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A Detailed View on the Proanthocyanidins in Ginkgo Extract EGb 761

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ABSTRACT

The Ginkgo extract EGb 761® manufactured with leaves of *Ginkgo biloba* has been continuously produced over decades at a large scale and is used as a clinically proven remedy for, among other things, the improvement of age-associated cognitive impairment and quality of life in patients with mild dementia. It belongs to the class of extracts addressed as quantified extracts according to the European Pharmacopeia. Accordingly, several compounds (e.g., flavone glycosides and terpene trilactones) are acknowledged to contribute to its clinical efficacy. Covering only about 30% of the mass balance, these characterized compounds are accompanied by a larger fraction of additional compounds, which might also contribute to the clinical efficacy and safety of the extract. As part of our systematic research to fully characterize the constituents of Ginkgo extract EGb 761, we focus on the structural class of proanthocyanidins in the present study. Structural insights into the proanthocyanidins present in EGb 761 and a quantitative method for their determination using HPLC are shown. The proanthocyanidins were found to be of oligomeric to polymeric structure, which yield delphinidin and cyanidin as main building blocks after acidic hydrolysis. A validated HPLC method for quantification of the anthocyanidins was developed in which delphinidin and cyanidin were detected after hydrolysis of the proanthocyanidins. The content of proanthocyanidins in Ginkgo extract EGb 761 was found to be approximately 7%.

ABBREVIATIONS

PACs	proanthocyanidins
Ph. Eur.	European Pharmacopeia
VIS	visual light detection

Introduction

The special Ginkgo extract EGb 761 [1] manufactured with leaves of *Ginkgo biloba* L. (Ginkgoaceae) is widely used for the improvement of age-associated cognitive impairment and quality of life in mild dementia, with proven clinical efficacy [2–4]. The Committee on Herbal Medicinal Products of the European Medicines Agency has published a monograph on *G. biloba* folium [5]. EGb 761 is registered as a drug in many countries worldwide. The herbal drug "Ginkgo leaf" is described in the monograph of the European Pharmacopeia [6] together with the monograph, *Ginkgo Dry Extract, Refined and Quantified* [7]. In accordance with the

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monograph, *Herbal Drug Extracts*, of the European Pharmacopeia [8], the classification as a quantified extract considers the fact that the specified active markers are likely to contribute to the clinical efficacy. However, in contrast to constituents with known therapeutic activity in standardized extracts, these active markers in quantified extracts cannot explain the clinical efficacy on their own [9]. In the case of the cited monograph of the European Pharmacopeia for quantified Ginkgo extract [7], the active markers are flavone glycosides, specified at 22.0 to 27.0%, and terpene trilactones, specified at 2.6 to 3.2% for bilobalide and 2.8 to 3.4% for ginkgolides A, B, and C (calculated on the dried extract). In sum, these compounds yield approximately 30% of the dry extract. As part of our systematic research on constituents of the extract EGb 761 from leaves of *G. biloba,* we are working on elucidation of compounds not covered by the monograph. In this study, we focus on the class of structures usually addressed as PACs.

