epi-(4β→6)-epi-(4β→8)-epi-(4β→6)-cat; triIII = cat-(4α→8)-cat-(4α→8)-epi; triIV = epi-(4β→6)-epi-(4β→8)-cat; triV = epi-(4β→6)-cat; triVI = epi-(4β→8)-cat; triVII = epi-(4β→8)-epi-(4β→8)-epi-(4β→8)-epi-(4β→8). Abbreviations for solvents: MeOH = methanol, H2O = water, ACN = acetonitrile, H3PO4 = orthophosphoric acid, HCO3H = formic acid, HCl = hydrochloric acid, HClO4 = perchloric acid, AcOH = acetic acid. \(^{a}\) not specified; \(^{b}\) ammonium buffer added.
The two crowded areas in the chromatograms around (+)-catechin and (-)-epicatechin are well resolved using MS detection. Analysis of extracts suggest that even further isomeric and higher molecular weight procyanidins elute in these areas of the chromatograms (see section 5.3.3).

8.3.5.2 TLC analyses
Lea's TLC method is well established for qualitative analysis [124,142]. The degree of polymerization of procyanidins up to the heptameric level can be determined from the $R_f$-values, which decrease with increasing molecular weight [50]. There is also a tendency of $4\rightarrow6$ linked procyanidins to exhibit higher $R_f$-values than corresponding procyanidins with $4\rightarrow8$ interflavanoid linkages. The same information can be obtained using the procyanidin TLC system (see Table 8.2). The latter system is found to be superior over Lea's method, because less poisonous solvents are used and $R_f$-values are higher. Table 8.4 gives an overview on the $R_f$-values with respect to the degree of polymerization of the procyanidins.

Table 8.4 $R_f$-values of procyanidins of different degrees of polymerization.

<table>
<thead>
<tr>
<th></th>
<th>Procyanidin TLC</th>
<th>Lea TLC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$R_f$-values$^a$</td>
<td></td>
</tr>
<tr>
<td>monomers</td>
<td>0.87</td>
<td>0.71</td>
</tr>
<tr>
<td>dimers</td>
<td>0.64 - 0.65</td>
<td>0.46</td>
</tr>
<tr>
<td>(B5: 0.74)</td>
<td></td>
<td>(B5: 0.54)</td>
</tr>
<tr>
<td>trimers</td>
<td>0.43 - 0.51</td>
<td>0.23</td>
</tr>
<tr>
<td>tetramers</td>
<td>0.26</td>
<td>0.19</td>
</tr>
</tbody>
</table>

$^a$ Experimental values, see Table 8.2.

The detection limit for (-)-epicatechin using different detection solvents was determined on silica TLC plates. The DMACA (1 % 4-dimethylaminocinnamaldehyde in 8 % methanolic sulphuric acid) and the VPA (1 % vanillin in 50 % aqueous ortho-phosphoric acid) reagents showed detection limits of 30 µg/ml. Sulphuric acid 50 % and EBS (0.5 % Echtblausalz (= diazotized benzeneanilide stain) in water) were equally sensitive, but are not specific for flavan-3-ols. Vanillin reagents prepared with
concentrated hydrochloric acid or 5 % ethanolic sulphuric acid were less sensitive (detection limits of approximately 60 µg/ml). The FeCl₃ reagent (10 % in water) showed the highest detection limit (120 µg/ml). The DMACA reagent was finally favored over the VPA reagent, because this reagent can be stored at 4 °C and chromatograms can be conserved under a self-adhesive foil (due to a lower concentration of acid).

8.3.5.3 Discussion on individual compounds
8.3.5.3.1 Monomers, dimers and trimers

(-)-Epicatechin is the predominating monomeric flavan-3-ol. (+)-Catechin was identified in the ethyl acetate extract using LC-MS and in purified fractions (F223-243) using HPLC-CRD. It was not detected by LC-MS analysis in freshly prepared samples of Crataegus leaves and flowers, obtained following the procedure described in section 2.1.2.1. This could be due to concentrations, which are below the detection limit. However, another possibility would be, that (+)-catechin is generated in older samples by epimerization of (-)-epicatechin. Epimerization occurs to a much greater extent with (-)-epicatechin than with (+)-catechin. Under acidic conditions conversions of 15 % to 17.5 % for (-)-epicatechin and 4 % to 4.5 % for (+)-catechin have been reported [49,143]. Epimerization also takes place in neutral or alkaline solutions [97]. Again, (-)-epicatechin shows much higher rates of conversion than (+)-catechin [144]. LC-MS analyses of methanolic solutions of (-)-epicatechin and (+)-catechin, which were stored at 4 °C for 19 and 15 months, respectively, yielded analogous results: (+)-Catechin was detected in the (-)-epicatechin solution (peak area corresponding to approximately 5 % of the one of (-)-epicatechin), while no (-)-epicatechin could be detected in the (+)-catechin solution.

However, there is strong evidence that (+)-catechin has not been formed exclusively by epimerization, since procyanidin B4 is detected in considerable concentrations also in freshly prepared samples. If dimers were only formed thermodynamically, other dimers showing (+)-catechin units would have to be detected, too. But no other (+)-catechin containing procyanidins except of procyanidin B4 have been found.
The identity of each dimer was established by NMR, MS and CD (circular dichroism) measurements (see section 10). Identification in additional fractions was based on elution features (Sephadex LH-20 column) and on comparison of retention times, UV ratios (220 nm / 280 nm), Rf-values and MS data from LC-MS analyses.

The identity of procyanidin C1 containing fractions was established in the same way as for the dimers. Procyanidin C1 and trimer I could be detected in freshly prepared samples by LC-MS (see section 5.3.3). Trimer II was isolated and gave a positive reaction in fractions from the first Sephadex LH-20 column using HPLC-CRD, but it was not detected in freshly prepared samples of Crataegus leaves and flowers with LC-MS. Like with (+)-catechin, the lack of this trimer could be due to the low concentration of this procyanidin in freshly prepared extracts. The late elution from the first Sephadex LH-20 column (slightly later than procyanidin B5) suggests that this trimer is genuinely present in Crataegus. But, it might be a degradation product of a higher molecular weight procyanidin, which would be expected to elute in late fractions from the Sephadex LH-20 column, too.

8.3.5.3.2 Artifact I
Artifact I was isolated and was detected in ethyl acetate extracts, fractions from Sephadex LH-20 columns and older sample solutions of (-)-epicatechin and (+)-catechin. It was not detected in freshly prepared samples of Crataegus leaves and flowers by LC-MS measurements. These data imply that this compound is an artifact. The molecular ion (m/z 591) suggests a methylene-bridged dimeric procyanidin, which could be confirmed by NMR and MS measurements (see section 10.4.9).

Another compound showing a molecular ion at m/z 591 (negative ionization mode) was detected by LC-MS in older sample solutions of procyanidin B2 and (+)-catechin. This second compound elutes much earlier and is therefore likely to be a structural isomer to artifact I. This hypothesis, however, would have to be proven unambiguously by additional experiments.
Compounds Pc A, Pc B and Pc C

Compounds Pc A, Pc B and Pc C have been isolated and show typical procyanidin UV spectra. Identification of Pc B in the different fractions was based on elution features from the first Sephadex LH-20 column and on comparison of retention times, UV ratios (220 nm / 280 nm), Rf-values and MS data from LC-MS analyses.

The low amounts and purities of Pc A and Pc C did not allow successful NMR measurements. Only the ^1H-NMR spectrum of free Pc B (isolated from F40-64) and ^1H-NMR and DQF-COSY spectra of acetylated Pc B could be recorded, but structure elucidation was not successful due to the lack of possible reference spectra.

Because of very low concentrations, no molecular weight information using LC-MS could be obtained for Pc A. The Rf-value in the procyanidin TLC system suggests a compound of low molecular weight (monomeric to dimeric). Since it is red-colored Pc A is considered as a degradation product. In any case, Pc A is a minor compound in Crataegus leaves and flowers, because it could only be isolated in very low quantities (< 1 mg).

Pc B is relatively unstable, because considerable degradation takes place during storage (over about two years). Rf-values suggest a dimeric compound, while the UV ratio 220 nm / 280 nm (6.1) is unusually low for a dimeric compound. LC-MS analysis reveals a molecular weight of 741, which is not attributable to a common procyanidin.

Rf-values for Pc C suggest a trimeric compound, while the UV ratio 220 / 280 nm (7.2) is unusually low for a trimeric compound. The molecular weight of 1028-1029 (determined by LC-MS) is again not characteristic for a common procyanidin. The lack of a response in the HPLC-CRD measurements is possibly due to a slow reaction rate of this compound. Variations in responses in HPLC-CRD due to structural differences of flavan-3-ols are well-known [126]. The positive response obtained in TLC analyses is explicable by the longer reaction times and more severe conditions that were applied compared to the HPLC-CRD procedure.

Compounds X and Y

Compound Y has not been isolated, while a first preparative chromatography of F7-12, containing compound X, was unsuccessful due to interferences from a phenolic acid derivative. HPLC chromatograms of fractions from the first column chromatography
over Sephadex LH-20 however show, that both compounds are present in significant amounts in the ethyl acetate extracts (see Figures 8.3A to 8.3D). Furthermore, compound X elutes in fractions, which would be expected to contain procyanidin B1. This latter procyanidin has been reported to be present in Crataegus by some authors (see also section 7) [8,74-75].

Rf-values and UV ratio 220 nm / 280 nm for compound X suggest a monomeric compound. The retention time in the fingerprint HPLC analysis (25 min) is between (-)-epicatechin and procyanidin B5. From the retention behavior of procyanidins on C18 columns (see section 8.3.5.1) procyanidin B1 would be expected to elute before procyanidin B4 (that is to say: retention time < 9.2 min under the conditions of the fingerprint HPLC). Moreover, LC-MS analysis provides a molecular weight of 452-453. All these data give enough evidence to be able to conclude that compound X is not procyanidin B1.

Compound Y shows analogous features like Pc B, but retention times in HPLC analyses are longer. Therefore, compound Y is possibly a structural isomer to Pc B.

8.3.5.3.5 Hypotheses on the nature of Pc B, Pc C, compounds X and Y

Pc B, Pc C, compound X as well as compound Y show typical (-)-epicatechin UV spectra, which implies that the flavan-3-ol chromophore still is present. However, the molecular weights of 452-453, 741 and 1028-1029, determined by LC-MS analyses, are untypical for procyanidins.

There are two hypotheses which may explain the nature of Pc B, Pc C, compound X and compound Y. The first one is summarized in Figure 8.4, taking compound X as an example. Chlorogenic acid, which is present in extracts of Crataegus, is oxidized by polyphenol oxidase (PPO) or by autoxidation to chlorogenic acid ortho-quinone. The quinone is reduced to chlorogenic acid via coupled oxidation involving water and thus generating hydrogen peroxide. This type of redox reaction has been demonstrated to occur in the enzymic oxidation of model solutions of caftaric acid, a phenolic acid contained in grapes [145]. The in situ generated hydrogen peroxide is a strong oxidant, which has been reported to be able to convert ethanol to acetaldehyde [94]. Methanol, which has been used as a solvent during the isolation procedure, can be oxidized to formaldehyde in an analogous way. Protonation of the aldehyde in acidic medium,
Figure 8.4 Hypothesis on the nature of Pc B, Pc C, compounds X and Y. Formation of a methylene bridge followed by retro-Diels-Alder fission. Example: compound X.
followed by an electrophilic attack leads to a methylol-substituted flavan-3-ol. This methanol adduct then looses a water molecule to give a new carbocation which can in turn crosslink to a second flavan-3-ol unit. Such a reaction sequence has already been described for the interaction of acetaldehyde with flavan-3-ols [146,147]. Tanaka et al. [148] even postulated that the loss of astringency in fruits is a result of such condensation reactions of proanthocyanidins with endogenous acetaldehyde formed from ethanol or by decarboxylation of pyruvic acid. Crosslinkage of flavan-3-ols via ethylene bridges is also of significant practical importance in the aging of wines [147]. A prerequisite for condensation is that the aldehyde is in its cationic form. Although (+)-catechin is reported to react with formaldehyde already at mild pH (pH 7) and ambient temperature conditions [36,149], the reaction rate is accelerated at more acidic pH [149,150]. Kiatgrajai et al. [151] reported condensation reactions between (+)-catechin and formaldehyde in model solutions also at alkaline pH. Since solutions of procyanidins show a pH value of 3-4 [25], reaction of flavan-3-ols with formaldehyde under the conditions applied during the isolation are considered to be possible.

In phenols, positions located in ortho and para position to the hydroxy groups are activated. Therefore, carbons 6 and 8 of the A-ring and, to a lesser extent, carbons 2', 5' and 6' of the B-ring are possible sites for a linkage. The methylene bridge must be located at one B-ring in order to enable retro-Diels-Alder cleavage and subsequent loss of the mass unit 138, if the structural hypothesis for compound X is correct. In an analogous reaction sequence the 2'-methylene-carbocation may react with a dimeric or trimeric procyanidin, followed by RDA-F (retro-Diels-Alder fission, see section 5, Figure 5.1) to yield molecules of masses 742 and 1030, respectively.

Facts opposing this first hypothesis are, that RDA-F has not been detected with regular procyanidins and no intermediates showing methylene linkages between the B-ring of one flavan-3-ol unit and the A-ring of another unit have been isolated. In the literature only linkages between the two A-rings have been reported so far for ethylene bridged [146] or methylene bridged [149,151] flavan-3-ols, too. Linkages between the A-ring and the B-ring have only been reported after oxidative coupling of (+)-catechin [152,153].
The second hypothesis for the occurrence of flavan-3-ols with unusual molecular weights is the presence of glycosides. Figure 8.5 shows examples of possible structural formulae for the unknown flavan-3-ols (assuming the occurrence of the most common procyanidin-3-O-glucopyranosides). Although glycosides of procyanidins have been found very rarely in nature, three species exhibiting a similar procyanidin pattern like *Crataegus* are known to contain them [38,40,59] (see section 6.1). Furthermore, *Crataegi* leaves and flowers have been lately reported to contain high amounts of polysaccharides, comparable to concentrations found in typical mucilage herbs [192]. D-glucose has also been detected after acid-catalyzed degradation of a hexameric procyanidin isolated from *Crataegus* sp. $^{13}$C-NMR data of the procyanidin oligomer were consistent with the presence of pyranose sugar residues, too [61].

Further proofs would be necessary to corroborate the presence of glycosides or artifacts like the ones described in the first hypothesis. Since measured masses in LC-MS analyses may vary by as much as ± 1 mass unit and measurements become even less reliable with higher masses, interpretation of mass spectra without additional structure elucidation by other methods can not be more than speculations. However, compounds following one of the two algorithms described above seem to be present in *Crataegus* after all, because LC-MS analysis of freshly prepared extracts of leaves and flowers as well as flowers revealed the presence of a total of four compounds showing molecular weights of 741-742, while in extracts of fruits three of these compounds could be detected.

### 8.3.5.3.6 Higher molecular weight procyanidins

Higher molecular weight procyanidins are expected to elute in late fractions from Sephadex LH-20. The HPLC chromatograms of F387-423 of the first column chromatography over Sephadex LH-20 show some discrete peaks at 10.8, 17.0, 19.4 and 20.7 min (see Figure 8.3D). R$_f$-values of DMACA active compounds of these fractions were below 0.38 in the procyanidin TLC system. The characterization of the compounds by LC-MS analysis was, however, not successful even after a second fractionation over Sephadex LH-20.
Figure 8.5  Hypothesis on the nature of Pc B, Pc C, compounds X and Y. Occurrence of procyanidin-3-O-glucopyranosides.
Since higher molecular weight procyanidins are scarcely extracted into ethyl acetate, the n-butanol extract (see Figure 8.1) was also analyzed by LC-MS. Numerous tetrameric up to hexameric peaks could be detected, but also high concentrations of (-)-epicatechin, dimeric and trimeric procyanidins, which indicates that considerable degradation must have occurred during storage (approximately three years at -20 °C). Peaks of ions implying the presence of tetrameric, pentameric and hexameric procyanidins were mainly detectable in the elution range of (+)-catechin (around 10 min), (-)-epicatechin (around 18 min) and between 25 to 29 min (HPLC fingerprint method). These results stand in line with the ones obtained for freshly prepared samples of Crataegus leaves and flowers (see section 5). However, unambiguous proof of the presence of such higher molecular weight procyanidins would have to be made by LC-MS-MS analyses.
9 Literature overview on the structure elucidation of procyanidins

The structure elucidation of procyanidins includes the determination of the following features:

- The stereochemistry at C2 and C3 of all units (i.e. identification of (+)-catechin or (-)-epicatechin units, respectively) and the sequential order of the different units.
- The bonding positions (i.e. 4 → 8 or 4 → 6) and the type of interflavanoid linkage (i.e. B-type linkages or occurrence of A-type ether linkages, respectively).
- The stereochemistry at C4 of all extension units (i.e. 3,4-cis or 3,4-trans).
- The degree of polymerization, and
- The presence of esters of gallic acid or other derivatives.

Traditionally, the structure elucidation of procyanidins is based on a combination of spectroscopic data (NMR, optical rotation, CD) and chemical degradation studies (i.e. thiolysis). The degree of polymerization of low molecular weight procyanidins is determined by mass spectrometry (preferably FAB-MS). The FAB mass spectra are characterized by the sequential loss of the constituent flavan-3-ol units and by fragments resulting from retro-Diels-Alder fission (RDA-F) of the heterocyclic rings (see section 5.3.1). The presence of doubly linked A-type procyanidins can readily be seen from the molecular ions, too [193]. Procyanidin gallates are most conveniently detected after enzymatic cleavage with tannase [118,128,138]. In the following sections an overview on the different aspects of the structure elucidation of procyanidins is given.

9.1 Spectroscopic methods

9.1.1 Nuclear magnetic resonance

Both, $^1$H- and $^{13}$C-NMR spectroscopy are useful tools for elucidating the structure of procyanidins. There are two characteristic regions that can be distinguished in the NMR spectra:
- the signals of the heterocyclic ring (C-ring) at $\delta = 2.6 - 5.5 \, (^1\text{H-NMR})$ and at $\delta = 25 - 85 \, (^{13}\text{C-NMR})$, respectively, and
- the signals of the phloroglucinol and catechol rings (A- and B-rings) at $\delta = 6.0 - 7.8 \, (^1\text{H-NMR})$ and at $\delta = 96 - 160 \, (^{13}\text{C-NMR})$, respectively [194].

The interpretation of the spectra is often severely complicated due to resonance multiplicity and broadening effects caused by overlapping signals. Furthermore, rotational isomerism due to hindered rotation about the interflavanoid linkages (atropisomerism) as well as conformational isomerism due to different geometries of the heterocyclic rings occur [194,195]. For this reason a complete and unambiguous assignment of all signals of phenolic procyanidins using two-dimensional NMR experiments has so far only been successful for dimeric procyanidins using 400 and 600 MHz apparatuses [166,196]. De Bruyne [197] discriminated between the almost equivalent chemical shifts of protons and carbons at positions 6 and 8 by the addition of traces of cadmium nitrate. This causes the separation of the broad phenolic signals into sharp singlets from which long-range heteronuclear correlations lead to the assignment of the 6 and 8 signals.

Since proton resonances are often considerably broadened in phenolic procyanidins due to proton exchange processes, most NMR studies are performed on their derivatives. For some peracetylated procyanidin dimers and trimer C2 [198,199] as well as for another peracetylated doubly-linked trimer [200] a complete assignment of proton and carbon resonances was achieved using two-dimensional NMR techniques. The signals of the A- and C-ring protons of the peracetylated trimer C1 have also been successfully assigned with $^1\text{H-}^1\text{H COSY}$ experiments [101]. The structure elucidation of another peracetylated tetrameric proanthocyanidin that is described in the literature was not only based on two-dimensional NMR but also on comparison with similar compounds of which the structures were derived from chemical degradation studies [201]. The structures of the vast majority of new pro(antho)cyanidins is still established by such combinations of NMR data and degradation studies (i.e. [23,101,166]).
9.1.1.1 Atropisomerism and conformational isomerism

Rotational isomerism occurs due to hindered rotation about the interflavan linkages. In dimers, an extended and a compact rotamer can be distinguished (see Figure 9.1, upper). The population of the two rotamers is temperature [202,203] and solvent-dependent. For procyanidin B4, for example, the more extended rotamer is preferred over the more compact rotamer in organic solvents, but in water the compact rotamer dominates [204]. The addition of water to organic solvents for NMR measurements has sometimes been used to simplify spectra via suppression of one of the two rotamers.

The problem of atropisomerism is commonly more pronounced in NMR spectra of procyanidin derivatives because of the bulky substituents. For dimeric peracetates duplication of signals generally occurs while for dimeric methyl ether acetates broadening of the signals can be observed at 30 °C. At higher temperatures spectra of methyl ether acetates become first order due to fast interconversion of the two rotational isomers with respect to the NMR time scale [194].

The relative rotamer populations of phenolic procyanidins as well as their peracetates have been studied with time resolved fluorescence spectroscopy. Using this technology rotamer populations can be measured even when they cannot be resolved by 1H-NMR spectroscopy. It could be demonstrated that the populations of the two rotamers were in the same order in the phenolic procyanidins and in the peracetates. The reduction of the energy barrier between the rotational isomers of the free phenolic forms in comparison to the peracetates causes interconversion of the isomers to become fast on the NMR time scale resulting in first order spectra (at room temperature; rotamer signals can however also be detected in phenolic procyanidins, if spectra are recorded at 0 °C) [205]. Molecular mechanics calculations (MM2) on procyanidin dimers also revealed that the differences in energies of the rotational minima are sufficiently small that both rotamers may be present in significant amounts [206]. Other studies came to the conclusion that some dimers may have an exclusive preference for only one rotamer [26].

Conformational isomerism is associated with the geometry of the heterocyclic rings [195]. In principle, two conformations can be distinguished: the E-conformer, in which the B-ring occupies an equatorial orientation and the A-conformer in which the B-ring adopts an axial orientation (see Figure 9.1, lower) [207]. X-ray studies and molecular
Figure 9.1  Atropisomerism and conformational isomerism.
Bottom: Conformations of the pyran heterocycle. Example: (+)-catechin.
Modified from [204,209].
mechanics calculations (MM2) on (-)-epicatechin [208] and derivatives of (+)-catechin confirmed the predominance of the E-conformer in the monomers [207] (E:A ratio (+)-catechin: 62:38, E:A ratio (-)-epicatechin: 86:14 [209]). The addition of an α-oriented phenyl group to C4 of (+)-catechin, however, favors the adoption of a sofa conformation (A-conformer) while the addition of a β-oriented phenyl group to C4 of (-)-epicatechin still favors the half-chair conformation (E-conformer) [207]. The energy barriers to rotation about the interflavanoid linkage is considerably higher in the A- than in the E-conformer [195]. This is reflected in the appearance of the $^1$H-NMR spectra: dimeric procyanidins with (-)-epicatechin upper units (i.e. B2 and B1) give broad but first order spectra in acetone-d$_6$ at room temperature (coalescence of rotamer signals), while dimeric procyanidins with (+)-catechin upper units (i.e. B3 and B4) show two sets of signals attributable to the two rotamers which are in slow exchange [8, 203]. The dependence of the heterocyclic ring conformation in dimeric procyanidins on the absolute configuration at C4 could also be confirmed by molecular mechanics calculations (MM2) [210].

