Dissertation

Charakterisierung von oligomeren Kakao-Proanthocyanidinen:

Extraktion und chromatographische Trennung mit simultaner Bestimmung der antioxidativen Kapazität

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2017

Charakterisierung von oligomeren Kakao-Proanthocyanidinen: Extraktion und chromatographische Trennung mit simultaner Bestimmung der antioxidativen Kapazität

Dissertation

zur Erlangung des naturwissenschaftlichen Doktorgrades (Dr. rer. nat.)
des Fachbereichs Chemie,
Fakultät für Mathematik, Informatik und Naturwissenschaften
der Universität Hamburg

vorgelegt von

Dipl. LMChem
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Hamburg

März 2017

Die vorliegende Arbeit wurde in der Zeit von November 2012 bis März 2017 unter der Leitung von Herrn Prof. Dr. Sascha Rohn, Institut für Lebensmittelchemie der Universität Hamburg, angefertigt. Die praktischen Arbeiten erfolgten an der Zürcher Hochschule für Angewandte Wissenschaften in Wädenswil in der Schweiz unter der Betreuung von Herrn Dr. Norbert Fischer.

Tag der Disputation: 30.10.2017

1. Gutachter der Dissertation: Prof. Dr. Sascha Rohn

2. Gutachter der Dissertation: Prof. Dr. Bernward Bisping

Kein zweites Mal hat die Natur
solche Fülle der wertvollsten Nährstoffe
auf einem so kleinen Raum zusammengedrängt
wie gerade bei der Kakaobohne.

Alexander von Humboldt

Veröffentlichungen

Publikationen

Pedan V, Weber, C, Do T, Fischer N, Reich E, Rohn S (2017) HPTLC fingerprint profile analysis of cocoa proanthocyanidins depending on origin and genotype. Food Chemistry. In press

Pedan V, Fischer N, Bernath K, Hühn T, Rohn S (2017) Determination of oligomeric proanthocyanidins and their antioxidant activity from different chocolate manufacturing stages using the NP-HPLC-online-DPPH methodology. Food Chemistry, 214, 523-532.

Pedan V, Fischer N, & Rohn S (2016) Extraction of cocoa proanthocyanidins and their fractionation by sequential centrifugal partition chromatography and gel permeation chromatography. Analytical and Bioanalytical Chemistry, 408, 5905-5914.

Pedan V, Fischer N, & Rohn S (2016) An online NP-HPLC-DPPH method for the determination of the antioxidant activity of condensed polyphenols in cocoa. Food Research International, 89, 890-900.

Vorträge

Pedan V, Weber C, Do T, Fischer N, Reich E, Rohn S (2016) High performance thin layer chromatography: Determination of oligomeric proanthocyanidins during different chocolate manufacturing stages. The 1st Food Chemistry Conference, 30. Oktober - 1 November 2016, Amsterdam, Niederlande.

Pedan V, Fischer N, Rohn S (2015) An online NP-HPLC-DPPH method for the determination of the antioxidant activity of condensed polyphenols in cocoa. 3rd International Congress on Cocoa Coffee and Tea, 22.-24. Juni 2015, Aveiro, Portugal.

Pedan V, Fischer N, Rohn S (2015) An online NP-HPLC-DPPH method for the determination of the antioxidant activity of condensed polyphenols from different chocolate manufacturing stages. 4th International Conference and Exhibition on Food Processing & Technology, 10.-12. August 2015. In: Proceedings of 4th International Conference and Exhibition on Food Processing & Technology 6 (8), ISSN: 2157-7110: 100.

Pedan V, Fischer N, Rohn S (2015) Monitoring of condensed polyphenols and their antioxidant activity in cocoa processing. 29th EFFoST International Conference, 28.-30.

November, Athen, Griechenland. In: Proceedings of 29th EFFoST International Conference, ISBN: 978-618-82196-1-8: 82-85.

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Poster

Pedan V, Weber, C, Do T, Reich E, Rohn S (2017) HPTLC fingerprint profile analysis of cocoa proanthocyanidins depending on origin and genotype. International Symposium for High-Performance Thin-Layer Chromatography, 4.-8. Juli, Berlin, Deutschland.

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Inhaltsverzeichnis

Abkürzungsverzeichnis

abs.	absolut
ABTS	2,2′-Azino-bis-(3-ethylbenzthiazolin-6-sulfonsäure)
ACN	Acetonitril
CyA	Cyanidinäquivalente
Da	Dalton
DAD	Dioden Array Detektor
dest.	Destilliert
DHAP	3-Deoxy-o-arabino-heptulosonat-7-phosphat
DMAC	4-Dimethylaminocinnamaldehyde
DMSO	Dimethylsulfoxid
DP	Degree of Polymerization
DPPH	2,2-Diphenyl-1-pikrylhydrazyl
ECÄ	Epicatechinäquivalente
ECG	Epicatechingallat
EGC	Epigallocatechin
EPA	Extrahierbare Proanthocyanidine
EPP	Extrahierbare Polyphenole
ESI	Elektrosprayionisation
EtOAc	Ethylacetat
EtOH	Ethanol
ff	Fettfrei
GAE	Gallussäureäquivalente
HAT	Wasserstoffatom-Transfer
k. A.	Keine Angaben
LC	Liquid Chromatography
LOD	Nachweisgrenze
LOQ	Bestimmungsgrenze
MeOH	Methanol
MS	Massenspektrometrie
MTBE	Methyl-*tert*-butylether
MW	Molekulargewicht
m/z	Masse-zu-Ladung-Verhältnis
NEPA	Nicht extrahierbare Proanthocyanidine
NP-LC	Normalphasen HPLC
PA	Proanthocyanidine
PEP	Phosphoenolpyruvat
PG	Polymerisationsgrad
PPO	Polyphenoloxidase
RP-LC	Umkehrphasen HPLC
rpm	Umdrehungen pro Minute
SCPC	Zentrifugale Gegenstromverteilungschromatographie
SET	Ein-Elektronen-Transfer
TEAC	Trolox Equivalent Antioxidant Capacity
TM	Trockenmasse
U/min	Umdrehungen pro Minute
UV	Ultraviolett
Vis	Sichtbares Spektrum

Kurzfassung

Kakaobohnen sind reich an phenolischen Substanzen, insbesondere an oligomeren und polymeren Proanthocyanidinen (PA), die bis zu 60% des Gesamtpolyphenolgehaltes im Kakao ausmachen. Die antioxidative Aktivität von monomeren Flavan-3-olen ist hinreichend bekannt, wobei es aber immer noch ein herausforderndes Thema ist, wenn es um die Bestimmung der antioxidativen Kapazität von oligomeren oder polymeren PA geht. Trotz des hohen wissenschaftlichen Interesses sind oligomere Kakao-PA mit einem Polymerisationsgrad (PG) ≤ 4 entweder teuer oder mit einem PG ≥ 5 nicht kommerziell zu erwerben.

Im ersten Teil der Arbeit wurde mittels schonender Extraktion, ein polyphenolreicher Kakaoextrakt aus der Matrix extrahiert. Hierfür wurde mittels eines kolorimetrischen *Assays* hinsichtlich des Extraktionslösungsmittels, der Extraktionstemperatur und dem Probe-zu-Lösungsmittel-Verhältnis eine Optimierung vorgenommen. Es konnte gezeigt werden, dass mittels 50%igem Aceton-Wasser-Gemisch, bei einer Extraktionstemperatur von 50 °C im Verhältnis 1:3 ein bestmögliches Ergebnis erreicht wurde. Der so gewonnene *Polyphenolreiche Kakaoextrakt* wurde im darauffolgenden Schritt mittels zentrifugaler Gegenstromverteilungschromatographie (engl. *sequential centrifugal partition chromatography*, SCPC) und dem Lösungsmittelsystem Ethylacetat-*n*-Butanol-Wasser (4/1/5, v/v/v) ein System zur Fraktionierung und Aufreinigung des gefriergetrockneten Kakaoextraktes geschaffen, welches es ermöglicht, die beiden Alkaloide Theobromin und Koffein von einer *Proanthocyanidinreichen Fraktion* abtrennen zu können. Für die weitere Isolierung einzelner PA wurde die *Proanthocyanidinreiche Fraktion* in eine Gelpermeationssäule gefüllt, mit Sephadex LH-20 injiziert und in einer sequentiellen Elutionsreihenfolge mittels Aceton-Wasser-Gemisch in seine individuellen PA aufgetrennt. Aus 40 mg der *Proanthocyanidinreichen Fraktion* konnte so ein Gemisch aus 5,6 mg von (+)-Catechin und Proanthocyanidin B2, 2 mg von Proanthocyanidin C1 und einem weiteren unbekannten Dimer und 2 mg des tetrameren Cinnamtannin A2 gewonnen werden.

Im zweiten Teil der Arbeit wurde darüber hinaus mittels Normalphasen-Hochleistungsflüssigkeitschromatographie und postchromatographischer Derivatisierung über 2,2-Diphenyl-1-pikrylhydrazyl (NP-HPLC-online-DPPH) eine Methode etabliert, welche es erlaubt, sowohl oligomere PA als auch die dazugehörige antioxidative Kapazität der Oligomere oder Oligomerfraktionen simultan zu messen. Durch das NP-HPLC-online-DPPH *Assay* konnten die oligomeren PA in ihre homologe Reihe angefangen vom Polymerisationsgrad (PG) PG = 1 bis PG = 13 getrennt und detektiert werden. Es konnte

gezeigt werden, dass die antioxidative Kapazität mit steigender homologer Reihe zunimmt, wobei das PA mit einem PG = 9 die höchste antioxidative Kapazität besitzt. Die antioxidative Kapazität bewegt sich nicht linear mit dem PG, sondern fällt mit dem Erreichen der polymeren PA-Struktur, wobei bei PA mit einem PG > 13 die antioxidative Kapazität wieder abnimmt. Nichtcharakterisierte polymere PA wurden im Chromatogramm als ein breiter Hügel detektiert, welcher sich von der Basislinie abhebt und zum Ende des Chromatogramms eluiert. Bei diesem Hügel handelt es sich um PA mit einem PG > 13. Der sogenannte ‘PA-Hügel’ mit seiner komplexen Zusammenstellung von Polyphenolen besitzt durch seine quantitative Menge zwar eine hohe antioxidative Aktivität aber nur eine geringe spezifische antioxidative Kapazität.

Die etablierte Polyphenolfraktionierung und -analytik wurde entlang des Schokoladenherstellungsprozesses angewandt, um Änderungen des Polyphenolgehaltes zu verfolgen. Hierbei verdeutlicht ein im Labormassstab durchgeführter Schokoladenherstellungsprozess die Änderung des Polyphenolprofils angefangen von der (1) rohen und frischen Kakaobohne, über die (2) fermentierte getrocknete Kakaobohne, zu der (3) gerösteten Kakaomasse, (4) 1 h conchierten Kakaomasse, (5) 4 h conchierten Kakaomasse bis hin zur (6) eingetafelten Schokolade. Hierbei wurde festgestellt, dass der Gesamtproanthocyanidin Gehalt ∑ (PG1 - PG13) von 30 mg (-)-Epicatechinäquivalente pro Gramm fettfreier Trockenmasse in rohen frischen Kakaobohnen auf 5 mg (-)-Epicatechinäquivalente in der finalen Schokolade abfällt. Die antioxidative Aktivität fällt dementsprechend von 25 mg (-)-Epicatechinäquivalente pro Gramm fettfreier Trockenmasse in den rohen frischen Kakaobohnen auf 4 mg (-)-Epicatechinäquivalente in der finalen Schokolade.

Insgesamt wurde während der Fermentation eine Verschiebung des Polyphenolprofils von oligomeren PA zu polymeren PA beobachtet, wobei oligomere PA bedingt durch die Polyphenoloxidase weiter kondensieren und so polymere Strukturen gebildet werden. Ein weiterer Einfluss auf die Verschiebung des Polyphenolprofils zeigte sich während der Röstung. Hierbei werden infolge der Hitzeeinwirkung sowohl monomere als auch polymere PA abgebaut. Der Gehalt an wertgebenden Inhaltsstoffen sinkt und die antioxidative Aktivität verringert sich hierbei.

Abstract

Cocoa beans are rich in monomeric flavan-3-ols and particularly epicatechin-based proanthocyanidin oligomers, which constitute approx. 60% of the total polyphenol content. Although the antioxidant activity of cocoa polyphenols is well known, it is still a challenging analytical field, especially, when it comes to the determination of condensed polyphenols. Despite the high scientific interest, oligomeric PA with a degree of polymerisation (DP) ≤ 4 are either expensive or with DP ≥ 5 not commercially available.

In the first part of the work, a gentle method was used to extract a polyphenol-rich cocoa extract from the matrix. A colorimetric assay was used to optimize the extraction conditions such as solvent, temperature and solvent-to-sample ratio. It was shown, that optimum extraction conditions were given with a 50% aqueous aceton mixture, a temperature of 50 °C and a solvent-to-sample ratio of 1:3. The polyphenol-rich cocoa extract was applied to the centrifugal partition chromatography with ethyl acetate-*n*-butanol-water (4/1/5, v/v/v) as solvent system for fractionation and purification. Here, a proanthocyanidin-rich fraction was obtained containing the cocoa polyphenols and the two alcaloids theobromine and caffeine. For further isolation of individual PA, gel permeation chromatography with sephadex LH-20 was used with a sequential elution order of aqueous acetone. Hereby, 40 mg of a PA rich cocoa extract yield 5.6 mg (+)-Catechin, PA B2, 2 mg PA C1 and 2 mg of the tetrameric cinnamtannin A2.

In the second part of the work, a NP-HPLC-online-DPPH system was developed by means of an online post-column derivatisation with 2,2-diphenyl-1-picrylhydrazyl (DPPH), to separate the homologous series of condensed polyphenols and to assess their antioxidant capacity in relation to their DP, simultaneously. In this context, several antioxidants were identified in the cocoa extract from monomers to tridecamers, whereby the results indicate that the radical scavenging activity of the homologous series of condensed PA is increasing with increasing DP.

A chocolate manufacturing process on a lab scale was monitored to visualize changes during processing on the content of oligomeric proanthocyanidins and their antioxidant capacity. Samples were collected from (1) raw fresh cocoa beans, (2) fermented, dried cocoa beans (3) roasted cocoa mass, (4) 1 h conched cocoa mass, (5) 4 h conched cocoa mass, and (5) molded chocolate bars. The sum of the total proanthocyanidin content ∑ (PG1 - PG13) decreased from 30 mg epicatechin equivalents per gram non-fat dry matter in raw fresh cocoa beans to 5 mg epicatechin equivalents per gram in the final chocolate. The antioxidant

capacity decreased accordingly from 25 mg epicatechin equivalents per gram nonfat dry matter in raw fresh cocoa beans to 4 mg/g in the final chocolate product.

The method described can be used for a rapid evaluation of condensed antioxidant components in complex matrices such as plant or food extracts. The online NP-HPLC-DPPH assay can also be considered as a promising technique for quality control in the chocolate manufacturing process.

1. Einleitung

Polyphenole sind Metabolite des sekundären Pflanzenstoffwechsels und stellen aufgrund ihrer gesundheitsfördernden Wirkung ein wichtiges Forschungsgebiet dar. Polyphenole kommen in hohen Konzentrationen in Lebensmitteln wie Obst, Früchten und Gemüse vor, aber auch in verarbeiteten Produkten wie Kaffee, Tee und Wein, sowie mit bedeutend hohen Gehalten in Kakao und seinem verarbeiteten Endprodukt Schokolade (Clarke & Vitzthum, 2001, Moreno-Arribas & Polo, 2009, Jalil & Ismail, 2008). In zahlreichen Untersuchungen konnten bereits die antioxidativen, entzündungshemmenden und sogar krebsvorbeugenden Eigenschaften von Polyphenolen nachgewiesen werden, wobei sich wirkungsspezifische *in vitro*- oder *in vivo* Messungen hauptsächlich auf monomere Polyphenole konzentrierten (Andújar *et al*., 2012). Sowohl einfache Extraktions- als auch Analysenmethoden zielen auf die Quantifizierung von monomeren Leitkomponenten ab. Obwohl der prozentuale Anteil an höhermolekularen Polyphenolen im Gegensatz zu niedermolekularen im Kakao um das 1,5-fache höher ist (Wollgast & Anklam, 2000), sind eindeutige wirkungsspezifische Nachweise dagegen nur schwer zugänglich. Die geringe Verfügbarkeit und die hohen Kosten von chemisch reinen Referenzsubstanzen machen es schwierig, substanzspezifische Messungen und einheitliche Aussagen zu tätigen. Viele *in vitro*- oder *in vivo* Studien arbeiten anstelle von Referenzsubstanzen mit polyphenolreichen Pflanzenextrakten, um eine wirkungsspezifische Aussage zu tätigen (Andújar *et al*., 2012). Um substanzspezifische Aussagen zu treffen, bedarf es jedoch einzelner Referenzsubstanzen, welche mittels Isolierung und Fraktionierung gewonnen werden können. Ein Ansatzpunkt hierzu bietet die zentrifugale Gegenstromverteilungschromatographie (engl. *sequential centrifugal partition chromatography*, SCPC). Diese ermöglicht es, Naturstoffe wie Flavonoide, abhängig von ihrem Verteilungskoeffizienten im ausgewählten Lösungsmittelsystem, in grösseren Mengen voneinander zu isolieren.

Um ein Verständnis für die biologische Wirkung zu bekommen, müssen einheitliche Messmethoden etabliert werden, die die Wirksamkeit von höhermolekularen Polyphenolen und ihrer antioxidativen Wirkung einander gegenüberstellen können. Die NP-HPLC mit postchromatographischer Derivatisierung bietet den Vorteil einer simultanen Bestimmung der oligomeren Proanthocyanidine (PA) und ihrer antioxidativen Kapazität in komplexen Matrizes.

Zunehmend rückt die Verfolgbarkeit von phenolischen Substanzen während der Lebensmittelbe- und -verarbeitung in den wissenschaftlichen Fokus. Gleichzeitig wird auf schonende Verfahren gesetzt, um nutritive Eigenschaften von wertgebenden Inhaltsstoffen

zu erhalten. Für eine ernährungsphysiologische positive Bilanzierung sollen somit im Endprodukt mehr genuine Inhaltsstoffe erhalten bleiben.

Die während des Promotionsvorhabens erbrachten Ergebnisse wurden in wissenschaftlichen Fachmagazinen wie *Food Chemistry*, *Food Research International* und *Analytical and Bioanalytical Chemistry* veröffentlicht. Auf Basis dieser wird die vorliegende Arbeit in Form einer kumulativen Dissertation präsentiert.

2. Theoretischer Hintergrund

Um das Verhalten der Polyphenole während der Schokoladenverarbeitung besser zu verstehen, werden im Folgenden die charakteristischen Prozessschritte erläutert. Im Mittelpunkt hierbei steht die Veränderung des Polyphenolprofils über den gesamten Schokoladenherstellungsprozess - von der ganzen Kakaobohne bis hin zur eingetafelten Schokolade. Im Detail wird das Polyphenolprofil auf der jeweiligen Verarbeitungsstufe anhand von Literaturdaten diskutiert. Daneben sollen Leitsubstanzen identifiziert werden, die zum Verständnis des jeweiligen Prozessparameters beitragen können.

2.1. Sekundäre Pflanzenstoffe des Kakaos

Pflanzenstoffe werden anhand ihres Stoffwechsels in primäre und sekundäre Metabolite unterteilt. Zu den primären Metaboliten zählen die energieliefernden Kohlenhydrate, Proteine und Fette. Sie sind ubiquitär verbreitet und essentiell für das Wachstum und die Entwicklung. Die sekundären Metabolite, zu denen u.a. die phenolischen Verbindungen zählen, sind hingegen z.T. selektiv verbreitet, haben aber meist eine essentielle Funktion für das Überleben der Pflanze im Ökosystem. Sie dienen der Pflanze z.B. als antimikrobieller Schutz, als Abwehrstoff gegen Fressfeinde oder sie sind Farb- und Duftstoffe der Pflanze. Die biologische Funktion vieler (polymerer) Polyphenole ist der Schutz der Pflanze im Falle einer Verletzung des Gewebes. Durch ihren bitteren und adstringierenden Geschmack dienen sie auch dem Schutz der Pflanze vor Fressfeinden.

Die grösste Untergruppe der Kakaopolyphenole stellen die Flavonoide dar, die widerrum in drei Gruppen unterteilt werden, wobei sie mengenmässig unterschiedlich in der Bohne vorliegen: Proanthocyanidine (PA) (~58%), Flavan-3-ole (~37%) und Anthocyane (~4%) (Wollgast und Anklam, 2000). Hierbei stellt laut Kim und Keeney (1984) das monomere (-)-Epicatechin mit 35% den grössten Anteil an Flavonoiden dar, was zwischen 34,7 und 43,3 mg/g fettfreier Trockenmasse in *Criollo*- oder *Forastero*-Bohnen entspricht. Aus den monomeren PA wie (-)-Epicatechin und (+)-Catechin (**Abbildung 1a**) setzen sich, über eine C4-C8 Bindungen, die oligomeren oder polymeren PA zusammen, die dann entweder über α- oder β-Konfiguration miteinander verknüpft sind (**Abbildung 1b**). Auf Grund der Vorrangstellung des (-)-Epicatechins ist das weit verbreitete PA B2, ein kondensiertes Dimer bestehend aus zwei (-)-Epicatechineinheiten. Als weitere phenolische Vertreter mit einer C4-C8 Bindung konnten im Kakao die Dimere B1, B3 und B4 identifiziert werden. Daneben existieren Dimere wie B5 und B6, die über eine C4-C6 Bindung miteinander verbunden sind. Das PA C1 ist das mengenmässig häufigste kondensierte Trimer und besteht aus drei (-)-Epicatechineinheiten, welche über eine C4-C8 Bindung miteinander verknüpft sind.

Sowohl C4-C8 als auch C4-C6 Dimere sind über eine Einfachbindung verknüft und werden als B-Typ zusammengefasst.

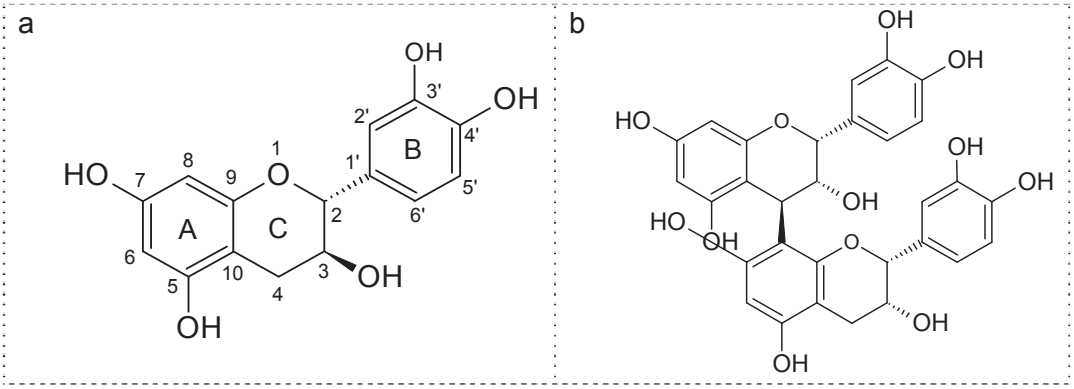


Abbildung 1: a. (+)-Catechin, b. Procyanidin B2

Das monomere Flavan-3-ol (-)-Epicatechin ist sowohl das dominanteste als auch das weit verbreitetste Polyphenol, welches sich in sämtlichen Genotypen der Gattungen *Theobroma cocoa* L. wieder finden lässt (Carrillo *et al*., 2014). Verschiedene Studien zeigen die Abhängigkeit des (-)-Epicatechingehaltes vom Genotyp, von der geographischen Herkunft oder Lage, wobei eindeutige Zusammenhänge nur schwer zu belegen sind (Elwers *et al*., 2009). Mit einem deutlich geringerem Gehalt, gilt auch das Diastereoisomer (+)-Catechin als weit verbreiteter Vertreter der Flavan-3-ole. Sowohl (-)-Epicatechin als auch (+)-Catechin können als Qualitätsmerkmal während der Schokoladenverarbeitung herangezogen werden (Clapperton *et al*., 1994b). Hurst *et al*. (2011) fanden in reifen Kakaobohnen einen (-)-Epicatechingehalt von 13,35 ± 2,24 mg/g, einen (+)-Catechingehalt von 0,56 ± 0,05 mg/g, jedoch kein (-)-Catechin. In unreifen Früchten wurde ein (-)-Epicatechingehalt von 9,77 ± 0,80 mg/g, ein (+)-Catechingehalt gleich den reifen Kakaobohnen von 0,57 ± 0,05 mg/g analysiert, jedoch kein (-)-Catechin (Hurst *et al*., 2011). Während der Fermentation bildet sich auf Grund der Wärmeinduktion (-)-Catechin. Während der Schokoladenverarbeitung sinkt der Gehalt an (-)-Epicatechin als auch (+)-Catechin, wobei (-)-Catechin im Spurenbereich zunimmt (Hurst *et al*., 2011).