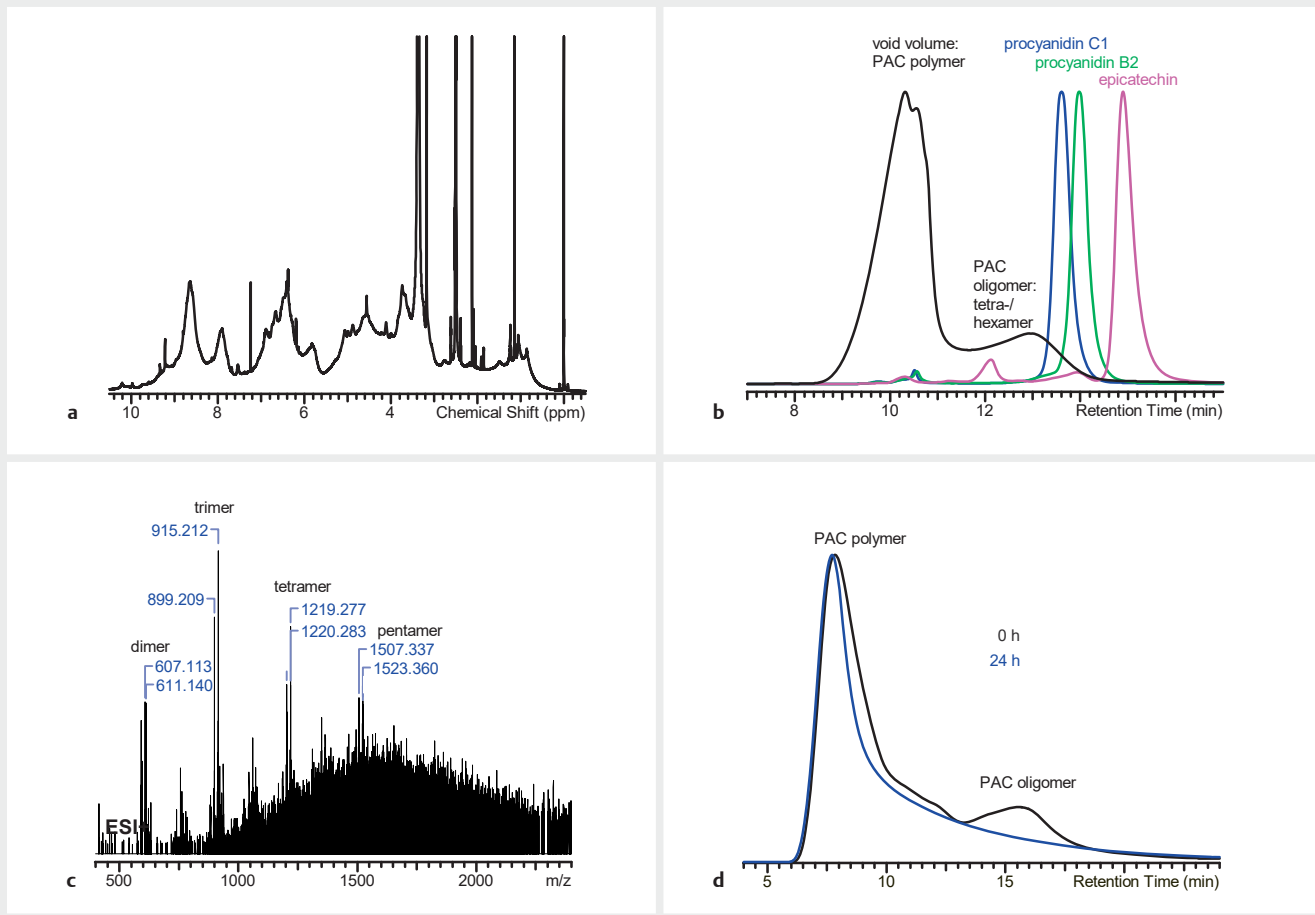
PACs are complex oligomeric and polymeric compounds in plants [10]. Depending on their degree of polymerization and the occurrence of different building blocks, fractions containing PACs show a multitude of compounds. Quantitative analytical approaches mainly applied classical methods such as color reactions and hydrolysis by hydrochloric acid followed by photometric assays [11] or used more modern methods like the (dimethylamino)cinnamaldehyde (DMAC) assay [12]. However, due to the manifold combination of the building blocks, linkage-types, stereochemical configuration, and polymerization degree, PACs are a highly challenging compound class that often require tailored methods for characterization [13]. The PACs in Ginkgo leaves were extensively studied by Qa'dan et al. [14] and were found to consist of B-type linkages ($4\beta \rightarrow 6$ or $4\beta \rightarrow 8$) of mainly (epi-)gallocatechins and some minor amount of (epi-)catechins. However, structures and quantity of PACs in Gingko extracts used for finished products like EGb 761 were not studied in detail.

In the present study, an authentic fraction of PACs in Ginkgo extract EGb 761 was purified and subjected to a phytochemical characterization to establish a robust quantification method for PACs in Ginkgo extract EGb 761 through liquid chromatography and photometric detection that could be applied for routine analysis. In the native fraction of PACs in Ginkgo extract EGb 761, the multitude of compounds does not allow a good peak separation. Thus, we decided to use an approach where PACs are hydrolyzed and quantification is done using the resulting peaks of defined anthocyanidins. As anthocyanidins are known to exhibit low stability as reference substances, we used procyanidin B2 as a reference substance for calibration purposes. This compound can be handled easily and is known to be sufficiently stable. Additionally, this compound is commercially available. For calibration purposes, reference solutions were prepared by hydrolysis of procyanidin B2, and the resulting peak of cyanidin was used for quantitation. Sample solutions of Ginkgo extract EGb 761 were treated accordingly, and resulting peaks of major anthocyanidins delphinidin and cyanidin were used. The method was appropriately validated for precision, intermediate precision, linearity, accuracy, selectivity, and robustness and is suitable for the intended application. Besides the analysis of 11 batches of Ginkgo extract EGb 761 for which the content of PACs was to be determined, we also analyzed the purified authentic fraction of PACs from Ginkgo extract EGb 761. Due to the phytochemical complexity of this authentic PACs fraction, we derived a response factor for recalculation of the content of PACs in this fraction and further calculation of PACs in Ginkgo extract EGb 761.