9.1.1.2 Structure elucidation based on comparative NMR studies
Traditionally the structure elucidation of procyanidins using NMR data is based on diagnostic shift parameters derived from studies on monomers and dimers as well as their derivatives. However, such analogies remain critical in view of the known occurrence of rotational isomers, temperature and solvent effects as well as the mostly very small differences in the characteristic chemical shifts. Some of the most widely encountered chemical shift criteria for the structure elucidation of procyanidins are discussed in the following sections together with some other structural clues.

Stereochemistry at C2 and C3
The relative stereochemistry at C2 and C3 is usually deduced from the coupling constants, which give information on the dihedral angle between the remaining vicinal protons through Karplus-type equations [211]. A large doublet (8 - 10 Hz) indicates a (+)-catechin unit while a broad singlet reveals the presence of an (-)-epicatechin unit [8, 21].
However, this rule has to be cautiously applied to oligomeric procyanidins, since studies on trimer C2 indicated that the coupling constant $J_{2,3}$ of a (+)-catechin unit can also be dramatically reduced due to conformational changes of the heterocyclic ring [198]. Enantiomers (i.e. (-)-catechin or (+)-epicatechin) seem not to obey this multiplicity rule either [47].

In $^{13}$C-NMR spectra (recorded in acetone-$d_6$ : water = 1:1) the chemical shift of C2 was found to be dependent on its orientation relative to the aryl substituent at C4. The C2 resonates at higher field when an axial group is placed at C4 than when an equatorial group is at this position. This 1,3 interaction is known as "γ-effect". Resonances near 83.7 ppm are therefore characteristic of units with 2,3-trans stereochemistry ((+)-catechin) and resonances near 76.5 ppm are characteristic of units with 2,3-cis stereochemistry ((-)-epicatechin) [194,203].

**Stereochemistry at C4**

The relative 3,4 stereochemistry can be deduced from the coupling constant of the proton at position 4 of the upper unit. If this proton resonates as a large doublet ($J = 7 - 10$ Hz) a trans stereochemistry can be postulated. However, in many cases the H4 signal represents a broad singlet from which no conclusions on the stereochemistry can be made (i.e. [8]).

In $^{13}$C-NMR spectra (in acetone-$d_6$ : water = 1:1) introduction of an aryl group at C4 results in a deshielding of C4 by 4.0 to 12.2 ppm. Generally the shift caused by equatorial substituents is greater than that of an axial aryl group [203].

Since the 2,3-trans-3,4-trans and 2,3-cis-3,4-trans stereochemistries are virtually ubiquitous for proanthocyanidins [207], the stereochemistry at C4 is generally found to be defined as soon as the stereochemistries at C2 and C3 are established. However, the isolation of 2,3-trans-3,4-cis procyanidins in the course of studies on the synthesis of procyanidins showed that such conclusions on the stereochemistry at C4 are not valid in any case [212,213]. The natural co-occurrence of 3,4-cis isomers in very low concentrations together with the 3,4-trans isomers has been first postulated [214] and has also been demonstrated later [215].
Determination of the type of linkage

Studies on a range of synthetic C6- and C8-substituted (+)-catechin derivatives led to the definition of absolute values for the chemical shifts of the residual A-ring protons. The H8 protons of 4 → 6 linked derivatives were found to resonate at lower field (δ = 6.30 - 6.47 [216] and around δ 6.6 [27] in chloroform-d4) than the H6 protons of 4 → 8 linked derivatives (δ = 6.07 - 6.24 in chloroform-d4) [216]. This phenomenon can be explained by the larger negative inductive effect of the cyclic ether bond at C8a in comparison to the analogous effect of the hydroxy group at C5 [217]. The validity of these characteristic shift parameters has also been extended to two trimers and a tetramer [101]. Although this rule is still widely used to determine the location of the interflavanoid linkage, its value has also been questioned because shift differences are small and steric effects are not considered [196,218]. Kolodziej [215], analyzing peracetates and methyl ether acetates of procyanidins B1-B8, indicated that this characteristic shielding effect is mainly restricted to the upper units showing 2,3-cis-3,4-trans configuration.

The proton at position 2 of the heterocycle was found to resonate between 4.37 and 5.01 ppm in 4 → 8 linked peracetylated dimers, whereas in 4 → 6 linked peracetylated dimers it resonates between 5.04 and 5.35 ppm [213,219]. This rule was again shown to be valid also for two trimeric and a tetrameric peracetylated procyanidin [101]. Other diagnostic shift parameters based on proton or carbon resonances at positions 2 or 4 of the heterocycles have been described in the literature (i.e. [27,219,220]), but these criteria have not been used very widely in the structure elucidation of procyanidins.

Rotamer populations in 1H-NMR spectra of dimeric peracetates and methyl ether acetates have also been used to distinguish among 4 → 8 and 4 → 6 linkages. In the latter almost equal proportions of the two rotamers are observed, while in the 4 → 8 linked procyanidins population of the two states differ substantially [27,221]. This rule is however only applicable to a narrow range of compounds and the experimental conditions have to be strictly obeyed [222]. Hör et al. [101] found an interesting feature of the catechol-ring proton resonances of minor rotamers in two peracetylated trimers and a tetramer. The chemical shifts of the E-ring protons were found to be dependent on the type of linkage. In oligomers which show a 4 → 8 linkage between the top unit and the second upper unit, the E-ring protons resonated between δ 6.67 and 6.99, while in
4 → 6 linked oligomers the E-ring protons resonated between δ 7.04 and 7.51 like all other catechol-ring protons. However, assignment of these resonances is difficult due to the generally poor resolution in this section of the spectra.

nOe difference spectroscopy has been used to distinguish between 4 → 8 and 4 → 6 linkages in methylated derivatives of procyanidins [223]. De Bruyne [193] could only apply nOe enhancement experiments successfully to native monomers (irradiation of 5-OH enhanced H6, while irradiation of 7-OH enhanced both H6 and H8). In phenolic dimers interpretation was hampered by the spectral complexity and therefore only two-dimensional NMR analysis yielded unambiguous assignments.

A-type procyanidins are recognizable in 1H-NMR spectra (recorded in acetone-ð6) from the characteristic AB-doublet (J = 3 - 4 Hz) of H4 of the upper unit [197,224]. In the DEPT 135 and 13C-NMR spectra (in acetone-ð6) A-type procyanidins only show one CH signal around 80 ppm, attributable to C2 of the lower unit. Furthermore, there is one additional quaternary carbon at 100 ppm due to the ketal function at C2 of the upper unit [197].

9.1.1.3 Derivatization for structure elucidation

Procyanidins are relatively unstable molecules and it is therefore often an advantage to stabilize them by suitable derivatization prior to spectroscopic studies. Furthermore, 1H-NMR spectra of phenolic procyanidins often show considerable broadening of the signals due to the exchange of rotational isomers. This behavior considerably hampers the interpretation particularly of two-dimensional spectra. The acetate and methyl ether acetate derivatives are mainly used for NMR analyses.

Acetylation in pyridine using acetic anhydride as reagent is the most widely used derivatization procedure for procyanidins. Losses are generally low, because no side reactions occur and reactions are nearly quantitative [75]. The bulky acetate groups cause the spectra of peracetates to display the effects of rotational isomerism. Since the population of each isomer is normally unequal, rotamer signals can easily be detected in the 13C-NMR spectra, if duplication of the signals occurs [219]. If the temperature is raised (170 °C in DMSO-ð6), signals are still broad, which indicates that no "fast" rotation about the interflavanoid linkages can be induced [65].
Balas et al. [198,218] unequivocally assigned all resonances of the $^1$H- and $^{13}$C-NMR spectra of some procyanidin dimers and trimer C2 by a combination of two-dimensional NMR techniques using a 500 MHz apparatus. The site of the interflavanoid linkage could be established in HMBC spectra by long range correlations of H4 of the upper unit with C8a of a lower unit (indicative for a 4 $\rightarrow$ 8 linkage) or with C5 of a lower unit (indicative of a 4 $\rightarrow$ 6 linkage), respectively.

Kolodziej [215,222] extensively studied $^1$H-NMR spectra of methyl ether acetates of procyanidin dimers and some trimers. To establish the bonding position he defined characteristic chemical shift parameters, based on remaining D-ring protons and resonances of C3-acetoxy protons and protons at position 2. A comparison of these relative shift differences with the ones obtained with the peracetate derivatives revealed that the shift differences are much more pronounced with the methyl ether acetates. The degree of polymerization can be derived from the number of 3-acetoxy proton resonances [225].

Although $^1$H-NMR spectral interpretation of procyanidins seems to be less equivocal with methyl ether acetates than with peracetates, this first derivatization method is not very widespread. This is mainly due to the several drawbacks of this method. Firstly, spectra are recorded in chloroform-$d_1$ at high temperatures (100 °C !) to overcome the effects of rotational isomerism [158,213]. Even higher temperatures are necessary, if 3,4-cis isomers must be analyzed (150 - 170 °C) [214]. Secondly, methylation of aromatic hydroxy groups using diazomethane is a relatively unsatisfactory procedure because side reactions (ring expansion) [226] and overmethylation (methylation of aliphatic hydroxyls at C3 and methylation of C6 and C8) [194] frequently occur. The conversion of procyanidins into methyl ether acetates therefore requires successive chromatographic purification at each step which results in substantial loss of material [215].

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9.1.2 Circular dichroism

The relative configuration at the chiral centers can largely be established by suitable NMR techniques. The absolute stereochemistry of the upper unit(s) can be determined with optical methods like optical rotation or CD (circular dichroism) measurements. The absolute configuration of the terminal (lower) unit can be assessed by nOe spectroscopy [47], selective esterification of the hydroxy groups (Horeau method) or by X-ray diffraction (crystallization of most procyanidins failed so far) [37]. This task however needs a lot of compound and still remains difficult.

The absolute configuration at C4 is nowadays commonly established by CD measurements. CD spectroscopy is based on the measurement of absorbances in wavelength regions where so called "Cotton effects" emerge. These describe the phenomenon of differing absorbances for left- and right circularly polarized light that occur, if a chromophore lies close to a chiral center. In CD spectra the differences in absorption coefficients for left- and right circularly polarized light, $\Delta \varepsilon$, or the molar ellipticity, $[\theta]$, are displayed versus the wavelength region at which the measurements are conducted. The molar ellipticity $[\theta]$ is defined as: $[\theta] = \frac{M \cdot \Psi}{b \cdot C}$, where $M$ represents the molar mass of the solute, $\Psi$ the ellipticity, $b$ the pathlength in decimeters and $C$ the solute concentration in units of g/100 ml of solution. The ellipticity $\Psi$ defines the ellipse of the electric vector which is described when the electric vectors of the left- and right circularly polarized light beams are combined after the passage through a chiroptical sample. For very small values of ellipticity the following approximation is valid: $[\theta] \approx 3300 \cdot \Delta \varepsilon$ [227-229].

The CD spectra of procyanidins display a small Cotton effect at 280 nm which is attributed to the chirality at C2 and C3. The contributions of different absolute stereochemistries at C2 and C3 have been studied with a number of monomeric flavanols. R-configurations at C2 and C3 were found to contribute negatively, while S-configurations were found to contribute positively to this band [230]. From the sign of the band an object-to-mirror relationship of procyanidin dimers B1 / B2 (positive Cotton effect) and B3 / B4 / B6 (negative Cotton effect) has been postulated, but no structural conclusions were drawn [8]. This quasi-enantiomeric behavior is also reflected in the sign of the specific rotations of these compounds [27].
The CD spectra are clearly dominated by the high-intensity Cotton effects between 200 and 240 nm which are due to the two phenyl A-rings adjacent to the chiral center at C4. Based on the sign of this band a chiroptical rule has been defined to establish the absolute configuration at C4. The rule states that a positive Cotton effect (positive band) indicates that the 4-aryl substituent is positioned above the plane of the fused A-ring and thus a 4R (= 4β) configuration is present. Conversely, if a negative Cotton effect (negative band) is measured, the 4-aryl substituent is below the plane of the A-ring and thus a 4S (= 4α) configuration exists [209,231]. Early works used the sign of the Cotton effect near 200 nm to define the R- or S-configuration, respectively [232,233]. But nowadays the sign of the band near 240 nm is preferentially interpreted, because the measuring error is smaller (at lower wavelengths the larger absorbance of the compounds and the smaller intensity of the lamp results in higher noise levels) [194]. The CD spectra of phenolic procyanidins as well as peracetylated and methyl ether acetate derivatives were found to show the same signs [232]. The measured Cotton effects become larger with increasing degree of polymerization and were found to also exhibit slight solvent dependence [26].

Westenhuizen et al. [234] demonstrated that the Cotton effect in the low-wavelength region is influenced by the conformation of the heterocycle of the 4-aryl-flavan-3-ols. Some flavan-3-ol derivatives which showed boat conformation in their heterocycles (established from the $J_{2,3}$ and $J_{3,4}$ coupling constants) were found to oppose to the chiroptical rule for the determination of the absolute configuration at C4. Peracetylated dimeric procyanthocyanidins which are substituted at position 3 of the lower unit are reported to also represent exceptions to the rule [235]. This clearly shows that the interpretation of CD spectra of trimers or even higher oligomers is very critical. Even if the conformations of the heterocycles are known, the many chromophores and chiral centers in close proximity may interact in a way which makes it very difficult to predict the sign of the Cotton effects.
9.2 Chemical degradation methods

The stereochemistry at C2 / C3 and the nature of the interflavanoid bond are accessible with current two-dimensional NMR techniques for monomers and dimers. Spectra of higher oligomeric procyanidins become increasingly complicated and attempts of interpretations almost exclusively rely on comparative NMR studies (see section 9.1.1.2). Chemical degradation methods therefore still remain the method of choice for the structure elucidation of procyanidins with a degree of polymerization above three.

Applying acid catalyzed degradation pro(antho)cyandidins are protonated at the point of linkage and the interflavanoid bond is subsequently cleaved. The upper part of the molecule carries the charge, while the lower part is released as an uncharged monomer or pro(antho)cyanidin, depending on the degree of polymerization. The lower parts may be analogously cleaved in the case of pro(antho)cyandidins, if the fragments are exposed longer to the acidic environment. In the presence of oxygen the carbocations of the upper parts are oxidized to produce anthocyanidins. This procedure is suitable to detect the different kinds of proanthocyanidin units that may be present in a compound (i.e. cyanidin results from procyanidin units and delphinidin results from prodelfphinidin units). A major drawback of this method however is that the stereochemistries at C2 and C3 in the heterocycles are lost [236].

For this reason methods have been developed to capture the carbocations with suitable nucleophiles right after the cleavage of the interflavanoid bond, thus preserving the stereochemistries at C2 and C3. The nucleophiles may attack the carbocations from the "upper" or "lower" side of the molecule. As a result two derivatives are formed which show different stereochemistry at C4. For steric reasons the two derivatives of (+)-catechin are both produced in significant amounts, while one thiol derivative is preferentially formed from (-)-epicatechin units [209]. The most widely used nucleophiles are toluene-α-thiol (synonyms: benzylmercaptan, phenylmethanethiol) and phloroglucinol (see Figure 9.2) [194].

A-type pro(antho)cyandidins resist acid-catalyzed cleavage. Steynberg et al. [224] lately introduced a procedure to cleave these acetal functionalities thus permitting the establishment of their configurations by means of a chemical degradation method, too.
Figure 9.2 Chemical degradation methods for the structure elucidation of procyanidins: thiolysis and formation of phloroglucinol adducts.
Thiolysis

Thiolytic cleavage is most commonly performed in ethanolic acetic acid and has first been described by Thompson et al. [8], who identified the degradation products by two-dimensional paper chromatography. Nowadays identification of the reaction products is performed by HPLC analysis (i.e. [101,118,237,238]), NMR analysis after isolation and purification (i.e. [14,17,46]) and more seldom by GC analysis [237].

Complete thiolysis (refluxing the reaction mixture for 24 hours [38]) yields monomers and 4-benzylthioethers of monomers. The average stereochemistry at C2 and C3 of the composing units can easily be established. The mean degree of polymerization of higher oligomers and polymers can also be determined from the ratio of the two types of reaction products (i.e. [236,239]).

Partial thiolysis (refluxing the reaction mixture for i.e. 5-6 hours [38]) produces oligomeric and monomeric fragments from which the position of the interflavanoid linkages can be deduced in oligomers. The 4-thiobenzyl derivatives may also be reduced with Raney-nickel as a catalyst to the respective flavan-3-ols (see Figure 9.3) [9,194,238].

The stability of the interflavanoid bond depends on the stereochemistry at C2 / C3 and even more on the type of linkage: 4 → 8 linked procyanidins are cleaved within 3 - 4 hours, while cleavage of 4 → 6 linked procyanidins takes 18-20 hours in ethanolic acetic acid on a boiling water bath [27,240]. This difference in reaction rate has extensively been used as a structural proof (i.e. [118]). However, prolonged heating leads to significant epimerization and disproportionation even under the moderately acidic conditions commonly applied [9,237]. It has also been demonstrated that cleavage of the C4 to C4a bonds may occur [241]. Rigaud et al. [238] could minimize such secondary reactions by the replacement of acetic acid by sulphurous acid (the reactions are carried out at 60 °C for 15 and 30 minutes, respectively).

The reaction yields range from 34 to 63 % for polymers. They were found to be also dependent on the temperature and the solvent. The addition of water, which is sometimes used to solubilize the samples, leads to a significant reduction of yields [237].

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Figure 9.3  Thiolysis and reduction of thiobenzyl derivatives (R = thiobenzyl).
Formation of phloroglucinol adducts

Matsuo et al. [242] introduced the odorless phloroglucinol as a scavenger for the carbocations produced from acid catalyzed cleavage of pro(antho)cyanidins. The reaction is carried out in ethanolic acetic acid solutions at elevate temperatures like thiolysis. The reaction products are identified by TLC (i.e. [243]) or HPLC (i.e. [15,16,118,165]). Reaction yields are reported to be four times lower than those obtained with toluene-α-thiol, what might be an explanation for the more limited distribution of this method compared to the thiolysis method [237].

9.3 Structure elucidation of higher molecular weight compounds

The structure elucidation of higher molecular weight oligomers and polymers becomes increasingly complicated. The samples are always a mixture of several isomers, because pure compounds are only present in low amounts and can not be purified in reasonable quantities. Polymeric samples furthermore are composed of compounds of differing degree of polymerization. The NMR spectra of such samples are characterized by broad and overlapping signals from which only limited information can be drawn. Chemical degradation methods give more precise information on the composition, but they are neither quantitative nor universally applicable. Therefore, only the combination of several analytical techniques makes it possible to characterize these compounds with respect to the degree of polymerization, the stereochemistry at the heterocycles and the procyanidin / prodelphinidin ratio. Estimates for the relative abundance of 4 → 8 and 4 → 6 interflavanoid linkages as well as for the extent of chain-branching have also been proposed based on $^{13}$C-NMR chemical shifts of C3, C4, C6 and C8 signals [244]. All these structural criteria have been established based on the data obtained for low molecular weight compounds (i.e. dimers and trimers). These clues may therefore also be of value in the analysis of oligomeric compounds.
Estimates for the degree of polymerization (molecular weight)

Several methods are used to estimate the mean degree of polymerization in a sample:

- Mass spectrometry is especially useful for compounds of lower molecular weight.
- Complete thiolysis releases flavan-3-ols from the lower unit and benzylthioethers from the upper units of a polymer. The two species may be quantified from $^1$H-NMR spectra (via integration of H2 signals) [14] or from HPLC chromatograms [16,49]. Estimates for the mean degree of polymerization are obtained by dividing the (molar)proportions of the derivatives by that of the flavan-3-ols. Phloroglucinol derivatives can analogously be used [245]. A limitation to estimates based on such degradation methods is, that up to one third of a polymer may resist hydrolysis, particularly when the samples originate from aged tissues [239].
- $^{13}$C-NMR (in D$_2$O / H$_2$O). A formula has been established which relates the relative proportions of 4 $\rightarrow$ 8 / 4 $\rightarrow$ 6 interflavanoid linkages ($\delta = 110$ - 111) and the free C6 ($\delta = 96$ - 98) as well as the free C8 ($\delta = 95$ - 98) of the terminal (lower) units [246].
- $^{13}$C-NMR (in acetone-d$_6$ / H$_2$O). C3 of the terminal (lower) unit resonates at higher field ($\delta = 67$ - 68) than the C3 of the upper units ($\delta = 72$ - 73). The ratio of the intensities of the signals is a measure for the degree of polymerization [5]. Due to line broadening this estimate can only be used for lower molecular weight oligomers [14,247].
- Estimates for the degree of polymerization have also been obtained from colorimetric assays (see section 1.4.1.2.4).

Generally, it is more characteristic to determine the molecular weight distribution of a sample instead of the mean degree of polymerization. The performance of gel permeation chromatography serves this purpose. Peracetylated procyanidins are commonly chromatographed on Ultrastyragel (Waters) [239,248], HP-PL gel Mixed-D (Hewlett Packard) [249], PL-gel (Polymer Lab.) [101] or TSK G (Tosohaas) [49,138] columns using tetrahydrofuran or methylene chloride as eluents. The gel permeation chromatography of phenolic procyanidins has only been successful on a PL-gel column (Polymer Lab.) at elevate temperatures using dimethylformamide as an eluent [250].
The systems are calibrated with linear polystyrene standards. However, molecular mechanics calculations (MM2) revealed that polymeric procyanidins form random coils which are smaller than the linear polystyrene standards of the same molecular weight [206]. If calibrations are performed with polystyrene standards, the molecular weights of pro(antho)cyanidins are therefore underestimated. Furthermore, the conformation of the polymers is likely to be influenced by the eluents, too [35]. The mean degree of polymerization determined by gel permeation chromatography has been reported to be both higher [16,49] as well as lower [101] than the one determined by complete thiolysis followed by HPLC analysis.

**Determination of the cis / trans ratio**

The cis / trans ratio is a measure for the relative proportions of (+)-catechin (and (+)gallocatechin) to (-)-epicatechin (and (-)-epigallocatechin) that are present in a sample. The following strategies for its determination are described in the literature:

- Chemical degradation methods using toluene-α-thiol or phloroglucinol [15] as scavengers yields flavan-3-ols and their derivatives which may be identified by HPLC.

- $^{13}$C-NMR (in acetone-d$_6$ / H$_2$O). The C2 signals of units with trans configuration resonate at 84 ppm while the C2 signals of units with cis configuration resonate at 77 ppm. The relative proportions can be calculated from the signal intensities [5].

- Units exhibiting a cis-configuration have a characteristic IR absorption band at 795 - 800 cm$^{-1}$, which is absent in units showing trans-configuration. The analysis of 26 polymers led to the establishment of the following distribution rules: If the band at about 800 cm$^{-1}$ is as intensive as the one at 780 cm$^{-1}$, the polymer contains over 70 % units with a cis-configuration; if it is only a shoulder, the polymer contains less than 30 % of cis-units [247,251].