Counet *et al*. (2006) fanden in Produkten wie Trauben, Wein, Oliven und dunkler Schokolade einen Anteil von ca. 71% an monomeren und dimeren PA im Vergleich zum Gesamtpolyphenolgehalt. Zum Gesamtpolyphenolgehalt trägt hauptsächlich (-)-Epicatechin als monomeres PA bei und PA B1 und B2 als dimere Vertreter. Tomas-Barberán *et al*. (2007) haben in einem konventionellen Kakaopulver ein PA B1 Gehalt von 1,12 ± 0,01 mg/g und PA B2 von 2,62 ± 0,14 mg/g detektieren können. Natsume *et al*. (2000) untersuchten Kakao aus sechs verschiedenen Ländern, wobei der Anteil am dimeren PA B2 mit 0,24 mg/g aus Kolumbien am niedrigsten und mit 1,97 mg/g aus Brasilien am höchsten war. Die gleiche

Studie hat einen durchschnittlichen Gehalt an PA C1 im Kakao ausmachen können, mit einem Anteil von 0,12 mg/g aus Kolumbien und 1,51 mg/g aus Brasilien.

Mengenmäßig geringer kommen dimere PA des A-Typs in der Kakaobohne vor. Diese besitzen eine Doppelbindung und sind mit dem jeweiligen monomeren (Epi)Catechinbaustein sowohl über eine C-C (4β→8) als auch über eine C-O-Etherbindung (2β→O-7) miteinander verknüpft. Im Kakao konnte hier das PA Dimer A1 (-)-Epicatechin-(2β→O-7,4β→8)-(+)-Catechin (Nonaka *et al*., 1987) und A2 (-)-Epicatechin-(2β→O-7,4β→8)-(-)-Epicatechin isoliert werden (Jacques *et al*., 1974).

Neben den beschriebenen monomeren und oligomeren Flavan-3-olen liegen Flavonoide in der Zelle nur selten in Aglykon Form vor, sondern sind meistens *O*- oder *C*-glykosidisch mit Zuckern wie Glucose, Arabinose, Galactose, Rutinose verbunden. Durch die Glykosylierung erhöht sich die Polarität des Moleküls in der Zelle, der Membrantransport verbessert sich, wodurch die Speicherfähigkeit der Pflanzenzellvakuolen zunimmt (Aherne & O'Brien, 2002).

Die beiden Diastereomere (+)-Gallocatechin und (-)-Epigallocatechin wurden erstmals durch Forsyth (1955) nachgewiesen. Eine quantitative Auswertung wurde bis dato noch nicht dokumentiert. Subhashini *et al*. (2010) haben jedoch in ihrem Versuch zur Bestimmung von phenolischen Substanzen im Trinkkakao einen Gehalt an (-)-Epicatechin-3-*O*-gallat von 43,07 mg/Portion, einen Gehalt an (-)-Epigallocatechin von 50,37 mg/Portion und einen Gehalt an (-)-Epigallocatechin-3-*O*-gallat von 140,16 mg/Portion analysiert.

Tabelle 1 gibt die strukturellen Merkmale der Flavanole, die Mengenangaben im untersuchten Pflanzenmaterial an und ordnet die Flavan-3-ole den jeweiligen Veröffentlichungen zu.

Tabelle 1: Verschiedene Flavanole und deren Gehalt in ausgewählten Kakaoerzeugnissen.

Name	Substituenten						Vorkommen	Gehalt [mg/g]	Referenzen
	3	5	7	3‘	4‘	5‘			
(+)-Catechin	β OH	OH	OH	H	OH	OH	Kakao Bohnen ungeröstete Bohnen	3,02 0,26	Ramli *et al*. (2001) Kothe *et al*. (2013)
(-)-Catechin	α OH	OH	OH	H	OH	OH	geröstete Bohnen	0,18	Kothe *et al*. (2013)
(-)-Epicatechin	α H	OH	OH	H	OH	OH	frische Bohnen ungeröstete Bohnen Kakao Bohnen	21,9-43,3 0,97-4,82 4,61	Kim & Keeney (1984) Kothe *et al*. (2013) Nazaruddin *et al*. 2001
(+)-Gallocatechin	β OH	OH	OH	OH	OH	OH	k. A.	Spuren	Forsyth (1955) Haslam (1998) Wollgast (2005)
(-)-Epicatechin-3-*O*-gallat	*O*-Gal	OH	OH	H	OH	OH	Kakaopulver	43,07 *	Subhashini *et al*. (2010)
(-)-Epigallocatechin	α OH	OH	OH	OH	OH	OH	k. A. Kakaopulver	Spuren 50,37 *	Wollgast (2005) Subhashini *et al*. (2010)
(-)-Epigallocatechin-3-*O*-gallat	*O*-Gal	OH	OH	H	OH	OH	Kakaopulver	140,16 *	Subhashini *et al*. (2010)
Procyanidin B1	(-)-Epicatechin-(4β-8)-(+)Catechin	OH	OH	H	OH	OH	ungeröstete Bohnen	0,027	Kothe *et al*. (2013)
Procyanidin B2	(-)-Epicatechin-(4β-8)-(-)-Epicatechin	OH	OH	H	OH	OH	ungeröstete Bohnen Kakaopulver	2,03 1,58	Kothe *et al*. (2013) Baba *et al*. (2007)
Procyanidin B5	(-)-Epicatehin-(4β-6)-(-)-Epicatechin	OH	OH	H	OH	OH	ungeröstete Bohnen	0,57	Kothe *et al*. (2013)
Procyanidin C1	(-)-EC-(4β-8)-(-)-EC-(4β-8)-(-)EC	OH	OH	H	OH	OH	Kakaopulver	0,96	Baba *et al*. (2007)

k.A. keine Angaben

* Gehalt angegeben in mg/Portion (7,3 g Kakaopulver in 100 mL dest. Wasser bei 100 °C)

Flavonole wie Quercetin und Quercetin-3-*O*-glucosid wurden erstmals von Rohan und Connell (1964) dokumentiert und aus den Kotyledonen der Kakaopflanze isoliert. Ein Jahrzehnt später wurde von Jalal und Collin (1977) das Quercetin-3-*O*-galactosid nachgewiesen und von Sánchez-Rabaneda *et al*. (2003) bestätigt. Quercetin-3-*O*-arabinosid konnte von Sanbongi *et al*. (1998) mittels NMR-Spektroskopie identifiziert werden. Quercetin-3-*O*-glucuronid wurde als letzter Vertreter erst 2001 von der Arbeitsgruppe um Lamuela-Raventós *et al*. nachgewiesen. Nach Wollgast (2005) macht Quercetin und seine Glucoside einen Anteil von 2 - 5% am Gesamtpolyphenolgehalt aus. **Abbildung 2a** zeigt das Grundgerüst der Flavonole, wobei in **Abbildung 2b-e** die jeweiligen Glucoside und mit der Tilde die Angriffsstelle der Dehydroxylierung dargestellt wird. **Tabelle 2** gibt die strukturellen Merkmale der jeweiligen Substanzklassen an und ordnet die Flavonole den jeweiligen Veröffentlichungen zu. Außerdem kann nach Wollgast (2005) das Quercetin als Leitkomponente im Schokoladenherstellungsprozess herangezogen werden. Dessen Zunahme im Laufe der Herstellung kann auf die Deglykosilierung des Quercetin-3-*O*-glucosids zurückgeführt werden. Flavonole sind gelbe Naturfarbstoffe, die vergleichsweise schwer wasserlöslich, aber leicht löslich in Dimethylsulfoxid (DMSO) sind.

Anthocyane sind die farbgebenden Komponenten, die in unterschiedlichen Konzentrationen in den Kotyledonen vorkommen und bei hohen Konzentrationen die unreife Kakaobohne rötlich erscheinen lassen. Mit fortschreitender Fermentation sinkt die Konzentration an Anthocyanen und die anfänglich intensive Rotfärbung geht in ein Braun über. Anthocyane können deswegen auch als Indikator für den Fermentationsgrad der Probe herangezogen werden. Bereits 1963 wurden in Studien von Forsyth und Quesnel ein Anthocyaningehalt in der Kakaobohne von durchschnittlich 4% quantifiziert. Forsyth und Quesnel (1957) haben hierbei das Cyanidin-3-*O*-galactosid und das Cyanidin-3-*O*-arabinosid nachweisen können. Darüber hinaus wurde auch das Cyanidin-3-*O*-rutinosid identifiziert. Cyanidin-glucoside sind gut wasserlöslich. Ihre Struktur ändert sich je nach pH-Wert, wobei sie im sauren Milieu bei pH < 3, auf Grund der Bildung des Flavyliumkations Anthocyanidine, rötlich erscheinen. Im neutralen Milieu bei pH 7-8 bilden sich durch Deprotonierung mesomeriestabilisierte ionische Anhydrobasen, welche tiefblau sind. Im basischen pH > 11 bilden sich gelbe Chalkon-Anionen. **Abbildung 3a** zeigt das Grundgerüst der Anthocyane, wobei in **Abbildung 3b-d** die jeweiligen Glucoside und mit der Tilde die Angriffsstelle der Dehydroxylierung dargestellt werden. **Tabelle 3** gibt die strukturellen Merkmale der jeweiligen Inhaltsstoffe an und ordnet die Anthocyane den jeweiligen Veröffentlichungen zu.

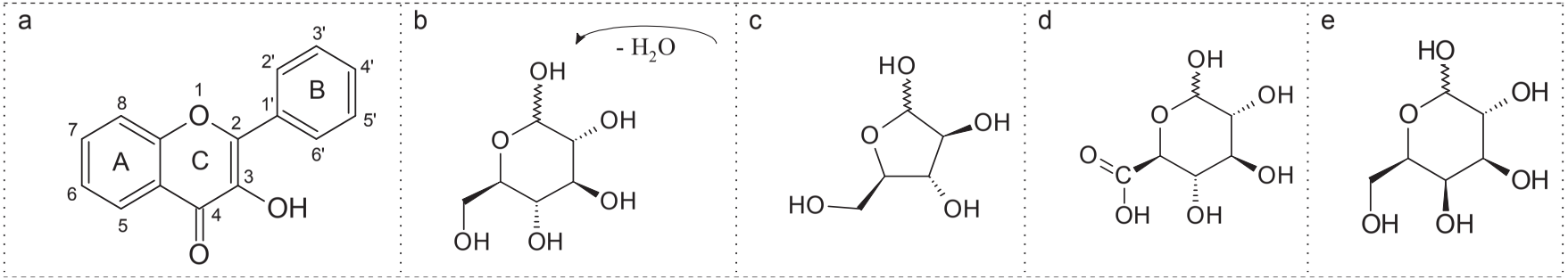


Abbildung 2: a. Grundgerüst der Flavonole, b. Glucosid, c. Arabinosid, d. Glucuronid, e. Galactose

Tabelle 2: Verschiedene Flavonole und deren Gehalt in der Kakaobohne

Name	Substituenten					Vorkommen	Gehalt [mg/g]	Referenzen
	3	5	7	3‘	4‘			
Quercetin	OH	OH	OH	OH	OH	Kotyledone	0,02 0,09	Lamuela-Raventós *et al*. 2001 Tomas-Barberán *et al*. 2007
Quercetin-3-*O*-glucosid	O-Gly	OH	OH	OH	OH	Kotyledone	0,23	Lamuela-Raventós *et al*. 2001
Quercetin-3-*O*-arabinosid	O-Arb	OH	OH	OH	OH	Kotyledone	0,3	Tomas-Barberán *et al*. 2007
Quercetin-3-*O*-glucuronid	O-Glu	OH	OH	OH	OH	Kotyledone	0,05	Lamuela-Raventós *et al*. 2001
Quercetin-3-*O*-galactosid	O-Gal	OH	OH	OH	OH	Kotyledone	Spuren	Stark *et al*. 2005 Wollgast 2005

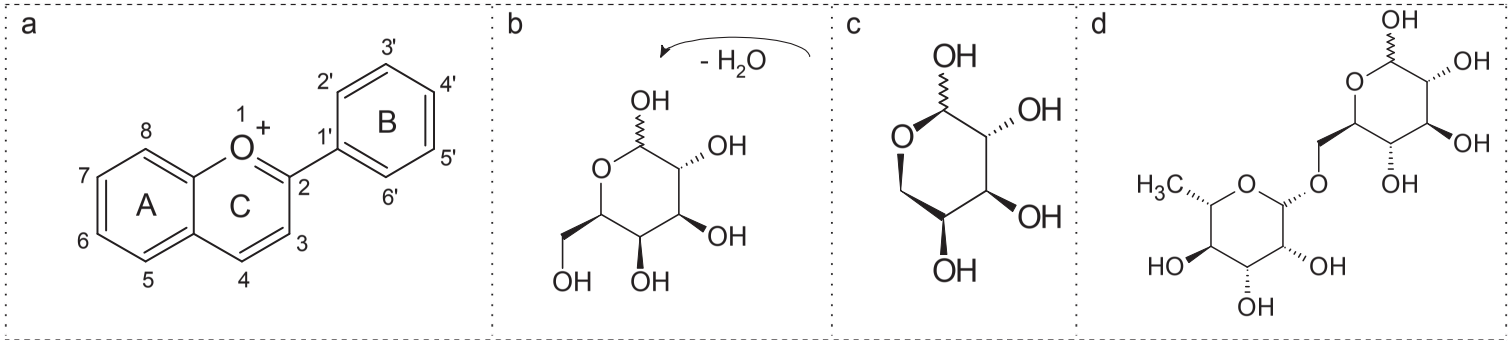


Abbildung 3: a. Grundgerüst der Anthocyane, b. Galactose, c. Arabinosid, d. Rutinosid

Tabelle 3: Verschiedene Anthocyane und deren Gehalt in der Kakaobohne

Name	**Substituenten**					**Vorkommen**	**Gehalt [mg/g]**	**Referenzen**
	3	5	7	3‘	4‘			
Cyanidin-3-*O*-galactosid	O-Gal	OH	OH	OH	OH	Kotyledone	Spuren	Forsyth & Quesnel, 1957
Cyanidin-3-*O*-arabinosid	O-Gly	OH	OH	OH	OH	Kotyledone	Spuren	Forsyth & Quesnel, 1957
Cyanidin-3-*O*-rutinosid	O-Arb	OH	OH	OH	OH	Kotyledone	Spuren	Cakirer, 2003 Wollgast, 2005

Die Gruppe der Flavone und Flavanone wurde bereits vor vier Jahrzehnten im Kakao als phenolische Substanzklassen nachgewiesen (**Abbildung 4a-b**, **5a-b**). Quantitative Angaben können bis dato jedoch nicht aus der Literatur entnommen werden. Mittels LC-MS Messungen von Sánchez-Rabaneda *et al*. (2003) konnten im Kotyledonargewebe von frischen Kakaosamen nur niedrige Gehalte an Apigenin, Apigenin-6-*C*-glucosid, sowie Luteolin, Luteolin-6-*C*-glucosid und Naringenin, Naringenin-7-*O*-glucosid ausfindig gemacht werden (**Tabelle 4**, **5**). Flavone und Flavanone kommen nur in Spuren vor und tragen somit nur wenig zum Gesamtpolyphenolgehalt bei. Denoch beeinflussen Flavanone wesentlich den sensorischen Sinneseindruck, wobei sie unter anderem für die Bitterkeit der Schokolade verantwortlich sind (Yoshikawa, 2009).

Einige dieser phenolischen Verbindungen wie Apigenin-7-*O*-glucosid und Chrysoeriol wurden von Jalal & Collin (1977) in Blättern des Kakaobaumes nachgewiesen. Hinweise auf deren Vorkommen im Kotyledonargewebe konnten jedoch nicht bestätigt werden.

Beide Gruppen sind gelbe Pflanzenfarbstoffe und nahezu unlöslich in Wasser, jedoch leicht löslich in Methanol oder Chloroform.

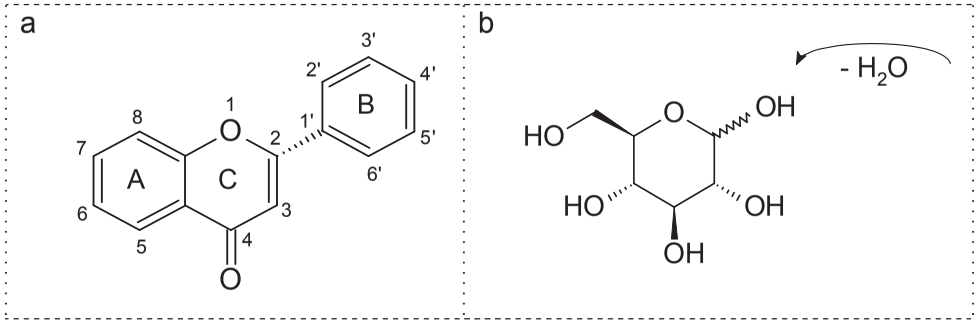


Abbildung 4: a. Grundgerüst der Flavone, b. Glucosid

Tabelle 4: Verschiedene Flavanone und deren Gehalt in der Kakaobohne

Name	Substituenten							Vorkommen	Gehalt [mg/g]	Referenzen
	5	6	7	8	3‘	4‘	5‘			
Apigenin	OH	-	OH	-	-	OH	-	Kotyledone	Spuren	Sánchez-Rabaneda *et al*., 2003
Apigenin-6-*C*-glucosid	OH	α-Gly	OH	-	-	OH	-	Blätter Kotyledone Kotyledone	Spuren	Jalal & Collin, 1977 Sánchez-Rabaneda *et al*., 2003 Stark *et al*., 2005
Apigenin-8-*C*-glucosid	OH	-	OH	β-Gly	-	OH	-	Blätter Kotyledone	Spuren	Jalal & Collin, 1977 Stark *et al*., 2005
Apigenin-7-*O*-glucosid	OH	-	O-Gly	-	-	OH	-	Blätter	Spuren	Jalal & Collin, 1977
Chrysoeriol	OH	-	OH	-	OCH_3	OH	-	Blätter	Spuren	Jalal & Collin, 1977
Luteolin	OH	-	OH	-	OH	-	-	Blätter Kotyledone	Spuren	Jalal & Collin, 1977 Sánchez-Rabaneda *et al*., 2003
Luteolin-6-*C*-glucosid	-	β-Gly	-	-	-	OH	OH	Kotyledone	Spuren	Sánchez-Rabaneda *et al*., 2003
Luteolin-7-*O*-glucosid	OH	-	O-Gly	-	OH	OH	-	Kotyledone	Spuren	Stark *et al*., 2005

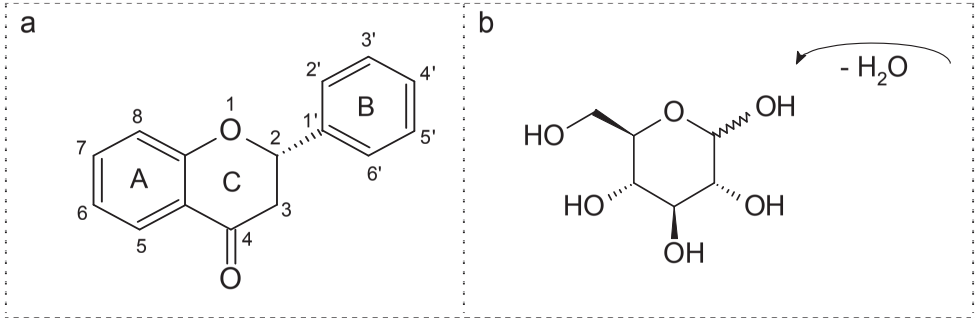


Abbildung 5: a. Grundgerüst der Flavanone, b. Glucosid

Tabelle 5: Verschiedene Flavanone und deren Gehalt in der Kakaobohne

Name	Substituenten							Vorkommen	Gehalt [mg/g]	Referenzen
	5	6	7	8	3‘	4‘	5‘			
Naringenin	OH	-	OH	-	-	OH	-	Kotyledone	Spuren	Sánchez-Rabaneda *et al*. 2003
Naringenin-7-*O*-glucosid	-	-	α -O-Gly	-	-	OH	-	Kotyledone	Spuren	Sánchez-Rabaneda *et al*. 2003 Stark *et al*. 2005

In den letzten fünf Jahrzehnten konzentrierte sich die Wissenschaft hauptsächlich auf die Identifizierung monomerer phenolischer Verbindungen, wobei sie nur 5 - 10% des Gesamtpolyphenolgehaltes ausmachen, wohingegen oligomere und polymere Polyphenole bis zu 90% ausmachen (Andres-Lacueva *et al*., 2008). Anhand ihres Polymerisierungsgrades (PG) werden PA in oligomere oder polymere Verbindungen unterteilt. Nach Gu *et al*. (2002) können sie weiter differenziert werden, in monomer PA einem PG = 1, oligomere PA mit einem PG = 2 - 10 und polymere PA mit einem PG > 10 (**Abbildung 6**).

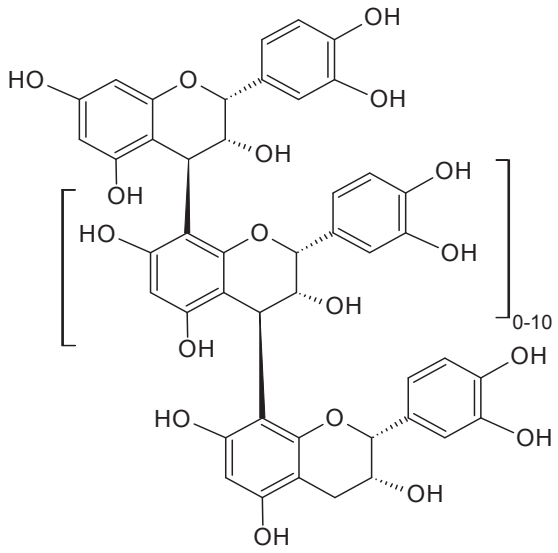


Abbildung 6: Oligomere Proanthocyanidine mit einer C4-C8-Interflavan Verbindung.

Der prozentuale Anteil an oligomeren Polyphenolen am Gesamtpolyphenolgehalt wird mit zunehmender Kettenlänge kleiner (**Tabelle 6**). Der grösste Teil der Literatur über kondensierte PA verweist vornehmlich auf oligomere PA wie Dimere, Trimere und Tetramere, auch weil die analytischen Bedingungen zur Identifizierung höher kondensierter PA limitiert sind (Pedan *et al*., 2016). Höhere Polymerisierungsgrade existieren zwar im Kakao, sind aber zusehends unlöslicher, wobei nach Bate-Smith & Swain (1962) wasserlösliche Phenole ein Molekulargewicht bis zu MW = 3000 besitzen. Insgesamt lässt sich sagen, daß mit zunehmendem PG die Wasserlöslichkeit der PA verloren geht, wobei laut Bate-Smith & Swain (1962) ab einem PG ≥ 10, es für die Extraktion einen Anteil an organischem Lösemittel im Extraktionsgemisch bedarf. Darüber hinaus existiert ein größerer Anteil an PA, die weder mit Wasser noch mit einem Alkohol-Wasser-Gemisch extrahiert werden können. Diese Klasse der *nicht-extrahierbaren Proanthocyanidine* (NEPA) verbleibt bei der Extraktion im Rückstand und ist um ein Vielfaches höher als der Anteil an *extrahierbaren Proanthyanidinen* (EPA).