Results

For molecular characterization and as the quantitation reference standard, a representative fraction of the PACs was purified by repeated column chromatography. The purified fraction was characterized by ^{1}H-NMR, and the distribution of the molecular weight was determined by analytical gel permeation chromatography and HRMS (▶ **Fig. 1**). The NMR spectrum featured broad signals that are characteristic for polymers in general and PACs in particular (▶ **Fig. 1 a**). Besides a considerable amount of water observed as a characteristic resonance at 3.3 ppm in DMSO-d_6, only minor sharp peaks of small-molecule impurities were observable, which were negligible in comparison to the integrals of the broad polymer signals. The water content was determined to be 9.19% by Karl Fischer titration using common standard procedures. In gel permeation chromatography, a large fraction of the proanthocyanidins appeared in the void volume, indicating a high polymerization degree. However, a considerable fraction appeared as a broad peak with a retention volume, which would correspond to tetra- to about hexameric procyanidins (▶ **Fig. 1 b**), referred to as oligomers in the following. The molecular weights of these oligomers could be observed up to pentamers in HRMS spectra (▶ **Fig. 1 c**). The observed mass patterns correspond to pure prodelphinidins or prodelphinidins with 1 procyanidin building block (mass difference of 16 amu), all with mainly B-type linkages and with A-type linkages only observed for dimers. Due to the mass range limitation of the Orbitrap detector and low signal-to-noise ratio, higher polymers could not be detected. However, the detected oligomers were not stable in solution. This was observed in gel permeation chromatography, where the oligomer peak disappeared after storage times of > 24 h in solution, yielding only polymers eluting in the void volume (▶ **Fig. 1 d**). This behavior indicates that the PACs undergo a transformation in solution, which might be oxygen-induced polymerization or non-covalent aggregation. Other tested protocols for characterization of the PACs such as Diol-phase separation [15] did not yield PAC clusters as distinct peaks as expected from literature data. This observation may be due to the nature of the Ginkgo PACs, which seem to be prone to polymerization in solution and are mainly composed of prodelphinidins instead of procyanidins, the latter being the class of compounds applied to Diol-phase separation in literature [15]. Due to the negligible impurity signals in NMR spectra and a chromatogram that is characteristic for oligomers and polymers with no absorption for retention times of small molecules, the purified PACs were set to a content of 100% HPLC purity minus the water content of 9.19%.

In order to confirm the flavan-3-ol monomer composition expected from the above HRMS data, the proanthocyanidin fraction was hydrolyzed with hydrochloric acid and analyzed by HPLC. For the characterization of the phenolic hydrolysis products, a gradient HPLC method was used, with a detection wavelength specific for anthocyanidins (▶ **Fig. 2**). Since anthocyanidins are the only compounds in the Ginkgo extract that exhibit absorption at 530 nm after hydrolysis, the method for quantification not only



▶ **Fig. 1** **a** ^{1}H-NMR spectrum of polymeric proanthocyanidins (PACs) purified from EGb 761. The broad signals are characteristic for polymers and comprise the major intensity of the spectrum. Sharp signals of small molecule impurities have low intensity compared to the polymer signals, and thus, can be considered as negligible. Only the water resonance at 3.3 ppm is of considerable intensity. **b** Gel permeation chromatograms of PACs (black), and proanthocyanidins of different size for calibration: procyanidin C1/trimer (blue), procyanidin B2/dimer (green), and epicatechin-monomer (pink) detected optically at a wavelength of 230 nm. A large fraction of the PACs elutes in the void volume at the retention time of 9–11 min. A minor fraction elutes at the retention time of 12–13 min, indicating a tetra- to hexameric state of the proanthocyanidins. In the PAC fraction, no absorption at 230 nm is observable for molecules smaller than procyanidin B2, indicating that the fraction consists mainly of PAC oligomers and polymers. **c** ESI+HRMS Spectrum of PACs. Mass patterns of dimers to pentamers are observable for prodelphinidins or prodelphinidins with 1 cyanidin monomer due to the mass difference of 1 oxygen atom (16 amu). The detected masses are characteristic for B-type linkage, with masses for A-type linkage only observable for the dimer (607 amu). **d** Gel permeation chromatograms of PACs detected at a wavelength of 230 nm. Measurement directly after dissolving the sample (black), and after 24 h in solution (blue). The oligomer fraction depletes over time in the solution, resulting in elution only in the void volume. This behavior indicates a polymerization of the proanthocyanidins in solution. The different retention times are due to 2 different columns used: MCX analytical 100 Å column (**b**) and PolySep-SEC GFC-P3000 column (**d**).