- The specific optical rotation $\alpha_D$ at 20 °C of a polymer is linked to the molar fraction of the units showing a cis-configuration by a linear relationship [5,247].

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Determination of the procyanidin / prodelphinidin ratio

Higher molecular weight oligomers and polymers are rarely exclusively composed of procyanidin units. Most common are mixed procyanidin / prodelphinidins (see section 6). These compounds are commonly characterized by the determination of the relative proportions of pyrogallol to catechol B-rings. Several strategies are described in the literature:

- **1H-NMR (in acetone-d₆).** The H2 resonances of flavan-3-ols and their benzylthioether derivatives (after thiolysis) occur at characteristic frequencies. The signals may be integrated to determine the relative amounts [14]. More conveniently, the degradation products may be quantified by HPLC.

- **13C-NMR (in D₂O / H₂O).** A single band for C1’ indicates pure catechol B-rings (δ = 130 - 132) or pure pyrogallol B-rings (δ = 132 - 135). If two signals occur, the relative proportions of the two hydroxylation patterns can be calculated by integration of the signals [246].

- **13C-NMR (in acetone-d₆ / H₂O).** The signals of C3’ and C4’ of procyanidins produce one signal close to 146 ppm. If two signals occur this is indicative for the presence of prodelphinidins. The ratio is estimated by integration of the signals [5].

- The specific absorbance determined at the maximum absorbance between 270 - 280 nm versus the mole fraction of procyanidin units that are present in the molecule follows a linear relationship [5].

- The position of the UV maximum after complete acid hydrolysis (λₚₓₚ delphinidin: 558 nm, λₚₓₚ cyanidin: 547 nm) gives an approximate idea of the relative proportions of procyanidin and prodelphinidin units [7,252].

- After partial acid hydrolysis monomer units may be detected by HPLC [128].

- Characteristic IR absorption bands for procyanidin units are at 1520 cm⁻¹ (skeletal stretching of the aromatic ring) and at 770 - 780 cm⁻¹ (out-of-plane deformations of the hydrogen atoms of the aromatic rings). The absorption bands of prodelphinidin units are at 1535, 1520 and 730 cm⁻¹. The characteristic double peak at 1520 - 1535 cm⁻¹ is only apparent, if a minimum of about 60 % prodelphinidin units are present in the polymer [62,247,251].
10 Structure elucidation of isolated compounds

10.1 Introduction

The structure elucidation of procyanidins is a challenging task. The difficulties encountered lie primarily in the structural diversity of this class of natural compounds. Besides the presence of several chiral centers the occurrence of compounds with differing degrees of polymerization is mainly responsible for the troubles which arise with structure elucidation. If all possible combinations for B-type procyanidins are considered, that is to say two possible monomer units ((-)epicatechin or (+)-catechin) and two types of interflavanoid linkages (4->8 or 4->6) that may be present, there are 8 dimers, 32 trimers and 128 tetramers which can be formed. Even more combinations are possible, if the occurrence of enantiomeric species, uncommon stereochemistries at C4 or A-type procyanidins are also considered. From these calculations it is obvious, that an unambiguous identification of isolated procyanidins asks for very sophisticated analytical techniques on one hand and a sufficient amount of pure sample on the other hand. In practice these two criterions are most seldomly met and therefore compromises are inevitable.

The goal of the present investigation was to identify the isolated compounds with the least amount of sample possible. For this reason chemical degradation methods were not applied because their performance generally needs a larger sample amount than the conductance of spectroscopic investigations. Furthermore, lacking any reference compounds, the structure elucidation based on NMR and CD (for dimers) was considered to be more reliable for the low molecular weight procyanidins that had been isolated.

10.2 Summary

The identity of the isolated (-)-epicatechin and the procyanidins B2, B5, B4, C1, trimer I and tetramer I could be established by spectroscopic methods. The $^1$H-NMR data of the native and peracetylated compounds were in agreement with the literature data. The assignments for the principal resonances in peracetylated dimers and (-)-epicatechin were additionally confirmed by $^1$H-$^1$H-COSY and heteronuclear (HMQC, HMBC)
experiments, respectively. From the $^1$H-$^1$H-COSY spectra of the trimers and the tetramer no additional information could be drawn due to the low concentrations used.

The identity of trimer II was established on the basis of NMR data received for the phenolic compound. The spectra of the peracetylated derivative exhibited considerable complexity due to rotational isomerism, which made the assignments of individual resonances impossible. This behavior however stands in line with the published data.

The MS spectra of (-)-epicatechin and all procyanidins were characteristic. The molecular ions were present in all spectra together with typical fragment ions which were due to RDA-F and loss of acetyl groups (in peracetylated derivatives), respectively.

The absolute configuration of the aryl substituent at C4 in the phenolic dimers was deduced from the CD spectra. The measurement of the optical rotation and comparison of the values with the literature data enabled the exclusion of the presence of enantiomeric species in monomers, dimers and procyanidin C1.

From the NMR and MS data the structure of artifact I was established to be bis-8-8’-epicatechinylmethane.

10.3 Experimental

Materials

Acetone, acetonitrile, chloroform and methanol were of HPLC quality (Romil Chemicals, GB-Shepshed). Benzene (p.a., Scharlau) was obtained from EGT Chemie (CH-Tägerig). Pyridine (puriss. p.a.) and 4-dimethylaminocinnamaldehyde (DMACA, purum) were purchased from Fluka (CH-Buchs). Acetic anhydride (p.a.) was from Merck (CH-Dietikon). Sulphuric acid was purchased from Hänseler (CH-Herisau). (-)-Epicatechin (HPLC grade) was obtained from Extrasynthèse (F-Genay), (+)-Catechin (CHR grade) was from Roth (CH-Reinach). De-ionized water was obtained using a NANO-pure cartridge system (Skan, CH-Basel).

The NMR solvents acetone-d$_6$, methanol-d$_4$, benzene-d$_6$ and chloroform-d$_4$ (isotopic purity: $>$ 99.5 %) were purchased from Dr. Glaser AG (CH-Basel). The samples were dissolved in 550 µl and were measured in NMR tubes of 5 mm o.d.
Instrumentation

NMR: Bruker AMX-300 and AMX-600 (Spectrospin, CH-Fällanden).

MS: ZAB-SEQ for FAB-MS.
Matrix: 3-NOBA (3-nitrobenzyl alcohol), mode: positive ionization.

CD: Jasco J-710/720 (Jasco Corporation, J-Tokyo).
Wavelength range: 210-300 nm, resolution: 0.2 nm, scan speed: 20 nm/min, number of scans: 3, response: 3 seconds, bandwidth: 2 nm, sensitivity: 20 millidegrees, temperature: 20.0 °C, cell: 0.1 cm.

Optical rotation: Polarimeter 241 (Perkin Elmer, D-Ueberlingen).
Wavelength: sodium D-line (589 nm), temperature: 20.0 °C.

Vacuum drying oven: Salvis Vacucenter (Salvis AG, CH-Reussbühl/LU)
Linomat: Model IV (Camag, CH-Muttenz)

Procedures

NMR. 
$^1$H-NMR spectra of the phenolic compounds (in acetone-$d_6$ and/or methanol-$d_4$) and of the peracetylated derivatives (in chloroform-$d_4$, procyanidin B5 and trimer II also in benzene-$d_6$) were recorded. All spectra were referenced to residual hydrogen or carbon resonances of the respective solvents. Residual water resonances could be detected in almost all spectra (acetone-$d_6$: ~2.8 ppm, methanol-$d_4$: ~4.8 ppm, chloroform-$d_4$: ~1.6 ppm, benzene-$d_6$: ~0.4 ppm). Solvent impurities were also obvious in some of the spectra (i.e. in acetone-$d_6$ signals < 1.6 ppm). The chemical shifts and multiplicities were compared to published data. The assignments of the proton signals of the peracetylated derivatives were confirmed by the interpretation of DQF-COSY spectra in the case of monomers and dimers (the two-dimensional spectra of the trimers and the tetramer were of little value due to low concentrations). DEPT, $^{13}$C-NMR as well as HMQC and HMBC experiments were additionally used to establish and confirm the principal assignments for procyanidins B2, B4 and artifact I.

MS. MS spectra of peracetylated compounds as well as of native (-)-epicatechin, procyanidin B2 and C1 were measured in 3-NOBA (3-nitrobenzyl alcohol) in the positive ionization mode by Dr. W. Amrein and his staff (Chemical Department of the Swiss Federal Institute of Technology Zurich, Switzerland).
CD. Measurements were performed with procyanidins B2 (1.065 mg/ml), B5 (1.080 mg/ml), B4 (1.025 mg/ml), C1 (1.105 mg/ml) and (-)-epicatechin (1.085 mg/ml). In preliminary tests methanol and acetonitrile were tested as solvents with procyanidin B2. Acetonitrile, which is optically more transparent than methanol in the low wavelength region, did not yield smoother CD curves. Methanol was chosen as solvent, because other optical parameters (specific rotation, see below, and molar absorption coefficients, see section 4.3.1) were determined with this solvent, too. Molar ellipticities, \([\theta]\), are reported of the most important extrema in deg \(\cdot\) cm\(^2\) \(\cdot\) decimole\(^{-1}\).

**Optical rotation.** The optical rotation of procyanidins B2 (1.065 mg/ml), B5 (1.080 mg/ml), B4 (1.025 mg/ml), C1 (1.105 mg/ml) and (-)-epicatechin (1.085 mg/ml) were determined in methanol at 20.0 °C.

**Acetylation.** (-)-Epicatechin and procyanidin peracetates were prepared by dissolving 4 - 14 mg of compound in 1 ml pyridine in 10 ml snapcap glass flasks. Subsequently 1 ml of acetic anhydride was added. A stream of nitrogen was applied for approximately 10 seconds before firmly closing the flasks and storing them in the dark at room temperature. The course of the reaction was monitored by TLC. Analyses were performed on silica gel 60F\(_{254}\) aluminium plates (Merck, CH-Dietikon) over a distance of approximately 8 cm. The eluent consisted of acetone – benzene (1:4, v/v). Detection was performed using a 1 % (w/v) solution of DMACA in methanolic sulphuric acid (8 ml H\(_2\)SO\(_4\) ad 100 ml methanol). Phenolic compounds remained at the start, while the derivatives were eluted. The reactions were completed after 20 to 30 hours. The derivatization of tetramer I was not finished even after 40 hours of reaction. The derivatization procedure was however interrupted after this time.

The reaction mixtures were cooled on ice and poured onto 5 ml of ice-cooled water in 20 ml snapcap glass flasks. The reaction flasks were rinsed with another 5 ml ice-cooled water. After several hours of storage at 4 °C the precipitates were filtered over a sintered glass filter (1 ml, porosity number 4). The derivatives were completely dried in a vacuum drying oven at 25 - 28 °C for several hours.

**Preparative TLC for peracetylated derivatives.** The peracetates of trimer II and tetramer I were purified by preparative TLC on silica gel 60F\(_{254}\) aluminium plates.
(Merck, CH-Dietikon) over a distance of approximately 18 cm. The solutions were sprayed onto the plates using a Linomat TLC application device (charge: approximately 1 mg per plate, bandshaped, width: 17 cm). The TLC was developed in the solvent system acetone – benzene (1:4, v/v). The relevant bands were detected by UV at 254 nm, were cut out and were macerated with chloroform over night. The extract solutions were filtered over a sintered glass filter (number 4) and were evaporated to dryness.

Figure 10.1A Structural formulae of isolated compounds. Part I.
Figure 10.1B Structural formulae of isolated compounds. Part II.
**Figure 10.1C** Structural formulae of isolated compounds. Part III.

**tetramer I**
(-)-epicatechin-(4β→8)-(-)-epicatechin-(4β→8)-
(-)-epicatechin-(4β→8)-(-)-epicatechin

**artifact I**
bis-(-)-epicatechinyl-methane
10.4 Results and discussion

Structural formulae of the different compounds are given in Figures 10.1A to 10.1C. Characteristic NMR and CD spectra are in Appendix I.

10.4.1 (-)-Epicatechin

NMR. The assignments for the protons at C6 and C8 in underivatized (-)-epicatechin are exchanged (or are reported to be interchangeable [45], respectively) in papers published before 1993. The first unambiguous assignment of these two resonances has been made by Shen et al. [253] using two-dimensional techniques in DMSO-d$_6$. De Bruyne [197] could confirm the reassignments for H6 and H8 by measurements in acetone-d$_6$. The unusually high chemical shift for H6 in [19] is possibly due to a typing error. The NMR data of the isolated (-)-epicatechin conform well with the published data (see Table 10.1).

The NMR values obtained for the peracetylated derivative are also in agreement with the literature data (see Table 10.2 and 10.3). The B-ring multiplicities are generally not anymore obvious due to overlapping of the signals in peracetylated proton spectra. The small coupling constant of the two A-ring protons was only detectable for H6 in the investigations presented here. This is attributable to the relatively low resolution of the 300 MHz NMR apparatus. For the same reason one of the double doublets of the two protons at C4 could not be detected either. The definite assignment of the H6 and H8 resonances could not yet be established even by two-dimensional experiments [199]. In most publications the H6 is reported to resonate upfield of the H8. However, since analogy to the phenolic data is assumed to be the basis for such assignments, these chemical shifts are not anymore in compliance with the reassigned resonances for H6 and H8 (see above). Therefore, the H6 and H8 resonances should most correctly still be reported as interchangeable. The assignments for the C6 and C8 resonances in $^{13}$C-NMR spectra may be interchanged for the same reason.
Table 10.1  \(^1\)H-NMR spectral data of (-)-epicatechin.

<table>
<thead>
<tr>
<th>H</th>
<th>Measured(^a)</th>
<th>Published data(^c)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>(\delta) (ppm) (J) (Hz)</td>
<td>[19](^d)</td>
</tr>
<tr>
<td>2</td>
<td>4.88 s</td>
<td>4.90 s</td>
</tr>
<tr>
<td>3</td>
<td>4.20 s(^{br})</td>
<td>4.23 s(^{br})</td>
</tr>
<tr>
<td>4</td>
<td>2.87 dd(^e)</td>
<td>2.7 - 3.1 m</td>
</tr>
<tr>
<td></td>
<td>((J = 4.5, 16.9))</td>
<td>((J = 4.4, 16.8))</td>
</tr>
<tr>
<td>6</td>
<td>6.02 d ((J = 2.28))</td>
<td>6.98 s(^{br})</td>
</tr>
<tr>
<td>8</td>
<td>5.92 d ((J = 2.26))</td>
<td>6.10 s(^{br})</td>
</tr>
<tr>
<td>2'</td>
<td>7.05 d ((J = 1.59))</td>
<td>6.80 - 6.90</td>
</tr>
<tr>
<td>5'</td>
<td>6.78 d ((J = 8.11))</td>
<td>6.80 - 6.90</td>
</tr>
<tr>
<td>6'</td>
<td>6.84 dd</td>
<td>7.08 s</td>
</tr>
<tr>
<td></td>
<td>((J = 1.9, 8.1))</td>
<td>((J = 8.4, 2, 0.5))</td>
</tr>
</tbody>
</table>

\(^a\)(-)-Epicatechin was dissolved in acetone-\(d_6\) (300 MHz, 298 K, internal standard: acetone-\(d_6\) = 2.05 ppm). \(^b\) broad signal; \(^c\) solvent: acetone-\(d_6\); \(^d\) in acetone-\(d_6\) + D\(_2\)O; \(^e\) signals are overlapping with water; \(^f\) assignments for H6 and H8 were made with a special NMR assignment technique using cadmium nitrate.
Table 10.2 $^1$H-NMR spectral data of peracetylated (-)-epicatechin.

<table>
<thead>
<tr>
<th>H</th>
<th>Measured$^a$</th>
<th>Published data$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\delta$ (ppm) $J$ (Hz)</td>
<td>[19]</td>
</tr>
<tr>
<td>2</td>
<td>5.10 s</td>
<td>5.39 $s^{br}$</td>
</tr>
<tr>
<td>3</td>
<td>5.38 $s^{br}$</td>
<td>5.11</td>
</tr>
<tr>
<td>4</td>
<td>2.87 s (2x)$^c$</td>
<td>2.90 - 2.96 m</td>
</tr>
<tr>
<td></td>
<td>2.97 dd</td>
<td>($J = 4.1, 17.9$)</td>
</tr>
<tr>
<td>6</td>
<td>6.56 d ($J = 1.13$)$^d$</td>
<td>6.57 d ($J = 2.2$)</td>
</tr>
<tr>
<td>8</td>
<td>6.66 s$^d$</td>
<td>6.67 d ($J = 2.2$)</td>
</tr>
<tr>
<td>2'</td>
<td>7.34 s</td>
<td>7.20 - 7.35</td>
</tr>
<tr>
<td>5'</td>
<td>7.19 d ($J = 8.35$)</td>
<td>7.20 - 7.35</td>
</tr>
<tr>
<td>6'</td>
<td>7.27 s (2x)</td>
<td>7.20 - 7.35</td>
</tr>
<tr>
<td>aliphatic acetate</td>
<td>1.91 s</td>
<td>-</td>
</tr>
<tr>
<td>aromatic acetates</td>
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<td>-</td>
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<tr>
<td>acetates</td>
<td>1.91 - 2.28</td>
<td>2.28 - 2.29</td>
</tr>
</tbody>
</table>

$^a$ (-)-Epicatechin was dissolved in chloroform-d$_4$ (300 MHz, 298 K, internal standard: chloroform-d$_4$ = 7.27 ppm). $^{br}$ broad signal; $^c$ solvent: chloroform-d$_4$; $^d$ chemical shifts of protons are interchangeable; $^e$ coupling constant ($J$) of the two singlets: 17.7 Hz; n.s. = not specified.
Table 10.3  $^{13}$C-NMR spectral data of peracetylated (-)-epicatechin.

<table>
<thead>
<tr>
<th>C</th>
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<th>Published data$^b$</th>
</tr>
</thead>
<tbody>
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<td></td>
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</tr>
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</tr>
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<td>4a</td>
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<td>109.7</td>
</tr>
<tr>
<td>5$^e$</td>
<td>149.7</td>
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<td>108.2</td>
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<tr>
<td>7$^e$</td>
<td>149.7</td>
<td>149.8</td>
</tr>
<tr>
<td>8</td>
<td>108.8$^g$</td>
<td>109.0</td>
</tr>
<tr>
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<td>136.1</td>
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<td>2'$^'$</td>
<td>122.0</td>
<td>122.4</td>
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<td>3'$^f$</td>
<td>142.0</td>
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<td>4'$^f$</td>
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<td>142.2</td>
</tr>
<tr>
<td>5'$^'$</td>
<td>123.2</td>
<td>123.5</td>
</tr>
<tr>
<td>6'$^'$</td>
<td>124.4</td>
<td>124.8</td>
</tr>
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<td>aliphatic</td>
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<tr>
<td>carbonyl</td>
<td>170.4</td>
<td>n.s.</td>
</tr>
<tr>
<td>aromatic</td>
<td>168.0 -</td>
<td>n.s.</td>
</tr>
<tr>
<td>carbonyls</td>
<td>169.0</td>
<td></td>
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<tr>
<td>carbonyls</td>
<td>168.0 -</td>
<td>167 - 171</td>
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<td>170.4</td>
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<tr>
<td>methyls</td>
<td>20.6 -</td>
<td>20 - 21</td>
</tr>
<tr>
<td></td>
<td>21.1</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Peracetylated (-)-epicatechin was dissolved in chloroform-d$_3$ (75.5 MHz, 298 K, internal standard: chloroform-d$_3$ = 77.0 ppm). $^b$ solvent: chloroform-d$_3$; $^c$ signals are not assigned to the respective carbons in the publication; $^d$ assignments were exchanged by mistake in the publication; $^e$ and $^f$ chemical shifts of carbons are interchangeable; $^g$ assignments may be interchanged (literature data are not consistent); n.s. = not specified.
Analogous ions as described for the breakdown of peracetylated (+)-catechin were detected [254]. The molecular ion of peracetylated (-)-epicatechin is measured at m/z 501. The fragment ion at m/z 441 is characteristic for the loss of an aliphatic acetoxy group (u 60). The principal signal at m/z 399 is produced by the loss of a keten fragment (u 42, characteristic of phenolic acetates) from m/z 441. Sequential loss of another three keten or acetyl (u = 43) fragments, respectively, yields the signals at m/z 357, 314 and 272.

The principal signal in the MS spectrum of (-)-epicatechin is the molecular ion at m/z 291. Adducts of water (m/z 309) and potassium (m/z 330) are also present. Fragments of RDA-fission (m/z 139, 153) can be detected as well. The spectrum is however clearly dominated by the signals of the 3-nitrobenzyl alcohol matrix (m/z 460, 307, 289, 154 and 136).

\[
\begin{align*}
\left[\alpha\right]_D \text{ Isolated (-)-epicatechin: } & \quad \left[\alpha\right]_{280.8} = -45.2^\circ \text{ (methanol, c = 0.1085, at 20.0 °C)} \\
\text{Reference standard: } & \quad \left[\alpha\right]_{578} = -59.0^\circ \text{ (methanol, c = 0.1305, at 20.0 °C)} \\
\text{Literature values: } & \quad \left[\alpha\right]_{298} = -57.6^\circ \text{ (methanol, c = 0.25) [40]} \\
& \quad \left[\alpha\right]_{589} = -43^\circ \text{ (methanol, c = 0.15) [102]}
\end{align*}
\]

The relatively low value determined for the isolated (-)-epicatechin is thought to be due to impurities (the isolated compound is beige colored, while the reference standard is off-white).

\[
\begin{align*}
\text{CD. Isolated (-)-epicatechin: } & \quad \left[\theta\right]_{280.8} = -1812 \quad \left[\theta\right]_{275.0} = -1777 \\
& \quad \left[\theta\right]_{240.6} = +3345 \\
\text{Reference standard: } & \quad \left[\theta\right]_{280.2} = -2595 \quad \left[\theta\right]_{278.0} = -2513 \\
& \quad \left[\theta\right]_{240.8} = +4944 \\
\text{Literature values [232]: } & \quad \Delta\left[\theta\right]_{3281} = -0.71 \quad (\equiv \left[\theta\right]_{281} = -2343) \\
& \quad \Delta\left[\theta\right]_{275} = -0.65 \quad (\equiv \left[\theta\right]_{275} = -2145) \\
& \quad \Delta\left[\theta\right]_{239.0} = +1.57 \quad (\equiv \left[\theta\right]_{239} = +5181)
\end{align*}
\]

The differences in absolute values are again thought to be due to impurities in the isolated (-)-epicatechin.
10.4.2 Procyanidin B2

NMR. The measured NMR data are in agreement with the reported data (see Tables 10.4 to 10.6). The assignments of H8 and H6 of the upper unit and H6 of the lower unit was done according to the published values of de Bruyne [197] in the spectra of procyanidin B2. In the peracetylated compound no meta-meta coupling of the aromatic H6 and H8 of the upper unit could be detected in the 300 MHz spectrum. In order to unambiguously confirm the identity, the $^1$H-NMR spectrum was also recorded on a 600 MHz apparatus. The characteristic doublets with low coupling constants (2.1 Hz) could readily be detected.