Tabelle 6: Oligomere Proanthocyanidine und deren Gehalt in ausgewählten Kakaoerzeugnissen

Name	Polymerisationsgrad	Vorkommen	Gehalt [mg/g]	Referenzen
Monomer	PG 1	Frische Kakaobohnen	14,24 ± 0,38	Gu *et al.*, 2002
		Kakaobohnen	13,81 ± k. A.	USDA Database, 2004
		Kakaopulver	3,16 ± 4,66	USDA Database, 2004
		Schwarze Schokolade	0,31 ± 0,002	Gu *et al* l. 2004
Dimer	PG 2	Frische Kakaobohnen	8,57 ± 0,51	Gu *et al.*, 2002
		Kakaobohnen	8,31 ± k. A.	USDA Database, 2004
		Kakaopulver	1,83 ± 3,47	USDA Database, 2004
		Schwarze Schokolade	0,31 ± 0,009	Gu *et al.*, 2004
Trimer	PG 3	Frische Kakaobohnen	8,10 ± 0,49	Gu *et al.*, 2002
		Kakaobohnen	7,85 ± k. A.	USDA Database, 2004
		Kakaopulver	1,59 ± 2,56	USDA Database, 2004
		Schwarze Schokolade	0,21 ± 0,008	Gu *et al.* 2004
Tetramer	PG 4	Frische Kakaobohnen	27,74 ± 0,56	Gu *et al.*, 2002
Pentamer	PG 5	Kakaobohnen	26,90 ± k. A.	USDA Database, 2004
Hexamer	PG 6	Kakaopulver	5,24 ± 7,27	USDA Database, 2004
		Schwarze Schokolade	0,55 ± 0,035	Gu *et al.*, 2004
Heptamer	PG 7	Frische Kakaobohnen	22,93 ± 0,37	Gu *et al.*, 2002
Octamer	PG 8	Kakaobohnen	22,24 ± k. A.	USDA Database, 2004
Nonamer	PG 9	Kakaopulver	1,88 ± 2,85	USDA Database, 2004
Decamer	PG 10	Schwarze Schokolade	0,38 ± 0,003	Gu *et al.*, 2004
Polymere	PG > 10	Frische Kakaobohnen	16,17 ± 0,80	Gu *et al.*, 2002
		Kakaobohnen	15,68 ± k. A.	USDA Database, 2004
		Schwarze Schokolade	0,68 ± 0,009	Gu *et al.*, 2004

k.A. keine Angaben

Daneben besitzen PA spezielle Eigenschaften, wie beispielsweise die Fähigkeit mit Alkaloiden oder Proteinen Präzipitate zu bilden. NEPA hingegen zeichnen sich einerseits durch ihr hohes Molekulargewicht und andererseits durch ihre Komplexbindung an Proteinen oder Polysacchariden aus (Saura-Calixto *et al*., 2007), was sie zu unlöslichen Molekülen macht. Diese Unlöslichkeit ist der Grund für die fehlenden Quantifizierungen des Gesamtpolyphenolgehaltes in Pflanzen. Die struktur- oder konzentrationsbestimmenden Analysen beschränken sich häufig auf löslische Polyphenole in den Pflanzenextrakten, wobei die gleichen Analysen für NEPA schwer umzusetzen sind und deshalb nicht im Fokus von wissenschaftlichen Arbeiten stehen.

Auch bei der homologen Reihe der PA erkennt man die farbliche Veränderung angefangen von weißen niedermolekularen Verbindungen wie beim (-)-Epicatechin, über rötlich erscheinende oligomere PA, zu den bräunlich werdenden höher kondensierten Flavonoiden wie am Beispiel des Tannins zu erkennen ist (Shirley, 1996). Niedermolekulare Verbindungen tragen geschmackphysiologisch zum bitteren Eindruck bei, erst bei höherem Polymerisationsgrad tritt ein adstringierender, stumpfer Geschmack auf (Lea und Arnold, 1978). Hierbei treten die *ortho*-Diphenylgruppen der Proanthocyanidine mit den Speichelproteinen im Mund in Verbindung, wobei es zu einer Fällungsreaktion kommt (Drewnowski und Gomez-Carneros, 2000). Vor allem bei Schokolade mit einem hohen Kakaoanteil wird in dem Zusammenhang von einem ‘pelzigen‘ Mundgefühl, der Adstringenz, gesprochen (Stark *et al*., 2006).

Speziell für oligomere PA glaubt man, dass sie höhere antioxidative Kapazitäten besitzen als monomere Verbindungen. Rösch *et al*. (2004) extrahierten Proanthocyanidine aus Sanddorn (*Hippophae rhamnoides*) und fanden die höchste antioxidative Kapazität für Tetramere. Untersuchungen an Äpfeln von Lu & Yeap Foo (2000) bestätigten für Trimere und Tetramere eine größere antioxidative Aktivität als für PA B2 und (-)-Epicatechin.

Neben der großen Gruppe der Flavonoide wurde im Kakao die Substanzklasse der Phenolcarbonsäuren mit den zwei Hauptvertretern Hydroxyzimtsäure und Hydroxybenzoesäuren detektiert. Bei den Hydroxyzimtsäuren konnten als Minorkomponenten phenolischen Substanzen wie Kaffeesäure, Ferulasäure, Sinapinsäure, Cumarinsäure im Kakaosamen ausfindig gemacht werden (Sánchez-Rabaneda *et al*. 2003). Bei der Gruppe der Hydroxybenzoesäuren konnte Gallussäure, Protocatechusäure, Vanillinsäure und *p*-Hydroxybenzoesäure detektiert werden (Belščak *et al*., 2009). Da ihr Anteil nur sehr klein ist, tragen sie kaum zum Gesamtpolyphenolgehalt bei.

Theobromin und Koffein haben auf Grund ihrer bitteren und adstringierenden Eigenschaft hauptsächlich als Abwehrfunktion gegenüber schädlichen Insekten eine Bedeutung. Wohingegen sie im menschlichen Körper eine stimulierende Wirkung auf das Zentralnervensystem besitzen (Mitchell *et al*., 2011). Obwohl sie nicht zu der Klasse der Polyphenole zählen, werden sie als wertgebende Inhaltsstoffe in dieser Arbeit mitbehandelt – auch weil ihre Anwesenheit sich nicht störend auf die Analytik auswirkt.

Laut Forsyth und Quesnel (1963) haben die beiden Alkaloide Theobromin und Koffein (**Abbildung 7**) einen Anteil von 14% in der Polyphenolspeicherzelle.

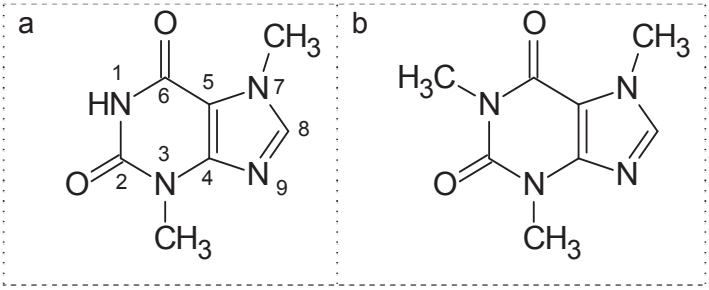


Abbildung 7: Struktur von a. Theobromin und b. Koffein

Mit dem Absterben des Kakaokeimlings, das nach 24 - 48h nach Fermentationsbeginn einsetzt, kommt es zu einer Diffusion der Methylxanthine von der Kakaobohne in die Schale. Laut Humphries (1943) kommt es zu einem Verlust von ca. 40% an Theobromin im Kakaosamen. Die Diffusion dauert so lange an, bis das Konzentrationsverhältnis in dem Kakaosamen und der Schale gleich ist. Genauso wie bei den phenolischen Substanzen ist der Gehalt an Theobromin und Koffein von Genotyp, ökophysiologischen Bedingungen und Pflanzenernährung abhängig (**Tabelle 7**). In Studien von Spiller (1998) wurden zehn Hybride des *Theobroma cacao* L. analysiert, wobei der Theobromingehalt zwischen 11,1 - 36,2 mg/g und der Koffeingehalt zwischen 1,1 - 11,3 mg/g lag.

Tabelle 7: Die wichtigsten Alkaloide und deren Gehalt in ausgewählten Kakaobohnen

Name	**Substituenten**			**Vorkommen**	**Gehalt [mg/g]**	**Referenzen**
	1	3	7			
Theobromin	H	CH_3	CH_3	Kakaobohnen (Malaysia) Kakaobohnen (Ghana)	16,23 ± 1,33 26,64 ± 2,78	Ramli *et al*. 2001
Koffein	CH_3	CH_3	CH_3	Kakaobohnen (Malaysia) Kakaobohnen (Ghana)	4,12 ± 0,04 4,98 ± 0,06	Ramli *et al*. 2001

Theobromin ist mit 0,3 g/L nur wenig in Wasser löslich, dafür aber besser in DMSO. Koffein ist gut löslich in Chloroform. Referenzsubstanzen wurden dementsprechend in passenden Konzentrationen mit diesen Lösemitteln angesetzt.

2.2. Die Biosynthese von Polyphenolen in Kakao

Um zelluläre Strukturen und komplexe Kompartimentierungen in der Pflanze besser zu verstehen, können Lokalisationsstudien herangezogen werden. Mit Hilfe von markierten Enzymen und deren Aktivitätsbestimmung kann somit eine gewebsspezifische Lokalisation und ein ausführlicher Biosyntheseweg für Polyphenole oder Alkaloide beschrieben werden. In den Kotyledonen der Kakaosamen lassen sich zwei Arten von Parenchymzellen unterscheiden. Zu über 90% existieren im Mesophyll die fettreichen Speicherparenchymzellen, welche Stärkekörner, Aleuronkörner und Fetttröpfchen enthalten (Biehl, 1973). Zwischen diesen Zellen liegen die deutlich größeren Polyphenolspeicherzellen, welche im Mesophyll des Kotyledonargewebes gespeichert werden. Phenolische Substanzen sind mit bis zu 8% im Kotyledon der Kakaopflanze enthalten (Belitz *et al*., 2008; **Tabelle 8**), wobei die monomeren PA den größten Teil mit bis zu 3% ausmachen. Die beiden Alkaloide ergeben demnach zusammen einen Anteil von 1,8%. Studien von Osman *et al*. (2004) geben für den Gehalt an phenolischen Substanzen in frischen Samen einen Anteil von 14 - 20% ihrer Trockenmasse an.

Tabelle 8: Bestandteile der Kotyledonen und der Polyphenolspeicherzellen bezogen auf die Trockenmasse (Belitz *et al*., 2008).

Bestandteile	Kotyledon [%]	Polyphenolspeicherzelle [%]
Gesamtpolyphenole	8,0	66,5
Theobromin	1,7	14,0
Koffein	0,1	0,5
Freie Zucker	-	1,6
Polysaccharide	-	3,0
Sonstige	-	14,4
Catechine	3,0	25,0
Proanthocyanidine	2,5	21,0
Polymere Proanthocyanidine	2,1	17,5
Anthocyane	0,4	3,0

Die im Kakao mengenmäßig größere Gruppe an polyphenolischen Metaboliten sind monomere Bausteine mit dem Flavonoid Grundgerüst C6-C3-C6 (**Abbildung 8**). Gemeinsam ist ihnen der Aufbau aus drei aromatischen Ringen, wobei das Grundgerüst aus zwei aromatischen Ringen (A- und B-Ring) und einem heterozyklischen C-Ring besteht. Durch Änderungen an diesem sogenannten C-Ring können die Flavonoide in fünf Majorgruppen unterteilt werden (Tsimogiannis *et al*., 2007): Flavonole (2,3-Doppelbindung, 3-Hydroxy-gruppe, 4-Ketogruppe), Flavone (2,3-Doppelbindung, 4-Ketogruppe), Dihydroflavonole (3-Hydroxygruppe und 4-Ketogruppe), Flavanone (4-Ketogruppe) und Flavanole (3-Hydroxygruppe).

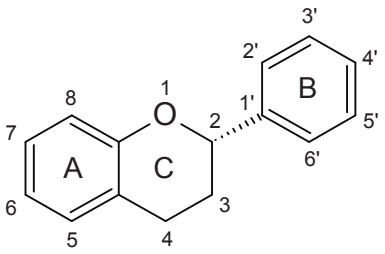


Abbildung 8: Flavonoid Grundgerüst.

Die Biosynthese aromatischer Verbindungen bleibt ausschließlich Pflanzen vorbehalten und erfolgt auf zwei verschiedenen Wegen. Der B- und C-Ring entsteht aus dem Shikimisäure Biosyntheseweg und dem Phenylpropanstoffwechselweg. Der A-Ring leitet sich aus dem Flavonoid Biosyntheseweg ab und entsteht aus der Reaktion von drei Molekülen, Malonyl-Co A des Glukose-Metabolismus und dem 4-Hydroxyzimtsäure-CoA (Wollgast und Anklam, 2000).

Der Shikimisäure Biosyntheseweg erzeugt mit dem 4-Hydroxyzimtsäure-CoA einen C9-Körper (**Abbildung 9**). Im Detail, das aus dem Kohlenhydratstoffwechsel stammende Startermolekül Phosphoenolpyruvat und D-Erythrose-4-phosphat bildet über eine Aldoladdition den C7-Körper 3-Dehydroxy-D-arabino-heptulososäure-7-phosphat (DHAP). Nach der Abspaltung der Phosphatgruppe cyclisiert die Verbindung zum C6-Körper 5-Dehydrochinasäure. Die Shikimisäure bildet sich als Zwischenprodukt durch Wasserabspaltung und Hydrierung aus dem vorherigen Pflanzenmetabolit (Dewick, 2002). Im Shikimisäure-Weg wird das C6-C3 Grundgerüst gebildet. Der Mechanismus der Biosynthese, welche zum B und C-Ring der Flavonoide führt, leitet sich aus der Glucose via Shikimisäure und Hydroxyzimtsäure ab.

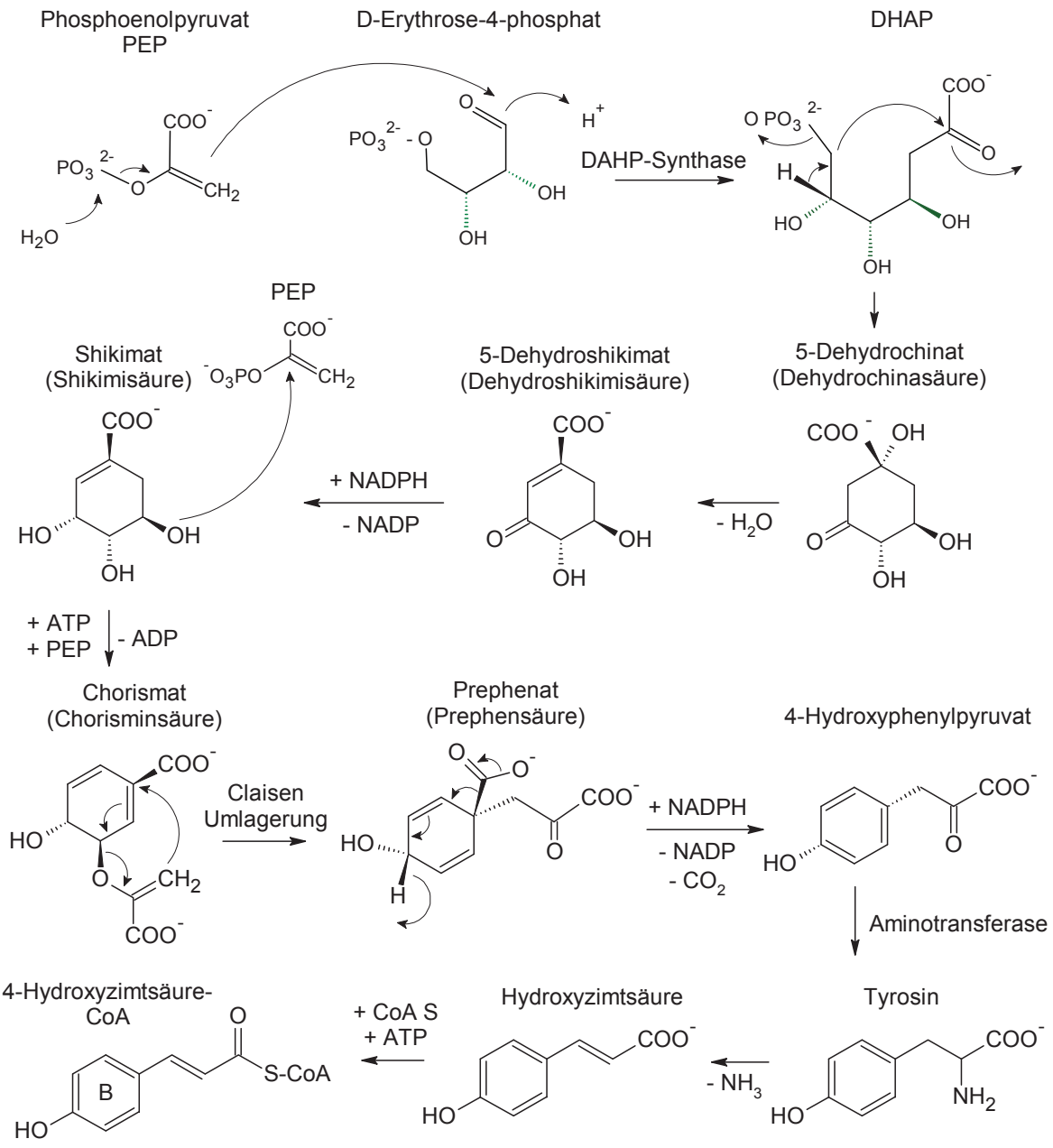


Abbildung 9: Shikimisäure-Biosyntheseweg (angelehnt an Dewick, 2002).

Eine Schlüsselrolle spielt dabei die Chorisminsäure, das Endprodukt des Shikimisäurewegs und eine reaktive Verzweigungsstelle, die bei der Bildung der Flavonoide und Lignine beteiligt ist. Durch Decarboxylierung, Wasserabspaltung und Transaminierung entsteht die aromatische Aminosäure Tyrosin, die wiederrum Ausgangsprodukt für den Phenylpropanstoffwechselweg ist. Aus Tyrosin entstehen über unterschiedliche Reaktionswege Hydroxyzimtsäurederivate wie Ferulasäure, Kaffeesäure, *p*-Coumarsäure. Diese kommen nur im Spurenbereich im Kakao vor.

Im nächsten Schritt wird der zweite aromatische Ring gebildet (**Abbildung 10**). Das gewonnene Hydroxyzimtsäure-CoA kondensiert nacheinander mit je drei Molekülen Malonyl-CoA, was ebenfalls ein Produkt des Kohlenhydratstoffwechsels ist, und formt dabei das C15-Molekül Chalkon. Auf diese Art entsteht der A-Ring des Flavonoids. Durch verschiedene enzymatische Zwischenschritte werden daraus die verschiedenen Flavonoide umgeformt (Formica & Regelson, 1995). Hierbei können verschiedene Formen gebildet werden, so wie Chalcone, Flavanone, Dihydroflavonole, Flavanone, Anthocyanidine, Flavonole, als auch Flavan-3-ole, und Proanthocyanidine.

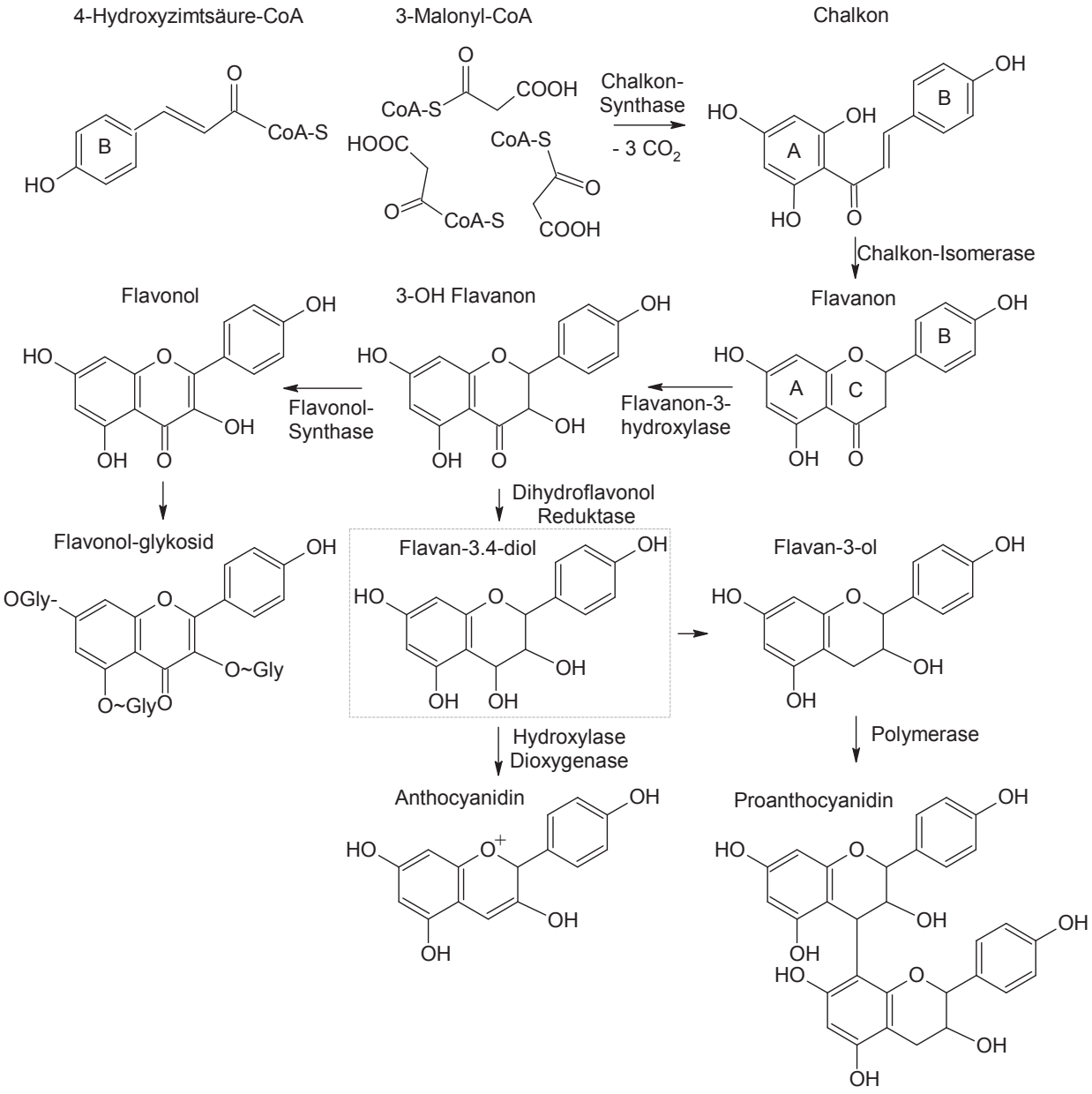


Abbildung 10: Flavonoidbiosyntheseweg (angelehnt an Shirley, 1996; Shirley, 2001).

Der Flavonoidbiosyntheseweg wird durch Licht induziert, wobei die phenolischen Substanzen in der Zellvakuole gespeichert werden. Wie erwähnt wird die Chalkonsynthase als Schlüsselenzym der Flavonoidsynthese bezeichnet. Über die Chalkonsynthese bilden sich während der Fermentation 3-Deoxyanthocyanidine, die in der polymerisierten Form die Phlobaphene darstellen (**Abbildung 11**). Die Endstufe der PA-Kondensation bilden die hochkondensierten, wasserunlöslichen Phlobaphene. Speziell Phlobaphene sind für die violettstichig braune Farbe der Kakaobohne verantwortlich, aber auch für die Milderung des bitteren und adstringierenden Geschmacks (Hansen *et al*., 1998).