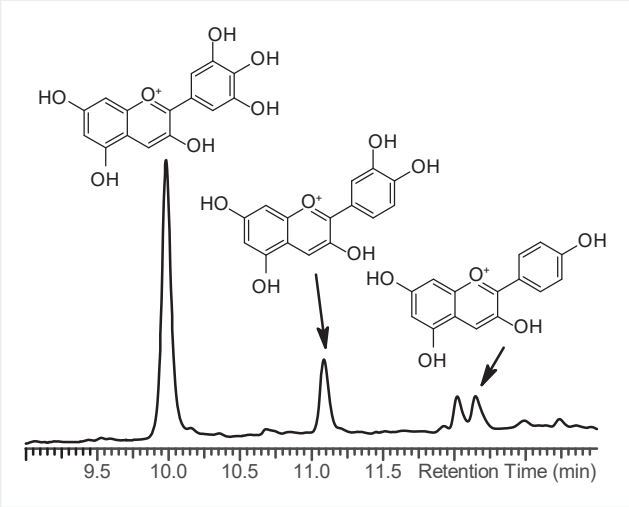
can be used for the purified fraction but also the whole extract. The detected anthocyanidins were delphinidin, cyanidin, pelargonidin, and an anthocyanidin compound that could not yet be identified, in the relative integral-ratio of 77.7%, 15.9%, 3.0%, and 3.4%, respectively. The hydrolysis compounds were identified by spiking with reference compounds. Although the anthocyanidins are in a charged state, the compounds could not be detected by ESI mass spectrometry. In this respect, the unknown hydrolysis compound could not be identified by a naïve approach. Spiking was tried with luteolinidin, fisetinidin, peonidin, petunidin, and malvidin, which, however, did not exhibit the retention time of the unknown peak (data not shown). The monomer composition observed after hydrolysis was consistent with the composition determined by HRMS, with (epi-)gallocatechin (or delphinidin after hydrolysis) being the major monomer with a minor content of (epi-)catechin (or cyanidin after hydrolysis). This observation is also consistent with literature data for Ginkgo PACs [14].

In summary, the EGb 761 PACs consist of polymers and dimers to about hexamers. The latter polymerize or aggregate in solution to a high polymeric form over time. The polymeric proanthocyanidins form mainly delphinidin and cyanidin, trace amounts of pelargonidin, and one unknown anthocyanidin compound as hydrolysis products.

Several HPLC methods were evaluated for quantitation of PACs in Ginkgo Extract EGb 761. In the monograph *Ginkgo Dry Extract, Refined and Quantified* by the European Pharmacopeia [7], a method is described for flavonoids after acidic hydrolysis of Ginkgo flavone glycosides using HPLC and photometric detection. During

our research, we observed that when using this original method of the European Pharmacopeia, a characteristic peak pattern was detectable at a wavelength of 530 nm within the first few min. The main emerging peaks were found to be the anthocyanidins delphinidin and cyanidin beside two smaller peaks at retention times of 5.8 and 6.3 min. As the original method focuses on quantitation of flavonoids, we used the method for additional quantitation of anthocyanidins within another retention timeframe and detection wavelength. For this reason, we adapted the method regarding the gradient elution to shorten the run time.

The purified PAC fraction as described above was subjected to the HPLC assay after hydrolysis. As the HPLC assay of the fraction is essential for the calculation of the content, this assay was done in replicates (n = 6) with completely independent sample preparations and was repeated with new samples (n = 6) after 1 wk. The result was the content for PACs of 43.4% (day 1) and 42.2% (day 2) in this fraction, calculated as procyanidin B2. As result, the mean value of 42.8% was taken, corresponding to 47.1% calculated on the water-free fraction considering the water content of 9.19%. The emerging peaks of delphinidin and cyanidin in this fraction were quantified as cyanidin, and the content of PACs in this fraction was calculated using a calibration of the emerging peak of cyanidin in the standard solutions of procyanidin B2 as described. Since the PAC fraction purified from Ginkgo Extract EGb 761 was set to 100% by convention, a conversion factor was needed for determining the content using procyanidin B2. The applied HPLC assay correlates to a response factor of 2.12 for the water-free fraction. To receive the corresponding content of PACs in Ginkgo extract EGb 761, this response factor must be considered. This means that the results of anthocyanidins for the extract must be multiplied by 2.12 to get the content of genuine EGb 761 PACs. In this context, 11 batches of Ginkgo extract EGb 761 were subjected to the HPLC assay, and the contents of PACs determined as described above are summarized in ▶ **Table 1**. Thus, the mean content of PACs in the batches of Ginkgo extract EGb 761 was 6.98%, with a range from 6.02% to 7.36%.