MS. Analogous ions as described in the literature for peracetylated procyanidin B2 [255] or peracetylated dimeric B-type procyanidins [121,254], respectively, could be detected. The molecular ion of peracetylated B2 is detected at m/z 999. A dimeric cluster ion is also detectable at m/z 1999. A first cascade (m/z 957, 897, 837, 795 and 753) is caused by sequential losses of aliphatic acetoxy (u 60) and keten fragments (u 42) from the molecular ion. A second route leads to the fragment ion at m/z 939 by the loss of an acetoxy group (u 60). Loss of acetyl (u 43) and keten (u 42) from this ion produces fragment ions at m/z 896, 853, 811 and 769. RDA-fission of fragment m/z 939 leads to the ion m/z 661 (m/z 939 - 278 u), which may sequentially loose keten fragments (m/z 619, 577, 535, 493) or which may break down to the fragment m/z 439 by fission of the (original) interflavanoid linkage (see also section 5, Figure 5.1). This same fragment m/z 439 may also be produced by fission of the interflavanoid linkage in fragment m/z 879 (m/z 939 - 60 u). Finally, sequential loss of keten groups from the ion at m/z 439 yields fragment ions at m/z 397, 355 and 313. The principal signals in the spectrum are between m/z 895 - 897.

The MS spectrum of procyanidin B2 shows the molecular ion at m/z 579. Fragments of RDA-fission can also be detected (m/z 579 - u 152 = m/z 427 - 18 u = m/z 409). The signal at m/z 329 is due to the cleavage of the interflavanoid linkage and addition of potassium to the resulting monomeric fragment. The spectrum is however clearly dominated by the intense signals of the 3-nitrobenzyl alcohol (m/z 613, 460, 307, 289, 154, 136). Because some of these matrix signals show similar or even the same (m/z 289) mass to charge ratio like characteristic fragments of procyanidin B2, the measurement of MS spectra of peracetylated compounds is to be preferred.
Table 10.4  $^1$H-NMR spectral data of procyanidin B2.

<table>
<thead>
<tr>
<th>H</th>
<th>Measured$^a$</th>
<th>Published data$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\delta$(ppm)</td>
<td>$J$(Hz)</td>
</tr>
<tr>
<td>2 upper</td>
<td>5.09 s</td>
<td>5.12 $s^{br}$</td>
</tr>
<tr>
<td>3 upper</td>
<td>3.98 d ($J=1.0$)</td>
<td>4.04 $s^{br}$</td>
</tr>
<tr>
<td>4 upper</td>
<td>4.72 s</td>
<td>4.75 $s^{br}$</td>
</tr>
<tr>
<td>6 upper</td>
<td>5.99 s</td>
<td>6.05 $s^{br}$</td>
</tr>
<tr>
<td>8 upper</td>
<td>5.97 s</td>
<td>6.02 $s^{br}$</td>
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<tr>
<td>2 lower</td>
<td>4.94 $s^{br}$</td>
<td>4.99 $s^{br}$</td>
</tr>
<tr>
<td>3 lower</td>
<td>4.27 $s^{br}$</td>
<td>4.34 $s^{br}$</td>
</tr>
<tr>
<td>4 lower</td>
<td>2.89 $dd^e$ ($J=4.6, 16.4$)</td>
<td>2.93 $dd$</td>
</tr>
<tr>
<td>6 lower</td>
<td>5.95 s</td>
<td>6.00 s</td>
</tr>
<tr>
<td>B / E rings</td>
<td>6.69 - 7.06</td>
<td>6.75 - 7.13</td>
</tr>
</tbody>
</table>

* Procyanidin B2 was dissolved in acetone-d$_6$ (300 MHz, 298 K, internal standard: acetone-d$_6$ = 2.05 ppm). $^{br}$ broad signal; $^c$ solvent: acetone-d$_6$; $^d$ in acetone-d$_6$ + D$_2$O; $^e$ signals are overlapping with water; n.s. = not specified.
Table 10.5  $^1$H-NMR spectral data of peracetylated procyanidin B2.

<table>
<thead>
<tr>
<th>H</th>
<th>Measured$^a$</th>
<th>Published data$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\delta$ (ppm) $J$ (Hz)</td>
<td>[121]</td>
</tr>
<tr>
<td>2 upper</td>
<td>5.59 s</td>
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</tr>
<tr>
<td>3 upper</td>
<td>5.18 s</td>
<td>5.18 m</td>
</tr>
<tr>
<td>4 upper</td>
<td>4.47 d ($J$ = 1.1)</td>
<td>4.46 d ($J$ = 1.8)</td>
</tr>
<tr>
<td>6 upper</td>
<td>6.00 d ($J$ = 2.1)</td>
<td>6.00 d ($J$ = 2.2)</td>
</tr>
<tr>
<td>8 upper</td>
<td>6.25 d ($J$ = 2.1)</td>
<td>6.23 d ($J$ = 2.2)</td>
</tr>
<tr>
<td>2 lower</td>
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<td>4.54 s</td>
</tr>
<tr>
<td>3 lower</td>
<td>5.12 m</td>
<td>5.12 m</td>
</tr>
<tr>
<td>4 lower</td>
<td>2.8 - 3.1 m</td>
<td>2.84 - 3.05 m</td>
</tr>
<tr>
<td>6 lower</td>
<td>6.67 s</td>
<td>6.66 s</td>
</tr>
<tr>
<td>B / E rings</td>
<td>6.89 - 7.37</td>
<td>6.67 - 7.36 m</td>
</tr>
<tr>
<td>acetates</td>
<td>1.57 - 2.33</td>
<td>1.56 - 2.31</td>
</tr>
</tbody>
</table>

$^a$ Peracetylated procyanidin B2 was dissolved in chloroform-d$_1$ (600 MHz, 303 K, internal standard: chloroform-d$_1$, $\delta$ = 7.27 ppm). $^b$ broad signal; $^c$ solvent: chloroform-d$_1$; n.s. = not specified.
<table>
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<tr>
<th>C</th>
<th>Measured&lt;sup&gt;a&lt;/sup&gt; δ (ppm)</th>
<th>Published data&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
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<td>73.6</td>
</tr>
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<td>71.1</td>
</tr>
<tr>
<td>4 upper</td>
<td>71.0</td>
<td>71.0</td>
</tr>
<tr>
<td>6 upper</td>
<td>73.9</td>
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<td>107.3</td>
</tr>
<tr>
<td>8 lower</td>
<td>110.3</td>
<td>110.3</td>
</tr>
</tbody>
</table>

<sup>a</sup> Peracetylated procyanidin B2 was dissolved in chloroform-d<sub>6</sub> (75.5 MHz, 298 K, internal standard: chloroform-d<sub>6</sub> = 77.0 ppm).

<sup>b</sup> Solvent: chloroform-d<sub>6</sub>; recorded at 30 °C; n.s. = not specified.
Isolated procyanidin B2:  
\[ \alpha \]_{578} = + 28.3^\circ \text{ (methanol, } c = 0.1065, \text{ at } 20.0 ^\circ C) 
\[ \alpha \]_{589} = + 30.2^\circ \text{ (methanol, } c = 0.15) \text{ [102]}

Literature values:  
\[ \alpha \]_{578} = + 23.2^\circ \text{ (methanol, } c = 0.25) \text{ [40]}

CD: Isolated procyanidin B2:  
\[ \theta \]_{289.8} = - 2640 \quad \text{[} \theta \text{]}_{279.8} = - 4593 
\[ \theta \]_{240.8} = + 24'328

Literature values [232]:  
\( \Delta \epsilon_{288} = - 0.43 \) \quad (\Rightarrow [\theta]_{288} = - 1419)
\( \Delta \epsilon_{275} = - 1.13 \) \quad (\Rightarrow [\theta]_{275} = - 3729)
\( \Delta \epsilon_{235} = + 9.25 \) \quad (\Rightarrow [\theta]_{235} = + 30'525)

From the sign of the CD band near 240 nm the \( \beta \)-configuration of the aryl substituent at C4 of the upper unit is defined. The comparison of absolute values obviously is useless. Possible explanations for the variations are the use of different equipment and measuring conditions. Furthermore, very tiny quantities are detected with CD spectroscopy (the difference of the molar absorption coefficients (\( \Delta \epsilon \)) for left and right circularly polarized light are approximately 0.4 to 9 (see above), while the molar absorption coefficient (\( \epsilon \)) for procyanidin B2 was determined to amount to 7850 (see section 4, Table 4.3).

10.4.3 Procyanidin B5

NMR. The measured NMR data are in agreement with the reported data (see Tables 10.7 and 10.8). The assignments for H6 and H8 of the upper unit of procyanidin B5 could not yet be accomplished using different solvents and two-dimensional techniques [166,197]. The spectrum of the procyanidin B5 is well resolved, while the spectrum of the peracetylated compound is characterized by extensive broadening of resonances due to rotational isomerism (see appendix I). Using a 400 MHz NMR apparatus the signals of two rotational isomers could be distinguished in the spectrum of the peracetylated derivative. The resonances of H6 and H8 of the upper unit could however not yet be assigned [257].

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It was attempted to obtain first order spectra of peracetylated procyanidin B5 by the use of a solvent with a higher boiling point than chloroform. First order spectra are reported in the literature for some peracetylated procyanidins in nitrobenzene-d$_5$ [202,203]. Since this solvent was not available, benzene-d$_6$ was used. Figure 10.2 shows $^1$H-NMR spectra of peracetylated procyanidin B5 in benzene-d$_6$ recorded at different temperatures. As can be seen, resonances turn out to be sharper and show more fine structure as the temperature is raised. However, interpretation of the two-dimensional spectra revealed that the clue resonances of H6 and H8 protons were partly masked by the B- and E-ring aromatic signals. This is a major drawback compared to the spectra recorded with the other solvents in which the H6 and H8 resonances are well separated from the remaining aromatic proton signals. Furthermore, the H3 and H2 resonances of the upper unit overlap and two rotational isomers occur. This approach was therefore not pursued further.

Table 10.7 $^1$H-NMR spectral data of procyanidin B5.

<table>
<thead>
<tr>
<th>H</th>
<th>Measured$^a$</th>
<th>Published data$^c$</th>
</tr>
</thead>
<tbody>
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<td></td>
<td>$\delta$ (ppm) $J$ (Hz)</td>
<td>[101,257]</td>
</tr>
<tr>
<td>2 upper</td>
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<td>4.98 $^b$s</td>
</tr>
<tr>
<td>3 upper</td>
<td>4.09 s</td>
<td>4.08 $^b$s</td>
</tr>
<tr>
<td>4 upper</td>
<td>4.67 s</td>
<td>4.66 d ($J$=1.8)</td>
</tr>
<tr>
<td>6 / 8 upper</td>
<td>6.10 s</td>
<td>6.08 d ($J$=2.55)</td>
</tr>
<tr>
<td>6 / 8 upper</td>
<td>6.11 s</td>
<td>6.10 d ($J$=2.55)</td>
</tr>
<tr>
<td>2 lower</td>
<td>4.85 s</td>
<td>4.84 $^b$s</td>
</tr>
<tr>
<td>3 lower</td>
<td>4.18 s</td>
<td>4.17 $^b$s</td>
</tr>
<tr>
<td>4 lower</td>
<td>2.63 - 2.82 m$^d$</td>
<td>2.66 dd ($J$=2.1, 16.5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(J = n.s., 16.8)</td>
</tr>
<tr>
<td>B / E rings</td>
<td>6.74 - 7.07 m</td>
<td>6.73 - 7.06</td>
</tr>
</tbody>
</table>

$^a$Procyanidin B5 was dissolved in acetone-d$_6$ (300 MHz, 298 K, internal standard: acetone-d$_6$ = 2.05 ppm). $^b$broad signal; $^c$solvent: acetone-d$_6$; $^d$signals are overlapping with water; n.s. = not specified.
Table 10.8  $^1$H-NMR spectral data of peracetylated procyanidin B5.

<table>
<thead>
<tr>
<th>H</th>
<th>Measured$^a$</th>
<th>Published data$^c$</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\delta$ (ppm) $J$ (Hz)</td>
<td>[257] major rotamer</td>
<td>minor rotamer</td>
</tr>
<tr>
<td>2 upper</td>
<td>5.19 s</td>
<td>5.12 s$^{br}$</td>
<td>5.16 s$^{br}$</td>
</tr>
<tr>
<td>3 upper</td>
<td>5.35 m$^{br}$</td>
<td>5.37 m</td>
<td>5.43 m</td>
</tr>
<tr>
<td>4 upper</td>
<td>4.18 s$^{br}$</td>
<td>4.10 s$^{br}$</td>
<td>4.33 s$^{br}$</td>
</tr>
<tr>
<td></td>
<td>4.39 s$^{br}$</td>
<td>4.33 s$^{br}$</td>
<td>4.10 s$^{br}$</td>
</tr>
<tr>
<td>6 / 8 upper</td>
<td>6.80 s$^{br}$</td>
<td>6.73 d ($J=2.25$)</td>
<td>6.73 d ($J=2.25$)</td>
</tr>
<tr>
<td></td>
<td>6.67 s$^{br}$</td>
<td>6.72 d ($J=2.25$)</td>
<td>6.72 d ($J=2.25$)</td>
</tr>
<tr>
<td>2 lower</td>
<td>5.23 s</td>
<td>5.16 s$^{br}$</td>
<td>5.17 - 5.18 m</td>
</tr>
<tr>
<td>3 lower</td>
<td>5.44 s</td>
<td>5.43 m</td>
<td>5.26 s$^{br}$</td>
</tr>
<tr>
<td>4 lower</td>
<td>2.99 m$^{br}$</td>
<td>2.81 - 2.97 m</td>
<td>2.81 - 2.97 m</td>
</tr>
<tr>
<td>8 lower</td>
<td>masked$^d$</td>
<td>6.60 s</td>
<td>6.60 s</td>
</tr>
<tr>
<td>B / E rings</td>
<td>7.20 - 7.41</td>
<td>7.13 - 7.33 m</td>
<td>7.13 - 7.33 m</td>
</tr>
<tr>
<td>acetates</td>
<td>1.89 - 2.40</td>
<td>1.5 - 2.33 m</td>
<td>1.5 - 2.33 m</td>
</tr>
</tbody>
</table>

$^a$ Procyanidin B5 was dissolved in chloroform-d$_4$ (300 MHz, 298 K, internal standard: chloroform-d$_4$ = 7.27 ppm). $^{br}$ broad signal; $^c$ solvent: chloroform-d$_4$; $^d$ signal is overlapping with H6 / H8 of the upper unit.
Figure 10.2 $^1$H-NMR spectra of peracetylated procyanidin B5 recorded at different temperatures. Temperature: 323 K, 320 K, 310 K, and 300 K (top to lower); solvent: benzene-$d_6$; recorded at 300 MHz.
The MS spectrum of the peracetylated procyanidin B5 shows similar ions and abundances like the spectrum of peracetylated procyanidin B2. The molecular ion is detected at m/z 999 and its sodium adduct at m/z 1022. The principal signals are again between m/z 895 - 897. Fragments which result from RDA-fission (m/z 661) or cleavage of the interflavanoid linkage (m/z 439), respectively, are present in the spectrum, besides many fragments resulting from loss of acetyl, acetoxy or keten groups (m/z 939, 895 - 897, 879, 853, 837, 811, 795, 753, 619, 577, 535, 397, 355, 313).

\[ \alpha \] Isolated procyanidin B5: \[ \alpha \] = +112.2° (methanol, c = 0.1061, at 20.0 °C)

Literature values:
\[ \alpha \] = +137° (methanol, c = 0.34) [40]
\[ \alpha \] = +119.2° (methanol, c = 1.35) [8]

CD: Isolated procyanidin B5: \[ \theta \] = +6713 \[ \theta \] = -8569
\[ \theta \] = +2107649

Literature values [232]:
\[ \Delta \theta \] = +1.2 \[ \theta \] = +3960
\[ \Delta \theta \] = -0.95 \[ \theta \] = -3135
\[ \Delta \theta \] = +23.50 \[ \theta \] = +77550

From the sign of the CD band at 235 nm the \( \beta \)-configuration of the aryl substituent at C4 of the upper unit is defined.

10.4.4 Procyanidin B4

NMR. The measured NMR data conform with the published values (see Tables 10.9 and 10.10). The occurrence of two sets of rotamer signals at ambient temperature in the spectrum of procyanidin B4 is indicative of the presence of an upper unit showing a 2,3-trans configuration. Furthermore, the coupling constant \( J_{2,3} \) (~10 Hz) of the upper unit in both the phenolic and peracetylated compounds also supports this view (H2 of an (-)-epicatechin upper unit usually appears as a broad singlet). The rotamer signals occur in a ratio of approximately 1 : 1.2 in the spectra of procyanidin B4 and in a ratio of approximately 1 : 3.5 in the spectra of the peracetylated derivative.
The assignments of the H6 and H8 protons were done in analogy to the literature data because two-dimensional spectra did not show the characteristic cross peaks (due to the low concentration applied). The signal at 6.74 ppm is the only resonance which could not be assigned by comparison with literature data and evaluation of the recorded two-dimensional spectra. Its intensity suggests that it might be the rotamer signal of H6 of the lower unit.

The following signals could be assigned in the $^{13}$C-NMR spectrum (75.5 MHz, 298 K, internal standard: chloroform-d$_1$ = 77.0 ppm, $\delta$ (ppm); u = upper unit, l = lower unit): 26.58 (4 l); 36.74 (4 u); 66.64 (3 l); 69.98 (3 u); 77.26 (2 l); 79.0 (2 u); 108.14 (8 u); 109.52, 110.08 and 115.03 (6 l, 8 l and 6 u).

**MS.** The MS spectrum of peracetylated B4 is typical of a dimeric procyanidin. Besides the molecular ion at m/z 999, a sodium adduct (m/z 1022) and a cluster ion (m/z 1999) can be detected. Ions between m/z 895 - 897 show the highest abundance. Ions characteristic of RDA-fission (m/z 661) and cleavage of the interflavanoid bond (m/z 439) are present. Again many signals occur due to the loss of acetoxy, acetyl and keten fragments (m/z 957, 939, 895 - 897, 879, 853, 837, 811, 795, 619, 577, 535).

$[\alpha]_D$ Isolated procyanidin B4: $[\alpha]_{578} = -205.4^\circ$ (methanol, c = 0.1013, at 20.0 °C)

Literature value: $[\alpha]_{578} = -193.5^\circ$ (ethanol, c = 1.0, at 20 °C) [8]

CD: Isolated procyanidin B4: $\theta_{279.9} = + 6788$ $\theta_{243.4} = -26'594$

[$\theta]_{279.9} = + 110'967$

Literature values [232]:

- $\Delta \theta_{286} = -0.64$ ($\equiv [\theta]_{286} = -2112$)
- $\Delta \theta_{267} = -0.78$ ($\equiv [\theta]_{267} = -2574$)
- $\Delta \theta_{231} = -12.20$ ($\equiv [\theta]_{231} = -40'260$)

From the sign of the CD band near 240 nm the $\alpha$-configuration of the aryl substituent at C4 of the upper unit is defined.
Table 10.9  

1H-NMR spectral data of procyanidin B4.

<table>
<thead>
<tr>
<th>H</th>
<th>Measured</th>
<th>Published data</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>major rotamer</td>
<td>major rotamer</td>
</tr>
<tr>
<td></td>
<td>minor rotamer</td>
<td>minor rotamer</td>
</tr>
<tr>
<td>2 upper</td>
<td>4.44 d (J = 9.7)</td>
<td>4.46 d (J = 9.6)</td>
</tr>
<tr>
<td></td>
<td>4.40 d (J = 9.7)</td>
<td>4.40 d (J = 9.6)</td>
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<td>4.54 s</td>
<td>4.56 m</td>
</tr>
<tr>
<td></td>
<td>4.37 m</td>
<td>4.37 m</td>
</tr>
<tr>
<td>4 upper</td>
<td>4.50 d (J = 7.4)</td>
<td>4.70 d (J = 7.6)</td>
</tr>
<tr>
<td></td>
<td>4.68 d (J = 7.5)</td>
<td>4.52 d (J = 7.6)</td>
</tr>
<tr>
<td>6 upper</td>
<td>5.85 d (J = 2.3)</td>
<td>5.85 d (J = 2.4)</td>
</tr>
<tr>
<td></td>
<td>5.87 - 5.94 m</td>
<td>5.94 d (J = 2.4)</td>
</tr>
<tr>
<td>8 upper</td>
<td>5.87 - 5.94 m</td>
<td>5.88 d (J = 2.4)</td>
</tr>
<tr>
<td></td>
<td>5.87 - 5.94 m</td>
<td>5.95 d (J = 2.4)</td>
</tr>
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<td>4.99 s</td>
<td>5.00 s&lt;sup&gt;br&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>4.88 s</td>
<td>4.88 s&lt;sup&gt;br&lt;/sup&gt;</td>
</tr>
<tr>
<td>3 lower</td>
<td>4.24 s</td>
<td>4.11 s&lt;sup&gt;br&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>4.11 s</td>
<td>4.26 s&lt;sup&gt;br&lt;/sup&gt;</td>
</tr>
<tr>
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<td>masked&lt;sup&gt;e&lt;/sup&gt;</td>
<td>2.94 d&lt;sup&gt;d&lt;/sup&gt; (J = 4.4, 16.8)</td>
</tr>
<tr>
<td></td>
<td>masked&lt;sup&gt;e&lt;/sup&gt;</td>
<td>2.85 d&lt;sup&gt;d&lt;/sup&gt; (J = 4.4, 16.8)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.82 d&lt;sup&gt;d&lt;/sup&gt; (J = 2.4, 16.8)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.73 d&lt;sup&gt;d&lt;/sup&gt; (J = 2.4, 16.8)</td>
</tr>
<tr>
<td>6 lower</td>
<td>6.04 s</td>
<td>6.04 s</td>
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<td>6.20 s</td>
</tr>
<tr>
<td></td>
<td>6.35 - 7.19 m</td>
<td>6.38 - 7.20</td>
</tr>
</tbody>
</table>

<sup>a</sup> Procyanidin B4 was dissolved in acetone-d<sub>6</sub> (300 MHz, 298 K, internal standard: acetone-d<sub>6</sub> = 2.05 ppm). Chemical shifts (δ) are given in ppm; coupling constants (J) are given in Hz. <sup>b</sup> broad signal; <sup>c</sup> solvent: acetone-d<sub>6</sub>; <sup>d</sup> signals are not assigned to the respective protons in the publication; <sup>e</sup> signals are overlapping with water.
Table 10.10 $^1$H-NMR spectral data of peracetylated procyanidin B4.