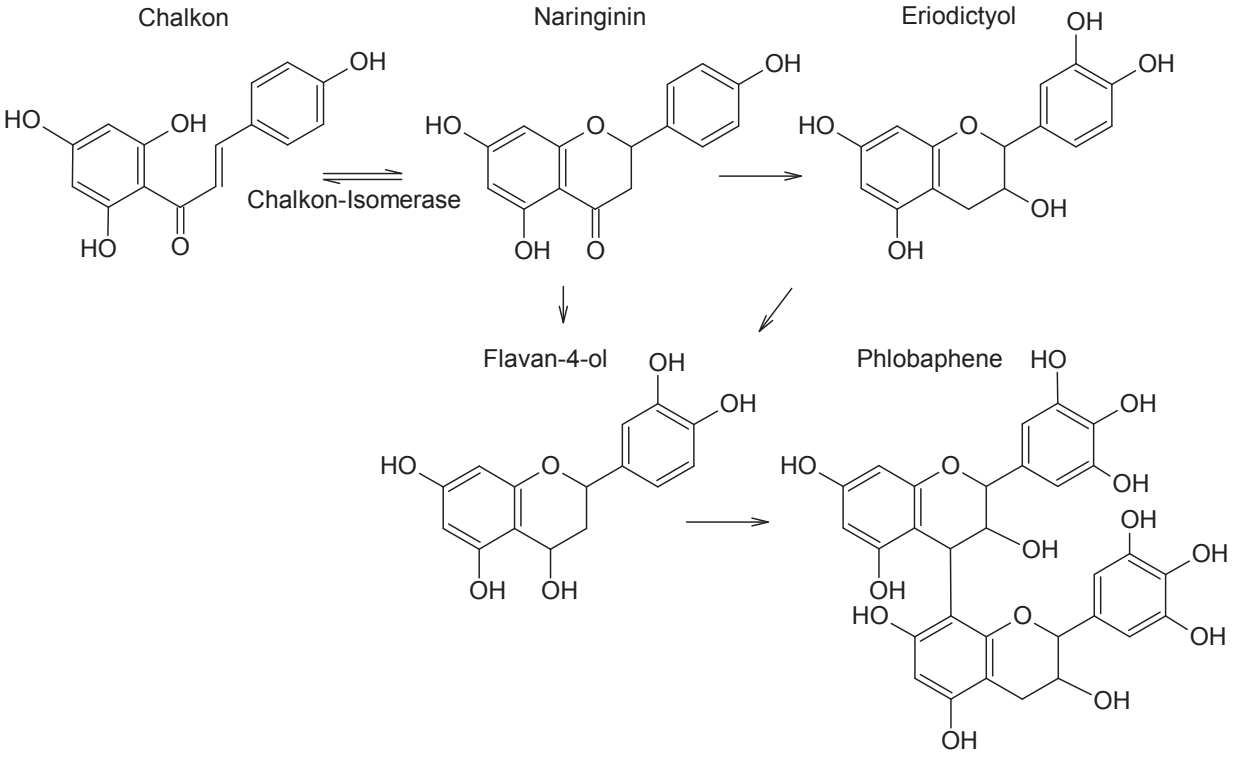


Abbildung 11: Bildung höherkondensierter Phlobaphene.

Ebenso wie den monomeren Polyphenolen, wird den Phlobaphenen bedingt, durch ihre strukturellen Merkmale, eine antifungale Wirkung nachgesagt (Shirley, 1996).

Neben den Flavonoiden stechen insbesonde die beiden Alkaloide Theobromin und Koffein hervor. Die biogenetische Herkunft der Alkaloide leitet sich vom Metabolismus der aliphatischen Aminosäuren ab, wobei die Aminogruppe der Aminosäuren den Stickstoff für den heterozyklischen Ring des Alkaloids liefert. Auch hier dient der Shikimisäure-Biosyntheseweg der Bildung einzelner biogener Vorstufen, die in den Aminosäuremetabolismus eingespeist werden. Der Biosyntheseweg für Theobromin und Koffein beginnt mit der Methylierung des Xanthosins, wobei über weitere enzymatische Schritte Theobromin und anschließend Koffein gebildet wird (**Abbildung 12**).

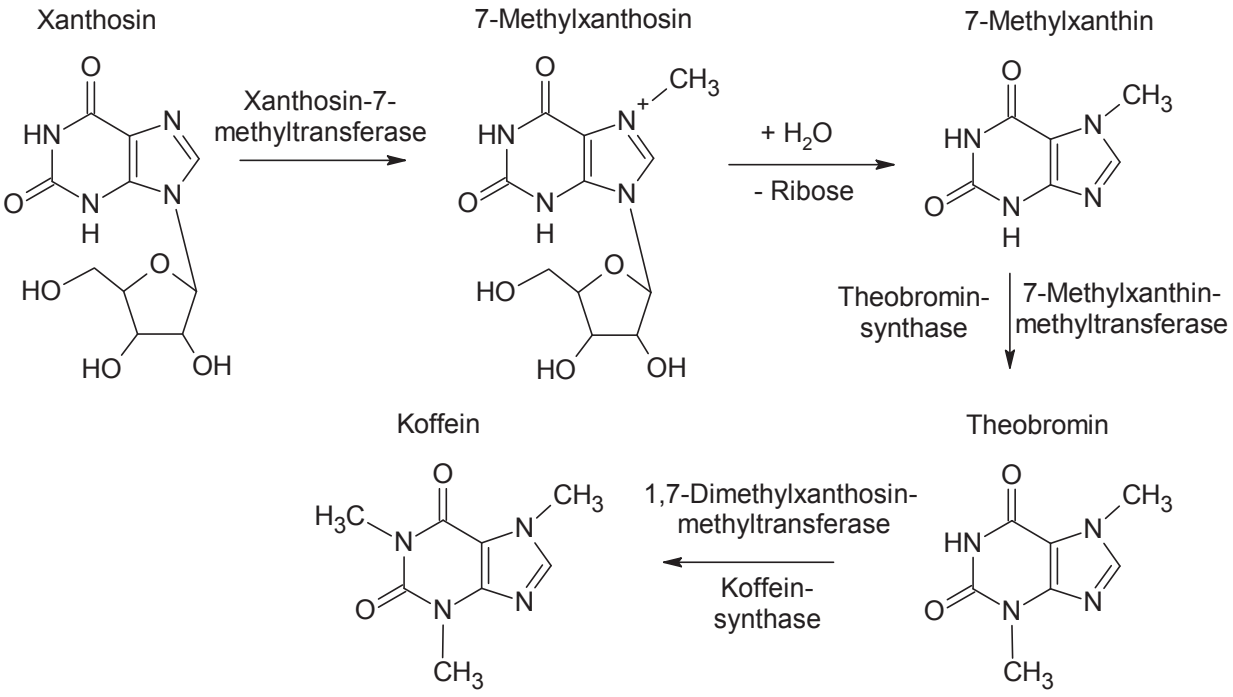


Abbildung 12: Alkaloid Biosyntheseweg (angelehnt an Ziegler & Facchini, 2008).

Hauptsächlich in jungen Kakaoblättern wurden die beiden Alkaloide Koffein und Theobromin nachgewiesen, wobei hier der chemische Schutz des jungen Gewebes vor Fressfeinden an erster Stelle steht. Mit dem Alter der Pflanze und der Entwicklung der Blätter sinkt auch gleichzeitig der Alkaloidgehalt an Theobromin (0,81 mg/g Frischmasse) und Koffein (0,15 mg/g Frischmasse) um bis zu 75% (Koyama *et al*., 2003). Bei älteren Blättern konnte keine Alkaloidsynthese beobachtet werden (Ashihara *et al*., 2008). Der Alkaloidgehalt variiert mit den unterschiedlichen Kakao-Genotypen *Criollo*, *Forastero* und *Trinitario*, wobei im Samen des *Criollo* die Konzentrationen leicht höher sind (Hammerstone *et al*., 1994). Daneben kann selbst die Kakaoschale zur Theobromin Gewinnung herangezogen werden (Belitz *et al*., 2008). Theobromin ist nämlich in der Kakaobohne leicht an Gerbsäure gebunden und setzt sich im Verlauf der Fermentation während der Essigsäureoxidation frei, so dass sie teilweise in die Kakaoschale wandert.

2.3. Verarbeitung von *Theobroma cacao* Linné

2.3.1. Genetik

Die Nutzung der Kakaopflanze ist schon seit 1.000 v. Chr. bekannt, als die Olmeken am Golf von Mexiko im Tiefland die Kakaobohne vermutlich nicht nur als herbes Gewürzgetränk tranken, sondern auch das zuckerhaltige Fruchtfleisch zu einem alkoholhaltigen Getränk verarbeitet haben. Das Schokoladengetränk erlebte mit der Besiedlung Mittelamerikas durch

die Spanier einen Durchbruch, wobei sie das heiße Getränk mit Rohrzucker versüßten. So wurde der Kakaobaum 1735 vom Botaniker Carl von Linné *Theobroma* genannt, welcher sich aus zwei zusammengesetzten griechischen Wörtern „theos“ (Gott) und „bromas“ (Speise) ergibt. Der Kakaobaum *Theobroma cacao* Linné war ursprünglich am Amazonasbecken beheimatet, wo sich ihm optimalen Wachstumsbedingungen boten, mit einer Durchschnittstemperatur von 20 - 30 °C, einer jährlichen Niederschlagsmenge von 2.000 Millimetern und einer Sonnenscheindauer von 2.000 Stunden. Bedingt durch wirtschaftliche Faktoren, hat sich das Anbaugebiet nach West-Afrika verschoben, dem heutzutage weltgrössten Kakaoproduktionsgebiet mit einem Weltmarktanteil von 72% (International Cocoa Organization ICCO, 2016). Die stetig steigende Nachfrage nach Kakao macht es zu einer wertvollen Handelspflanze, sowohl in den Kakaoanbauländern als auch in den Verarbeitungsländern. In Europa werden 1,6 Mio Tonnen Kakaobohnen verarbeitet, was einem Weltmarktanteil von 39% entspricht (ICCO, 2016). Die größten Nutzer sind hierbei nicht nur die Süsswarenindustrie, sondern auch die Lebensmittel-, Getränke-, und Kosmetikindustrie.

Der Kakaobaum gehört zu der Familie der Malvengewächse (*Malvaceae*). Von *Theobroma cacao* Linné sind drei Varietäten besonders wichtig. Ein Vertreter, mit einem Weltproduktionsanteil von 5% ist der würzige Edelkakao *Criollo* (*criollo*: einheimisch), der mit seiner milden und außergewöhnlichen Aromavielfalt besticht. Der als Konsumkakao bekannte herbe *Forastero* (*forastero*: fremd), besitzt einen Weltmarktanteil von 80% und wird als widerstandsfähigste und ertragreichste Sorte angesehen. Einen Weltmarktanteil von 15% hat der für sein feines sensorisches Aromaprofil bekannte fruchtige *Trinitario*, ein Hybrid von *Criollo* und *Forastero,* der ertragreicher und krankheitsresistenter ist als der *Criollo*. Je nach Genotyp variieren sie in Grösse, Form und Farbe. Der Kakaobaum wird bis zu 15 Meter groß und entwickelt ein paar Dutzend Früchte, in welchen bis zu 50 Samen heranreifen können. Bei den Früchten handelt es sich um 15 - 20 cm große und 300 - 500 g schwere, eiförmige, kurzgestielte Trockenbeeren, die ein faseriges festes Perikarp besitzen. Bei der Reife wechseln sie ihre Farbe von grün zu gelb nach rotbraun. Das essbare, weiße Fruchtfleisch, welches als Fruchtpulpa bezeichnet wird, umgibt die 2 - 3 cm großen Samen. Von der Bestäubung der Blüte bis zur Frucht dauert es 5 - 8 Monate. Die Zusammensetzung der Kakaobohnen variiert bezüglich Genotyp und ökophysiologischen Bedingungen, wie geographische Lage, Temperatur, Bodenbeschaffenheit und Pflanzenernährung. Nach Lange und Fincke (1970), sowie Hansen *et al*. (2000) besitzen *Criollo* Kakaobohnen ungefähr zwei Drittel weniger Gesamtpolyphenole als *Forastero*. Dahingegen konnten bei Studien von Elwers *et al*. (2009) keine Unterschiede zwischen den Kakaosorten *Criollo*, *Forastero* und *Trinitario* und dem Gesamtpolyphenol- bzw. (-)-Epicatechingehalt festgestellt

werden. Kim und Keeney (1984) zeigten bei ihren Untersuchungen von Kakaobohnen unterschiedlichen Ursprungs einen 6-fachen Unterschied im (-)-Epicatechingehalt auf, wobei sich der Gehalt von 2,7 mg/g fettfreier Trockenmasse aus Jamaica bis hin zu 16,5 mg/g aus Costa Rica bewegt. Eine ähnliche Studie von Elwers *et al*. (2009) zeigte einen negativen Zusammenhang zwischen starker Bodendüngung und dem Anteil am Gesamtpolyphenol-, Flavan-3-ol- oder Anthocyangehalt auf. Hingegen wurden größere Mengen an Kaffeesäure auf ungedüngtem Boden gefunden. Carrillo *et al*. (2014) untersuchte den Einfluss der geographischen Lage auf den Polyphenolgehalt und konstatierte, dass je niedriger die Höhenlage, desto mehr Polyphenole werden in der Kakaopflanze gebildet. Genetische Unterschiede wurden von Clapperton *et al*. (1994a) in frisch geernteten Kakaobohnen mit bis zu 4-fachen Flavanolgehalten in verschiedenen Genotypen ausfindig gemacht. Kim und Keeney (1984) beobachteten bei frisch geernteten Kakaobohnen verschiedenen genetischen Ursprungs einen Unterschied im (-)-Epicatechingehalt zwischen 21,9 und 43,3 mg/g fettfreier Trockenmasse. Niemenak *et al*. (2006) analysierten neunzehn frisch geerntete Klone aus der kamerunischen Datenbank und konnten einen (-)-Epicatechingehalt von 14,4 bis 43,9 mg/g fettfreie Trockenmasse aufzeigen. Der Gesamtpolyphenolgehalt wurde von Tomas-Barberán *et al*. (2007) mittels FOLIN-CIOCALTEU-*A*ssay zur Bestimmung von sieben Kakaosorten verschiedenen geographischen Ursprungs und unterschiedlicher genetischer Variation herangezogen. Hierbei hatte der CCN51 Klon aus Ecuador den höchsten Gesamtpolyphenolgehalt mit 8,42 ± 0,87 mg GAE/100g Trockengewicht, wohingegen der Genotyp *Criollo* aus der Dominikanischen Republik den niedrigsten Gehalt aufwies. Gu *et al*. (2013) analysierte den Gesamtpolyphenolgehalt von unfermentierten Kakaobohnen aus Papua-Neuguinea, Indonesien und China, wobei die Konzentration im Bereich von 8,1 bis 30,1 mg GAE /g fettfreier Trockenmasse lag, mit dem niedrigsten Gehalt an phenolischen Substanzen in Kakaobohnen aus Papua-Neuguinea und dem höchsten Gehalt bei Bohnen aus China.

2.3.2. Fermentation

Nach Reifung werden die Früchte mit Macheten aufgeschlagen und die Samen mit Fruchtpulpa für 5 - 7 Tage in Kisten/Bottiche/Körbe gelagert (Aikpokpodion & Dongo, 2010). In den ersten zwei Tagen der Fermentation wird die Fruchtpulpa unter anaeroben Bedingungen von Hefepilzen zersetzt. Die anhaftende zuckerhaltige Fruchtpulpa, welche aus 10% Glucose und Fructose besteht (Belitz *et al*. 2008), beginnt sich zu verflüssigen. Aus einem Teil des Zuckers entsteht zunächst Ethanol, der die beginnende Keimung des Samens unterdrückt. Die in der Pulpa enthaltene Citronensäure wird von der Milchsäurebakterien metabolisiert, wobei es in Folge zu einem Anstieg des pH-Wertes in der Fermentationsmasse kommt. Durch Enzyme der Hefe wird die Pulpa verflüssigt und

abgebaut. Durch das Abfließen des Pulpensafts entstehen Höhlräume zwischen den fermentierten Kakaosamen, wobei Luft eindringen kann. Ab dem dritten Tag ändert sich das Fermentationsmilieu, wobei in der nun ablaufenden aeroben Phase das Ethanol weiter zur Essigsäure oxidiert wird. Die Temperatur steigt auf 50 °C an, dadurch beschleunigt sich auch das Eindringen der Essigsäure in das Pflanzengewebe, was das Absterben des Kakaosamens zur Folge hat. Phenolische Substanzen treten im Zuge der Dekompartimentierung aus der Polyphenolspeicherzelle aus. Der Gehalt an (-)-Epicatechin sinkt um 80 - 95% im Verlgeich zum Ausgangswert (Kim und Keeney, 1984). Kealey *et al*. 2001 dokumentierten einen Abbau des Gesamtpolyphenolgehaltes von 60,7 mg/g fettfreier Trockenmasse zum Zeitpunkt t = 0 Stunden auf 22,9 mg/g zum Zeitpunkt t = 120 Stunden. Vor allem der Gehalt an monomeren Proanthocyanidinen sinkt von 21,9 auf 8,5 mg/g. Ähnliche Werte konnten auch durch Fermentationsversuche von Di Mattia *et al*. (2013) bestätigt werden, wobei unterschiedliche Versuchsanordnungen einen anfänglichen Gesamtpolyphenolgehalt von ca. 115 - 135 mg GAE/g fettfreie Trockenmasse zum Zeitpunkt t = 0 h ergaben und in einem nicht-linearen Verlauf der Gehalt auf ca. 105 mg GAE/g zum Zeitpunkt t ≈ 125 h sank.

Während der Fermentation werden die Aromavorstufen gebildet. Generell gilt, dass Schokolade aus unfermentierten Kakaobohnen sehr bitter und adstringent ist und keinen ansprechenden Geruch besitzt (Beckett, 1994). Beim Fermentationsprozess erhält die anfänglich violette Kakaobohne ihr typisches bräunliches Aussehen, entfaltet ihr würziges, malziges Aroma und vermindert den adstringierend und bitter schmeckenden Gerbstoffgehalt. Zum Ende der Fermentation hin kommt es zu einer enzymatischen Oxidation der Polyphenole, bedingt durch die Polyphenoloxidase (PPO). Durch die oxidative Vergerbung reagieren Polyphenole mit freien Amino- bzw. Iminogruppen der Kakaospeicherproteine, wobei es zu polymeren Bräunungsprodukten kommt. Weiterhin kann die PPO enzymatische, als auch nicht-enzymatische Bräunung katalysieren, wobei *ortho*-Dihydroxyverbindungen zu *ortho*-Chinon polymerisieren (**Abbildung 13**). Daneben können durch Polymerisationsreaktionen, Epicatechin- oder Catechineinheiten mit weiteren monomeren PA polymerisieren und so höher molekulare Verbindungen formen (Albertini *et al*., 2015). Durch die beiden Prozessschritte wird ein Teil der niedrig molekularen Polyphenole in unlösliche, höhermolekulare Verbindungen überführt. Nach Fincke und Fincke (1965) besitzen die wasserunlöslichen Polymere eine typische braune Farbe, was der fermentierten Kakaobohnen ihre charakteristische Färbung verleiht.

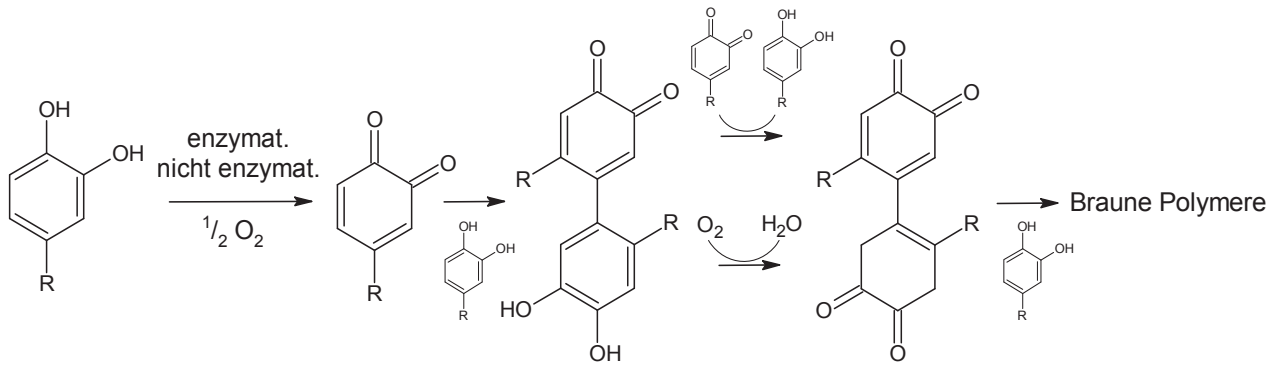


Abbildung 13: Oxidations- und Kondensationsprodukte polyphenolischer Komponenten (angelehnt an Foo & Karchesy, 1989).

Nach dem circa sechstägigen Fermentationsprozess und der optimalen Ausprägung der Kakaoaromavorstufen, können die Samen als Kakaobohnen bezeichnet werden. Gut fermentierte Kakaobohnen weisen eine braune Farbe auf und besitzen eine definierte Struktur. Wenig fermentierte Kakaosamen erscheinen violett und haben eine wachsartige Konsistenz.

2.3.3. Trocknung

Der Abbruch der Fermentation erfolgt durch die Trocknung der Bohnen. Hierbei werden die fermentierten Kakaosamen auf Bastmatten in einer dünnen Schicht ausgebreitet und in der Sonne zum Trocknen hingelegt. Die Trocknung stellt eine Konservierung dar, wobei der Wassergehalt auf 5% sinkt und sich dadurch die Gefahr eines Schimmelpilzbefalls vermindert. Bei der Trocknung oxidiert ein Grossteil der Essigsäure an der Luft und wird unter Hitzeentwicklung zu Wasser und Kohlenstoffdioxid zersetzt. Trocknungstemperaturen von 65-70 °C dürfen hierbei nicht überschritten werden (Jacquet *et al*., 1980), da ein erhöhter Säuregehalt und eine stärkere Adstringenz zur Qualitätsminderung des Rohkakaos führen würden. Die Trocknungszeit dauert ungefähr 7 Tage, wonach im Anschluss der getrocknete Rohkakao in Jutesäcken verpackt und zumeist per Schiff in die Verarbeitungsländer transportiert wird. Die Studie von Di Mattia *et al*. (2013) ergab für die Trocknung im Rahmen des traditionellen Prozesses mit spontaner Fermentation einen anfänglichen Gesamtpolyphenolgehalt von ca. 105 mg GAE/g ff TM zum Zeitpunkt t = 0 h und sank innerhalb 100 h auf ca. 25 mg GAE/g, was eine Reduktion des Gesamtpolyphenolgehaltes um ca. 75% bedeutet.

Die fermentierte und getrocknete Kakaobohne besteht zu 54% aus Fett, 11,5% aus Proteinen und zu 6% aus Polyphenolen (**Tabelle 9**). Die anregende Wirkung im Kakao beruht auf den beiden Alkaloiden Theobromin und Koffein, wobei diese zu 1,2% bzw. zu

0,2% vorkommen. Auch in der Kakaoschale, welche als teeähnlicher Aufguss genutzt wird, kommt Theobromin bis zu 1,4% vor.

Tabelle 9: Zusammensetzung von fermentierten, luftgetrockneten Kakaokernen, Kakaoschalen und Kakaokeimlingen (Belitz *et al*., 2008).

Bestandteile	Kakaokern [%]	Kakaoschale [%]	Kakaokeimling [%]
Wasser	5,0	4,5	8,5
Fett	54,0	1,5	3,5
Polyphenole	6,0	-	-
Koffein	0,2	-	-
Theobromin	1,2	1,4	-
Rohprotein	11,5	10,9	25,1
Mono- und Oligosaccharide	1,0	0,1	2,3
Stärke	6,0	-	-
Pentosane	1,5	7,0	-
Cellulose	9,0	26,5	4,3
Carbonsäuren	1,5	-	-
Sonstige Stoffe	0,5	-	-
Asche	2,6	8,0	6,3

2.3.4. Rösten

Bis zu diesem Zeitpunkt durchlaufen die Kakaobohnen einen nicht-standardisierten Prozess. Die drei Schritte über Ernte, Fermentation und Trocknung differenzieren sich je nach Ursprungsland, wodurch auch der Polyphenolgehalt stark von den unterschiedlichen Bedingungen abhängt. In den Verarbeitungsländern dagegen passieren die Kakaobohnen einen kontrollierten und reproduzierbaren Prozess. Hierbei werden sie sterilisiert, geröstet, gebrochen und zu einer Kakaomasse vermahlen, welche direkt in der Schokoladenproduktion weiterverarbeitet wird. Aus den freien Aminosäuren und den reduzierenden Zuckern aus der Fermentation, entstehen beim Rösten über die MAILLARD Reaktion und den STRECKER-Abbau die typischen Kakaoaromen. Hohe Temperatur und niedrige Feuchtigkeit beschleunigen die MAILLARD-Reaktion. Der Röstprozess dauert je nach Manufaktur zwischen 15 - 45 min und variiert zwischen 70 - 140 °C. Während des Röstens löst sich die Kakaoschale und wird vom Kakaokern getrennt. Beim Vermahlen entsteht ein dickflüssiger Brei, der als Kakaomasse zur Herstellung von unterschiedlichen Produkten weiterverwendet werden kann. Einen grossen thermischen Einfluss erfahren Polyphenole während des Röstvorganges. So haben Studien von Kothe *et al*. (2013) eine Minderung des Flavanolgehaltes um 40% während einer 30-minütigen Röstung bei 120 °C ergeben, wobei er von 7,75 mg/g in der ungerösteten auf 4,77 mg/g in der gerösteten Kakaobohne sank. Auch das monomere (-)-Epicatechin ist von 4,77 ± 0,08 mg/g auf 3,35 ± 0,2 mg/g gesunken.

Abbildung 14: Querschnitt durch eine ungeröstete und eine geröstete Kakaobohne.