▶ **Fig. 2** HPLC chromatogram of the polymeric proanthocyanidins (PACs) purified from EGb 761 after acidic hydrolysis detected at 530 nm. The major hydrolysis product is delphinidin eluting at 10.0 min, with some minor fraction of cyanidin (eluting at 11.1 min) and pelargonidin (eluting at 12.2 min). The elution peak at 12.0 min could not yet be characterized.

▶ **Table 1** Results for PACs in 11 batches of Ginkgo extract EGb 761. Results calculated as procyanidin B2 are transferred to the authentic PACs fraction by multiplication with the conversion factor 2.12.

Batch Ginkgo Extract EGb 761	Assay PACs calculated as procyanidin B2	Assay PACs calculated as authentic PACs fraction
1	3.46%	7.34%
2	3.44%	7.29%
3	3.38%	7.17%
4	3.35%	7.10%
5	3.47%	7.36%
6	3.06%	6.49%
7	2.84%	6.02%
8	3.15%	6.68%
9	3.45%	7.31%
10	3.32%	7.04%
11	3.30%	7.00%
Mean	**3.29%**	**6.98%**
Standard deviation	**0.20%**	**0.42%**

Discussion

The polymeric PACs present in *G. biloba* extract EGb 761 exhibit an uncommon characteristic composition of hydrolysis products with the major compounds being delphinidin and cyanidin. The extent of polymerization seems to include an oligomeric fraction of mainly B-type dimers to hexamers that were, however, unstable in solution and form higher polymers over time. Since the polymerization takes place in solution, it is possible that the major polymeric fraction is not the genuine state of the PACs but rather a reaction product happening during extraction and isolation. PACs derived from hawthorn, cranberries, or grape seed are mainly composed of (epi-)catechin building blocks (or cyanidin after hydrolysis). In contrast, the PACs present in Ginkgo extract have (epi-)gallocatechin as the major building block (or delphinidin after hydrolysis). This difference may be responsible for the fact that some methods for characterization of PACs reported in literature, like Diol-phase separation, do not lead to usable results in the case of Ginkgo PACs.

Due to the complexity of this fraction with polymeric structures, the objective of this study was to provide a new method for easy and robust quantitation of PACs in Ginkgo extract EGb 761 by HPLC and photometric detection. The method developed in this study is based on the pharmacopeial method for flavonoids in the monograph *Ginkgo Dry Extract, Refined and Quantified* from the European Pharmacopeia [7], which we adopted to quantify PACs. Instead of delphinidin or cyanidin, procyanidin B2 is suggested as a standard substance since it shows sufficient stability

for handling at room temperature and is easily commercially available. The analytes delphinidin and cyanidin are formed *in situ* during the hydrolysis of both standard solution and sample solutions and are analyzed together within the same chromatographic run within appropriate run times. A product-specific correlation of the results was derived by analysis of the authentic fraction of EGb 761-PACs. The new method is highly selective and supports the obtained results by additional information concerning the building blocks of PACs in Ginkgo. As a chromatographic method, automation is easily possible in contrast to classical methods based on photometry that require more manual steps.

Our data show that PACs were present in the batches tested with a mean quantity of approximately 7%. Thus, PACs can now be considered to be a quantitatively relevant portion of natural compounds within the Ginkgo extract EGb 761. Due to the well-defined production process, the content of PACs in EGb 761 is maintained within a constant narrow range, including the batches tested in clinical trials for demonstration of clinical efficacy of EGb 761. The polymerization degree and building blocks of PACs are relatively well known, but the exact structures still require elucidation.

Materials and Methods

Purification of PACs

One kg of Ginkgo extract EGb 761 was dissolved in 2 L deionized water at 60 °C. After cooling the solution to room temperature (25 °C), the solution was filtered through a porous glass filter with a pore size of 10–16 µm (G4) to remove a small amount of insoluble residue. The insoluble residue was suspended in about 100 mL deionized water, sonicated for 5 min, and filtered again. The insoluble residue consists mainly of ginkgolides (~ 12 g). The extract solution was applied on water-presoaked Sephadex LH-20 (GE Healthcare) material in a column, with a gel bed volume of 12.5 L. The elution started with 30 L water to remove all hydrophilic compounds. The upper 4.5 L of the gel bed containing the PACs were removed, suspended in aqueous 25% vol. ethanol, and placed into a separate column. A serial elution followed with 14 L of aqueous 25% vol. ethanol, followed by 10 L of 96% vol. ethanol. These 2 fractions were discarded. The PACs were eluted with 8 L of an aqueous 70% vol. acetone solution. This protocol was repeated with another 1 kg of Ginkgo extract EGb 761. The PAC fractions from the first and second purification batch were combined, and the solvent was evaporated. For final purification, the solid residue (78 g) was dissolved in 0.7 L aqueous 50% vol. ethanol and applied once again on a fresh LH-20 material presoaked with aqueous 50% vol. ethanol and a gel bed volume of 5.4 L. The elution started with 5.9 L aqueous 50% vol. ethanol to remove remaining impurities. The PACs were eluted with 14.6 L of an aqueous 70% vol. acetone solution, yielding 69 g dry residue.