<table>
<thead>
<tr>
<th>H</th>
<th>Measureda</th>
<th>Published datac</th>
<th>[258]</th>
<th>[213]</th>
<th>[255]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>major rotamer</td>
<td>minor rotamer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 upper</td>
<td>4.83 d ($J = 10.0$)</td>
<td>-</td>
<td>4.85 d ($J = 9.8$)</td>
<td>4.82 d ($J = 9.6$)</td>
<td>4.86 d ($J = 10$)</td>
</tr>
<tr>
<td>3 upper</td>
<td>5.73 t ($J = 9.8, 9.8$)</td>
<td>-</td>
<td>5.67 t</td>
<td>5.72 t</td>
<td>5.82 d (2x)</td>
</tr>
<tr>
<td>4 upper</td>
<td>4.54 d ($J = 9.6$)</td>
<td>5.07 d ($J = 9.5$)</td>
<td>4.52 d ($J = 9.8$)</td>
<td>4.53 d ($J = 9.6$)</td>
<td>4.56 d ($J = 10$)</td>
</tr>
<tr>
<td>6 upper</td>
<td>6.54 d ($J = 2.3$)</td>
<td>6.57 d ($J = 2.4$)</td>
<td>6.52 d ($J = 2.5$)</td>
<td>6.58</td>
<td>6.57 d ($J = 2-3$)</td>
</tr>
<tr>
<td>8 upper</td>
<td>6.59 d ($J = 2.3$)</td>
<td>6.68 d ($J = 2.2$)</td>
<td>6.55 d ($J = 2.5$)</td>
<td>6.58</td>
<td>6.61 d ($J = 2-3$)</td>
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<td>2 lower</td>
<td>5.01 s</td>
<td>-</td>
<td>5.01 s</td>
<td>5.01 s</td>
<td>5.01 s</td>
</tr>
<tr>
<td>3 lower</td>
<td>5.19 - 5.24 m</td>
<td>5.55 - 5.66 m</td>
<td>5.18 m</td>
<td>5.22 m</td>
<td>5.27 d (3x)</td>
</tr>
<tr>
<td>4 lower</td>
<td>2.73 - 2.97 m$^d$</td>
<td>3.02 dd ($J = 5.0, 18.0$)</td>
<td>2.91 m</td>
<td>n.s.</td>
<td>(J = 1.8, &lt; 2, 5)</td>
</tr>
<tr>
<td>6 lower</td>
<td>6.64 s</td>
<td>6.74 s$^e$</td>
<td>6.60 s</td>
<td>6.63</td>
<td>6.67 s</td>
</tr>
<tr>
<td>B / E rings</td>
<td>6.86 - 7.47 m</td>
<td>6.86 - 7.47 m</td>
<td>6.85 - 7.30 m</td>
<td>n.s.</td>
<td>6.9 - 7.53 m</td>
</tr>
<tr>
<td>acetates</td>
<td>1.65 - 2.35 m</td>
<td>1.65 - 2.35 m</td>
<td>1.60 - 2.40 m</td>
<td>n.s.</td>
<td>1.62 - 2.30 m</td>
</tr>
</tbody>
</table>

Procyanidin B4 was dissolved in chloroform-d$_1$ (300 MHz, 298 K, internal standard: chloroform-d$_1$ = 7.27 ppm). Chemical shifts ($) are given in ppm; coupling constants ($J$) are given in Hz. $^b$ broad signal; $^c$ solvent: chloroform-d$_1$; $^d$ one doublet ($J = 18.0$) at 2.76 ppm is present; $^e$ tentatively assigned (from rotamer populations); n.s. = not specified.
10.4.5 Procyanidin C1

NMR. The measured NMR data of procyanidin C1 in methanol-\textit{d}_4 are similar to the one reported in acetone-\textit{d}_6 (see Table 10.11). The chemical shifts of the peracetylated procyanidin C1 are in agreement with the reported data (see Table 10.12).

Hör et al. [101,257] succeeded in assigning all A- and C-ring proton resonances of the three flavan-3-ol units in peracetylated procyanidin C1 by long-range $^1$H-$^1$H-COSY experiments on a 400 MHz apparatus. For the first time, the appearance of two rotamer sets in a ratio of 1 : 2 is described. The identity of procyanidin C1 was additionally confirmed by partial thiolytic degradation.

Based on these published data, all resonances in the spectrum of procyanidin C1 could be assigned. The presence of two rotamer populations in the peracetate in an approximate ratio of 1 : 2-3 could also be confirmed.

MS. The molecular ion of peracetylated procyanidin C1 appears at m/z 1497. The sodium adduct (m/z 1520) is also detectable. Loss of an aliphatic acetoxy (u 60) followed by the loss of acetyl (u 43) and keten (u 42) fragments of aromatic acetates leads to the ions at m/z 1437, 1394, 1351, 1309 and 1267. The ion at m/z 1437 undergoes RDA-fission after the elimination of one keten fragment (m/z 1395 - u 278 = m/z 1117). Sequential elimination of an acetoxy followed by acetyl and keten groups from the same fragment ion (m/z 1437) yields the ions at m/z 1377, 1335, 1292, 1249 and 1207. The loss of a keten group and elimination of water produces ions at m/z 1455 and 1437 from the molecular ion (m/z 1497). The principal signals in the spectrum are between m/z 1393 - 1395.

In the MS spectrum of procyanidin C1 the molecular ion (m/z 867) and a dimeric fragment (m/z 579) are present. Ions which are due to RDA-fission (m/z 409 and 425) are also detectable in low abundances. The signal at m/z 329 is due to the cleavage of the lower interflavanoid linkage and addition of potassium to the resulting monomeric fragment. Again, the spectrum is dominated by matrix signals originating from 3-nitrobenzyl alcohol.
Table 10.11 $^1$H-NMR spectral data of procyanidin C1.

<table>
<thead>
<tr>
<th>H</th>
<th>Measured$^a$</th>
<th>Published data$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\delta$ (ppm) $J$ (Hz)</td>
<td>[101]</td>
</tr>
<tr>
<td>2 upper</td>
<td>4.99 s, 5.08 s or 5.22 s$^b$</td>
<td>5.06 s$^b$ or 5.15 s$^b$</td>
</tr>
<tr>
<td>3 upper</td>
<td>4.02 m$^b$</td>
<td>4.07 s$^b$</td>
</tr>
<tr>
<td>4 upper</td>
<td>4.71 s</td>
<td>4.80 s$^b$ or 4.82 s$^b$</td>
</tr>
<tr>
<td>6 upper</td>
<td>5.93 - 6.00 m$^b$</td>
<td>5.96 - 6.03 m</td>
</tr>
<tr>
<td>8 upper</td>
<td>5.93 - 6.00 m$^b$</td>
<td>5.96 - 6.03 m</td>
</tr>
<tr>
<td>2 middle</td>
<td>4.99 s, 5.08 s or 5.22 s$^b$</td>
<td>5.06 s$^b$ or 5.15 s$^b$</td>
</tr>
<tr>
<td>3 middle</td>
<td>4.02 m$^b$</td>
<td>4.07 s$^b$</td>
</tr>
<tr>
<td>4 middle</td>
<td>4.71 s</td>
<td>4.80 s$^b$ or 4.82 s$^b$</td>
</tr>
<tr>
<td>6 middle</td>
<td>5.93 - 6.00 m$^b$</td>
<td>5.96 - 6.03 m</td>
</tr>
<tr>
<td>2 lower</td>
<td>4.99 s, 5.08 s or 5.22 s$^b$</td>
<td>5.06 s$^b$ or 5.15 s$^b$</td>
</tr>
<tr>
<td>3 lower</td>
<td>4.32 s$^b$</td>
<td>4.33 s$^b$</td>
</tr>
<tr>
<td>4 lower</td>
<td>2.78 - 3.08 m</td>
<td>~2.7 - 2.8$^d$</td>
</tr>
<tr>
<td>6 lower</td>
<td>5.93 - 6.00 m$^b$</td>
<td>5.96 - 6.03 m</td>
</tr>
<tr>
<td>B / E / H rings</td>
<td>6.50 - 7.13 m$^b$</td>
<td>6.68 - 7.17 m</td>
</tr>
</tbody>
</table>

$^a$ Procyanidin C1 was dissolved in methanol-d$_4$ (300 MHz, 298 K, internal standard: methanol-d$_4$ = 3.31 ppm). $^b$ broad signal; $^c$ solvent: acetone-d$_6$; $^d$ signals overlapping with water.
Table 10.12 ¹H-NMR spectral data of peracetylated procyanidin C1.

<table>
<thead>
<tr>
<th>H</th>
<th>Measured assignments according to:</th>
<th>Published data⁹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[101,257] major rotamer</td>
<td>minor rotamer</td>
</tr>
<tr>
<td>2 upper</td>
<td>5.41 m</td>
<td>5.71 s</td>
</tr>
<tr>
<td>3 upper</td>
<td>5.41 m</td>
<td>4.96 s</td>
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<tr>
<td>4 upper</td>
<td>4.79 s</td>
<td>4.51 s</td>
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<td>6 upper</td>
<td>6.66 s</td>
<td>6.27 s</td>
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<td>8 upper</td>
<td>6.77 s&lt;sup&gt;br&lt;/sup&gt;</td>
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<tr>
<td>2 middle</td>
<td>5.41 m</td>
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<tr>
<td>3 middle</td>
<td>5.41 m</td>
<td>5.12 s</td>
</tr>
<tr>
<td>4 middle</td>
<td>4.71 s</td>
<td>4.67 s</td>
</tr>
<tr>
<td>6 middle</td>
<td>6.66 s</td>
<td>6.90 s or 6.61 s</td>
</tr>
<tr>
<td>2 lower</td>
<td>5.21 s</td>
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</tr>
<tr>
<td>3 lower</td>
<td>5.48 s</td>
<td>5.41 m</td>
</tr>
<tr>
<td>4 lower</td>
<td>2.94 - 3.07 m&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2.94 - 3.07 m&lt;sup&gt;d&lt;/sup&gt;</td>
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<td>(&lt;i&gt;J&lt;/i&gt; = 18.0)</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 lower</td>
<td>6.72 s</td>
<td>6.61 s or 6.90 s</td>
</tr>
<tr>
<td>B / E / H rings</td>
<td>6.77 - 7.37 m</td>
<td>6.77 - 7.37 m</td>
</tr>
<tr>
<td>acetates</td>
<td>1.39 - 2.38 m</td>
<td>1.39 - 2.38 m</td>
</tr>
</tbody>
</table>

* Procyanidin C1 was dissolved in chloroform-<i>d</i><sub>1</sub> (300 MHz, 298 K, internal standard: chloroform-<i>d</i><sub>1</sub> = 7.27 ppm). Chemical shifts (δ) are given in ppm; coupling constants (<i>J</i>) are given in Hz. <sup>b</sup> broad signal; <sup>c</sup> solvent: chloroform-<i>d</i><sub>1</sub>; <sup>d</sup> one doublet (<i>J</i> = 17.8) at 2.97 ppm is present; <sup>e</sup> signals are overlapping with the ones of the major rotamer.
[\alpha]_D Isolated procyanidin C1: 
\[ \alpha \]_{378} = +71.6° (methanol, c = 0.1105, at 20.0 °C)

Literature values: 
\[ \alpha \]_{378} = +76.4° (acetone, c = 0.86, at 27 °C) [101]

CD: Isolated procyanidin C1:
\[ \theta \]_{289.6} = -4067
\[ \theta \]_{241.8} = +42'416

Literature values [232]:
\( \Delta \varepsilon_{284} = +0.47 \) (methanol)
\( \Delta \varepsilon_{276} = -1.25 \)
\( \Delta \varepsilon_{230} = +16.40 \)
\( \equiv [\theta]_{284} = +1551 \)
\( \equiv [\theta]_{276} = -4125 \)
\( \equiv [\theta]_{230} = +54'120 \)

The sign of the CD band at 242 nm suggests the β-configuration of the aryl substituents at the C4 of the upper and middle flavan-3-ol units. But, CD spectra of trimers and higher oligomers do not necessarily follow the spectroscopic rule which has been established for dimers (see section 9.1.2). Interpretations therefore have to be done with great caution.

10.4.6 Trimer I

NMR. The identification of trimer I by Hör et al. [101,257] is based on thiolytic degradation. The assignments of the proton resonances of the peracetylated derivative were established by \(^1\)H-\(^1\)H-COSY experiments and comparison of chemical shift parameters with literature values.

Based on these published data, all resonances in the spectrum of trimer I could be assigned (see Tables 10.13 and 10.14). The multiplet of the protons at C4 of the lower unit were masked by water in the spectrum recorded in acetone-\(d_6\) in the investigation presented here. The multiplet however readily showed up when the spectrum was recorded in methanol-\(d_4\). In the peracetylated derivative some minor signals are detectable which are also present in the spectrum published by Hör [257]. The existence of a minor rotamer can therefore not definitely be ruled out, although Hör postulated that peracetylated oligomers with an upper 4 \(\rightarrow\) 6 interflavanoid linkage only show one conformer in chloroform-\(d_1\).
Table 10.13 \(^1\)H-NMR spectral data of trimer I.

<table>
<thead>
<tr>
<th>H</th>
<th>Measured(^a)</th>
<th>Published data(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(\delta) (ppm)</td>
<td>(J) (Hz)</td>
</tr>
<tr>
<td>2 upper</td>
<td>4.92 s(^{br})</td>
<td>4.93 s(^{br})</td>
</tr>
<tr>
<td>3 upper</td>
<td>3.97 m(^{br})</td>
<td>3.95 m or 3.98 s(^{br})</td>
</tr>
<tr>
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<tr>
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<td>5.97 - 6.09 m</td>
<td>5.95 - 6.10 m</td>
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<td>5.97 - 6.09 m</td>
<td>5.95 - 6.10 m</td>
</tr>
<tr>
<td>2 middle</td>
<td>4.92 s(^{br})</td>
<td>4.93 s(^{br})</td>
</tr>
<tr>
<td>3 middle</td>
<td>3.97 m(^{br})</td>
<td>3.98 s(^{br}) or 3.95 m</td>
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<td>4.67 s or 4.58 s</td>
<td>4.68 s(^{br}) or 4.58 s(^{br})</td>
</tr>
<tr>
<td>8 middle</td>
<td>5.97 - 6.09 m</td>
<td>5.95 - 6.10 m</td>
</tr>
<tr>
<td>2 lower</td>
<td>4.92 s(^{br})</td>
<td>4.93 s(^{br})</td>
</tr>
<tr>
<td>3 lower</td>
<td>4.26 s(^{br})</td>
<td>4.26 m</td>
</tr>
<tr>
<td>4 lower</td>
<td>masked(^e)</td>
<td>2.68 d(^{br}) ((J = n.s.))</td>
</tr>
<tr>
<td>6 lower</td>
<td>5.97 - 6.09 m</td>
<td>5.95 - 6.10 m</td>
</tr>
<tr>
<td>B / E / H rings</td>
<td>6.65 - 7.09 m</td>
<td>6.65 - 7.09 m</td>
</tr>
</tbody>
</table>

\(^a\) Trimer I was dissolved in acetone-\(d_6\) (300 MHz, 298 K, internal standard: acetone-\(d_6\) = 2.05 ppm). 
\(^{br}\) broad signal; \(^c\) solvent: acetone-\(d_6\); \(^d\) in acetone-\(d_6\) + D\(_2\)O; \(^e\) signals are overlapping with water.

**MS.** The MS spectrum of peracetylated trimer I is similar to the one of peracetylated procyanidin C1. The molecular ion and a sodium adduct are at m/z 1497 and 1520, respectively. Losses of acetoxy, acetyl and keten fragments from the molecular ion leads to the ions at m/z 1437, 1394, 1351, 1309 and 1267. Analogous losses from the ion at m/z 1437 produces the fragments at m/z 1377, 1335, 1293, 1249 and 1207. RDA-fission after elimination of one keten fragment (m/z 1437 - u 42 = m/z 1395 - u 278) produces the ion at m/z 1117. The principal signals in the spectrum are between m/z 1393 - 1395. Some impurities seem to be present, since fragments with higher masses can not be assigned (m/z 1629 and 1686).

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Table 10.14  $^1$H-NMR spectral data of peracetylated trimer I.

<table>
<thead>
<tr>
<th>H</th>
<th>Measured* $\delta$ (ppm) $J$ (Hz)</th>
<th>Published data*</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 upper</td>
<td>5.70 s</td>
<td>5.67 s$^{br}$</td>
</tr>
<tr>
<td>3 upper</td>
<td>4.96 m</td>
<td>4.94 m</td>
</tr>
<tr>
<td>4 upper</td>
<td>4.50 s</td>
<td>4.48 d ($J = 1.6$)</td>
</tr>
<tr>
<td>6 upper</td>
<td>6.59 d (-)</td>
<td>6.57 d ($J = 2.25$)</td>
</tr>
<tr>
<td>8 upper</td>
<td>6.67 ($J = 2.0$)</td>
<td>6.65 d ($J = 2.25$)</td>
</tr>
<tr>
<td>2 middle</td>
<td>5.48 s</td>
<td>5.46 s$^{br}$</td>
</tr>
<tr>
<td>3 middle</td>
<td>5.29 m</td>
<td>5.27 m</td>
</tr>
<tr>
<td>4 middle</td>
<td>4.41 s</td>
<td>4.39 s$^{br}$</td>
</tr>
<tr>
<td>8 middle</td>
<td>6.86 s</td>
<td>6.84 s</td>
</tr>
<tr>
<td>2 lower</td>
<td>5.17 s</td>
<td>5.14 s$^{br}$</td>
</tr>
<tr>
<td>3 lower</td>
<td>5.48 s</td>
<td>5.46 s$^{br}$</td>
</tr>
<tr>
<td>4 lower</td>
<td>2.88 - 3.08 m</td>
<td>2.88 - 3.12 m</td>
</tr>
<tr>
<td>6 lower</td>
<td>6.49 s</td>
<td>6.47 s</td>
</tr>
<tr>
<td>B/E/H rings</td>
<td>7.07 - 7.54 m</td>
<td>7.04 - 7.51 m</td>
</tr>
<tr>
<td>acetates</td>
<td>1.23 - 2.41 m</td>
<td>1.25 - 2.38 m</td>
</tr>
</tbody>
</table>

* Trimer I was dissolved in chloroform-d$_3$ (300 MHz, 298 K, internal standard: chloroform-d$_3$ = 7.27 ppm). $^{br}$ broad signal; * solvent: chloroform-d$_3$.

10.4.7 Trimer II

$NMR$. In the literature, the composing units of trimer II have been established from the results of thiolysis studies, while the position of linkage (4→8 or 4→6) has been determined based on chemical shift parameters [39,70].

The identity of trimer II could only be established on the basis of the data reported for the native compound (see Table 10.15). The spectrum of the peracetylated derivative is characterized by the phenomenon of extensive rotational isomerism which makes it impossible to assign any individual resonances (see Table 10.16). The appearance of the spectrum did not change even after additional clean up of the
derivative by a second preparative TLC. The recording of the spectrum in benzene-d$_6$ on a 600 MHz NMR apparatus did not yield better resolution either. Based on the spectroscopic data of the phenolic compound the identity of trimer II is considered to be tentatively established.

The NMR spectra of most peracetylated procyanidins show a better resolution than the spectra of their native compounds. Trimer II shows a rather unusual behavior, in that the spectrum of the peracetylated derivative exhibits much more line broadening and overlapping resonances than the spectrum of the phenolic compound. However, this same behavior is also found with procyanidin B5 and the presence of 4→6 interflavanoid linkages in trimer II is therefore very likely.

Table 10.15 $^1$H-NMR spectral data of trimer II.

<table>
<thead>
<tr>
<th>H</th>
<th>Measured$^a$</th>
<th>Published data$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\delta$(ppm)</td>
<td>[39]</td>
</tr>
<tr>
<td>2 upper</td>
<td>4.91 s</td>
<td>2.9 - 5.0 m</td>
</tr>
<tr>
<td>3 upper</td>
<td>3.96 s or 4.01 s</td>
<td>2.9 - 5.0 m</td>
</tr>
<tr>
<td>4 upper</td>
<td>4.84 s</td>
<td>2.9 - 5.0 m</td>
</tr>
<tr>
<td>6 upper</td>
<td>6.09 - 6.14 m</td>
<td>6.0 - 6.2 m</td>
</tr>
<tr>
<td>8 upper</td>
<td>6.09 - 6.14 m</td>
<td>6.0 - 6.2 m</td>
</tr>
<tr>
<td>2 middle</td>
<td>4.89 s</td>
<td>2.9 - 5.0 m</td>
</tr>
<tr>
<td>3 middle</td>
<td>4.01 s or 3.96 s</td>
<td>2.9 - 5.0 m</td>
</tr>
<tr>
<td>4 middle</td>
<td>4.49 s</td>
<td>2.9 - 5.0 m</td>
</tr>
<tr>
<td>8 middle</td>
<td>6.09 - 6.14 m</td>
<td>6.0 - 6.2 m</td>
</tr>
<tr>
<td>2 lower</td>
<td>4.89 s</td>
<td>2.9 - 5.0 m</td>
</tr>
<tr>
<td>3 lower</td>
<td>4.31 s$^{br}$</td>
<td>2.9 - 5.0 m</td>
</tr>
<tr>
<td>4 lower</td>
<td>masked$^e$</td>
<td>2.9 - 5.0 m</td>
</tr>
<tr>
<td>8 lower</td>
<td>6.09 - 6.14 m</td>
<td>6.0 - 6.2 m</td>
</tr>
<tr>
<td>B / E / H rings</td>
<td>6.62 - 7.14 m</td>
<td>6.6 - 7.2 m</td>
</tr>
</tbody>
</table>

$^a$ Trimer II was dissolved in acetone-d$_6$ (300 MHz, 298 K, internal standard: acetone-d$_6$ = 2.05 ppm).
$^{br}$ broad signal; $^c$ solvent: acetone-d$_6$; $^d$ in acetone-d$_6$ + D$_2$O; $^e$ signals are overlapping with water.
Table 10.16 $^1$H-NMR spectral data of peracetylated trimer II.

<table>
<thead>
<tr>
<th>H</th>
<th>Measured$^a$</th>
<th>Published data$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\delta$ (ppm)</td>
<td></td>
</tr>
<tr>
<td>heterocyclic (C, F, I rings)</td>
<td>3.9 - 5.7 m</td>
<td>4.2 - 5.5 m</td>
</tr>
<tr>
<td>4 lower</td>
<td>2.9 - 3.1 m</td>
<td>2.9 m</td>
</tr>
<tr>
<td>aromatic (A, D, G rings)</td>
<td>6.5 - 6.9 m</td>
<td>6.6 - 6.7 m</td>
</tr>
<tr>
<td>aromatic (B, E, H rings)</td>
<td>7.0 - 7.5 m</td>
<td>7.0 - 7.5 m</td>
</tr>
<tr>
<td>acetates</td>
<td>1.6 - 2.3 m</td>
<td>1.6 - 2.4 m</td>
</tr>
</tbody>
</table>

$^a$ Trimer II was dissolved in chloroform-d$_4$ (300 MHz, 298 K, internal standard: chloroform-d$_4$ = 7.27 ppm). $^c$ solvent: chloroform-d$_4$.

**MS.** The spectrum of peracetylated trimer II looks similar to the ones of the derivatives of trimer I and procyanidin C1. The molecular ion and its sodium adduct are detectable at m/z 1497 and 1520, respectively. Losses of acetoxy, acetyl and keten fragments lead to the fragments at m/z 1437, 1394, 1377, 1351, 1309, 1293, 1267, 1249 and 1207. The fragment at m/z 1117 results from RDA-fission of the ion at m/z 1437 after elimination of one keten. m/z 1057 results from loss of an acetoxy group from the fragment at m/z 1117. The principal signals in the spectrum are again between m/z 1393 - 1395.