Bei der Röstung intensiviert sich die braune Farbe der Kakaobohne und verliert endgültig ihr violettes Aussehen (**Abbildung 14**).

2.3.5. Alkalisierung

Häufig wird in der Schokoladenindustrie das Verfahren der Alkalisierung angewandt, die diese dunkler aussehen lässt und den bitteren Geschmack der Schokolade mildert, was mehr den heutigen Konsumentenbedürfnissen entspricht. In Detail wird hierbei der Kakaobruch gemahlen und mit einer warmen wässrig-alkalischen Lösung zu Kakaopulver aufgeschlossen, indem Kaliumcarbonat (K_2CO_3), Kaliumhydroxid (KOH) oder Ammoniumhydroxid (NH_4OH) verwendet wird. Die Alkalisierung setzt zwar den natürlichen Säuregehalt der Kakaobohnen von pH 5,0-5,6 auf pH 7,0-8,0 herauf und reduziert den Essigsäuregehalt um bis 67% (Kostic, 1997), laugt aber auch gleichzeitig wertgebende Inhaltsstoffe wie die Alkaloide und Flavanoide aus (Li *et al*., 2012). Der Gesamtpolyphenolgehalt sinkt von 31,8 mg GAE/g auf bis zu 19,4 mg/g (Li *et al*., 2012). Dadurch gehen der Schokolade auch die gesundheitsfördernde und antioxidative Wirkung verloren. Laut Meursing (2009) können im alkalischen Medium Polyphenole zu Phenoxiden transformieren, die wiederrum leicht zu *ortho*-Chinonen oxidiert werden können (**Abbildung 15**).

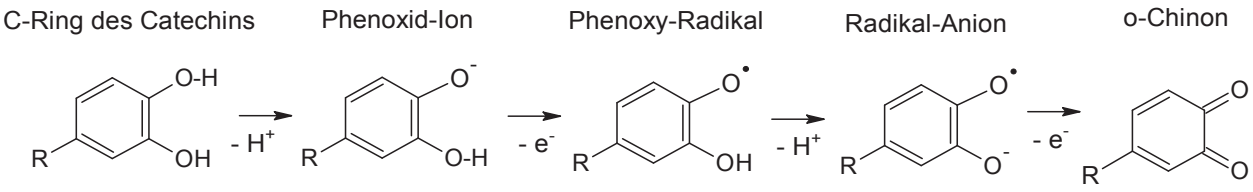


Abbildung 15: Oxidation des Polyphenols über ein Phenoxid-Ion zum *ortho*-Chinon (Meursing, 2009).

Eine Information zum Verfahren der Alkalisierung braucht vom Hersteller nicht angegeben zu werden.

2.3.6. Conchieren

Die Kakaobohnen werden durch Walzen in Kakaonibs gebrochen und weiter zur Kakaomasse verarbeitet. Durch das intensive Walzen wird das Zellgewebe zerstört und die Kakaobutter wird freigelegt. Beim Conchieren wird die Schokolade umgerührt und erwärmt, wobei Aromastoffe zusätzlich aus dem Fett herausgelöst und von Zuckerpartikeln aufgenommen werden, was den Geschmack harmonischer gestaltet. Der Hauptgrund des Conchierens ist jedoch das Austragen von unerwünschten, leicht-flüchtigen Aromaverbindungen wie Essigsäure, Propionsäure und Buttersäure. In westeuropäischen Manufakturen beträgt eine adäquate Conchierdauer bis zu 72 Stunden, wobei die meisten amerikanischen Chocolatiere nur 18 bis 20 Stunden benötigen (Alberts & Cidell, 2006). Studien von Di Mattia *et al*. (2014) haben für eine Kurzzeitconchierung ein Gesamtpolyphenolgehalt von 39,88 mg GAE/g und für eine Langzeitconchierung einen Gesamtpolyphenolgehalt von 39,11mg GAE/g gemessen. Ausgehend von dieser Studie scheint der Einfluss der Conchierung auf den Gesamtpolyphenolgehalt nicht groß zu sein.

2.3.7. Schokolade

Der Pro-Kopf-Konsum an Schokolade ist vor allem in der Schweiz mit ca. 11 kg pro Jahr sehr hoch (ICCO, 2016). Kleine Manufakturen sind in der Schweiz verbreitet und produzieren unterschiedliche Sorten an Schokolade. Gu *et al*. (2006) verglichen verschiedene Schokoladen unterschiedlicher Hersteller und fanden einen Gesamtpolyphenolgehalt zwischen 8,5 und 19,8 mg/g. Zusätzlich wurden von Cooper *et al*. (2007) 68 verschiedene Schokoladen auf ihren (-)-Epicatechingehalt untersucht und gefunden wurde eine Spanne von 0,07 - 1,94 mg/g. Letztendlich spielt die vorhergehende Verarbeitung bei den jeweiligen Prozessschritten eine wesentliche Rolle, die die Endqualität der Schokolade und damit auch ihren gesundheitliche Wirkung beeinflusst.

2.4. Kolorimetrische Messmethoden

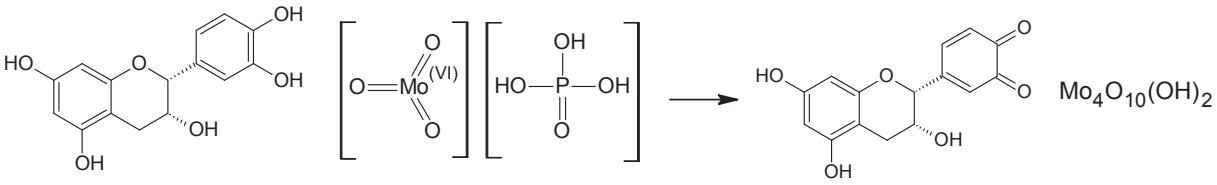
Quantitative Bestimmungen können anhand von Summenparametern (Gesamtpolyphenol-Flavonoid- oder Proanthocyanidingehalt) beschrieben werden. Hierzu werden kolorimetrische Messmethoden angewandt, die sowohl einfache als auch schnelle Verfahren zur Konzentrationsbestimmung sind. Über eine geeignete Reaktion, in der die Substanz in eine gefärbte Verbindung überführt wird, kann die Konzentration über eine Farbmessung durch den Vergleich mit einer Lösung von bekannter Konzentration bestimmt werden. Um das allgemeine Verständnis für die Handhabung aber auch eventuelle Schwachstellen der

Untersuchungsmethoden zu verstehen, ist es unabdingbar sich die ablaufenden chemischen Reaktionen vor Auge zu führen. Die aufgeführten Methoden werden ubiquitär zur Bestimmung von extrahierbaren Polyphenolen in Lebensmitteln genutzt.

Laut Haslam (1989) ist ein großer Teil der Polyphenole nicht mit Wasser und den üblichen organischen Lösungsmitteln extrahierbar. Zurückzuführen ist das auf eine kovalente Bindung polymerer PA mit Kohlenhydraten, Proteinen oder anderen Polymeren in der Pflanzenzelle. Somit verbleibt ein Grossteil der Polyphenole unlöslich im Rückstand zurück. Durch diesen Sachverhalt wurden die kolorimetrischen Methoden in dieser Arbeit aufgeteilt in die Bestimmung von extrahierbaren und nicht-extrahierbaren phenolischen Substanzen. Extrahierbare Phenole können demzufolge mit einem Alkohol-Wasser-Gemisch aus der Probe herausextrahiert werden, wohingegen nicht-extrahierbare Phenole im Rückstand verbleiben.

2.4.1. Gesamtpolyphenolbestimmung mittels FOLIN-CIOCALTEU-*Assay*

Die am häufigsten angewandte Methode zur Gesamtpolyphenolbestimmung ist die Methode nach FOLIN-CIOCALTEU. Hierbei reagieren Phosphormolybdän- und Phosphorwolframsäure in Lösung zu einem Komplex aus $3H_2O \cdot P_2O_5 \cdot 13WO_3 \cdot 5MoO_3 \cdot 10H_2O$ mit phenolischen und polyphenolischen Verbindungen (Peterson, 1979). Das Testverfahren beruht auf einem Elektronentransfer im basischen Milieu durch reduzierende Stoffe. Hierbei wird aus dem gelben FOLIN-CIOCALTEU-Reagenz mit Molybdän(VI) und Wolfram(VI) das blaue Molybdänoxidhydroxid mit der allgemeinen Formel $Mo_xO_y(OH)_2$ gebildet wird, wobei Molybdän und Wolfram im niedrigen pH-Bereich niedrigere Oxidationsstufen einnehmen (Prior *et al*., 2005). Zur quantitativen Bestimmung des Gesamtpolyphenolgehalts wird das blaue Farbpigment herangezogen. Hierbei ist die Intensität der Blaufärbung proportional zum Gehalt an reduzierenden Verbindungen (**Abbildung 16**).



(-)-Epicatechin

Phosphormolybdänsäure
Mo (VI)
gelb

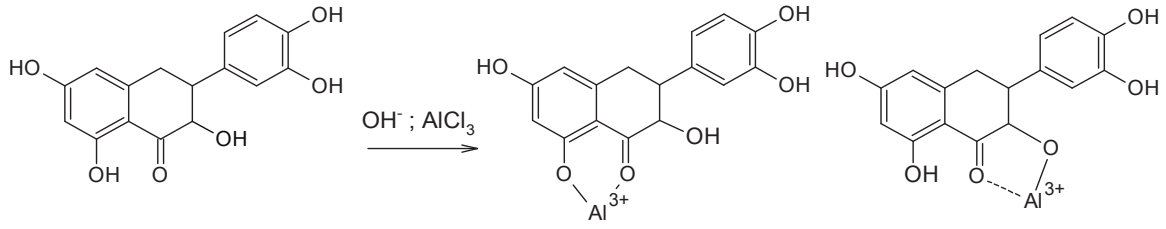
Reduziertes Chinon
Mo (V)
blau

Abbildung 16: Oxidation des (-)-Epicatechins beim FOLIN-CIOCALTEU-*Assay.*

Je größer die Anzahl der freien phenolischen Hydroxylgruppe einer Verbindung ist, desto größer ist die Reaktivität zum Assay. Die Extinktion für Lebensmittel wird bei einer Wellenlänge von 750 nm gemessen. Der Nachteil des Assays besteht darin, dass neben Polyphenolen auch andere Verbindungen mit Hydroxylgruppen erfasst werden, wie reduzierende Zucker oder Proteine. Die kolloidale Lösung von Molybdänblau baut sich langsam auf, ist erst in einem Zeitraum von 120 bis 240 Minuten stabil und baut sich anschliessend wieder ab (Pomory, 2008).

2.4.2. Gesamtflavonoidbestimmung mittels Aluminiumchlorid-*Assay*

Der Gesamtflavonoidgehalt wurde mittels Aluminium(III)chlorid-*Assay* gemessen, der speziell dem qualitativen Nachweis von monomeren Flavanoiden dient. Laut Deng & van Berkel (1998) basiert die chemische Reaktion auf einer Komplexierung des Al^{3+}-Ions am A-Ring des Favonoids, wobei eine freie Bindung über die 3-Hydroxy-4-keto-Funktion ausschlaggebend ist (**Abbildung 17**).



Flavonoid

pinkes Kondensationsprodukt
Absorptionsmaximum bei 510 nm

Abbildung 17: Bildung eines Polyphenol-Metall-Komplexes durch den Angriff des Al^{3+}-Ions an den A-Ring des Flavonoids.

Als Flavanoide im Kakao wurde die Gruppe der Flavan-3-ole, Flavonole, Flavone und Flavanone zusammengefasst, wobei das Ergebnis die Summe dieser Polyphenolklassen widergibt.

2.4.3. Proanthocyanidinbestimmung mittels Butanol-HCl-*Assay*

Die Analyse von kondensierten PA ist auf Grund der Komplexität der Substanzen eher schwierig. Es werden viele Methoden zur Quantifizierung von Tanninen angewandt, wobei einige *Assays* Depolymerisierung von PA beinhalten, indem der A-ring des Flavonols mit einer aromatischen Aldehydgruppe reagiert und es zu einer Redox-Reaktion kommt (Waterman & Mole, 1994). Der Butanol-HCl-*Assay* wird ubiquitär zur Bestimmung von

kondensierten extrahierbaren Proanthocyanidinen in Lebens- und Futtermitteln verwendet (Makkar *et al*., 1999).

Hierbei werden polymere PA im sauren Milieu depolymerisiert und das Spaltprodukt photometrisch vermessen (Bate-Smith, 1973). Da interflavanoid Verbindungen säurelabil sind, spalten sich während alkoholischer Säurebehandlung PA in ihre Untereinheiten (+)-Catechin oder (-)-Epicatechin auf. Die kationischen Produkte Anthocyanidin und Xanthylium-Ion weisen eine Rotfärbung auf, wodurch sie photometrisch detektierbar werden. Im Detail werden hierbei kondensierte PA durch saure Butanolyse an der säurelabilen C4-C8-Bindung in ein Flavan-3-ol und Carbokation gespalten (**Abbildung 18**). Für die rote Färbung des Eisen-Phenol-Komplexes ist das farbige Anthocyanidin Molekül verantwortlich, welches sich unter Luftsauerstoff in ein Hydrid-Ion abspaltet (Steinegger *et al*., 1999), das widerrum bei 550 nm photometrisch vermessen werden kann. Je längerkettiger das gespaltene PA ist, desto intensiver ist auch die rote Färbung und desto höher ist die gemessene Extinktionsenergie.

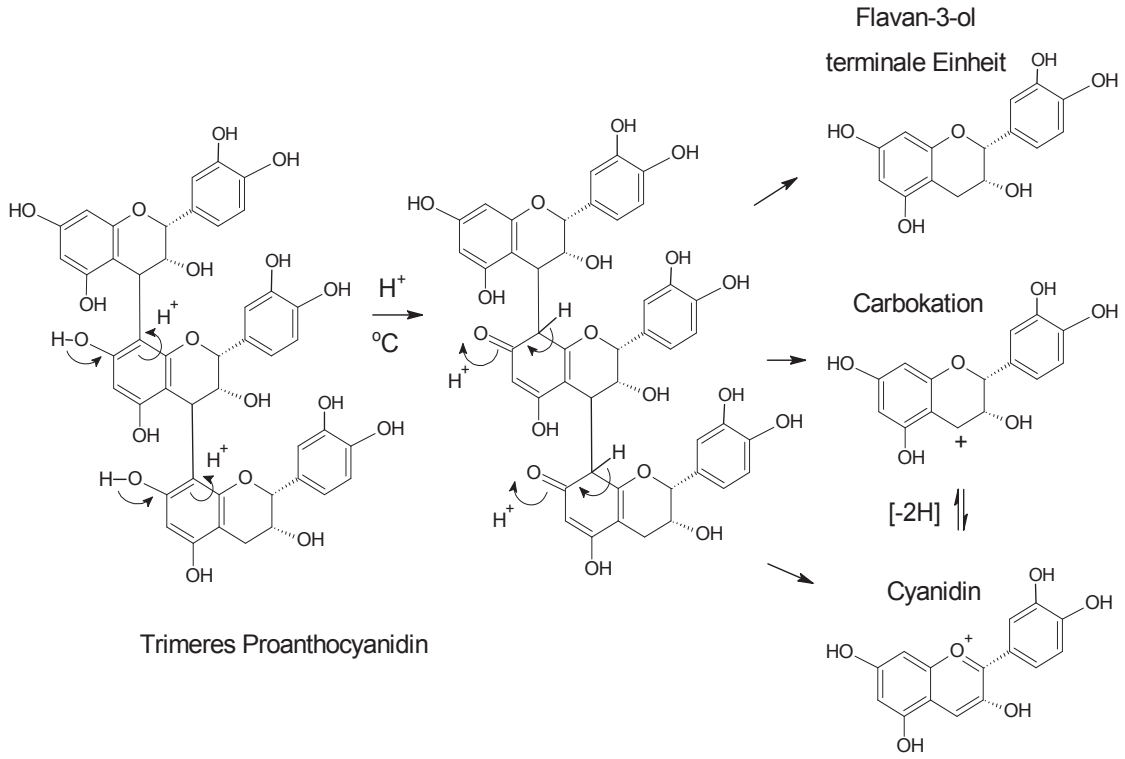


Abbildung 18: Säurekatalysierte Spaltung eines trimeren PA.

Eine Schwachstelle in dem aufgeführten Assay ist der unterschiedliche Verlauf der Spaltung, wobei PA nicht vorzugsweise in die monomeren Bausteine gespalten werden, sondern bevorzugt in dimere oder trimere PA, was in unterschiedlichen Farbverläufen resultiert (Rohr, 1999). Studien von Schofield *et al*. (2001) weisen darauf hin, dass C4-C6 Verbindungen der PA schwerer gespalten werden, als C4-C8 Verbindungen. Weiterhin wird angegeben, dass

aus höhermolekularen PA mehr Cyanidin Spaltprodukte entstehen, als aus niedermolekularen PA. Daneben ist auf Grund der Heterogenität der kondensierten PA in Lebensmitteln eine Festlegung auf eine geeignete Referenzsubstanz nur schwer durchzusetzen.

Zur Bestimmung oligomerer und polymerer PA werden neben der Säurespaltung mittels BuOH-HCl-*Assay* Präzipitationsversuche angesetzt, sowie enzymatische Spaltreaktionen oder gravimetrische Untersuchungsmethoden verwendet.

2.5. Antioxidative Kapazität von Polyphenolen

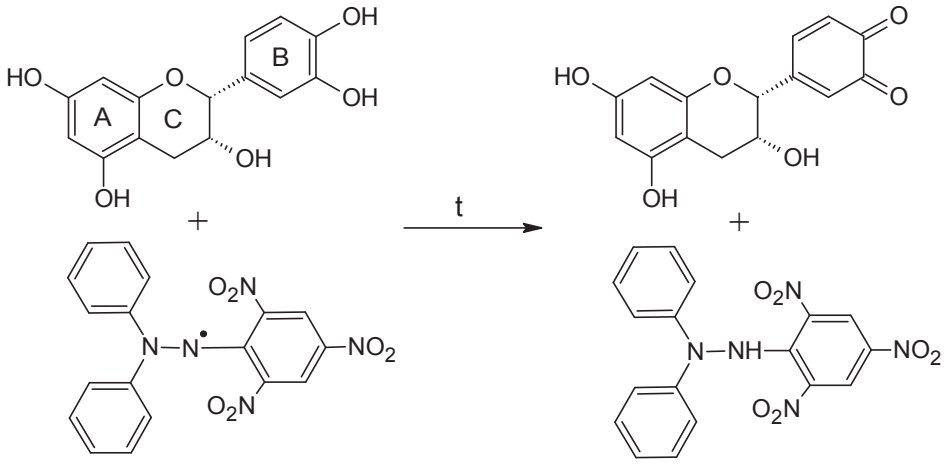
Polyphenole sind für das menschliche Immunsystem speziell als Antioxidantien von Bedeutung. Mittels *in vitro*-Studien zur Bestimmung der antioxidativen Aktivität wurde die positive Wirkung von Polyphenolen auf den menschlichen Körper postuliert, wobei der eigentliche Metabolismus im Körper ein anderer ist. So besitzen Polyphenole als Antioxidantien die Eigenschaft, reaktive Sauerstoffspezies im Körper abzufangen und radikal-induzierte Kettenreaktionen zu unterbrechen und leisten somit einen Beitrag zum Zellschutz. Einige freie Radikale sind mutagen und können die DNA schädigen, wie beispielsweise das Superoxid-Anion (O_2^-), das Hydroxyl- ($OH^•$), das Peroxyl- (ROO•) und Alkoxylradikal (RO•). Erste *in vivo*-Studien zeigen, dass monomere Flavan-3-ole und ihre Dimere vom Körper absorbiert werden können, wobei jedoch die Resorption mit steigendem Molekulargewicht abnimmt. Die Absorption ist ebenfalls davon abhängig, ob ein Molekül als Aglykon vorliegt oder glykosidisch mit Zuckerresten verknüpft ist. PA bis zu einem Polymerisationsgrad von PG > 10 können in (-)-Epicatechineinheiten hydrolysiert und über den Dünndarm absorbiert werden. Rios *et al*. (2002, 2003) konnten zeigen, dass PA den sauren pH-Bereich des Magens (pH = 2) unverändert passieren. Die Zersetzung von Kakao-Flavanolen findet somit erst im oberen Abschnitt des Dünndarms statt (Kwik-Uribe & Bektash 2008). PA, welche durch Verdauungsenzyme nicht gespalten wurden, erreichen den Dickdarm, wo sie durch die Mikrobiota im grossen Umfang zu monomeren Verbindungen gespalten werden, wie den Phenolsäuren Phenylessig-, Phenylpropion- und Phenylbuttersäure (Kwik-Uribe & Bektash, 2008; Urpi-Sarda *et al*., 2009; Saura-Calixto *et al*., 2010). Die mit der Nahrung aufgenommenen phenolischen Substanzen werden zu 90% vom menschlichen Körper im Dünn- oder Dickdarm verstoffwechselt (Saura-Calixto *et al*., 2007). Monomere Polyphenole glykosylieren im Dünndarm und werden im Blutstrom absorbiert. Eine genaue Klärung zur Absorption, Verteilung, Metabolisierung und Ausscheidung *in-vivo* ist jedoch durch die geringe Verfügbarkeit von käuflichen Referenzsubstanzen nur schwer

durchführbar. Die Absorption ist prinzipiell abhängig von der Molekülgrösse, der Molekülkonfiguration, der Lipophilie und der Säurekonstante des jeweiligen Moleküls.

2.5.1. Bestimmung der antioxidative Kapazität mittels DPPH

Eine Vielzahl von kolorimetrischen Nachweisreaktionen wird zur Bestimmung der antioxidativen Kapazität angewandt. Hierbei werden im Grunde zwei Mechanismen bedient, wobei das Radikal entweder über den Wasserstoffatom-Transfer HAT (engl. *hydrogen atom transfer*) oder den Ein-Elektronen-Transfer SET (engl. *single electron transfer*) reagiert. Das Endresultat ist zwar das gleiche, aber die Kinetik und die Möglichkeit Nebenreaktionen einzugehen variiert (Prior er al., 2005). HAT basierte Methoden messen die Fähigkeit eines Antioxidans freie Radikale über Wasserstoffdonation zu inhibieren. Laut Prior *et al*. (2005) sind HAT Methoden Lösungsmittel- und pH-Wert abhängig. Sie reagieren aber recht schnell und sind meistens in wenigen Sekunden oder Minuten abgeschlossen. Demgegenüber messen SET basierte Methoden die Fähigkeit eines Antioxidans Elektronen auf Verbindungen zu übertragen und diese zu reduzieren. SET Reaktionen laufen langsam ab und benötigen lange, um vollständig abzureagieren, so dass die Berechnung hierbei eher über die prozentuelle Abnahme als über die Kinetik geschieht.

Ein Radikal, welches sich beide Mechanismen zunutze macht, ist das 2,2-Diphenyl-1-pikrylhydrazyl (DPPH). Dieses Radikal kann somit als Wasserstoffakzeptor oder Elektronendonator fungieren. Das DPPH ist ein stabiles Radikal, welches kommerziell erwerblich ist und nicht vor Gebrauch noch erstellt werden muss. Die Methode basiert auf dem Verschwinden der tief violetten Farbe des DPPH Radikals (**Abbildung 19**).



Absorption bei 515 nm
violett

Absorption bei 324 nm
gelblich

Abbildung 19: Reaktion des DPPH Moleküls bei Anwesenheit eines Antioxidants.

Laut Osman (2011) wird in einem ersten schnellen Reaktionsschritt das Catechin zum *ortho*-Chinon oxidiert. Das *ortho*-Chinon kann in einem weiteren Schritt entweder mit einem Catechin zu einem dimeren Produkt kondensieren oder ein Addukt mit einem DPPH Molekül bilden. Die unterschiedlichen Reaktionswege kommen über die verschiedenen Polaritäten und Konzentrationsunterschiede zustande.

Tatsächlich besitzt jedes Polyphenol strukturbedingt eine unterschiedliche antioxidative Aktivität, wobei die eigentliche Rolle auf die Grundstruktur des C6-C3-C6 Moleküls zurückzuführen ist. Hierbei spielt die *ortho*-ständigen Hydroxygruppen am B-Ring, die konjugierten Doppelbindungen am A- und B-Ring, sowie die 3-Hydroxylgruppe am C-Ring eine wichtige Rolle. Durch Abgabe der Wasserstoffatome durch die verschiedenen Hydroxylgruppen können reaktive Sauerstoffspezies abgefangen werden. Durch die OH-Gruppe am aromatischen Ring entsteht ein zusätzliches freies Elektronenpaar, welches leicht ein Elektron abgeben kann, dadurch reduktiv und somit als Antioxidans wirkt (Rice-Evans *et al*., 1996; Kuhnert, 2013).