Molecular characterization of PACs

NMR spectra were acquired on a Bruker Avance III HD 600 MHz system equipped with an inverse Prodigy TCI cryoprobe. We dissolved 24.5 mg of PACs in 600 µL DMSO-d_6. 1D-^{1}H NMR spectra were measured using tetramethylsilane as internal standard with 32 accumulated scans with a spectral width of 18 ppm, an offset of 8 ppm for the transmitter frequency, and a digital resolution of 64 k data points. The spectra were recorded and processed with Bruker Topspin software (v3.6pl7) and analyzed with ACD/Labs Spectrus Processor (v2017.2.1).

ESI-HRMS spectra were acquired on a Thermo Orbitrap Fusion mass detector. PACs were dissolved to a concentration of 1 mg/mL in a mixture of acetonitrile-water-formic acid 49.75 : 49.75 : 0.50 (v/v/v) and injected with 5 µL/min directly into the ESI source by a syringe pump. Spectra were measured in positive ion mode with an ionization voltage of 3500 V, a high mass range of 400–6000 m/z, and a resolution of 30 k. The spectra were recorded and processed by Thermo Xcalibur software (v4.3) and analyzed by ACD/Labs Spectrus Processor (v2017.2.1).

HPLC analysis of the molecular weight by gel permeation chromatography was carried out on a Hitachi LaChrom System, consisting of an autosampler, a high pressure mixing pump, a column oven, and a diode array detector coupled in-line with a refractive index detector. Two methods were used for analysis. One method used a Polymer Standards Service MCX analytical 100 Å column (5 µm, 8 × 300 mm) with a total run time of 30 min, and the other method used a Phenomenex PolySep-SEC GFC-P3000 column (7.8 × 300 mm) with a total run time of 45 min. Both methods applied an isocratic elution of an aqueous 40% vol. acetonitrile solution with a flow rate of 0.8 mL/min; column temperature: 50 °C; detection: UV at 230 nm; injection volume: 10 µL; sample concentration: 4.5 mg/mL in elution solvent. For calibration of the retention times, epicatechin 0.8 mg/mL (monomer), procyanidin B2 1.0 mg/mL (dimer), and procyanidin C1 0.8 mg/mL (trimer) were used, respectively. Standards for higher oligomeric proanthocyanidins were not commercially available. The molecular weight of the sample was estimated by extrapolating the elution volumes of the mono-, di- and trimer.

Water content was determined by Karl Fischer titration using common standard procedures.

Qualitative HPLC analysis of the hydrolysis products was carried out on a Thermo UltiMate 3000 system with autosampler WPS-3000 TFC, pump HPG-3400 RS, detector DAD-3000 RS, and column oven TCC-3000 RS. The anthocyanidin hydrolysis products were analyzed using a Waters Cortecs UPLC C18 column (1.6 µm, 2.1 × 150 mm); solvent system A: water/acetonitrile/formic acid 98.7 : 1 : 0.3 (v/v/v), solvent system B: acetonitrile-water-formic acid 98.7 : 1 : 0.3 (v/v/v); 15–50% B over 17 min, 50–100% B over 2 min following rinsing with 100% B for 2 min, 100–15% B for 1 min and equilibration with 15% B for 5 min; flow rate: 0.2 mL/min; column temperature: 25 °C; detection: 530 nm; injection volume: 5 µL; sample concentration: 5 mg/mL in elution solvent. For calibration of the retention times, analytical grade standards were used; delphinidin, cyanidin, pelargonidin, peonidin, and malvidin were purchased from Sigma Aldrich, luteolinidin and fisetinidin were obtained from Extrasynthese, and petunidin was purchased from Cayman Chemical.

The HPLC method that was used for quantification and verification of the composition is described below.

Quantification of PACs

Test samples

Eleven batches of Ginkgo extract EGb 761 were used to analyze the quantity of PACs with the method described. Specimens are deposited in the archive of Dr. Willmar Schwabe GmbH & Co. KG, Germany. The results were used for additional calculation with an extract-specific response factor for the purified authentic PACs fraction of Ginkgo extract EGb 761 (see below).

Solvents, reagents, and chemicals

Methanol (p. a.), *o*-phosphoric acid, and hydrochloric acid were purchased from Merck. The deionized water was obtained by a water purification system (Evoqua, Water Technologies). For the preparation of the hydrolysis solution, 1 part methanol and 1 part 1.5 M hydrochloric acid were mixed (v/v).