**10.4.8 Tetramer I**

**NMR.** Hör et al. [101,257] established the structure of tetramer I by thiolysis and HPLC analysis of the degradation products. The proton resonances of the peracetylated compound were assigned based on long-range $^1$H-$^1$H-COSY couplings using a 400 MHz NMR apparatus. Two rotamers could be detected which occur in an approximate ratio of 3 : 2. Tetramer I has been isolated and named cinnamtannin A$_1$ by other researchers, but no spectroscopic data are reported [59].

Based on the data published by Hör, all resonances in the spectrum of tetramer I could be assigned (see Tables 10.17 and 10.18). The multiplet of the protons at C4 were detectable when the spectrum was recorded in methanol-d$_4$. The peracetylated
The derivative was additionally cleaned up by TLC, but the resolution of the signals (i.e. signals around 3 ppm, attributable to the protons at position 4 of the lower unit) could not be improved. The presence of two rotamer populations in the peracetate in an approximate ratio of 2:3-4 could be confirmed.

### Table 10.17 ¹H-NMR spectral data of tetramer I.

<table>
<thead>
<tr>
<th>H</th>
<th>Measuredᵃ</th>
<th>Published dataᶜ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>δ (ppm) J (Hz)</td>
<td></td>
</tr>
<tr>
<td>2 upper</td>
<td>5.08 sᵇʳ, 5.15 sᵇʳ or 5.30 sᵇʳ</td>
<td>5.07 sᵇʳ, 5.15 sᵇʳ or 5.29 sᵇʳ</td>
</tr>
<tr>
<td>3 upper</td>
<td>4.11 sᵇʳ</td>
<td>4.08 sᵇʳ or 4.12 sᵇʳ</td>
</tr>
<tr>
<td>4 upper</td>
<td>4.83 sᵇʳ or 4.90 sᵇʳ</td>
<td>4.83 sᵇʳ or 4.90 sᵇʳ</td>
</tr>
<tr>
<td>6 upper</td>
<td>6.00 mᵇʳ</td>
<td>5.96 - 6.03 m</td>
</tr>
<tr>
<td>8 upper</td>
<td>6.00 mᵇʳ</td>
<td>5.96 - 6.03 m</td>
</tr>
<tr>
<td>2 upper middle</td>
<td>5.08 sᵇʳ, 5.15 sᵇʳ or 5.30 sᵇʳ</td>
<td>5.07 sᵇʳ, 5.15 sᵇʳ or 5.29 sᵇʳ</td>
</tr>
<tr>
<td>3 upper middle</td>
<td>4.11 sᵇʳ</td>
<td>4.08 sᵇʳ or 4.12 sᵇʳ</td>
</tr>
<tr>
<td>4 upper middle</td>
<td>4.83 sᵇʳ or 4.90 sᵇʳ</td>
<td>4.83 sᵇʳ or 4.90 sᵇʳ</td>
</tr>
<tr>
<td>6 upper middle</td>
<td>6.00 mᵇʳ</td>
<td>5.96 - 6.03 m</td>
</tr>
<tr>
<td>2 lower middle</td>
<td>5.08 sᵇʳ, 5.15 sᵇʳ or 5.30 sᵇʳ</td>
<td>5.07 sᵇʳ, 5.15 sᵇʳ or 5.29 sᵇʳ</td>
</tr>
<tr>
<td>3 lower middle</td>
<td>4.11 sᵇʳ</td>
<td>4.08 sᵇʳ or 4.12 sᵇʳ</td>
</tr>
<tr>
<td>4 lower middle</td>
<td>4.83 sᵇʳ or 4.90 sᵇʳ</td>
<td>4.83 sᵇʳ or 4.90 sᵇʳ</td>
</tr>
<tr>
<td>6 lower middle</td>
<td>6.00 mᵇʳ</td>
<td>5.96 - 6.03 m</td>
</tr>
<tr>
<td>2 lower</td>
<td>5.08 sᵇʳ, 5.15 sᵇʳ or 5.30 sᵇʳ</td>
<td>5.07 sᵇʳ, 5.15 sᵇʳ or 5.29 sᵇʳ</td>
</tr>
<tr>
<td>3 lower</td>
<td>4.35 sᵇʳ</td>
<td>4.35 sᵇʳ</td>
</tr>
<tr>
<td>4 lower</td>
<td>maskedᵈ</td>
<td>~ 2.7 - 2.8ᵈ</td>
</tr>
<tr>
<td>6 lower</td>
<td>6.00 mᵇʳ</td>
<td>5.96 - 6.03 m</td>
</tr>
<tr>
<td>B / E / H / L rings</td>
<td>6.69 - 7.19 m</td>
<td>6.68 - 7.17 m</td>
</tr>
</tbody>
</table>

ᵃ Tetramer I was dissolved in acetone-d₆ (300 MHz, 298 K, internal standard: acetone-d₆ = 2.05 ppm).
ᵇʳ broad signal; ᶜ solvent: acetone-d₆; ᵈ signals are overlapping with water.
Table 10.18  $^1$H-NMR spectral data of peracetylated tetramer I.

<table>
<thead>
<tr>
<th>H</th>
<th>Measured$^a$</th>
<th></th>
<th>Published data$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>major rotamer</td>
<td>minor rotamer</td>
<td>major rotamer</td>
</tr>
<tr>
<td>2 upper</td>
<td>5.45 s</td>
<td>5.75 s</td>
<td>5.42 s$^{br}$</td>
</tr>
<tr>
<td>3 upper</td>
<td>5.32 s</td>
<td>4.97 s</td>
<td>5.29 m</td>
</tr>
<tr>
<td>4 upper</td>
<td>4.78 s</td>
<td>4.52 s</td>
<td>4.75 s$^{br}$</td>
</tr>
<tr>
<td>6 upper</td>
<td>6.66 s</td>
<td>6.26 d ($J=2.03$)</td>
<td>6.63 d ($J=2.25$)</td>
</tr>
<tr>
<td>8 upper</td>
<td>6.78 d ($J=2.27$)</td>
<td>5.90 s</td>
<td>6.75 d ($J=2.25$)</td>
</tr>
<tr>
<td>2 upper middle</td>
<td>5.45 s</td>
<td>4.57 s</td>
<td>5.42 s$^{br}$</td>
</tr>
<tr>
<td>3 upper middle</td>
<td>5.36 s</td>
<td>5.16 s</td>
<td>5.33 s$^{br}$</td>
</tr>
<tr>
<td>4 upper middle</td>
<td>4.85 s</td>
<td>4.81 s</td>
<td>4.82 s$^{br}$</td>
</tr>
<tr>
<td>6 upper middle</td>
<td>6.75 s</td>
<td>6.90 s</td>
<td>6.73 s</td>
</tr>
<tr>
<td>2 lower middle</td>
<td>5.29 s</td>
<td>5.36 s</td>
<td>5.26 s$^{br}$</td>
</tr>
<tr>
<td>3 lower middle</td>
<td>5.34 s</td>
<td>5.29 s</td>
<td>5.31 m</td>
</tr>
<tr>
<td>4 lower middle</td>
<td>4.68 s</td>
<td>4.63 s</td>
<td>4.65 s$^{br}$</td>
</tr>
<tr>
<td>6 lower middle</td>
<td>6.71 s</td>
<td>6.63 s</td>
<td>6.69 s</td>
</tr>
<tr>
<td>2 lower</td>
<td>5.21 s</td>
<td>5.21 s</td>
<td>5.18 s$^{br}$</td>
</tr>
<tr>
<td>3 lower</td>
<td>5.49 s</td>
<td>5.49 s</td>
<td>5.46 s$^{br}$</td>
</tr>
<tr>
<td>4 lower</td>
<td>3.05 m$^{br}$</td>
<td>3.05 m$^{br}$</td>
<td>2.94 d$^{br}$($J=18.0$)</td>
</tr>
<tr>
<td>6 lower</td>
<td>6.66 s</td>
<td>6.59 s</td>
<td>6.63 s</td>
</tr>
<tr>
<td>B/E/H/L rings</td>
<td>6.94 - 7.38 m</td>
<td>6.94 - 7.38 m</td>
<td>6.95 - 7.34 m</td>
</tr>
<tr>
<td>acetates</td>
<td>1.27 - 2.39 m</td>
<td>1.27 - 2.39 m</td>
<td>1.33 - 2.36 m</td>
</tr>
</tbody>
</table>

$^a$ Tetramer I was dissolved in chloroform-d$_4$ (300 MHz, 298 K, internal standard: chloroform-d$_4$ = 7.27 ppm). Chemical shifts ($\delta$) are given in ppm; coupling constants ($J$) are given in Hz. $^{br}$ broad signal; $^c$ solvent: chloroform-d$_4$.  

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MS. The molecular ion of peracetylated tetramer I is detectable at m/z 1998 (one additional mass unit is due to the natural abundance of the $^{13}$C isotop). The sodium adduct occurs at m/z 2021. The loss of an acetoxy group and two hydrogens leads to the mass fragment at m/z 1936. Losses of another acetoxy group, keten and acetyl fragments, respectively, lead to the ions at m/z 1893, 1936, 1876, 1850, 1833, 1808, 1791, 1765, 1749, 1723 and 1706. The ion at m/z 1731 originates from the loss of water from fragment m/z 1749. RDA-fission takes place with the fragment m/z 1791 and yields the ion at m/z 1513. The principal signals are between m/z 1893 - 1892.

10.4.9 Artifact I

NMR. The appearance of the $^1$H-NMR spectrum of artifact I is almost identical to the one of (-)-epicatechin (see Table 10.19 and Appendix I). The following deviations are obvious in the spectrum of artifact I: The methylene proton resonances, which are absent in the spectrum of (-)-epicatechin, occur at $\delta = 3.84$ ppm. The doublet due to the proton at C8 is missing, and the doublet at position 6 appears as a singlet due to the lack of a proton in meta position in the aromatic A-ring. The downfield shift of the residual proton at the A-ring is typical of compounds that are substituted at position 6 or 8, respectively [216]. All these features are also obvious in the couple bis-8-8'-catechinylmethane / (+)-catechin (see Table 10.19).

The assignments for the proton and carbon resonances in the peracetylated derivative (see Table 10.20) was accomplished by interpreting the $^{13}$C-NMR, DEPT as well as two-dimensional spectra (DQF-COSY, HMQC, HMBC). The $^1$H-NMR spectrum exhibited the same features compared to the spectrum of peracetylated (-)-epicatechin as outlined above. The assignments for the B-ring protons could not be established unambiguously. The following assignments were tentatively made: H2': 6.94 ppm d ($J = 1.7$), H5': 6.98 ppm d ($J = 7.1$) and H6': 7.08 ppm s and 7.11 ppm s.

The chemical shifts of the carbon resonances of peracetylated artifact I are almost identical to the ones of (-)-epicatechin (see also Table 10.3). Only the chemical shift of the carbon at position 8 is significantly shifted downfield by 10 ppm, which suggests that the methylene group is linked at this position. From the DEPT it can be concluded that the carbon at position 8 is quaternary. The resonance of the methylene bridge is detectable at 19.17 ppm. It shows an intensity which is about half as high as the one of
the other carbon signals. This indicates the presence of a symmetrical dimer with respect to this group. In the HMBC spectrum correlations between the methylene proton resonances and C8, C5 / C7 and C8a are visible. The calculation of the chemical shift of the methylene bridge in a 8-8'-linked analogous phenolic dimer with the SpecTool® software revealed a value of 17.7 ppm. Calculations assuming 8-6'- and 6-6'-linkages gave even lower chemical shift values. All these data suggest that the methylene bridge is located between the positions 8 of the two monomeric units. This stands in line with the literature data: studies on the polymerization of (+)-catechin with formaldehyde also revealed that the C8 position is more reactive than the C6 position [151].

Because artifact I has never been detected in freshly prepared samples it is assumed that this compound has been formed during the isolation procedure (see section 8.3.5.3.2). An analogous reaction sequence as described in section Isolation 8.3.5.3.5 and illustrated in Figure 8.4 is conceivable. The electrophilic attack of formaldehyde (presumably generated from methanol by coupled oxidation with chlorogenic acid) to position 8 of (-)-epicatechin leads to the 8-methylol-derivative. After loss of water the carbocation thus produced may crosslink to a second (-)-epicatechin, of which position 8 seems to be the most reactive site. Such methylene-bridged derivatives of procyandinins have also been reported in studies on condensation of (+)-catechin with formaldehyde [149,151].

MS. The molecular ion of peracetylated artifact I is detectable at m/z 1013 and its sodium adduct at m/z 1035. Fission of the interflavanoid linkage in the molecular ion leads to the fragment m/z 513, which is a monomer fragment with a methylene group attached to it. Sequential loss of keten fragments leads to the ions at m/z 471, 429, 387 and 345. Loss of the methylene group produces m/z 331 from which another keten fragment is cleaved leading to the fragment at m/z 289. Analogous cascades take place after initial loss of an acetoxy group from the fragments at m/z 471 (producing ions at m/z 411, 397, 355, 313, 271) and m/z 429 (producing ions at m/z 369, 327, 285, 271). Loss of a keten fragment from the molecular ion generates the fragment at m/z 971 which undergoes RDA-fission. The resulting fragment at m/z 693 looses a keten fragment (m/z 651) before the interflavanoid linkage is cleaved thus releasing the ion at m/z 439. This ion in turn looses another four keten fragments (m/z 397, 355, 313 and
Table 10.19  $^1$H-NMR spectral data of artifact I and related compounds.

<table>
<thead>
<tr>
<th>H</th>
<th>Artifact I</th>
<th>(-)-Epicatechin</th>
<th>bis-8-8'-Catechinyl-methane</th>
<th>(+)-Catechin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>measured$^a$</td>
<td>measured$^a$</td>
<td>according to [151]$^c$</td>
<td>measured$^{a,d}$</td>
</tr>
<tr>
<td>2</td>
<td>5.03 s</td>
<td>4.88 s</td>
<td>4.69 d ($J = 8$)</td>
<td>4.55 d ($J = 7.8$)</td>
</tr>
<tr>
<td>3</td>
<td>4.25 s$^{br}$</td>
<td>4.20 s$^{br}$</td>
<td>4.07 m</td>
<td>3.31 - 4.02 m</td>
</tr>
<tr>
<td>4</td>
<td>masked$^e$</td>
<td>2.87 dd ($J = 4.5, 16.9$)</td>
<td>2.92 dd ($J = 5, 15$)</td>
<td>masked$^e$</td>
</tr>
<tr>
<td>6</td>
<td>5.97 s</td>
<td>6.02 d ($J = 2.28$)</td>
<td>5.98 s</td>
<td>6.02 s</td>
</tr>
<tr>
<td>8</td>
<td>missing</td>
<td>5.92 d ($J = 2.26$)</td>
<td>missing</td>
<td>5.87 d ($J = 1.7$)</td>
</tr>
<tr>
<td>methylene bridge C9</td>
<td>3.84 s</td>
<td>missing</td>
<td>3.60 s</td>
<td>missing</td>
</tr>
<tr>
<td>2'</td>
<td>7.16 s</td>
<td>7.05 d ($J = 1.59$)</td>
<td>6.79 s</td>
<td>6.89 s</td>
</tr>
<tr>
<td>5'</td>
<td>6.82 d ($J = 8.1$)</td>
<td>6.78 d ($J = 8.11$)</td>
<td>6.94 s</td>
<td>6.72 - 6.80 m</td>
</tr>
<tr>
<td>6'</td>
<td>6.93 d ($J = 8.0$)</td>
<td>6.84 dd ($J = 1.9, 8.1$)</td>
<td>6.79 s</td>
<td>6.72 - 6.80 m</td>
</tr>
</tbody>
</table>

$^a$ Compounds were dissolved in acetone-d$_6$ (300 MHz, 298 K, internal standard: acetone-d$_6$ = 2.05 ppm). Chemical shifts (δ) are given in ppm; coupling constants (J) are given in Hz. $^b$ broad signal; $^c$ solvent: acetone-d$_6$; $^d$ assignments according to [197]; $^e$ signals are overlapping with water.
Finally, losses of acetoxy, keten and acetyl groups from the molecular ion at m/z 1013 produce the fragments at m/z 953, 910, 893, 867, 851, 825 and 809. The principal signals are between m/z 471 - 472.

Table 10.20 $^1$H-NMR and $^{13}$C-NMR spectral data of peracetylated artifact I.

<table>
<thead>
<tr>
<th>H / C</th>
<th>$^{13}$C-NMR&lt;sup&gt;a&lt;/sup&gt;</th>
<th>$^1$H-NMR&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\delta$ (ppm)</td>
<td>$\delta$ (ppm) $J$ (Hz)</td>
</tr>
<tr>
<td>2</td>
<td>77.2</td>
<td>4.29 s</td>
</tr>
<tr>
<td>3</td>
<td>66.5</td>
<td>5.18 s&lt;sup&gt;br&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td>26.1</td>
<td>2.78 s (2x)&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>4a</td>
<td>109.5</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>147.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>108.8</td>
<td>6.53 s</td>
</tr>
<tr>
<td>7</td>
<td>147.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
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<tr>
<td>8a</td>
<td>153.2</td>
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<td>methylene bridge C9</td>
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<td>3.86 s</td>
</tr>
<tr>
<td>1'</td>
<td>136.2</td>
<td>-</td>
</tr>
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<td>2'</td>
<td>122.3</td>
<td>6.94 - 7.11 m</td>
</tr>
<tr>
<td>3'</td>
<td>141.6&lt;sup&gt;d&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>4'</td>
<td>141.4&lt;sup&gt;d&lt;/sup&gt;</td>
<td>-</td>
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<tr>
<td>5'</td>
<td>123.2</td>
<td>6.94 - 7.11 m&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>6'</td>
<td>125.3</td>
<td>6.94 - 7.11 m&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>aliphatic carbonyls /acetates</td>
<td>170.6</td>
<td>1.87 s</td>
</tr>
<tr>
<td>aromatic carbonyls /acetates</td>
<td>167.8 - 168.7</td>
<td>2.18 - 2.30 m&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>methyls</td>
<td>20.6, 20.7, 20.8</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup> Peracetylated artifact I was dissolved in chloroform-d$_4$ ($^{13}$C-NMR: 75.5 MHz, 298 K, internal standard: chloroform-d$_4$ = 77.0 ppm; $^1$H-NMR: 300 MHz, 298 K, internal standard: chloroform-d$_4$ = 7.27 ppm).<br><sup>br</sup>broad signal; <sup>a</sup> and <sup>d</sup> chemical shifts of carbons are interchangeable; <sup>e</sup> coupling constant ($J$) of the two singlets: 18.0 Hz; <sup>f</sup>tentative assignments are given in the text.
Part III: Stability of procyanidins

11 Literature overview

Many significant features of procyanidins are linked to their chemical reactivity. Complexation reactions besides chemical and enzymatic transformations are mainly responsible for the color development in wines during aging [260,261], the browning of fruits and fruit juices [262] and the development of hazes in beer and juices [263,264]. During the fermentation of tea leaves, flavan-3-ol condensation products are formed, among them the well-known theaflavins and thearubigins. The fermentation products are very complex [265].

Oxidations are the most important reactions procyanidins may undergo. Three different types of oxidation reactions can be distinguished: autoxidation, enzymatic and coupled oxidations. The intermediate products are reactive and are involved in numerous condensation reactions.

Procyanidins are known to undergo numerous other types of degradation reactions besides the oxidation reactions mentioned above. In acidic media the interflavanoid bonds are cleaved which produces highly electrophilic carbocations that may attack nucleophiles like thiols, thioethers, amines and hydroxyls of polypeptides, polysaccharides and nucleic acids. But also the phenolic hydroxyl groups of the procyanidins themselves and water may act as nucleophiles ([33], see also sections 1.4.1.2 and 9.2). The interflavanoid bonds of procyanidins are also cleaved in alkaline media. In stronger basic solutions (pH > 9) flavan-3-ol monomers, which are liberated from the terminal end of procyanidins, are rearranged to catechinic acid [97,194,266]. The degradation reaction starts with the formation of a quinone methide in the B-ring which results in the opening of the pyran ring followed by condensation of C2 onto C8 of the phloroglucinol ring [36]. Studies on procyanidin B2 in mildly alkaline solutions (0.1 M sodium hydrogen carbonate) revealed that a low percentage of procyanidin B2 is converted to A-type procyanidins. In addition the migration of an (-)-epicatechin moiety from C4 to C2, isomerization and the formation of phlobatannins were observed [267]. In general, the reaction rates are slowest near neutral pH because ionization of the
electrophilic or nucleophilic groups in the molecules is minimal under these conditions [36].

The epimerization of (-)-epicatechin to a mixture of itself and ent-catechin, or (+)-catechin to a mixture of itself and ent-epicatechin proceeds easily in the pH range of 4 to 8 [197,268]. The reaction rate of (-)-epicatechin was found to be much faster than the one of (+)-catechin [195]. The reaction mechanism in neutral to acidic media proceeds through ionization of the 4’-hydroxyl group and a B-ring quinone-methide intermediate [269]. In alkaline solutions an analogous radical mechanism is proposed because the presence of oxygen is mandatory [97].

There are quite a number of studies that prove that degradation reactions are accelerated at higher temperatures (i.e. [191,270]), in the presence of metal ions as catalysts [179,271] and by light [272]. The photolytic rearrangement products were found to consist of higher oligomeric open chain derivatives of the monomeric units [273]. Light-induced epimerization has also been reported [234].

Complexation reactions may lead to an apparent loss of detectable individual procyanidins which is not associated with a structural alteration. Besides complexations via the ortho-diphenolic B-rings flavan-3-ols may act as co-pigments. Because a planar structure is a prerequisite for this type of complexation reaction (-)-epicatechin is a better co-pigment than (+)-catechin [260]. For example, cyanidin glycosides co-pigmented by proanthocyanidins and quercetin glycosides are responsible for the coloration of apple skins [183]. The formation of yellow xanthylum salts composed of an anthocyanin and a procyanidin has also been postulated [260,261,274].

11.1 Oxidation reactions
Procyanidins may undergo enzymatic, non-enzymatic as well as coupled oxidations. Autoxidation of phenolic compounds like flavan-3-ols involves oxygen and proceeds via semiquinone radicals. The radicals may add molecular oxygen and subsequently decay to the corresponding ortho-quinones or they may undergo condensation reactions [33,94,275]. Ortho-quinones are very reactive in aqueous media. They are powerful electrophiles as well as strong oxidizing agents. The condensation of a catechol-quinone with any electron supplying groups (like i.e. phenyl nucleis) lowers the
oxidation-reduction potential and therefore increases the oxidizability of the condensed molecule in comparison to the original catechol. In this way the oxidation of procyanidins is autocatalytical [94]. Semiquinone radicals and/or ortho-quinones are also produced in enzymatic and coupled oxidation reactions.