Deswegen werden Quercetin und Epigallocatechingallat, im Gegensatz zu (-)-Epicatechin als besonders aktiv angesehen. Auch gibt es Hinweise darauf, dass höher kondensierte PA eine größere antioxidative Kapazität besitzen als niedrig kondensierte oder monomere PA. So haben Studien von Arteel und Sies (1999) Hinweise gefunden, dass längerkettige PA effektivere Radikalfänger sind, als kurzkettige PA, wobei im Besonderen das Tetramer eine höhere antioxidative Kapazität gegen Oxidationsreaktionen besitzt. Ramljak *et al*. (2005) zeigten für pentamere Kakao-PA sogar einen hemmenden Effekt auf das Wachstum von Brustkrebszellen. In einem *offline*-assay von Rösch *et al*. (2003) wurde eine ähnliche Reihenfolge bezüglich der antioxidativen Kapazität und oligomeren PA aus Sanddorn (*Hippophae rhamnoides*) gefunden, wobei im Speziellen tetramere PA die höchste antioxidative Aktivität besitzen.

2.6. Verwendung chromatographischer Trenntechniken für die Matrix Kakao

2.6.1. Bestimmung der antioxidativen Kapazität oligomerer Proanthocyanidine mittels NP-LC-online-DPPH

Neben dem kolorimetrischen DPPH-*Assay* zur Bestimmung der antioxidativen Kapazität von Lebensmittelproben als Summenparameter, können auch einzelne oligomere PA und deren antioxidative Kapazität bestimmt werden. Mittels NP-LC-online-DPPH werden oligomere PA im ersten Schritt chromatographisch voneinander getrennt und in einem zweiten Schritt über

eine Nachsäulenderivatisierung ihre antioxidative Kapazität ermittelt. Die Bestimmung mittels NP-LC-online-DPPH erlaubt es, einzelne PA in ihrer gesamten Matrix zu bewerten.

Die in dieser Arbeit angewandte Methode lehnt sich an dem LC-online-TEAC (*Trolox Equivalent Antioxidant Capacity*) Grundaufbau nach Koleva *et al*. (2001) an. Hierbei wurden Pflanzenextrakte über ein RP-LC System injeziert und deren antioxidative Kapazität mittels Nachsäulenderivatisierung über ABTS bestimmt. Bei der LC-online-TEAC Methode wird auf den Gebrauch eines internen Standards zurückgegriffen, wobei Trolox als relative Bezugsgrösse zur Berechnung herangezogen wird (Riehle *et al*., 2013). Phenolische Substanzen werden somit als Troloxäquivalente wiedergegeben. Die Verwendung eines internen Standards wird häufig zur Quantifizierung eingesetzt, wobei er nicht nur die Qualität des Verfahrens einschätzen soll, sondern auch eventuelle Probenverluste während des Analysenverfahrens. Die Konzentration des Analyten kann über die Wiederfindungsrate des internen Standards korrigiert werden und so systematische oder zufällige Fehler aufdecken.

Die qualitative Bestimmung von PA mittels RP-Säulen gestaltet sich als schwierig, da sie wegen ihrer Grösse und ihren komplexen Verzweigungsstellen nur ungenügend mit dem Säulenmaterial wechselwirken und eine Separation deswegen unmöglich macht (Hellström und Mattila, 2008). Für die Separation von oligomeren PA werden NP-Säulen herangezogen, wobei PA hier in ihre homologe Reihe von PG = 1 bis PG ≥ 10 voneinander getrennt werden können (Rigaud *et al*., 1993; Hammerstone *et al*., 1999; Gu *et al*., 2002; Robbins *et al*., 2009; Robbins *et al*., 2012).

Die nun voneinander getrennten PA werden in eine Reaktionskapillare geleitet, in der die beschriebenen Reaktionen stattfinden. Durch die Wasserstoff- und Elektronenübertragung kommt es zu einem Abbau des violetten DPPH-Radikals, welches bei einer Wellenlänge von 515 nm gemessen werden kann. Vordergründig werden zwei Signale detektiert und in der Software verarbeitet, wobei ein positives Signal bei 280 nm für die Bestimmung der oligomeren PA steht und ein negatives Signal bei 515 nm die antioxidative Kapazität der isolierten PA wiedergibt.

2.6.2. Fraktionierung oligomerer Proanthocyanidine mittels semipräparativer NP-LC

Eine Methode zur vollständigen Identifizierung phenolischer Substanzen stellt die Isolierung mittels semipräparativer NP-LC und anschliessender Charakterisierung mittels LC-MS dar. Auf Basis des zuvor beschriebenen analytischen Systems kann über ein *up-scaling* eine semipräparative LC aufgestellt werden. Hierbei können die gesammelten Fraktionen

aufgefangen und für weitere Analysenzwecke bereitgestellt werden. Dafür muss sowohl die Probenaufarbeitung, die Probenbeladung und die anschließende Fraktionierung auf das semipräparative NP-LC angepasst werden.

2.6.3. Fraktionierung phenolischer Substanzen mittels SCPC

Eine Möglichkeit der präparativen Fraktionierung und Isolierung wurde mittels Zentrifugaler Gegenstromverteilungschromatographie durchgeführt. Diese Trenntechnik wird vor allem zur Aufreinigung, Fraktionierung und Isolierung von labilen Naturstoffreagenzien eingesetzt. Die erste Anlage für die Gegenstromverteilungschromatographie (engl. *counterurrent chromatography*, CCC) wurde in den 60er Jahren zur Isolierung von Lymphozyten entworfen (Ito *et al*. 1966) und seitdem stetig weiterentwickelt. Wobei verschiedene Firmen wie Dynamics Extractions (UK), Armen Instruments (Saint-Avé, Frankreich) und Kromaton Technologies (Frankreich) sie zu einem schnellen, robusten und skalierbaren Instrument macht, welches langsam seinen Weg in die industrielle Produktion gefunden hat (Sutherland, 2007). Im Allgemeinen wird zwischen einer chromatographischen Trennung mit einer hydrodynamischen Säule (CCC-Chromatographie) respektive einer hydrostatischen Säule (CPC-Chromatographie) (engl. *centrifugal partition chromatography*, CPC) unterschiedenen (Sutherland *et al*., 2008).

Für eine CPC Trennung gebraucht man den üblichen LC-Geräteaufbau, wobei die LC-Säule von der hydrostatischen Säule ersetzt wird (**Abbildung 20**). Die CPC-Säule besteht aus vielen aneinander gereihten identischen Scheiben, die übereinandergestapelt sind. Jede Scheibe besteht aus einer in Serie miteinander verbundenen eingravierten kleinen Zellen. Die letzte Zelle der einen Scheibe ist mit der ersten Zelle der nächsten Scheibe verbunden. Die Säule wird auf einen Zentrifugenrotor platziert.

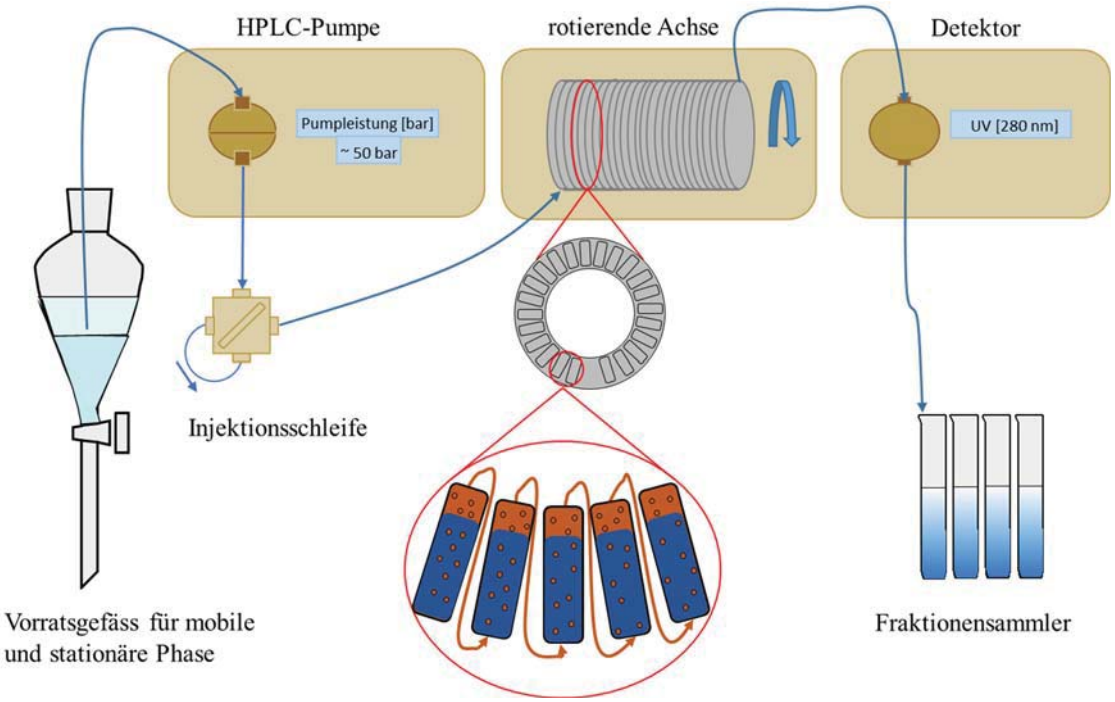


Abbildung 20: Aufbau einer SCPC-Anlage.

Die stationäre und mobile Phase kann durch die LC-Pumpe über Rotationsdichtungen in das System hineingepumpt werden. Hat man ein geeignetes Lösungsmittelsystem gefunden, wird die Säule zuerst mit der stationären Phase befüllt. Der Rotor wird gestartet und nachdem die Flussrate, sowie die Umdrehungszahl eingestellt wurden, kann die Säule mit der mobilen Phase befüllt werden. Die mobile Phase beginnt die stationäre Phase zu verdrängen, bis sich ein Gleichgewicht einstellt. Nach diesem Moment wird keine stationäre Phase mehr aus der Säule gespült, da diese von der Zentrifugalkraft im Rotor gehalten wird. Die Probe kann nun injiziert werden. Die Analyten in der Probe durchlaufen die in Serie verbundenen Zellen, wobei die Trennung auf Verteilungsvorgängen des Analyten in dem Lösungsmittelsystem basiert. Im Detail kommt es hierbei zu permanenten Mischungs- und Entmischungsvorgängen der mobilen Phase mit der stationären Phase. Aus dieser Verteilung des Analyten zwischen mobiler und stationärer Phase resultieren tausende Verteilungsvorgänge pro Stunde. Die theoretische Bodenzahl *N* kann als Funktion der Flussrate der mobilen Phase wiedergegeben werden, wobei bei dem in der Arbeit verwendeten System (siehe Anhang) mit einer Flussrate von ca. 2 mL/min eine Bodenzahl *N* von ca 400 - 600 besteht (Rodrigues *et al*., 2015).

Eine erfolgreiche Trennung hängt mit der Auswahl eines geeigneten Lösungsmittelsystems zusammen (Winterhalter, 2007). Die Grundanforderung stellt in erster Linie ein Lösungsmittelsystem, bestehend aus zwei nicht miteinander mischbaren Flüssigkeiten, dar. Eine Auswahl an typischen Zweiphasen Systemen sind Mischungen aus Hexan-Ethylacetat-

Methanol-Wasser oder MTBE-n-Butanol-Acetonitril-Wasser (Ito, 2005; Oka *et al*., 1991). Darüber hinaus sollen die Zielanalyten unterschiedliche Verteilungskoeffizienten im System besitzen. Bei der Gegenstromverteilungschromatographie gilt das Nernst'sche Verteilungsgesetzt. Damit sich ein Analyt optimal auftrennt, sollte der Verteilungskoeffizient *k* zwischen 0,5 und 1,5 liegen. Ist der Verteilungskoeffizient kleiner 0,5, kommt es zu einer verkürzten Trennzeit in der Säule und zu einer mangelhaften Auftrennung der Analyten. Ist der Verteilungskoeffizient größer, bleibt der Analyt länger in der Säule, wodurch es zu einer Peakverbreiterung kommt.

$$k = \frac{c_s}{c_m}$$

$C_{\text{stationäre Phase}}$ = Konzentration des gelösten Stoffes in der stationären Phase
$C_{\text{mobile Phase}}$ = Konzentration des gelösten Stoffes in der mobilen Phase

Daneben ist der Verteilungskoeffizient abhängig vom verwendeten Lösungsmittelsystem, wobei hier zwischen zwei Elutionsmodi unterschieden wird. Beim *descending* Modus fungiert die Phase mit der geringeren Dichte als stationäre Phase und die mit der grösseren Dichte als mobile Phase. Beim *ascending* Modus verhält es sich umgekehrt. Vergleichbar sind diese Einstellungen mit der Einteilung eines LC-Systems in Normalphasen- und Umkehrphasenchromatographie, wobei im häufig verwendeten *descending* Modus ein unpolares Lösemittel als stationäre Phase und ein schweres wässriges Lösemittel als mobile Phase dient.

Die gesammelten Fraktionen wurden den jeweiligen UV-Signalen im Chromatogramm zugeordnet, miteinander vermengt und anschließend gefriergetrocknet. Die Reinheit der resultierenden Feststoffe wurde mittels Referenzsubstanzen abgeglichen und mittels LC-DAD-ESI-MS überprüft. Die verwendeten Geräteparameter des SCPC-Systems sind im Anhang aufgeführt.

2.6.4. Isolierung individueller oligomerer PA mittels Gelpermeationschromatographie

Trotz der vielen Vorteile der SCPC, gestaltet es sich schwierig einzelne Probenkomponenten aus einer komplexen Matrix zu isolieren. Im Allgemeinen dient die SCPC vor allem der verlustfreien und schonenden Aufreinigung des *gefriergetrockneten Kakaoextraktes* und ermöglicht somit eine Vorfraktionierung von Probenkomponenten. Deswegen wurde in einem nachgeschalteten Schritt, die aus der SCPC gewonnen Vorfraktionen weiter mittels Gelpermeationschromatographie und Sephadex LH-20, aufgereinigt. Der zweite Schritt dient somit der weiteren Auftrennung einzelner Probenkomponenten, um sowohl die Ausbeute als auch die Reinheit zu erhöhen (Green und Cousineau, 1980; Kantz und Singleton, 1991). Die

für die Auftrennung der einzelnen oligomeren PA verwendeten Geräteparameter der Gelpermeationschromatographie sind im Anhang aufgeführt.

2.6.5. Identifizierung monomerer Polyphenole mittels LC-DAD-ESI-MS

Die Flüssigkeitschromatographie wird in 65% aller Methoden zur Polyphenolanalytik angewandt (Kartsova & Alekseeva, 2008), da sie eine höhere Detektionsempfindlichkeit von komplexen Matrizes garantiert. Die Analytik der Polyphenole wurde im Rahmen dieser Arbeit mittels LC-DAD-ESI-MS betrieben. Auf Basis ihrer Struktur, können aromatische Verbindungen, wie Polyphenole durch UV/Vis-Anregung, qualitativ in ihre jeweiligen Substanzklassen eingeteilt werden. Bei der UV/Vis-Spektroskopie wird über delokalisierte Elektronenpaare, die bei konjugierten Doppelbindungen am A- und C-Ring vorkommen, Energie aufgenommen, wobei die angeregten Valenzelektronen vom bindenden in ein antibindendes Orbital wechseln, was ein $\pi \rightarrow \pi^*$ Übergang zur Folge hat. Beim Verlassen des antibindenden Orbitals zurück in das bindende, kann Extinktionsenergie gemessen werden. Diese Extinktionsenergie ist jeweils individuell für die jeweilige polyphenolische Substanzklasse, wobei ein Emissionsspektrum charakteristisch für eine Polyphenolklasse steht. Ein typisches UV/Vis-Spektrum von Flavonoiden beinhaltet zwei Absorbionsbanden. Im Detail zeigen Flavone in methanolischer Lösung eine Absorption zwischen 310 - 350 nm (Bande I) und bei 250-280 nm (Bande II). Die Bande I wird dem B-Ring und Bande II dem A-Ring zugeordnet. Flavan-3-ole absorbieren hingegen zwischen 270-290 nm (Bande I) und bei 220 - 240 nm (Tsimogiannis *et al*., 2007). Zusätzliche Informationen über den C-Ring können hieraus nicht abgeleitet werden. Die Detektion der Flavan-3-ole erfolgte somit mittels DAD bei einer Wellenlänge von $\lambda = 280$ nm, die Gruppe der Flavonole wurde bei $\lambda = 320$ nm und die Gruppe der Anthocyane bei $\lambda = 520$ nm gemessen.

Innerhalb der LC-MS-Analytik werden monomere phenolischen Substanzen sowohl im positiven als auch im negativen Modus gemessen. Generell gelten für Messungen im negativen Modus ein niedrigeres Hintergrundrauschen und dadurch verbunden eine empfindlichere und sensitivere Messung (Watson, 2014). Dahingegen kann bei Anwesenheit von Methoxyl-, Hydroxyl- oder Polymethoxylflavonen nur im positiven Ionenmodus gemessen werden. Gleiches gilt für positiv geladene Anthocyane im niedrigen pH-Bereich (Watson, 2014). Zur Identifizierung unbekannter monomerer phenolischer Subtanzen wurde in dieser Arbeit im positiven Fullscanmodus zwischen m/z = 100 - 2000 Dalton (Da). gemessen.

Zur massenspektroskopischen Identifizierung von kondensierten phenolischen Substanzen wurde ebenso im positiven Ionenmodus gemessen, wobei hier im *selected ion modus*

gemessen wurde. Durch den limitierten Scanbereich können nur PA mit einem PG ≤ 5 bestimmt werden. Die verwendeten Geräteparameter des LC-DAD-ESI-MS-Systems sind im Anhang angegeben.

2.6.6. Quantifizierung monomerer Polyphenole mittels LC-DAD-ESI-MS

Für die Quantifizierung monomerer Polyphenole im Kakaoextrakt wurden kommerzielle Referenzsubstanzen mit einer Konzentration von 1 mg/mL hergestellt. Dafür wurden die meisten Referenzsubstanzen in Methanol gelöst, wohingegen die schwer wasserlöslichen Flavonole, Flavone, Flavanone und das wasserunlösliche Theobromin in DMSO gelöst wurden. Die quantitative Bestimmung erfolgte mittels externer Kalibrationsgeraden mittels der jeweilige Referenzsubstanz (s. Anhang). Die Kakaoproben wurden dreifach aufgearbeitet und analysiert. Die Ergebnisse sind entsprechend als Mittelwert und zugehörige Standardabweichung der Dreifachbestimmung dargestellt.

3. Problemstellung und Zielsetzung

Aufgrund fehlender Referenzsubstanzen im Bereich kondensierter Kakao PA gestaltet sich eine quantitative Bestimmung ihrer antioxidativen Kapazität als schwierig. Wie vorgängig erwähnt, sind oligomere PA entweder teuer oder PA mit einem PG ≥ 5 nicht als Referenzsubstanzen käuflich zu erwerben. Daher befasst sich diese Arbeit mit der Isolierung von kakaospezifischen Referenzsubstanzen, die für weiterführende Wirksamkeits- oder Bioaktivitätstests zur Verfügung gestellt werden können (‚Ziel 1'). Die Grundlage hierfür bildete die semipräparative Gegenstromverteilungschromatographie, auf deren Basis eine Vorfraktionierung phenolischer Subtanzen erreicht werden sollte. Im zweiten Schritt sollte mittels Gelpermeationschromatographie die zuvor vorfraktionierten Substanzen in individuelle oligomere PA isoliert werden. Die Kombination aus zwei Trenntechniken erlaubt es, ausgewählte Inhaltsstoffe im Labormassstab und mit hoher Reinheit zu isolieren. Die so gewonnenen Einzelsubstanzen, können für weitere Analysenverfahren oder Bioaktivitätstests eingesetzt werden. Daneben sollte eine leistungsstarke Analytik zur Identifizierung phenolischer Substanzen mittels LC-DAD-ESI-MS aufgebaut werden. Darüber hinaus sollte eine geeignete Probenaufarbeitung entwickelt werden, die es erlaubt, konzentrierte Proanthocyanidinfraktionen zu erhalten. Mittels einfacher kolorimetrischer Messmethoden sollte hier der Einfluss von unterschiedlichen Extraktionsparametern wie Lösungsmittel, Partikelgröße, Temperatur, Reaktionszeit, Lösungsmittelverhältnis auf die Extraktionsausbeute näher untersucht und auf schonende Extraktionsbedingungen hin optimiert werden.

Daneben war es Ziel dieser Arbeit eine geeignete Messmethode zu etablieren, die es ermöglicht, wirkungsorientierte Eigenschaften von hochmolekularen PA näher zu charakterisieren. Zu diesem Zweck sollte ein geeignetes LC-Verfahren entwickelt werden, welches es erlaubt, sowohl kondensierte PA und ihre antioxidative Kapazität mit Hilfe von Nachsäulenderivatisierung simultan zu bestimmen (‚Ziel 2'). Die dazu entwickelte Messtechnik könnte eine Möglichkeit bieten, ausgehend von der Molekülstruktur der PA, die antioxidative Aktivität abzuleiten.

Ein dritter Schwerpunkt lag in der quantitativen Bestimmung oligomerer PA und ihrer Veränderungen im Laufe des Schokoladenverarbeitungsprozesses (‚Ziel 3'). Die Problematik bei einer Verfolgung von wertgebenden Inhaltsstoffen über die gesamte Wertschöpfungskette, liegt in der Zugänglichkeit von Proben, sowie einem ungenügenden Einblick in chemische oder physikalische Messparameter. Deswegen sollte im Labormaßstab ein Schokoladenprozess simuliert, von der reifen Kakaofrucht bis hin zur

verzehrfertigen Schokolade, um Veränderungen von oligomeren PA exakt nachverfolgen zu können. Es sollte nicht nur Veränderungen des Polyphenolprofils, sondern auch der Einfluss von chemischen und physikalischen Parametern auf die antioxidative Kapazität von oligomeren PA untersucht werden.

4. Kumulativer Teil der Dissertation

Drei bereits veröffentlichte Publikationen bilden die Grundlage dieser Dissertation und werden in den darauffolgenden Kapiteln kumulativ zusammengefasst.

Ein zentraler Aspekt des Promotionsvorhabens beruhte auf der Etablierung einer Trennungstechnik, welche es erlaubt, aus einer komplexen Matrix pflanzlichen Ursprungs sekundäre Pflanzenstoffe mittels Gegenstromverteilungschromatographie (engl. *sequential centrifugal partition chromatography*, SCPC) zu fraktionieren und anschließend mittels Gelpermeationschromatographie (engl. *gel permeation chromatography*, GPC) in ihre individuelle Proanthocyanidine zu isolieren. Diese Kopplungstechnik ermöglicht es, Reinsubstanzen zu generieren. Neben einer optimierten Probenvorbereitung wird der Schwerpunkt auf die Gewinnung von oligomeren PA gelegt. (Publikation 1)

Daneben lag der Fokus dieser Arbeit in der Erarbeitung einer Messmethode zur simultanen Bestimmung der antioxidative Kapazität individueller oligomerer PA. In Anlehnung an das etablierte RP-LC-online-TEAC Messsystem, wurde in diesem Abschnitt ein chromatographisches Trennverfahren für die Bestimmung der antioxidativen Kapazität oligomerer PA erarbeitet. Die generierten Ergebnisse der NP-LC-online-DPPH Methode erlauben es, Aussagen über die antioxidative Kapazität der jeweiligen Molekülstruktur zuzuordnen. (Publikation 2)

Während eines Schokoladenverarbeitungsprozesses im Labormaßstab konnten die Änderungen des Polyphenolprofils und die Auswirkung auf die antioxidativen Aktivität mit Hilfe des NP-LC-online-DPPH Systems erfasst werden. Die Ergebnisse dienen dem näheren Verständnis der chemischen Veränderung bei der jeweiligen Verarbeitungsstufe und können für weiterführende Versuche zur Optimierung eingesetzt werden. Die Ergebnisse dienen somit sowohl wissenschaftlichen als auch industriellen Fragestellungen. (Publikation 3)

Im abschließenden Teil werden themenübergreifende Ergebnisse diskutiert und Schlussfolgerungen gezogen. Aus den vollständigen Autorenangaben und Danksagungen in den Publikationen können jeweils alle Mitwirkenden eindeutig zugeordnet werden.