Reference standard

Procyanidin B2 (CAS number 29 106–49–8) with purity ≥ 98 % was purchased from Cayman Chemical Company. The reference standard procyanidin B2 was hydrolyzed in the reference solution to form the reference peak cyanidin *in situ* (see below).

Preparation of standard solutions

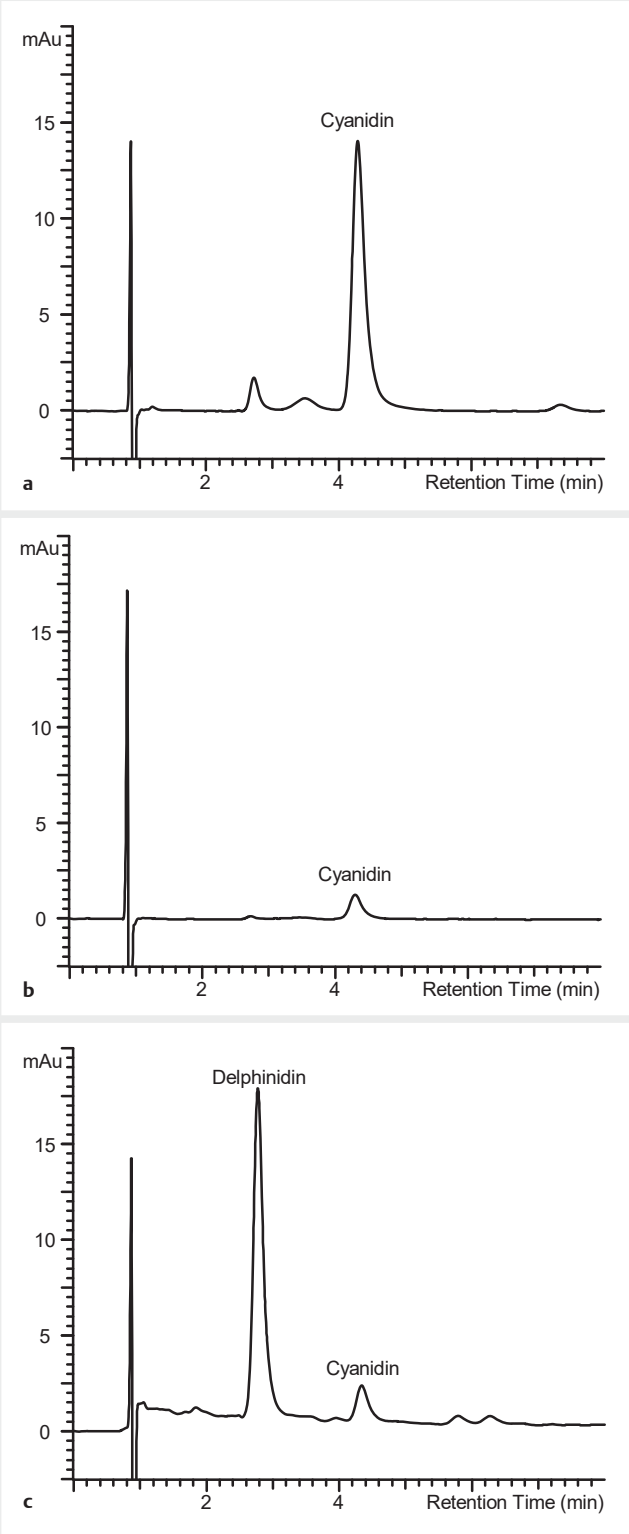
Reference standard (procyanidin B2) is weighed exactly into appropriate volumetric flasks and dissolved in the hydrolysis solution to obtain a standard solution with 100 µg procyanidin B2/mL (example chromatogram, see ▶ **Fig. 3 a**). This standard solution was diluted with the hydrolysis solution to receive an additional standard solution with 10 µg procyanidin B2/mL (example chromatogram, see ▶ **Fig. 3 b**). The standard solutions were hydrolyzed in parallel and in the same way as described for the sample solutions (see below). The standard solutions appeared clear after hydrolysis and were used directly for HPLC analysis without prior filtration.

Preparation of sample solutions

Samples of 100 mg Ginkgo extract EGb 761 were weighed exactly into 25 mL volumetric flasks, filled up to volumes with hydrolysis solution, and dissolved in an ultrasonic bath (SONOREX Super RK510, 160/320 W, 35 kHz, Company Bandelin) for 10 min at room temperature. The obtained test solutions were hydrolyzed in a boiling water bath in tightly closed 10 mL hydrolysis tubes with screw caps remaining above the surface. We determined 45 min to be the optimal duration to yield complete hydrolysis. After hydrolysis, the closed tubes were cooled in an ice bath and stored at ambient conditions for tempering to room temperature (23 °C). The sample solutions appeared clear after hydrolysis and were used directly for HPLC analysis without prior filtration (example chromatogram, see ▶ **Fig. 3 c**).