**Enzymatic oxidation**

Enzymatic oxidation is noticeable from the browning in fruit and vegetables after bruising, cutting or during storage. There are four general classes of enzymes that are known to be involved in the oxidation of polyphenolic compounds: the catecholases (= catechol oxidases, diphenol oxidases, diphenol oxygen oxidoreductases, EC 1.10.3.1), the laccases (EC 1.10.3.2), the tyrosinases (= monophenol mono-oxygenases, EC 1.14.18.1) and the peroxidases (EC 1.11.1.7). The tyrosinases mainly catalyze the hydroxylation of monophenols but they also show catecholase activity which consists of the catalysis of the oxidation of ortho-phenols to the respective ortho-quinones by a two-electron transfer. Because the reactions of both enzymes with respect to ortho-diphenols are the same the term "polyphenol oxidases" is commonly used to address both the tyrosinases and the catecholases [33,94,95,276,277].

The four classes of enzymes differ in their physical and chemical properties. They all contain metal ions in their active centers (polyphenol oxidases and laccases copper, peroxidases iron). The polyphenol oxidases appear almost universally in fungi and plants. In the latter they are located in the chloroplasts. The physiological role remains obscure. Their involvement in photosynthesis by acting as an oxygen buffer or scavenger is discussed. The laccases are detectable in tissues which are infected with molds. The peroxidases are also rarely found in intact plant tissues. Like the laccases they show a broader spectrum of possible substrates which includes ortho-diphenols. Like the polyphenol oxidases they catalyze the oxidation of phenols, but the reaction proceeds in a one-electron transfer which yields semiquinone radicals. In contrast to the peroxidases the laccases do not produce hydrogen peroxide. Semiquinone radicals may also be formed from quinones through a one-electron reduction by several flavoenzymes such as NADPH-cytochrome P-450 reductase [33,94,95,276,277].

Studies on the enzymatic oxidation of individual compounds with polyphenol oxidases in model solutions revealed that phenolic acids are much better substrates than flavan-3-ol monomers [278,279]. The ortho-quinones of (-)-epicatechin [280] and

298
(+)-catechin [134] were however found to be much less stable than those formed from chlorogenic acid. The flavan-3-ol quinones apparently very quickly react further which is evidenced by the fact that the reactions are only reversible by the addition of ascorbic acid in the beginning [134].

There is no final consensus on whether oligomeric procyanidins are substrates of polyphenol oxidases at all. Lee [135] who studied the enzymatic browning of individual compounds in model systems by measurement of the UV/VIS absorbance found that the reaction of the procyanidins B2, B3 and C1 was very slow in the beginning but continuously increased, reaching a maximum after 48 hours. The monomers showed a very fast initial rate of browning, reaching a maximum within 6 hours. Procyanidins were reported to be substrates of polyphenol oxidases but with a relatively poor affinity compared to phenolic acids by other authors, too [281]. In contrast, Cheynier and Ricardo da Silva [282] who determined the concentration profiles of native phenols and ortho-quinones (trapped as sulfones by benzenesulfonic acid [278]) during the enzymatic oxidation in wine model solutions by HPLC came to another conclusion. They found that the individual procyanidins (dimers, trimers and procyanidin gallates) were not oxidized, if they were incubated alone with the grape polyphenol oxidase. Rapid oxidation however occurred in the presence of caffeoyltartaric acid and a coupled oxidation was therefore postulated.

**Coupled oxidation**

Procyanidins have been recognized as one of the main sources of browning in foods [262,283], but at the same time they have been found to be no or relatively poor substrates of the polyphenol oxidases. The clue to this contradiction was found in the ability of ortho-quinones of enzymatically oxidized phenolic acids to be involved in coupled oxidation reactions [145,278]. Studies on this subject have been performed in wine model solutions containing flavan-3-ols, grape polyphenol oxidase and an acid ([281] using caftaric acid, [282] using caffeoyltartaric acid) on one hand, and in model solutions containing flavan-3-ols, an oxidase and chlorogenic acid on the other hand ([284] using commercially available tyrosinase, [280] using apple polyphenol oxidase). The flavan-3-ols were always degraded faster in the presence of the acids. At the same time, the oxidation of the acids proceeded at a slower rate than if they were incubated
alone with the enzymes due to the recovery of the hydroquinone forms in the non-enzymatic redox reactions. A slower degradation rate of caffeoyltartaric acid in musts has also been determined upon addition of (-)-epicatechin and procyanidin B2 [96]. The increase of the oxidation rates induced by ortho-quinones of phenolic acids was found to be larger for galloylated procyanidins than for non-galloylated procyanidins [281,282]. The addition of (+)-catechin to a wine model solution containing grape polyphenol oxidase also increased the oxidation rates of dimeric procyanidins, but (+)-catechin was less efficient than the phenolic acid [281].

In extract solutions many different substrates for polyphenol oxidases (or other oxidizing enzymes) as well as many compounds which may be involved in coupled oxidation reactions are co-present. The nature of the compounds that are predominating in the course of the oxidation process is dependent on the activity of the different enzymes, the concentration and nature of the compounds in the extract solution, the redox potentials of the different quinone / phenol couples and the storage conditions (i.e. availability of oxygen). In this way an increase as well as a decrease of the concentration of individual procyanidins is possible during the course of time. In the biological aging of sherry white wine, for example, a significant decrease of the levels of (+)-catechin, (-)-epicatechin, procyanidins B2 and B4 was determined, while an increase of the concentration of procyanidin B1 was measured [285].

11.2 Reactions of ortho-quinones and semiquinone radicals

The ortho-quinones and semiquinone radicals are recognized as the principal reactive species that are formed from the different oxidation reactions in which flavan-3-ol monomers and procyanidins are involved. Ortho-quinones may react with hydroquinones (native monomers and procyanidins) via 1,4-Michael-type nucleophilic additions (see Figure 11.1) [153,276]. The electron-withdrawing conjugated carbonyls in ring B reduce the electron density at C6' which makes this position to the most preferred site for a nucleophilic attack [286,287]. On the other hand, positions C8 and C6 are activated in the phloroglucinol A-rings due to the phenolic groups which act as electron donors. Because C8 is sterically less hindered the linkages predominantly
Figure 11.1  Condensation reactions: 1,4-Michael-type nucleophilic addition.
occur at this position [36,287]. The first such dimeric C6’→C8 condensation product, dehydrodicatechin, has been described by Weinges et al. [288]. Analogous mixed (-)-epicatechin / (+)-catechin dimers have for example been isolated from Quercus sp. [289]. Noteworthy is that these dimeric compounds are structural isomers of B-type procyanidins but do not yield cyanidin upon heating in mineral acids [288].

The B-rings of the condensation products may again be oxidized to the respective ortho-quinones. These quinones may attack other nucleophilic sites of flavan-3-ols and thus initiate polymerization reactions. In the absence of external nucleophiles the B-ring ortho-quinones may react with the phloroglucinol A-ring and/or ring C to produce cyclic ethers. The resulting compounds which contain only one ether-bridge are structural isomers of A-type procyanidins [152,153]. The best studied product of this reaction series is dehydrodicatechin A [286].

The Michael-type additions are especially favored at high pH because the nucleophilic character of the molecules is increased due to higher proportions of phenolate anions which are present. At lower pH condensation reactions more likely follow a radical mechanism (see Figure 11.2) [153,290]. The semiquinone radicals may be produced via direct oxidation of a hydroquinone or via reverse disproportionation of an ortho-quinone and a hydroquinone [290]. Both mesomeric forms of the resulting semiquinone radicals are involved in radical reactions which may lead to carbon-, ether- as well as peroxy-linked species of differing degree of polymerization [94,153,291].

The most favored sites for the formation of radicals are the positions C6’, C2’ and O-C3’ of the B-ring [25,152]. Again, the radicals preferentially attack the nucleophilic A-ring at position C8 and less frequently at the sterically more hindered position C6. The formation of A-ring radicals has been described but their existence has also been questioned by other researchers [152,288].

Besides the most common A-ring – B-ring condensation product linked at C8→C6’, linkages between C6→C6’ and C8→O-C3’ or O-C4’, respectively, have also been postulated [153]. The coupling of two A-rings or two B-rings is less frequent. The acetaldehyde induced polymerization results in the formation of CH3–CH bridges between the A-rings of two flavan-3-ols [147]. This condensation reaction has been proposed as a possible explanation for the loss of astringency in fruits during
Figure 11.2  Condensation reactions: reverse disproportionation and examples of possible radical reactions.
maturation [148]. The possibility of a linkage between the two B-rings has been evidenced by the isolation of condensates of flavan-3-ol monomers with flavonoid compounds from black tea [265] and willow bark [292].

The many condensation and polymerization reactions of ortho-quinones and semiquinone radicals of oxidized procyaniidins and monomers increase the complexity of the resulting products dramatically. In extract solutions, the number of possible reaction products is even larger, because many other compounds may act as possible nucleophiles besides the flavan-3-ols. In example, the formation of co-polymers of (+)-catechin and caftaric acid [281] as well as chlorogenic acid [284] has been proposed. The reaction of procyaniidins as well as flavan-3-ol monomers with acetaldehyde on one hand and anthocyanins (malvidin [293], malvidin-3-glucoside [274,294-296], cyanidin-3-glucoside [297]) on the other hand, has been studied by several researchers. (+)-Catechin and (-)-epicatechin were found to react directly with the anthocyanins as well as with the acetaldehyde [293]. Derivatives of (+)-catechin or procyaniidins, respectively, linked via an acetaldehyde bridge to an anthocyanin could be detected by FAB-MS [295] and by thiolysis [296]. Most important is however that all these possible condensation reactions regenerate or preserve (in the case of the acetaldehyde induced polymerization) hydroquinones which are in this way susceptible to a renewed oxidation.
12 Stability of selected procyanidins in solution

12.1 Introduction

The vast majority of studies on the stability of procyanidins in solution deal with their possible involvement in the development of hazes and precipitates in juices or their role in the development of color and taste in the ageing of wines. Most of these studies are conducted with model solutions which not only contain flavan-3-ols but also buffer salts besides other marker compounds (like i.e. anthocyanins or phenolic acids). The results of these studies are only of limited value in the evaluation of the stability of procyanidin reference standards which are dissolved in pure solvents for chromatographic analyses.

The primary scope of this investigation on the stability of selected procyanidins was to find suitable storage conditions for reference standard solutions which are intended to be used for quantitative analysis. Aqueous methanol was taken as a solvent although water is known to be a quite aggressive solvent (the oxidation of vicinal diphenols produces superoxide anions which are converted to hydrogen peroxide in weakly acidic solutions [94]). This choice was made in view of the intended quantitative chromatographic analysis. As could be demonstrated, unusual chromatographic behavior occurs, if the solvent exhibits a too strong elution strength (like i.e. pure methanol; see section 1.4.3.2.2). In addition, the study on the stability of selected procyanidins was intended to give information on the appearance and nature of the degradation products by HPLC-DAD and LC-MS analysis of the degraded solutions.

12.2 Summary

Solutions of the procyanidins B2, B5 and C1 in 50 % (v/v) aqueous methanol were stored at room temperature in daylight, darkness and UV 254 nm as well as in a fridge and at -80 °C (both in the dark). Aliquots of these solutions were analyzed by HPLC-UV / DAD to monitor the degradation products. All three procyanidin solutions proved to be stable at -80 °C over a period of at least one year. The solutions stored in the fridge showed less than 5 % degradation during the first five days of storage (procyanidin C1) or eight to ten days of storage (procyanidins B2 and B5, respectively).
Degradations were much faster at room temperature. Storage at UV 254 nm was found to accelerate the degradation rates of the 4→8 linked procyanidins B2 and C1, while the degradation rate of procyanidin B5 remained practically unchanged. The trimeric procyanidin C1 was less stable than the dimeric procyanidins B2 and B5. The principal degradation products formed under the different conditions were largely the same for one particular procyanidin as could be judged from the retention features. The LC-MS analysis revealed no reliable molecular weight information in most of the analyzed samples due to too low concentrations of the individual compounds.

12.3 Experimental

Materials and instrumentation
The procyanidins B2, B5 and C1 were purified from *Crataegus* leaves and flowers as described in section 8. Methanol (HPLC grade) was purchased from Romil Chemicals (UK-Shepshed). Ortho-phosphoric acid was obtained from Hänseler (CH-Herisau). Water was de-ionized using a NANOpure cartridge system (Skan, CH-Basel). Parafilm was purchased from Merck (CH-Dietikon).

The different solutions were stored at room temperature (air conditioned; approximately 23 °C), in a refrigerator (= fridge, model FKS 2600 by Liebherr; approximately 8 °C) and in a freezer (model Ultima II by Ismatec SA, CH-Glattbrugg; approximately -80 °C). For the experiments under UV 254 nm an UV cabinet by Camag (CH-Muttenz) was used.

Chromatographic conditions
The instrumentation and chromatographic conditions were the same as described in sections 8.3.1 and 8.3.2. The Fingerprint HPLC method was applied to monitor the degradation reactions. The evaluation was based on area counts at 280 nm. The injection volumes were chosen to be 5 μl in the beginning and were raised to 20 μl where necessary towards the end of the investigation. The following integration parameters were employed: initial area reject: 1.0, initial peak width: 0.5, initial threshold: -2.0, shoulders: off.
The solutions which were stored at room temperature were investigated using the HPLC method for the analysis of the wash-out peaks at the end of the investigations (for conditions see section 8.3.2). Selected samples were subjected to LC-MS analysis (for conditions see section 5.2).

**Procedures**

Solutions of the procyanidins in 50 % aqueous methanol (v/v) were prepared (concentrations: procyanidin B2: 0.431 mg/ml and 0.498 mg/ml; procyanidin B5: 1.42 mg/ml; procyanidin C1: 0.498 mg/ml and 0.464 mg/ml). Aliquots of the solutions were filled into HPLC vials and were stored under the following conditions: room temperature at darkness; room temperature at daylight; room temperature under UV 254 nm; fridge at darkness and -80 °C at darkness. The solutions were analyzed over time periods of 518 days (procyanidins B2 and C1), 344 days (procyanidin B5) or until no sample solutions were left, respectively. The samples which were stored in the fridge and at -80 °C were kept at room temperature in the dark for 10 to 15 minutes before analysis and were again stored at the original conditions right after sample injection. All the vials were kept in the dark during the HPLC analyses. All the lids of the vials were additionally wrapped with parafilm after the analyses to prevent volatilization through the pierced septa.

At each day of analysis an (−)-epicatechin reference solution was also injected to be able to identify the different degradation products via their retention times over the whole time period of the investigation.

**12.4 Results and discussion**

The procyanidins degraded under all storage conditions except at -80 °C. The degradations roughly follow a first order kinetic. The semi-logarithmic plots of the detector responses vs. time yield straight lines (the linear regressions revealed correlation coefficients between 0.93 and 1.00). A first order kinetic has also been determined for the degradation of procyanidin dimers and trimers in red wine [191]. In contrast, de Freitas et al. [179], who studied the kinetics of decomposition of several dimeric and trimeric procyanidins in model solutions of wine in the presence of
transition metal catalysts, found very complex mechanisms of decomposition which did not allow the determination of a simple kinetic order.

As expected, a decrease in temperature yielded slower degradation rates. The effect of light at a constant temperature on the degradation rate was much less pronounced than the effect of the temperature. The influence of light however is more complex because the individual procyanidins seem to be affected differently (see below).

It is a common knowledge, that the presence of oxygen is mainly responsible for the degradation of procyanidins. For example, thirteen times higher concentrations of procyanidins were measured in apple juices which had been produced anaerobically compared to aerobically manufactured juices [263]. An attempt to study the influence of an oxygen atmosphere in comparison to an argon atmosphere in vials containing the procyanidin solutions during the present investigation were unsuccessful due to the incomplete gas-tightness of the sample vials.

For a particular procyanidin it can be concluded from the retention times of the eluting peaks that the same principal degradation products were formed under all storage conditions. Intermediate compounds were produced with all three procyanidins which again disappeared in the course of the study. Few degradation products in relatively low concentrations were built up very slowly and seem to be more stable since they were detectable until the end of the investigations. For the procyanidins B2 and C1 the experimental data imply that at the most severe conditions, at room temperature in UV 254 nm, the principal degradation products react very rapidly to compounds which can not anymore be chromatographed (see below).

In the vast majority of studies on degradation products of procyanidins the identification of individual compounds is based on retention times in HPLC chromatograms and UV / DAD spectra (i.e. [274,284,297]). However, a lot of possible degradation products are structurally very closely related to natural flavan-3-ols and therefore may show similar retention features as well as UV / DAD spectra. An unambiguous identification is only possible by applying more specific detection modes like i.e. MS or NMR. This approach has so far only been followed in a study on the enzymatic oxidation of (+)-catechin [153]. The off-line MS analysis of purified fractions of the degraded solutions revealed the presence of dimeric compounds of the procyanidin A- and B-type. Additional NMR analysis led to the establishment of structure hypotheses for the dimeric degradation products in which one of the
monomeric flavan-3-ol units is predominantly linked via its B-ring with the second unit (and not via its A- or C-ring like in the naturally occurring procyanidins). LC-MS analysis of the degraded solution of procyanidin C1 justifies the view that HPLC-DAD data yield insufficient proof of the identity of degradation products (see below). Unfortunately, the concentrations of individual compounds were too low to obtain molecular weight information on degradation products for the other procyanidin solutions analyzed.

During the wash cycles (elution with pure methanol) additional compounds which show an (-)-epicatechin spectrum were eluted towards the end of the investigation periods in all degraded sample solutions. These peaks were analyzed with another chromatographic method (see section 8.3.2, HPLC for the analysis of wash-out peaks). No additional peaks could be detected in significant concentrations with none of the sample solutions. However, the baselines showed a small hump from approximately tR 5 to 40 minutes in the chromatograms of the sample solutions compared to the chromatogram of pure methanol. It is concluded that the intermediate degradation products are mainly converted to other compounds of higher molecular weight which can not be chromatographed and/or which occur in too low concentrations to be detected as single peaks.

In the following paragraphs the individual procyanidins that have been studied are discussed in more detail.

**Procyanidin B2**

After one day of storage at room temperature the peak areas had decreased by 9.3 % (darkness), 3.6 % (daylight) and 7.9 % (UV 254 nm), respectively. No procyanidin B2 could anymore be detected after: 41 days (darkness), 33 days (daylight) and 16 days (UV 254 nm), respectively (see Figure 12.1, lower). The faster degradation rate at 254 nm is thought to be due to accelerated radical reactions which are more likely to occur with light of higher energy. The experimental data imply that the solution kept in daylight degrades at a slower rate than the one that was kept in the dark. The reason for this behavior remains obscure.

After eight days of storage in the fridge the area of the procyanidin B2 peak had decreased by 3.8 %. The procyanidin B2 was detectable for 390 days under these conditions. After 518 days of storage at -80 °C the area of the procyanidin B2 peak had
decreased only by 3.2%. No degradation products were detectable after this time of storage. The course of the degradation under these conditions in comparison to the one at room temperature in darkness is shown in Figure 12.1 (upper).

**Figure 12.1** Degradation of procyanidin B2 under different conditions.
The degraded solutions first turn yellow and become brown in the course of the study. The occurrence and disappearance of the main degradation products for the solution kept at room temperature in the dark is depicted in Figure 12.2. Examples of chromatograms of the solution stored at room temperature in daylight are shown in Figure 12.3. The first degradation product which is detectable shows a retention time of approximately 30 minutes, followed by one which elutes at approximately 22 minutes. These two compounds are the main degradation products and can be detected in all test solutions (except the one stored at -80 °C). Table 12.1 gives an overview on all the main detectable degradation products under the different experimental conditions. It is assumed that the same degradation products are formed at room temperature (in daylight and in darkness) and in the sample solutions stored in the fridge. Not all of these compounds were detected at all conditions because the reaction rates were too slow and / or the sample solutions were used up. The peaks of the degradation products eluting after approximately 13 and 33 minutes respectively, show a remarkable peak tailing.

The solution which had been stored at room temperature under UV 254 nm showed a slightly different behavior. The principal two degradation products were also formed but besides these no other compounds were produced in sufficient amounts to be detectable. After 53 days of storage no peaks were detected at all anymore. Obviously, the principal degradation products react in a different way under these most severe conditions. Presumably, inhomogenous higher molecular weight compounds are formed at a faster rate which do not elute as peaks with defined k’-values.

Besides the principal compounds listed in Table 12.1 many very small peaks can be detected in the course of the study, especially during the first ten minutes of the chromatogram.
Figure 12.2 Appearance of the degradation products of procyanidin B2. Experimental conditions: room temperature, darkness.
Figure 12.3  Typical chromatograms of the stability solution of procyanidin B2.

Experimental conditions: room temperature, daylight; day 154: injection volume 20 µl.
**Table 12.1** Detection intervals for the principal degradation products of solutions of procyanidin B2 stored at different conditions.

<table>
<thead>
<tr>
<th>Degradation product&lt;sup&gt;a&lt;/sup&gt; (t&lt;sub&gt;R&lt;/sub&gt; in min)</th>
<th>Degradation product detected during the time interval at:</th>
<th>RT UV 254 nm (days)</th>
<th>RT daylight (days)</th>
<th>RT darkness (days)</th>
<th>fridge darkness (days)</th>
<th>-80 °C darkness (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td></td>
<td>1 - 16</td>
<td>5 - 120</td>
<td>1 - 213</td>
<td>33 - 518</td>
<td>b</td>
</tr>
<tr>
<td>22</td>
<td></td>
<td>3 - 35</td>
<td>14 - 155</td>
<td>6 - 276</td>
<td>219 - 518</td>
<td>n.d.</td>
</tr>
<tr>
<td>5.9</td>
<td></td>
<td>b</td>
<td>14 - 77</td>
<td>b</td>
<td>6 - 146</td>
<td>120 - 390</td>
</tr>
<tr>
<td>8.0</td>
<td></td>
<td>b</td>
<td>b</td>
<td>b</td>
<td>33 - 216</td>
<td>n.d.</td>
</tr>
<tr>
<td>13</td>
<td></td>
<td>n.d.</td>
<td>77 - 155</td>
<td>70 - 381</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>33</td>
<td></td>
<td>b</td>
<td>b</td>
<td>b</td>
<td>70 - 381</td>
<td>n.d.</td>
</tr>
<tr>
<td>38</td>
<td></td>
<td>n.d.</td>
<td>b</td>
<td>b</td>
<td>178 - 381</td>
<td>n.d.</td>
</tr>
<tr>
<td>last analysis</td>
<td></td>
<td>53</td>
<td>155</td>
<td>381</td>
<td>518</td>
<td>518</td>
</tr>
</tbody>
</table>

<sup>a</sup> listed in the order of evolution during the course of the investigation; <sup>b</sup> visible in chromatograms, but peak is not integrated; RT = room temperature; n.d. = not detected.
Procyanidin B5

After one day of storage at room temperature the peak areas had decreased by 5.1 % (darkness), 6.4 % (daylight) and 9.0 % (UV 254 nm), respectively. No procyanidin B5 could anymore be detected after: 62 days (darkness), 44 days (daylight) and 37 days (UV 254 nm), respectively. The degradation rates are very similar for all three storage conditions (see Figure 12.4, lower). This contrasts the findings made with procyanidins B2 and C1 (see below), where an acceleration of the degradation rates were noticeable at UV 254 nm compared to the storage conditions in the dark. From these results it can be concluded that light induced and/or light accelerated degradation reactions are less important with procyanidin B5 than with the procyanidins B2 and C1. Differences in the degradation rates which is dependent on the type of monomeric unit as well as the type of linkage have also been reported in a study on oxidation of procyanidin dimers in wine model solutions [179].