4.1. Extraktion von Kakaoproanthocyanidinen und deren Fraktionierung mittels Gegenstromverteilungschromatographie und Gelpermeations-chromatographie

Die Probenvorbereitung muss für jeden Analyten angepasst werden. Sie dient in erster Linie dazu, Analyten aus fester Matrix in Lösung zu bringen und störende Komponenten abzutrennen. Ziel war es hierfür, Optimierungen bezüglich verschiedener Extraktionsparameter wie Lösemittel, Temperatur, Extraktionszyklus und -zeit durchzuführen. Die qualitative und quantitative Beurteilung der Parameter wurde mittels kolorimetrischen Messmethoden durchgeführt. Die Messmethoden unterscheiden sich in ihrer Fähigkeit mit unterschiedlichen funktionellen Stoffklassen zu reagieren, wodurch eine Differenzierung von verschiedenen phenolischen Substanzklassen möglich ist. Zusammengenommen konnten so quantitative Aussagen zu den kakaospezifischen phenolischen Substanzklassen getätigt werden.

Darüber hinaus wurden zwei semipräparative chromatographische Trennsysteme, wie die Gegenstromverteilungs- und Gelpermeationschromatographie, miteinander gekoppelt, um Substanzklassen zu fraktionieren und Einzelsubstanzen voneinander zu isolieren.

Eigenanteil

Experimenteller Versuchsaufbau	80%
Durchführung der Versuche	80%
Durchführung und Auswertung der chemischen Analysen	80%
Verfassen des Manuskripts	70%

Analytical and Bioanalytical Chemistry (2016)

Vol. 408, Pgs. 5905-5914

Extraction of cocoa proanthocyanidins and their fractionation by sequential centrifugal partition chromatography and gel permeation chromatography

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4.1.1. Abstract

Cocoa beans contain secondary metabolites ranging from simple alkaloids to complex polyphenols with most of them believed to possess significant health benefits. The increasing interest in these health effects has prompted the need to develop techniques for their extraction, fractionation, separation, and analysis. This work provides an update on analytical procedures with a focus on establishing a gentle extraction technique. Cocoa beans were finely ground to an average particle size of < 100 µm, defatted at 20 °C using n-hexane and extracted three times with 50% aqueous acetone at 50 °C. Determination of the total phenolic content was done using the Folin-Ciocalteu assay, the concentration of individual polyphenols was analyzed by electrospray ionization high performance liquid chromatography-mass spectrometry (ESI-HPLC/MS).

Fractions of bioactive compounds were separated by combining sequential centrifugal partition chromatography (SCPC) and gel permeation column chromatography using Sephadex LH-20. For SCPC, a two-phase solvent system consisting of ethyl acetate/n-butanol/water (4:1:5, v/v/v) was successfully applied for the separation of theobromine, caffeine, and representatives of the two main phenolic compound classes flavan-3-ols and flavonols. Gel permeation chromatography on Sephadex LH-20 using a stepwise elution sequence with aqueous acetone has been shown for effectively separating individual flavan-

3-ols. Separation was obtained for (-)-epicatechin, proanthocyanidin dimer B2, trimer C1, and tetramer cinnamtannin A2. The purity of alkaloids and phenolic compounds was determined by HPLC analysis and their chemical identity was confirmed by mass spectrometry.

Keywords: Cocoa proanthocyanidins; Sequential centrifugal partition chromatography; Gel permeation chromatography; Total phenolic content.

4.1.2. Introduction

The frequently reported relationship between plant phenolic compounds, often referred to as polyphenols, and health has accelerated the research into these bioactive compounds. Promisingly, proanthocyanidins (PAs) in plant food are investigated because of their reported antioxidant, antiinflammatory, and antimicrobial activity. Especially, PAs from cocoa (*Theobroma cacao* L.) are assumed to possess health benefits [e.g. 1]. In general, cocoa beans contain compounds ranging from simple alkaloids such as theobromine and caffeine to complex polyphenols, which can be classified into the main groups of phenolic acids (hydroxybenzoic and hydroxycinnamic acids), and the quite large group of flavonoids comprising flavan-3-ols (catechin, epicatechin), anthocyanins (cyanidins), flavonols (quercetin), flavones (luteolin), and flavanones (naringenin). Especially higher oligomeric PAs, resulting from a condensation of flavan-3-ols, are assumed to have a higher antioxidant capacity than the monomers or lower oligomeric PAs. Despite the high scientific interest in compounds like cocoa PAs, the majority of them are either not commercially available or are very expensive.

It is generally consent, that at the moment it is impossible to develop an extraction method suitable for all bioactive compounds from plant materials. Nevertheless, a technique that generates significant amounts of purified compounds is essential for analytical purposes or bioavailability and related bioactivity studies. Accordingly, one objective of this work was to evaluate the highest content of total phenols obtained with different extraction conditions. Folin-Ciocalteu method was used for the quantification of the total phenolic content. The proximate composition of the total flavonoid content and the total PA content were also determined photometrically.

In order to further fractionate complex phenolic extracts, preparative techniques in various modes are frequently used to separate bioactive compounds. Separation of PAs in cocoa according to their molecular size has already been reported for normal phase HPLC [2]. Preparative purifying processes for catechins and PAs on reversed phase HPLC of root bark have been also described [3], as well as size exclusion chromatography for PAs obtained from various plant bodies including cocoa beans [4]. In the case of phenolic compounds

solid-liquid chromatography suffers from major drawbacks. The recovery rate and the obtained amounts are much less than the other separation mechanisms. To overcome these problems, separation using liquid-liquid chromatography has attracted researches as a more efficient approach. Shibusawaa, Yanagidaa, Isozakia, Shindoa, and Ito [5] separated apple PAs with regard to degree of polymerization (DP) using *high speed countercurrent chromatography* (HSCCC). Liquid-liquid chromatographic techniques such as HSCCC or SCPC (*sequential centrifugal partition chromatography*) are covering an important niche among the chromatographic methods applied for the isolation of secondary plant metabolites. A significant advantage can be seen in the avoidance of irreversible adsorption interactions on solid stationary phases, the limited risk of sample denaturation, the possibility for a total sample recovery, a comparatively large load capacity, and the possibility to perform separations on a semi-preparative scale [6]. SCPC is a relatively new continuous cyclic liquid-liquid chromatographic separation technology [7]. In contrast to CCC, CPC chromatography enables a fast chromatographic run and an almost unlimited upscaling.

A study by Esatbeyoglu, Wray, and Winterhalter [8] showed the possibility of isolating dimeric, trimeric, tetrameric, and pentameric PAs from unroasted cocoa beans using HSCCC with *tert*-butylmethlyether/n-butanol/water (4.3:0.7:5, v/v/v) as solvent system. Further, different modes of operation do however not permit a simple transfer of a HSCCC solvent system for isolation of cocoa polyphenols using to a SCPC separation. A complete separation of the main cocoa compounds using SCPC has not been described, yet. Furthermore, as the separation efficiency of liquid-liquid chromatographic systems is typically not sufficient for achieving a complete separation of complex samples in one single chromatographic run, the combination of SCPC with other methods such as preparative column chromatography is necessary because of their complementary properties.

Combinations of HSCCC and gel permeation chromatography using Sephadex LH-20 have already been shown to be an efficient separation technique. In a study by Yang, Li, and Wan [9], the main individual tannins from black tea were purified, whereby theaflavin, theaflavin-3-gallate, and theaflavin-3,3-digallate were obtained in a separation process using a combination of HSCCC and a chromatographic separation with Sephadex LH-20. Also, Zhou, Liang, Zhang, Zhao, Guo, and Shi [10] obtained a purification of glucosidase inhibitors from *Polygonatum odoratum* by HSCCC and a following separation with Sephadex LH-20. A study done by Cheel, Minceva, Urajová, Aslam, Hrouzek, and Kopecký [11] used also preparative CPC separation followed by gel permeation chromatography to obtain on 50 mg of crude soil cyanobacteria extract a yield of 3.5 mg Aeruginosin-865, with a purity over 95% as determined by HPLC.

Therefore, the aim of the present study was first to find an efficient extraction method for cocoa PAs. For this purpose, various extraction solvents were studied and extraction

conditions such as temperature, solid-liquid ratio, and extraction time were optimized. The second objective was to establish a new method for the separation and purification of cocoa bean extracts using sequential centrifugal partition chromatography combined with gel permeation chromatography. Critical parameters such as the two-phase solvent system and the sample load for the SCPC separation were optimized. The chemical structures of the purified compounds obtained were confirmed by mass spectrometry.

4.1.3. Materials and Methods

4.1.3.1. Materials

The phenolic reference substances (-)-epicatechin and (+)-catechin were purchased from Sigma Aldrich Chemie GmbH (Buchs, Switzerland). Proanthocyanidin B2, proanthocyanidin C1, and cinnamtannin A2 were purchased from PhytoLab GmbH & Co. KG (Vestenbergsgreuth, Germany). Proanthocyanidin B3 and B4 were purchased from TransMIT GmbH (Giessen, Germany). Further reference substances such as theobromine, caffeine, (-)-epicatechin, (+)-catechin, proanthocyanidin B1, quercetin, quercetin-3-*O*-arabinoside, and quercetin-3-*O*-glycoside were obtained from Extrasynthese (Lyon, France). Anhydrous sodium carbonate, aluminium chloride hexahydrate, n-butanol, Folin-Ciocalteu reagent, sodium hydroxide, and sodium nitrite were purchased from Sigma-Aldrich (Buchs, Switzerland). HPLC grade acetonitrile, water, ethanol, acetone, methanol, 1-propanol, 2-propanol and formic acid were obtained from Sigma Aldrich Chemie GmbH (Buchs, Switzerland). Double-distilled water (Merck & Cie KG, Schaffhausen, Switzerland) was used throughout the whole study. Column chromatography was carried out with Sephadex LH-20 gel (GE Healthcare Bio-Sciences AB, Glattbrugg, Switzerland)

4.1.3.2. Sample Preparation

Dried and fermented cocoa beans were from a genuine Trinitario variety grown and harvested in Ghana. Optimization of sample preparation was performed as described previously [12]. Primarily, frozen cocoa beans were freeze dried (Alpha 2-4 LDplus, Martin Christ, Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany), manually dehulled and crushed in a knife mill (A 11 basic Analytical Mill, IKA®-Werke GmbH & Co. KG, Staufen, Germany) for approx. 10 s to minimize heat development. In order to remove lipids, cocoa beans were extracted five times with the five-fold volume (v/w) of n-hexane for 5 min at 20 °C. The hexane phase was removed by centrifugation at 2,880 x g for 5 min (Centrifuge 5810, Vaudaux-Eppendorf AG, Schönenbuch, Switzerland). This procedure of defatting and grinding was done 4 times until the n-hexane phase appeared colorless. After defatting,

cocoa bean powder with an average particle size of < 100 µm was further used for the extraction of target compounds. For the analysis of the total phenolic content, 2 g of defatted and dry cocoa powdered samples were weighed into a 50 mL centrifuge vessel and extracted three times with 6 mL of aqueous acetone mixture for 8 min at 50 °C (Thermomixer MHR 23, Ditabis AG, Pforzheim, Germany). After each extraction step, the mixture was centrifuged and the combined supernatant was considered as *liquid cocoa extract* and directly used for spectrophotometric assays, or freeze dried for SCPC analysis and named *freeze dried cocoa extract*.

4.1.3.3. Solvent and Temperature Optimization

A range of solvents were tested for their efficacy in extracting target compounds from ground cocoa bean extract, including methanol, ethanol, 1-propanol, 2-propanol, and acetone, and their respective mixtures with water. Thirteen extraction temperatures (20-140 °C) were evaluated for their effect on the extraction of valuable cocoa compounds.

Generally, 2 g of cocoa bean powder were extracted three times with 6 mL of solvent / water mixtures for 8 minutes in a 50 mL centrifuge tube at different temperatures using a benchtop shaker (Thermomixer MHR 23, Ditabis AG, Pforzheim, Germany) at 800 rpm. The liquid cocoa extract obtained was analyzed for the total phenolic content using the photometric Folin-Ciocalteu assay, and for individual components using RP-HPLC/MS. Subsequently, individual compounds of the maintained liquid cocoa extracts were measured by RP-HPLC/MS. All extraction experiments were done in triplicate.

4.1.3.4. Determination of the total phenolic content (TPC) using Folin-Ciocalteu assay

The photometric method for determining the total phenolic content is used frequently in food analysis and is applicable to polar solvents. However, there are other reducing substances present, such as sugars or proteins, which are also able to react with the Folin-Ciocalteu reagent [13]. The total phenolic content (TPC) of cocoa beans was determined according to Blois [14] with some minor modifications: For *liquid cocoa extract* suspected of having high reactivity, the following dilutions were made: 1:10, 1:100. Following, a 1 mL aliquot of the *liquid cocoa extract* was mixed with 1 mL of Folin-Ciocalteu reagent (2 N reagent diluted 1:3 with dest. H_2O); 2 mL distilled water were added and the sample incubated for 3 min at room temperature. Thereafter, 2 mL of anhydrous sodium carbonate solution (20% Na_2CO_3, w/v) were added. The solution was kept for 2 h at room temperature

for color formation, and the absorption of the blue colored sample was measured at 750 nm using an UV/Vis spectrophotometer (Genesys^{TM} 10S, Thermo Fisher Scientific AG, Reinach, Switzerland) against a blank sample containing the same reagents and 1 mL distilled water. (-)-Epicatechin was used as a calibration standard and final results were expressed as milligrams of (-)-epicatechin equivalent per gram of non-fatty dry matter (mg ECE/g). The calibration curve was linear from 5 mg/L to 50 mg/L with a linear regression line of y = 18.821x + 0.0357 and R^2 = 0.998.

4.1.3.5. Determination of the total flavonoid content (TFC) using the aluminum chloride assay

Besides the determination of the TPC, the total flavonoid content (TFC) of the cocoa extract gives an indication of the phenolic composition of cocoa beans and the yield of the different extraction procedures. The determination of the TFC was done photometrically according to the method described by Zzaman, Bhat, and Yang [15] using the same extract as described before: For *liquid cocoa extract* suspected of having high reactivity, the following dilutions were made: 1:10, 1:100. Further on, an aliquot (1 mL) of the *liquid cocoa extract* or the standard solution was added to a test tube containing 4 mL distilled water. Afterwards, 0.3 mL sodium nitrite solution (5% $NaNO_2$, w/v) were added and the sample was incubated for 6 min at room temperature. 0.3 mL aluminum trichloride solution (10% $AlCl_3$, w/v) were added and the sample incubated for another 6 min. 2 mL of a 1 M sodium hydroxide solution (4% NaOH, w/v) were added and the sample was filled up to 10 mL using distilled water. After 15 minutes, the absorption of the pink solution was measured at 510 nm. A linear calibration line was obtained using (-)-epicatechin at concentration range of 25 to 225 mg/L with a linear regression line y = 4.1072x - 0.0025 and R^2 = 0.998, whereby the total flavonoid content was expressed in milligrams of (-)-epicatechin equivalent per gram non-fatty dry matter (mg ECE/g).

4.1.3.6. Determination of the total proanthocyanidin content (TPAC) using the acid-butanol assay

The acid butanol assay is widely used to determine the total proanthocyanidin content (TPAC) in food. The method by Porter, Hrstich, and Chan [16] and a modified one by Pérez-Jiménez, Arranz, and Saura-Calixto [16] were used during this study. For *liquid cocoa extract* suspected of having high reactivity, the following dilutions were made: 1:5, 1:10. Further on, in a screw cap tube 6 mL of acid butanol (5 mL 37% HCl plus 95 mL n-butanol) reagent were

added to 1 mL aliquot of the *liquid cocoa extract*. As a catalyst, 1 mL of a 2% solution of ammonium iron(III)sulfate in 2 N HCl (2% $NH_4Fe(SO_4)_2 \cdot 12H_2O$) was added. The tube was sealed and incubated at 95°C for 50 min using a benchtop shaker, followed by an immediate cool down with cold water. Afterwards the absorption of the pink color was measured photometrically at 550 nm against a blank. The proanthocyanidin content was calculated using cyanidin at concentration range of 10 to 70 mg/L using the following equation: y = 13.137x - 0.009 and R^2 = 0.9982. The proanthocyanidin content was expressed in milligram of cyanidin equivalent per gram non-fatty dry matter (mg CyE/g).

4.1.3.7. Sequential Centrifugal Partition Chromatography (SCPC)

The separation was carried out on a semi-preparative SCPC liquid-liquid partition chromatography system (Armen Instruments, Saint-Avé, France) with a total volume of 250 mL. A HPLC pump (Pump Model SD-300, Rainin Instrument Co. Inc., Oakland, California, USA) was used to deliver the mobile phase, in ascending and descending mode, respectively. The maximum rotational speed used was 3,000 rpm and a maximum back pressure of 60 bar. The effluent was continuously monitored at 275 nm using an UV detector (UV1, Rainin Instrument Co. Inc. Oakland, California, USA). A fraction collector (Foxy 200, Teledyne Isco, Lincoln, Nebraska, USA) was connected to the detector outlet. A manual injection valve (10 mL Rheodyne loop) allowed injections ranging up to 3 grams. The choice of the biphasic solvent system and the elution mode are crucial for successful liquid-liquid chromatographic separations. The biphasic solvent system ethyl acetate/butanol/water with 4:1:5 (v/v/v) was prepared by mixing defined portions of ethyl acetate, butanol, and water and degassing vigorously. After degassing, the solvent was equilibrated at room temperature for 30 minutes. In the meantime, the freeze dried cocoa extract (700 mg) was dissolved in 2 mL 40% aqueous 2-propanol supported by the use of an ultrasonic bath for 5 minutes at room temperature. The sample solution was then partitioned between 4 mL each of the mobile and the stationary phase and filtered through a 5.0 µm syringe filter (Nylon 66, BGB Analytik AG, Boeckten, Switzerland). Afterwards, sample solution was injected into the SCPC column through the manual injection valve. The chromatogram was continuously monitored and fractions were collected for a period of 5 min each with the fraction collector.

For the SCPC fractionation, the column was initially filled with the stationary phase (organic phase: upper phase) in the *descending* mode at a flow rate of 1.5 mL/min and a revolution speed of 500 rpm. The mobile phase (aqueous phase: lower phase) was then pumped at a revolution speed of 1,900 rpm until an equilibrium was established after 90 mL dead volume. After the separation of the first major peaks in the chromatogram was achieved, the column mode was turned to *ascending* after 220 min, in order to recover the

components that were not eluted under these conditions. The preparative separation of individual substance classes was carried out in a single run. The peak fractions (I-IV) were collected automatically, correspondingly to the chromatogram obtained and subjected to further HPLC-MS analyses.

4.1.3.8. Gel permeation chromatography

As applying a single separation technique typically is not sufficient to obtain individual components covering a wide range of polarities, a combination of chromatographic methods needs to be performed. Therefore, semi-preparative SCPC was selected as first and most important separation technique for its lack of a solid stationary phase that can lead to irreversible adsorption of phenolic components (e.g., on reversed phase chromatographic material) and a complete sample recovery [18]. The second purification step applied for isolating fractions or even single compounds of high purity was done on a lipophilic Sephadex LH-20 gel. Traditional column chromatography has been already proven for being suitable of separating a number of phenolic compounds. Especially, alkylated crosslinked dextran Sephadex LH-20 is suitable for a separation of polyphenolic polymers when using aqueous acetone as eluent [19, 20].

In the present study, a glass column 10 x 120 mm with a volume of 6 mL (ECO10/120V0V, YMC Europe GmbH, Dinslaken, Germany) was packed with 1 g of Sephadex LH-20. For purification, 40 mg of the *freeze dried cocoa extract* were dissolved in 100 mL 40% aqueous 2-propanol and directly subjected to Sephadex LH-20. Saturation and equilibration was done with pure water before adding the sample. The elution was performed by stepwise elution, starting with 100 mL defined solvent at a moderate flow rate of 0.6 mL/min. The effluents were collected in 100 mL volumetric flasks. Subsequently, the elution step was performed starting with 100 mL dest. H_2O as mobile phase, continuing with 100 mL 10% aqueous acetone, and further with 100 mL 15% aqueous acetone etc. until 100 mL 50% aqueous acetone. The fractions containing target compounds were freeze dried and dissolved in 40% aqueous 2-propanol for RP-HPLC/MS analysis. Regeneration of the column was done with 40% aqueous 2-propanol for at least two fold column volume before exposing again to sample.

4.1.3.9. Determination of individual cocoa compounds using RP-HPLC/MS analyses

Samples were analyzed with a liquid chromatograph coupled to a quadrupole mass spectrometer with electrospray ionization interface (LC/MS 6120, Agilent Technologies AG, Waldbronn, Germany). The fractions were analyzed in ESI-MS as total ion chromatogram in the positive mode *m*/*z* 100-2,000. Detailed conditions of the HPLC system and gradient elution are given in Pedan, Fischer, and Rohn [12]. All data obtained was processed with LC/MSD ChemStation software version Rev. B.04.03-SP1 (Agilent Technologies AG, Waldbronn, Germany).

4.1.4. Results and Discussion

4.1.4.1. Effect of extraction solvents on cocoa polyphenols

As already mentioned, cocoa extract is rich in monomeric and oligomeric PAs. The yield of PAs varies with the two main extraction parameters: type of solvent and extraction temperature. Initially, the efficiency of the various extraction solvents was investigated at room temperature. The results of the TPC are summarized in **Fig. 1**, whereby the different extraction yields of PAs are shown in relation to the type of solvent used. The yield of extracted PAs increases within the homologous series of alcohols such as methanol, ethanol, and 1-propanol and is influenced by a decreasing polarity. It can also be pointed out that extraction yield is more intense with increasing chain length of the solvent and a decrease of the amount of water in the extraction solvent. The results show that the mixture of 50% aqueous acetone was the most efficient in extracting the phenolic compounds.

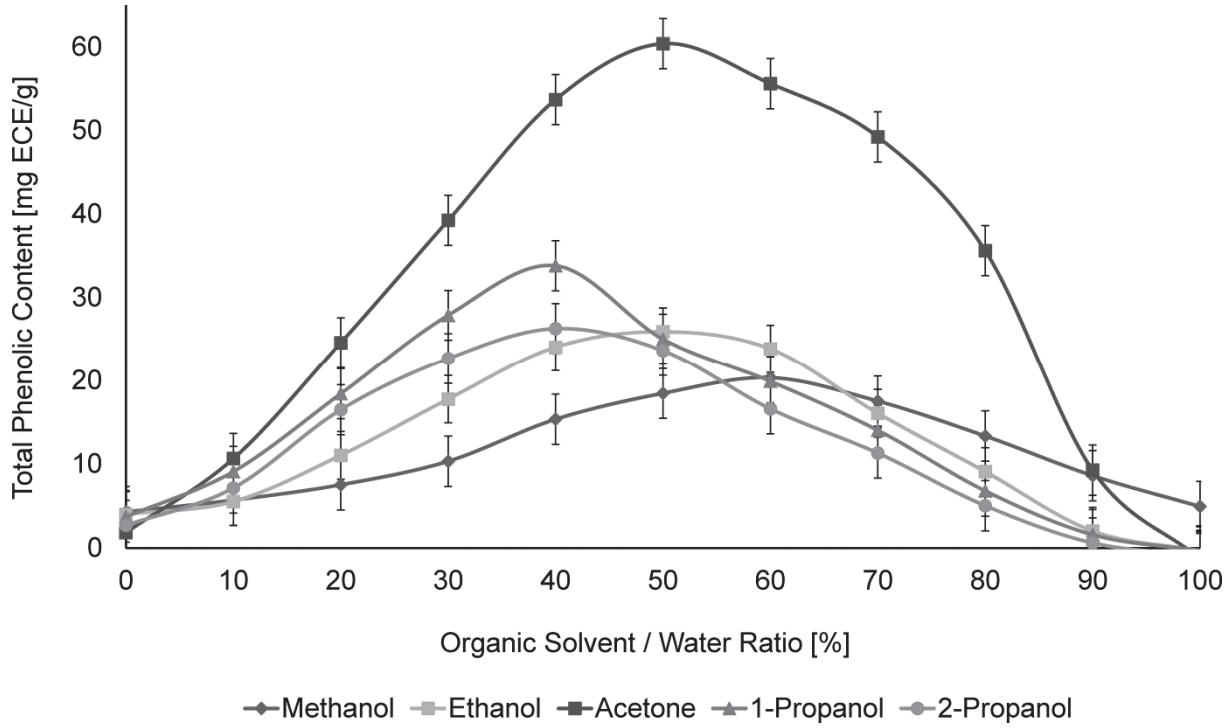

Figure 1 The influence of extraction solvent on the total phenolic content (TPC). Values are expressed as mg (-)-epicatechin equivalents per gram non-fat dry matter (mg ECA/g).