Analysis of PACs in samples of Ginkgo extract EGb 761 and EGb 761 PACs

The hydrolyzed standard solutions and sample solutions of the purified PACs fraction and of the 11 batches of Ginkgo extract EGb 761 were transferred directly into individual vials without prior filtration and were submitted for HPLC analysis.



▶ **Fig. 3** **a** HPLC chromatogram of the standard solution with approximately 100 µg procyanidin B2/mL after hydrolysis, detected at 530 nm. **b** HPLC chromatogram of the standard solution with approximately 10 µg procyanidin B2/mL after hydrolysis, detected at 530 nm. **c** HPLC chromatogram of one sample solution after hydrolysis, detected at 530 nm. The retention times differ from the method used for molecular characterization (▶ **Fig. 2**). The integrals of the measured peaks are within the range of the calibration.

Chromatography

HPLC was performed on a Thermo UltiMate 3000 system with an autosampler WPS-3000 TRS, a pump LPG-3400 RS, a detector MWD-3000 RS, and a column oven TCC-3000 SD using a Kromasil C18 (5 µm, 4 × 125 mm) column (MZ-Analysentechnik) without pre-column. The mobile phase consisted of water adjusted to pH 2.0 with *o*-phosphoric acid 85 % solution (phase A) and methanol (phase B). The following gradient was applied at a flow rate of 1.0 mL/min: isocratic from 0.00–1.00 min at 60 % Eluent A, from 1.00–8.00 min linear from 60 % Eluent A to 54.5 % Eluent A following 8.00–9.00 min linear 0 % Eluent A, 4 min column wash with 0 % Eluent A, from 13.00–13.50 min to 60 % Eluent A and 6.5 min equilibration period with 60 % Eluent A, resulting in a total run time of 20.00 min. VIS detection wavelength of 530 nm, a column temperature of 25 °C, and an injection volume of 10 µL were applied.

The retention time for delphinidin was approximately 2.8 min, for cyanidin 4.3 min, and for pelargonidin 6.3 min, respectively. Quantitation was done for the peaks of delphinidin and cyanidin individually using the standard solutions of hydrolyzed procyanidin B2, and both results were added for further calculation. Delphinidin was calculated as cyanidin. Smaller peaks like pelargonidin were not considered in the sample solutions for quantitation, due to being off of the dynamic detection range.

Data analysis

Data processing and analysis were carried out using Chromeleon 7.2 SR5 software (Thermo).

Validation data

The method was comprehensively validated addressing the parameters precision, intermediate precision, linearity, accuracy, selectivity, and robustness. A test for precision with n = 6 individual sample preparations of Ginkgo extract EGb 761 showed a relative standard deviation of 6.46 %. The test was repeated after 1 week for evaluation of intermediate precision with new sample preparation and new calibration and showed a relative standard deviation of 5.61 %. Linearity was tested by linear regression with 12 concentrations between 0.602 µg procyanidin B2/mL to 120.400 µg procyanidin B2/mL. Correlation coefficient was 0.999946 with y-intercept − 0.0181 area and slope 0.0353 area/µg/mL. As the reference substance cyanidin is formed *in situ* by acidic hydrolysis of procyanidin B2, accuracy was addressed by the addition of procyanidin B2 to an accurately weighed quantity of Ginkgo extract EGb 761. The quantity of Ginkgo extract EGb 761 was half as described in the test procedures and was supplemented by the addition of procyanidin B2 to a final quantity of 80 % and 120 % of the theoretical content of PACs in the sample of Ginkgo extract EGb 761. The test was done twice with recovery rates for cyanidin of 97.3 % and 95.7 % for the 80 % concentration level and 95.9 % and 95.7 % for the 120 % concentration level. Selectivity was shown with view on the peak identification of delphinidin, cyanidin, and pelargonidin and their good chromatographic separation. The robustness of the method was tested by variation of method parameters described in the test procedures like column temperature and pH value of the mobile phase A within relevant ranges and was considered to be suitable. Also, the time required for an analysis series was suitable to guarantee the stability of reference and sample solutions.

Contributors' Statement

Purification of the material: B. Röck; data collection: T. Ritter, J. Elsäßer, H. Schneider; design of the study: Z. Kulic, S. Germer; analysis and interpretation of the data: Z. Kulic, S. Germer; drafting the manuscript: Z. Kulic, S. Germer; these authors contributed equally: Z. Kulic, S. Germer.

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Conflict of Interest

All authors are employees of Dr. Willmar Schwabe GmbH & Co. KG, Germany.

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