The peak area of the procyanidin B5 peak had decreased by 3.3 % after ten days of storage in the fridge. The procyanidin B5 was detectable for 216 days under these conditions. In contrast to the procyanidins B2 and C1 (see below) there seems to exist an initial time interval in which the degradation proceeds at a slower rate (see Figure 12.4, upper). After 344 days of storage at -80 °C the area of the procyanidin B5 peak had decreased only by 1.5 %.

The degraded solutions first turn reddish and become yellow-brown upon prolonged storage. The occurrence and disappearance of the main degradation products for the solution kept at room temperature in the dark is shown in Figure 12.5. Representative chromatograms of the solution stored at room temperature in daylight are given in Figure 12.6. The degradation products showing retention times of 12 and 15 minutes appear simultaneously under all storage conditions. The principal degradation product is however detectable later and elutes approximately at tR 39 minutes. The degradation product which elutes after 23 minutes shows a fronting, while the one which elutes after approximately 13 minutes shows a tailing. All these five main degradation products can be detected in all degraded solutions (see Table 12.2). Analogous degradation routes can therefore be postulated for all storage conditions. Besides these principal degradation products again many very small peaks can be detected during the course of the study.
Figure 12.4 Degradation of procyanidin B5 under different conditions.
Figure 12.5 Appearance of the degradation products of procyanidin B5.
Experimental conditions: room temperature, darkness.
Figure 12.6  Typical chromatograms of the stability solution of procyanidin B5.
Experimental conditions: room temperature, daylight; day 149: injection volume 20 µl.
Table 12.2 Detection intervals for the principal degradation products of solutions of procyanidin B5 stored at different conditions.

<table>
<thead>
<tr>
<th>Degradation product ( \text{RT} ) (in min)</th>
<th>Degradation product detected during the time interval at:</th>
<th>( \text{RT} ) UV 254 nm ( \text{days} )</th>
<th>( \text{RT} ) daylight ( \text{days} )</th>
<th>( \text{RT} ) darkness ( \text{days} )</th>
<th>fridge darkness ( \text{days} )</th>
<th>(-80 \degree \text{C} ) darkness ( \text{days} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td></td>
<td>0 - 10</td>
<td>0 - 20</td>
<td>0 - 20</td>
<td>1 - 149</td>
<td>( b )</td>
</tr>
<tr>
<td>15</td>
<td></td>
<td>0 - 6</td>
<td>0 - 10</td>
<td>0 - 15</td>
<td>0 - 34</td>
<td>( b )</td>
</tr>
<tr>
<td>39</td>
<td></td>
<td>3 - 62</td>
<td>3 - 216</td>
<td>3 - 149</td>
<td>34 - 344</td>
<td>n.d.</td>
</tr>
<tr>
<td>23</td>
<td></td>
<td>3 - 10</td>
<td>6 - 10</td>
<td>6 - 20</td>
<td>34 - 216</td>
<td>n.d.</td>
</tr>
<tr>
<td>last analysis</td>
<td></td>
<td>97</td>
<td>344</td>
<td>216</td>
<td>344</td>
<td>344</td>
</tr>
</tbody>
</table>

* listed in the order of evolution during the course of the investigation; \( b \) visible in chromatograms, but peak is not integrated; \( \text{RT} \) = room temperature; n.d. = not detected.
Procyanidin C1
After one day of storage at room temperature the peak areas had decreased by 7.5 % (darkness), 14.0 % (daylight) and 13.2 % (UV 254 nm), respectively. No procyanidin C1 could anymore be detected after: 24 days (darkness), and 14 days (daylight, UV 254 nm), respectively (see Figure 12.7, lower). From these results it can be concluded that the degradation is accelerated in the presence of light. Radical reactions might therefore be likely to be involved in the degradation of procyanidin C1.

After five days of storage in the fridge the area of the procyanidin C1 peak had decreased by 4.9 %. The procyanidin C1 was detectable for 219 days under these conditions. After 518 days of storage at -80 °C the peak area had decreased only by 1.3 %. No degradation products were detectable after this time of storage. The course of the degradation under these two conditions in comparison to the one at room temperature in the dark is shown in Figure 12.7 (upper). Procyanidin C1 is degraded at a faster rate than the dimeric procyanidins under all conditions. This is in compliance with the literature data. Degradation studies in wine model solutions [179,274] and wine [191] revealed faster degradation rates for trimers compared to dimers or monomers, too.

The degraded solutions of procyanidin C1 first turn yellow-brown and become reddish-brown in the course of the study. The occurrence and disappearance of the main degradation products for the solution kept at room temperature in the dark is displayed in Figure 12.8. Examples of chromatograms of the solution stored at room temperature in daylight are shown in Figure 12.9. The first degradation product which appears is likely to be procyanidin B2 based on the retention features (tR 12.5 min). The peaks at tR 22 and 30 minutes would therefore most likely correspond to the principal degradation products of procyanidin B2 which already had been determined for the pure compound (see above). The fact that these two compounds and the presumable procyanidin B2 reach maximum concentrations at the same time is confusing. The LC-MS analysis of the sample solution stored in the fridge at a later stage of the degradation study (day 154) revealed a possible explanation. In the UV / DAD chromatogram of the concentrated solution the peak at 12.5 minutes is still present in significant concentrations besides procyanidin C1 and some other minor degradation products. The LC-MS analysis revealed the presence of low amounts of procyanidin C1 (m/z 864.8) as expected. The MS spectrum of the presumable procyanidin B2 peak
however only showed an abundance of less than 5% for the dimeric ion at m/z 577.3. The spectrum is clearly dominated by the ion at m/z 862.8 (see Figure 12.10). The mass of this principal signal points to the presence of a quinoid or an A-type trimeric procyanidin. Quinones of (-)-epicatechin have been shown to be extremely unstable which makes their detection unlikely in the present investigation [136]. The formation of an A-type procyanidin which is therefore more likely has already been postulated for oxidation products of (+)-catechin in model solutions [153]. If this trimeric compound (which shows the same retention time like procyanidin B2) is already present in the early stage of the degradation process, the time of the maximum concentration of the procyanidin B2 could be masked by this compound. It is therefore postulated that the maximum concentration of the procyanidin B2 peak is actually reached earlier and that the curve which displays the appearance of the peak eluting at 12.5 minutes shows the cumulative concentrations of the two degradation products.

The degradation products eluting at 24 and 31 minutes resemble the principal degradation products of procyanidin B2 with respect to retention features and temporal appearance. It is likely that these two degradation products are analogous compounds to the degradation products of procyanidin B2, only differing in their degree of polymerization.

Table 12.3 gives an overview on all the principal degradation products which are detectable under the different experimental conditions. Most of the main degradation products can be detected in all degraded solutions. The compound eluting after 27 minutes is only integrated in the chromatograms of the solution stored in the fridge. This compound appears in similar concentration as other main degradation products under these conditions and it is therefore also considered as a principal degradation product. Under the other storage conditions the peak at t_R 27 min is much less abundant or not even detectable (at room temperature in daylight). This points to the fact that the degradation route, which leads to the peak eluting after 27 minutes, is followed to different extents depending on the storage conditions. Like in the degradation process of procyanidin B2 a relatively early eluting peak (t_R 12.4 min) which shows a remarkable peak tailing, is not detectable in the solutions stored at UV 254 nm. In contrast to the other storage conditions no peaks could anymore be detected at all after 51 days of storage at UV 254 nm. The principal degradation products are obviously converted to other compounds, presumably of higher molecular weight, under these
most severe conditions. This behavior was noticed with the solutions of procyanidin B2 as well. Besides the principal compounds listed in Table 12.3 many very small peaks can be detected in the course of the study like with the other procyanidins, too.

Figure 12.7 Degradation of procyanidin C1 under different conditions.
**Figure 12.8** Appearance of the degradation products of procyanidin C1.

Experimental conditions: room temperature, darkness.
Figure 12.9  Typical chromatograms of the stability solution of procyanidin C1.

Experimental conditions: room temperature, daylight; day 55: injection volume 20 µl.
Figure 12.10 LC-MS analysis of the refrigerated solution of procyanidin Cl after 154 days of storage.

Top: LC-MS spectrum of the procyanidin Cl peak
Molecular ion of procyanidin Cl: m/z 864.8.

Bottom: LC-MS spectrum of the peak eluting at the retention time of procyanidin B2.
Molecular ion of procyanidin B2: m/z 577.3.

For conditions, see Experimental.
Table 12.3 Detection intervals for the principal degradation products of solutions of procyanidin C1 stored at different conditions.

<table>
<thead>
<tr>
<th>Degradation product*</th>
<th>Degradation product detected during the time interval at:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RT UV 254 nm (days)</td>
</tr>
<tr>
<td>(t&lt;sub&gt;R&lt;/sub&gt; in min)</td>
<td></td>
</tr>
<tr>
<td>12.5</td>
<td>1 - 14</td>
</tr>
<tr>
<td>30</td>
<td>1 - 9</td>
</tr>
<tr>
<td>22</td>
<td>2 - 9</td>
</tr>
<tr>
<td>12.4</td>
<td>n.d.</td>
</tr>
<tr>
<td>27</td>
<td>b</td>
</tr>
<tr>
<td>last analysis</td>
<td>51</td>
</tr>
</tbody>
</table>

* listed in the order of evolution during the course of the investigation; b visible in chromatograms, but peak is not integrated; RT = room temperature; n.d. = not detected.
Conclusions

Standard solutions of procyanidins should be stored at -80 °C to prevent degradation. The stability data obtained for the refrigerated solutions imply, that the HPLC vials containing calibration solutions of procyanidins could be used more than only once, if cooled sample trays for HPLC analysis were employed. Procyanidins contained in extract solutions are degraded at a slower rate (no reduction of the areas of the procyanidin peaks was detectable after 23 days of storage of an ethyl acetate extract in the fridge). This suggests the presence of other compounds that are even more oxidizable and which are able to protect the procyanidins from degradation to a certain extent. Based on the results of this investigation it is however recommended to make all manipulations with procyanidin containing samples whenever possible at low temperatures, and, to reduce unnecessary light exposure during work up procedures as well as HPLC analyses.

For the characterization of degradation products LC-MS spectra proved to be more valuable than the UV / DAD spectra. The latter do not show any obvious differences between the numerous compounds. The identification of degradation products in HPLC-DAD chromatograms can only be based on the retention features and must therefore be considered as highly speculative. LC-MS-MS and LC-NMR should be employed for an unambiguous identification. For this kind of analyses the starting procyanidin solutions should however be more concentrated.

From the general knowledge on the reactivity of procyanidins (see section 11) there is no doubt that degradation reactions include the cleavage of interflavanoid bonds which leads to naturally occurring flavan-3-ols (i.e. procyanidin C1 is cleaved to produce procyanidin B2 and (-)-epicatechin). But, the significance of these primary degradation products can not be predicted only from the UV / DAD chromatograms. In the study presented here, (-)-epicatechin has never been detected as a principal degradation product with none of the procyanidins, as can be judged from the lack of a significant peak at the retention time of (-)-epicatechin. Obviously, the monomer produced by the cleavage of interflavanoid bonds was rapidly captured by intermediate carbocations. In conclusion, the degradation of procyanidins can be understood as a simultaneous bond breaking and bond making process which in the end leads to countless isomeric compounds of differing degrees of polymerization occurring in very low concentrations.
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Epilogue

During the past years the demands for a proper documentation of the pharmaceutical quality of phytopharmaceuticals has risen constantly. Unlike in synthetically produced pharmaceuticals which mostly only contain one active ingredient, the active principle in phytopharmaceuticals is a plant extract which is composed of a multitude of different compounds. The combination of all these compounds is understood to be responsible for the pharmacodynamic actions and the pharmacokinetic properties. The assurance of a constant pharmaceutical quality for such multicomponent systems is a very challenging task for phytochemists. Progresses in the chromatographic techniques have rendered group determining procedures, like i.e. acid dye assays, almost to antique techniques. Standardization on individual compounds which have been demonstrated to contribute to the overall action in pharmacological tests has become a common practice. Lacking such active ingredients of proven and significant activity, a standardization on lead compounds which are typical for a given species is a generally accepted alternative. However, it may never be lost out of sight that any standardization only considers a tiny proportion of the active principle, which still is the total of the extract itself.

The choice on which compounds a standardization should be performed has to be done with great care. A clever decision which takes into account not only aspects of activity but also the demands of regulatory authorities with respect to documentation, feasibility and last but not least economical as well as marketing arguments, is mandatory. From the view of the phytochemist, the target compounds should be restricted to a small number and they should appear in reasonable concentrations which both simplifies analytical method development and validation procedures. The reference material should commercially be available in a constant quality and to reasonable cost. In view of the increasing demand for the conductance of stability tests, i.e. under stress conditions as specified in the ICH guidelines, a suitable stability of the target compounds in the extracts as well as during work up procedures is also a prerequisite.

The pharmaceutical quality of phytopharmaceuticals can not be assured sufficiently by standardization on compounds which only represent a proportion of a few percents or even per milles of the total extract, which in the end is the true active principle. A
full documentation of the pharmaceutical quality should therefore include a procedure which would give quality information on this overwhelming "rest". One possible solution consists of the recording of fingerprints of the main classes of compounds like i.e. polyphenols, alkaloids, terpenes etc., but also primary plant products like carbohydrates, lipids, amino acids and minerals could be documented. Such fingerprints could be generated using different kinds of chromatographic techniques like TLC, HPLC or GC among other suitable techniques. All the information could be put together to render a completer picture of a given extract. Such an approach is rather costly and therefore probably only justified in some product development stages and possibly during stability testing.

A second approach to obtain a more complete picture of an extract would be to find a suitable model for the intended therapeutic effect. This approach is based on the recognition that the goal of an adequate pharmaceutical quality is to assure a constant therapeutic quality which in the end consists of specific pharmacological effects. The declaration of an activity is quite unusual for phytopharmaceuticals but in other fields, like i.e. in the enzyme biochemistry, it is a common practice. Extracts of many medicinal plants have been shown to be more active than isolated individual compounds in pharmacological models. A standardization on a minimum target activity would make sense on this background. In order to be credible models for such a standardization of phytopharmaceuticals should be based on actual pharmacological as well as pathophysiological knowledge.

In my opinion, the documentation of the pharmaceutical quality of phytopharmaceuticals could include both a standardization as well as a declaration of a minimum pharmacological target activity. The standardization would lead to the declaration of minimum contents of compounds in phytopharmaceuticals which are known to be of therapeutic relevance or alternatively, which are especially characteristic for a specific plant extract. On the other hand, the sum of synergistic and additive pharmacological effects of the total extract could be accounted for in the declaration of a specific minimum pharmacological activity.

One great benefit of the declaration of a specific pharmacological activity would be that the effects of phytopharmaceuticals could not anymore be reduced to the few compounds on which standardization is performed. Such an approach would clearly delimitate phytopharmaceuticals from classical, mostly mono-component pharma-
ceuticals, which in turn would justify the demand for special regulatory standards. These might include reasonable validation specifications (which could be the same as for residue analysis with respect to i.e. accuracy) as well as protocols for stability testing which take into account the special situation of phytopharmaceuticals (in the end, nobody would ever test enzyme stability under stress conditions at 40 °C). Furthermore, the highly sophisticated pharmacokinetic studies with one or even more selected compounds could be avoided. Such studies are currently demanded in accordance to the regulatory standards established for synthetic drugs. The conductance of such studies does however not show any clinical relevance with phytopharmaceuticals. Last but not least, the declaration of a specific pharmacological target activity would represent a step in the direction of a more holistic view of phytopharmaceuticals which still stands on a firm scientific basis.

How should the pharmaceutical quality of preparations from hawthorn be guaranteed based on all these considerations and the results of the present work? Both, the flavonoids as well as the procyanidins have proved to be pharmacologically active. A standardization on either one of these classes of compounds would therefore be reasonable. A standardization on flavonoids is considered to be more feasible than a standardization on procyanidins for several reasons. The quantitative determination of procyanidins following the presented HPLC UV/DAD protocol remains critical in view of the poor selectivity of the detection and the delicate chromatographic separation. The inherent instability of the procyanidins, which at the same time is thought to be responsible for their beneficial pharmacological effects, complicates the analytical procedures. Furthermore, alterations of the absolute as well as relative concentrations of the procyanidins under the commonly applied ICH conditions for stability testing is more than likely (if such alterations have an effect on the therapeutic quality has not yet been studied).

Besides these more analytical arguments there are also other considerations that make a standardization on procyanidins less reasonable. Procyanidins are very widespread in the plant kingdom. Very similar procyanidin patterns like in Crataegus sp. can for example be found in food plants like Malus sylvestris (apple), Theobroma cacao (cocoa) and Dioscorea cirrhosa (yams). There is not one other medicinal plant described in the literature which shows a comparable procyanidin pattern and at the
same time is used in cases of declining heart function like hawthorn. A standardization
on procyanidins might therefore raise the question on how the beneficial effects of
preparations of hawthorn differ from the ones of other "healthy" foodstuffs like for
example apples. On the other hand, flavonoids are also very widespread but, at least the
C-glycosides contained in *Crataegus* sp. are not as ubiquitous as the procyanidins and
preparations standardized on these compounds would therefore clearly stand out from
many other flavonoid containing medicinal plants or foods.

The secret of the active principle(s) in preparations of hawthorn has not yet been
revealed. There is a traditional knowledge which has made hawthorn to a medicinal
plant in contrast to the closely related apple which at the same time has been used as a
food plant for centuries. Flavonoids and procyanidins which have been recognized as
pharmacologically active compounds in hawthorn are to be found in many other
nutraceuticals like i.e. green tea or wine, too. A standardization on either one of these
classes of compounds will at most consider one possible aspect of the therapeutic
activity of preparations of hawthorn and will therefore always remain unsatisfactory.

As an alternative, preparations of hawthorn could be standardized on a specific
pharmacological effect which is correlated with the intended therapeutic use. For
example, the determination of the antioxidative or radical scavenging activities would
allow to measure the effects of the flavonoids and procyanidins at the same time. Both,
antioxidative as well as radical scavenging properties are known to be valuable in
competing degenerative processes (i.e. atherosclerosis) which also play an important
role in the development of the coronary heart disease. The right selection of a suitable
pharmacological model should be done with great care and would be subject to another
basic research project. Such a pharmacological model would have to document the
uniqueness of preparations of hawthorn in cases of declining heart function and at the
same time would have to clearly separate these effects from the ones achievable by
nutraceuticals.
Appendix I

NMR and CD spectra of isolated compounds

$^1$H-NMR spectrum of phenolic (-)-epicatechin

$^1$H-NMR spectrum of peracetylated (-)-epicatechin

$^{13}$C-NMR and DEPT 135 spectra of peracetylated (-)-epicatechin

$^1$H-NMR spectrum of phenolic procyanidin B2

$^1$H-NMR spectrum of peracetylated procyanidin B2

$^{13}$C-NMR and DEPT 135 spectra of peracetylated procyanidin B2

$^1$H-NMR spectrum of phenolic procyanidin B5

$^1$H-NMR spectrum of peracetylated procyanidin B5

$^1$H-NMR spectrum of phenolic procyanidin B4

$^1$H-NMR spectrum of peracetylated procyanidin B4

$^{13}$C-NMR and DEPT 135 spectra of peracetylated procyanidin B4

$^1$H-NMR spectrum of phenolic procyanidin C1

$^1$H-NMR spectrum of peracetylated procyanidin C1

$^1$H-NMR spectrum of phenolic trimer I

$^1$H-NMR spectrum of peracetylated trimer I

$^1$H-NMR spectrum of phenolic trimer II

$^1$H-NMR spectrum of peracetylated trimer II

$^1$H-NMR spectrum of phenolic tetramer I

$^1$H-NMR spectrum of peracetylated tetramer I

$^1$H-NMR spectrum of phenolic artifact I

$^1$H-NMR spectrum of peracetylated artifact I

$^{13}$C-NMR and DEPT 135 spectra of peracetylated artifact I

CD spectra of procyanidins B2, B5, B4 and C1
$^1$H-NMR spectrum of phenolic (-)-epicatechin in acetone-d$_6$ (300 MHz, 298 K)
$^1$H-NMR spectrum of peracetylated (-)-epicatechin in chloroform-d$_3$ (300 MHz, 298 K)
$^{13}$C-NMR and DEPT 135 spectra of peracetylated (-)-epicatechin in chloroform-$d_1$ (75.5 MHz, 298 K)
$^1$H-NMR spectrum of phenolic procyandin B2 in acetone-d$_6$ (300 MHz, 298K)
'H-NMR spectrum of peracetylated procyanidin B2 in chloroform-d₁ (600 MHz, 303 K)
$^{13}$C-NMR and DEPT 135 spectra of peracetylated procyanidin B2 in chloroform-$d_4$ (75.5 MHz, 298 K)
$^1$H-NMR spectrum of phenolic procyanidin B5 in acetone-$d_6$ (300 MHz, 298 K)
$^1$H-NMR spectrum of peracetylated procyanidin B5 in chloroform-d$_1$ (300 MHz, 298 K)
$^1$H-NMR spectrum of phenolic procyanidin B4 in acetone-$d_6$ (300 MHz, 298 K)
$^1$H-NMR spectrum of peracetylated procyanidin B4 in chloroform-d$_1$ (300 MHz, 298 K)
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List of oral presentations

May 17th 1995      Chiroptical methods with emphasis on analytical applications of circular dichroism.
                   (phytochemical seminar, Departement of Pharmacy, ETH Zurich)

July 5th 1996      Technology of liquid chromatography – mass spectrometry and applications in natural products chemistry.
                   (phytochemical seminar, Departement of Pharmacy, ETH Zurich)

October 11th 1996  Chromatographisches Verhalten und Detektionsprobleme mit Procyanidinen in der RP-HPLC.
                   (Weissdorn-Workshop, Departement of Pharmacy, ETH Zurich)
Curriculum vitae

1963  Born on December 11th in CH-Basel
      as daughter of Jürg K. Rohr and Liz H. Rohr-Höriger

1970 - 1983  Schools in Basel
1984  Jacottet Küng award

1983 - 1989  Study of pharmacy at the University of Basel

1990  Industrial practicum at Sandoz Pharma AG, Basel
      (inverse gas chromatography)

1990 - 1992  Study director at RCC Umweltchemie AG, CH-Itingen/BL
             (head of the Food Contact Materials Laboratory)

1992 - 1994  Anthroposophic teachers' training college, CH-Dornach

1994 - 1998  Ph.D. candidate at the Swiss Federal Institute of Technology Zurich

1997  Marriage to Hans-Peter Fritschi

since 1998  Employment at Emil Flachsmann AG, CH-Wädenswil
            (head of the Analytical Development and Pharma Application Laboratories)