Besides the determination of the TPC, also the TFC and the TPAC were measured in the different aqueous acetone mixtures. **Fig. 2** shows the extraction yields for all three assays. Again, the 50% aqueous acetone mixture showed the best efficiency for extracting the different polyphenol classes in cocoa. The content of extracted total phenols of an unroasted cocoa extract was 60.4 mg ECE/g, the TFC was up to 37.2 mg ECE/g, and the TPAC was up to 25.4 mg CyE/g for 50% aqueous acetone.

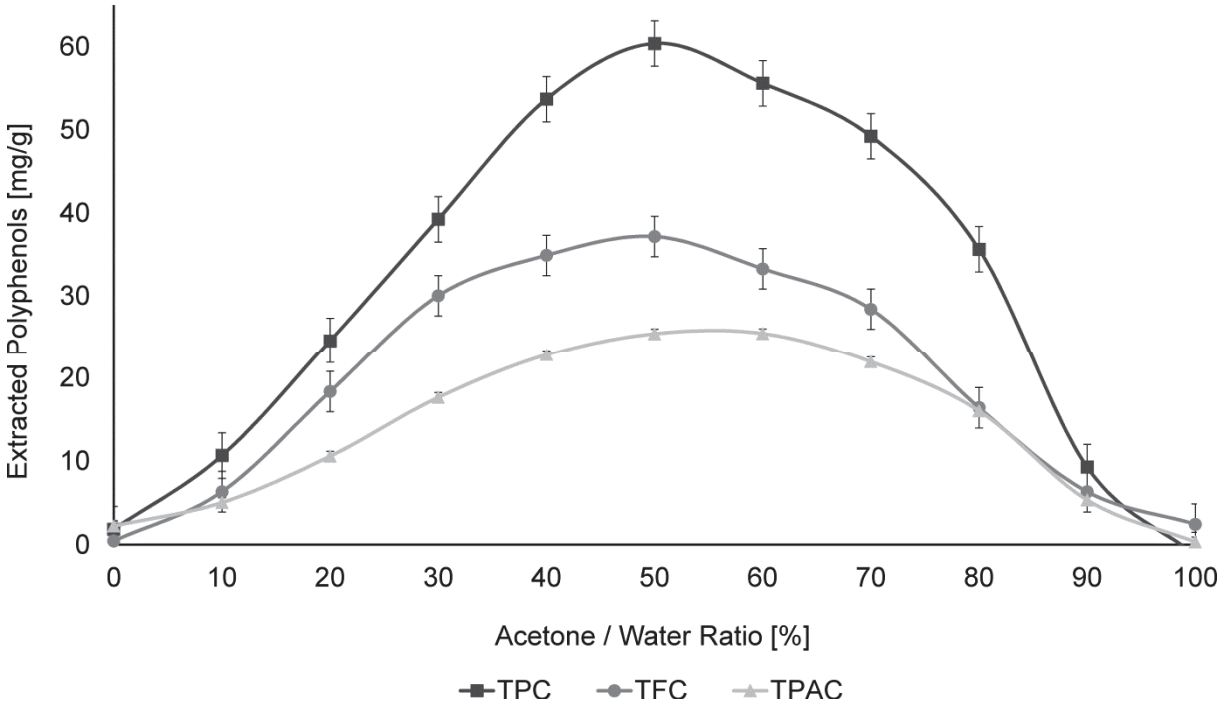

Figure 2 The influence of 50% aqueous acetone mixture on the total phenolic content (TPC), total flavonoid content (TFC), and total proanthocyanidin content (TPAC).

In order to substantiate the colorimetric results, RP-HPLC/MS analyses of the *liquid cocoa extracts* were carried out in addition. Cocoa bean compounds were identified based on retention times and mass spectra of reference compounds. In detail, it could be shown that 100% acetone as extraction solvent selectively extracts the two alkaloids theobromine and caffeine. With increasing water content (e.g., 10%, 90% aqueous acetone) (+)-catechin and (-)-epicatechin, followed by PA dimer were extracted. Extraction efficiency was improved for PA trimers and tetramers with solvent concentrations between 20% and 80% aqueous acetone and culminates with the highest extraction yield for condensed polyphenols using 50% aqueous acetone. The obtained cocoa extracts consist of at least oligomeric PA with DP=4. Generally, same order of solvent efficiency as shown in **Fig. 1** (50% > 40%, 60% etc.) was obtained when using RP-HPLC/MS analysis for identifying the individual phenolic compounds. Extraction efficiency with lower percentage of acetone/water mixture, or even water, was more selective for extracting single or low molecular weight polyphenols. On the other side, an indication for high oligomeric polyphenols can be observed through the additional peak eluted at the end of the chromatogram. Therefore, at a combination of acetone/water (50%), preferentially more PA oligomers are extracted (see ‘PA hump’).

Separation of complex mixtures typically fails when using reversed phase columns. Generally known, the efficiency of a reversed phase column for separating higher oligomeric PAs is limited due to the separation of complex polyphenols. In some cases, only peak broadening occurs, whereas in many cases, a group of higher oligomeric PAs occurs as a

'hump' at the end of the chromatographic run. Tarascou *et al*. [21] proved that high polymerized polyphenols eluting as a hump at the end of the chromatographic profiles. Kuhnert [21] stated that a chromatographic hump is generally a consequence of a complex mixture containing a too large number of compounds to be chromatographically resolved. Similar observations in the chromatographic behavior were observed in previous studies of the so-called 'PA hump' during the analysis of unroasted cocoa beans [12]. In case of the so-called 'thearubigin hump', containing condensed black tea polyphenols, major efforts have been undertaken in recent years by Kuhnert [22] to resolve this into several dozens of individual components consisting of quite similar structural subunits. It is reasonable to assume that a similar situation exists in cocoa, based on the similarity of the polyphenol (epicatechin) subunits and the proven occurrence of linearly condensed higher PAs.

In the present study, acetone-based solvents were most efficient for extracting oligomeric PAs, as compared to other extractions solvents e.g. alcohol/water mixtures. The extraction is therefore preferably carried out with 50% aqueous acetone. Hammerstone and Chimel [23] found acetone and ethanol being the most effective solvents in extracting higher oligomeric cocoa PAs. Especially for PAs with DP5-10, the efficiency increases significantly when comparing different water to solvent ratios, like e.g. the average percentage recovery of PA decamer increased from 0% using 100% acetone to more than 100% when using 50% aqueous acetone. Monrad, Howard, King, Srinivas, and Mauromoustakos [24] investigated red grape pomace and found 50% aqueous ethanol to be optimal for extracting total procyanidins than other ethanol/water compositions.

4.1.4.2. Effect of temperature on the extraction of cocoa polyphenols

An extraction can be carried out at temperatures ranging from chilled solvents up to the boiling points of the solvents. Since polyphenols are heat labile components, flavon-3-ol losses occur at elevated temperatures. Further, investigations on temperature conditions for comparing changes in content and composition are mandatory. In the present study, the total phenolic content was measured by Folin-Ciocalteu extracted three times for eight minutes using 50% aqueous acetone as solvent. Hereby, the concentration of cocoa polyphenols increased significantly with increasing temperature in the range of 20-110 °C, in detail 88 mg ECE/g at 20 °C to 116 mg ECE/g at 110 °C and began to decrease again to 99 mg ECE/g at 140 °C (**Fig. 3**). The data obtained, confirmed the significant effect of temperature on decreases or increases of TPC dependent on the applied temperature. In addition to the photometric assays, RP-HPLC/MS analyses of the *liquid cocoa extracts* from the different extraction procedures were carried out. In general, the yield of individual bioactive compounds such as (+)-catechin, (-)-epicatechin, PA dimer B2, trimer C1 increased only

slightly within the temperature range (data not shown). In contrast to another study dealing with the temperature influence [25], no degradation or loss could be observed at temperatures from 20 to 140 °C during an 8 minute extraction period. However, according to the present results, the extraction temperature was kept at 50 °C to ensure a reproducible polyphenol extraction below the boiling point of acetone as part of the extraction solvent. In addition, the moderate conditions help to preserve most of the initial phenolic compounds and to obtain the genuine flavan-3-ol composition.

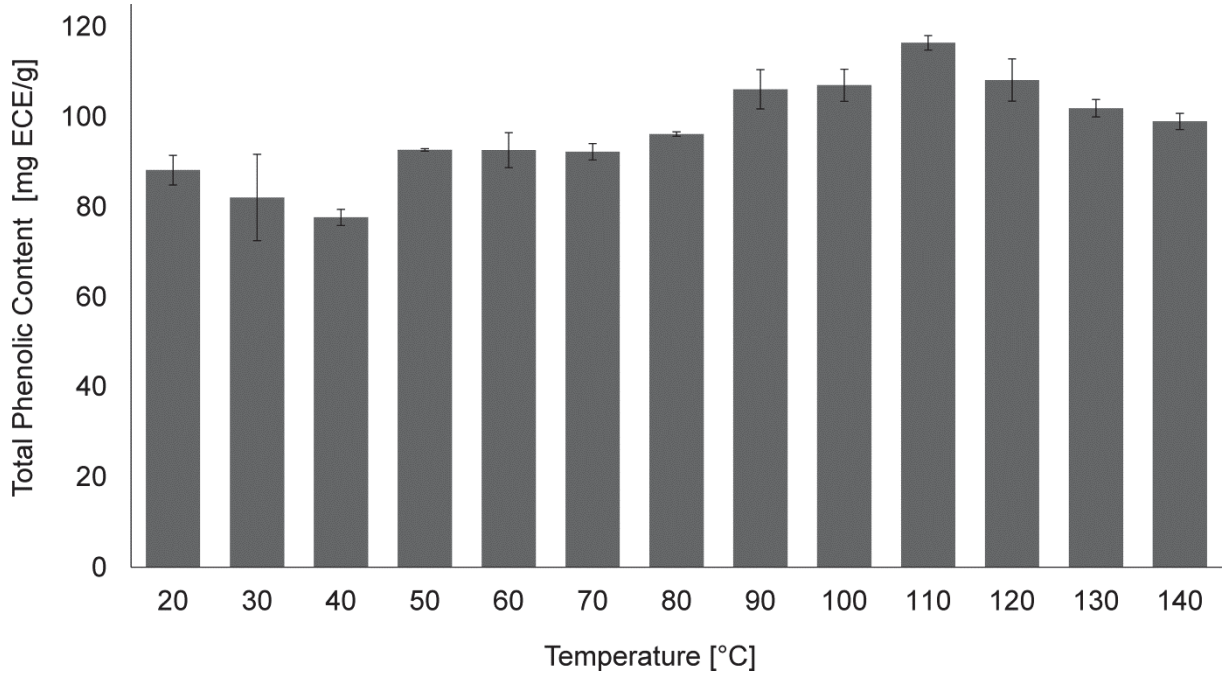

Figure 3 Influence of temperature extraction (20-140 °C) on the total phenolic content (TPC) as determined by using 50% aqueous acetone and 8 min of extraction.

Studies by Kothe *et al*. [25] claimed for epicatechin and PA dimer B1, B2, B5 progressive changes within a temperature range from 100 to 140 °C. Temperatures above 140 °C did not have an accelerated influence on further degradation.

Hammerstone and Chimel [23] also observed a temperature effect on the recovery of oligomeric cocoa PAs and recommended an elevated extraction temperature at 50 °C with an aqueous acetone mixture. Furthermore, it was pointed out in that study that the effect of temperature is non-significant for monomeric PAs and is significantly pronounced for more complex oligomeric PAs.

As already stated above, phenolic compounds' degradation depends on roasting time and temperature. The product pattern, resulting from thermal treatment at high temperatures under non-aqueous conditions (roasting) is quite different. For some flavonol mechanism for degradation under roasting conditions is a deglycosylation to the corresponding aglycone. The main aglycone product remains stable during further roasting. Compared to the cooking

process, flavonol glycosides showed several reaction products. In this context, studies observed degradation for flavonol glycosides in aqueous solution when heating up to 100 °C, whereby glycosides showed an intensive breakdown but did not form aglycones [26, 27].

4.1.4.3. Structural elucidation of isolated PAs with RP-HPLC/MS

The chromatogram of the SCPC separation gave four fractions (**Fig. 4**) and the coil fraction. Separation was completed within about 2.5 h so that at least two chromatographic runs could be performed within one day. Fraction I and II eluted in sharp peak starting at a retention time of about 70-80 min respectively 85-120 min. Fraction III eluted in a broad peak between 155 min followed by return to baseline at about 220 min. By switching the elution mode fraction IV eluted within 220-250 min. Several SCPC runs were repeated under the same condition, whereby the retention times were consistent between chromatographic runs.

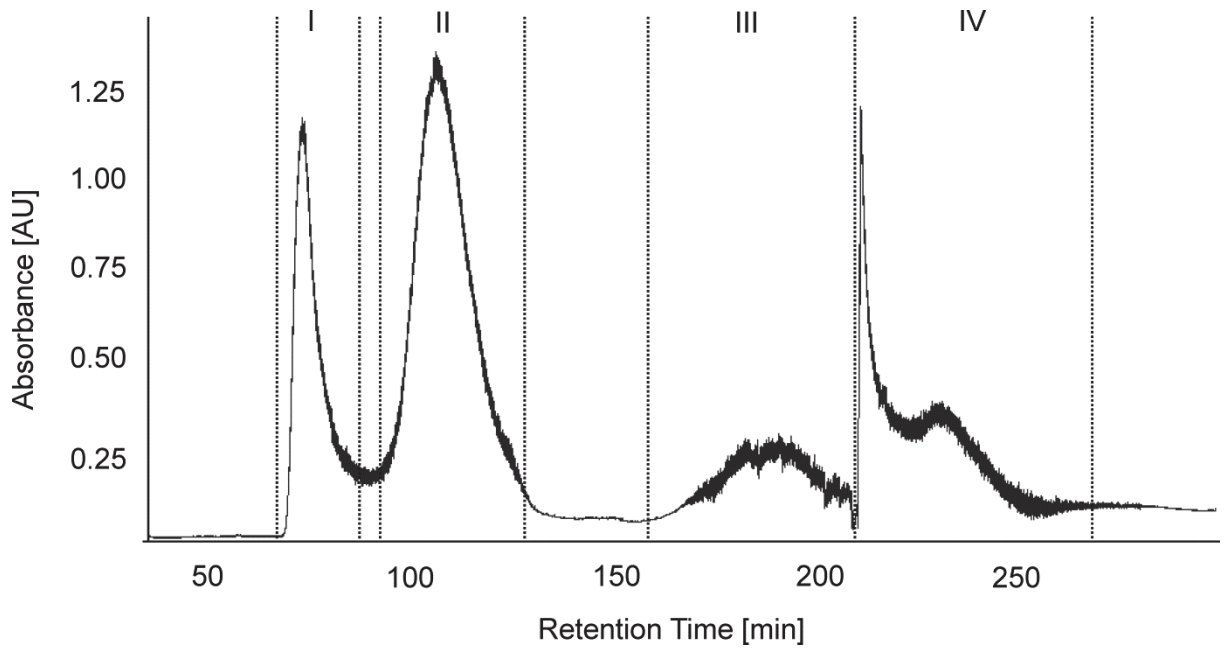

Figure 4 SCPC chromatogram when applying 700 mg cocoa extract. SCPC conditions: Solvent system: EtOAc/BuOH/H2O 4:1:5 (v/v/v); stationary phase: upper phase; revolution speed: 1,900 rpm; detection wavelength: 275 nm; flow rate: 1.5 mL/min. Switchover from descending to ascending after 220 minutes. Fr I-IV with (I) hydrophilic complex compounds; (II) theobromine; (III) caffeine; (IV) flavonols and proanthocyanidins.

The following compounds were identified according to their retention time and confirmed by mass spectrometry and authentic reference compounds. PAs were detected at 280 nm. In **Fig. 5**, the peaks numbered with arabic numerals correspond to the compounds assigned in the following text. During the separation in *descending* elution mode, polar compounds

eluted earlier. The polar fraction I from the partition of crude *freeze dried cocoa extract* contained mainly a mix of hydrophilic, largely complex, mostly branched molecules. An indication might be the 'PA hump' explained earlier in the study.

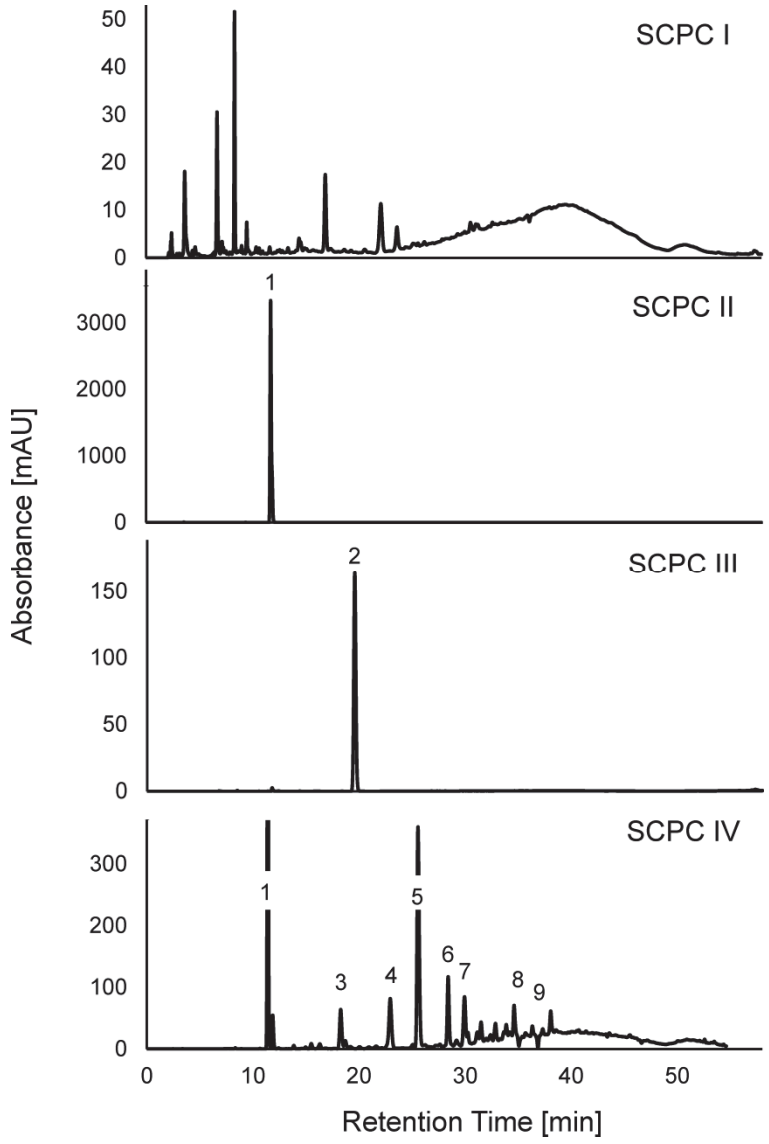

Figure 5 HPLC chromatograms of the SCPC fractions Fr I-IV. Peaks numbered with arabic numbers are in the order of increasing retention time and correspond to the compounds explained in the text. SCPC separation of fraction IV was operated three times before analyzing. (I) Hydrophilic complex compounds; (II) theobromine; (III) caffeine and (IV) flavonols and proanthocyanidins.

For the numbered peaks, it was possible to obtain a MS signal and in some cases MS fragmentation pattern. The resulting data are shown in **Table 1**. Based on the experiments and knowledge the fragmentation pattern $[M+H]^+$ of the identified compounds were summarized here as well.

Table 1. RP-HPLC-ESI/MS determination of phenolic compounds in an extract of unroasted cocoa beans. Compounds with peak no. 1-17 were detected in positive ion mode.

peak no	compound	t_R [min]	molecular weight [g/mol]	molecular ion $[M+H]^+$	major fragments $[M+H]^+$
1; 10	theobromine	11.7	180.16	181.1	n.d.
2; 11	caffeine	19.6	194.19	195.1	n.d.
3; 13	(+)-catechin	18.3	290.26	291.1	139.0; 165.1
4; 14	PA B2	22.9	578.52	579.2	291.1
5; 12	(-)-epicatechin	25.5	290.26	291.1	139.0; 165.1
6; 15	PA C1	28.4	866.77	867.2	579.2; 1155.5
7; 17	cinnamtannin A2	30.0	1155.04	1155.3	579.2; 867.0
8	quercetin-3-*O*-glucoside	34.6	464.38	465.1	303.1
9	quercetin-3-*O*-arabinoside	38.1	434.35	435.1	303.0
16	unknown PA dimer	34.8	866.77	867.2	579.2; 1155.5

n.d. not detected

In detail, a major portion of polar compounds eluted preferably at the beginning of the HPLC run. Furthermore, an unresolved 'PA hump' was observed for fraction I eluting in a retention timeframe of 35-45 min. Fraction II has been identified as theobromine (1, *m/z* 181.1). Fraction III contained caffeine (2, *m/z* 195.1).

In contrast, the fraction obtained with butanol (fraction IV) contained compounds with a high structural diversity such as flavan-3-ols with up to four epicatechin subunits and flavonol aglycones, with the latter also glycosylated with arabinose, galactose, and glucose. However, the fractions were still very complex in their composition and presence of residual theobromine (1). This underlines that the partition coefficient of flavan-3-ols and flavonols like quercetin and their glycosylated products are comparatively similar, being the main reason for not finding complete separation conditions.

In detail, fraction IV contained eight major peaks identified as target phenolic compounds (Peaks 3-9). Based on the HPLC/MS analysis, they were determined as (+)-catechin (3, *m/z* 291.1), PA dimer B2 (4, *m/z* 579.1, with a dominant fragment ion *m/z* 288.9), (-)-epicatechin (5, *m/z* 291.1), PA trimer C1 (6, *m/z* 866.5), as well as PA tetramer A2 (7, *m/z* 1155.1). Further compounds were UV-active at 360 nm and therefore assigned as negative peaks in the chromatogram (Peak 8, 9). They were identified as quercetin-3-*O*-glucoside (8, *m/z*

465.1, with fragment ions *m/z* 303.1) and quercetin-3-*O*-arabinoside (9, *m/z* 435.1, with fragment ions *m/z* 303.0), tentatively. However, their amount is low compared to flavan-3-ols.

Nevertheless, this method separates of about 700 mg *freeze dried cocoa extract* (I) 232 mg of hydrophilic complex compounds, (II) 88 mg theobromine, (III) 19 mg caffeine, (IV) 149 mg flavones and flavan-3-ols and 90 mg retained in the coil.

On the basis of a high PA accumulation in fraction IV, this fraction is called 'PA fraction'. The occurrence of theobromine (1) in fraction IV is significant and due to carry-over effects in the partition cells. Nevertheless, this effect of sample carry-over is still limited compared to conventional separatory-funnel partition. Nevertheless, an additional sample clean-up is required to minimize such "contamination effects".

However, the SCPC run resulted in four fractions according to the corresponding peaks separated. A typical color pattern can be observed for the eluted fractions. Fraction I had an intensive brown color. According to Fincke & Fincke [28], the brown color typically indicates water-insoluble phlobaphene. These compounds are structurally complex (see 'PA hump') and are responsible for the pure brown to the violet tinged color of the fermented cocoa bean. Moreover, it was recently shown that the color intensity increases from monomeric to polymeric PAs [12]. During cocoa fermentation, anthocyanidins are oxidized by polyphenol oxidase to quinones. The quinones can covalently react with amino acids and proteins or polymerize to form tannins. High molecular weight tannins can also interact non-covalently with proteins through hydrogen bonding, resulting as well in a brown, water insoluble pigment that gives cocoa its characteristic brown color [29]. With increasing retention time, the color appeared more violet-like for fraction II and fraction III. Upon turning the column mode to *ascending* mode, a quick color change appeared to a bright red for fraction IV, containing the flavan-3-ols and flavonols. The occurrence of the reddish color can be explained by the presence of purple anthocyanidin pigments (e.g. cyanidin arabinoside, -galactoside) in fresh cocoa beans. Although during fermentation these pigments are mostly hydrolyzed by glycosidases, resulting in a more pale purple color [30], they still occur in the extracts of unroasted cocoa.

4.1.4.4. Isolation of PAs from the freeze dried cocoa extract obtained by gel permeation chromatographie using Sephadex LH-20

In order to compare the purification grade of the main cocoa PAs with SCPC, a further clean-up step by gel permeation chromatography on Sephadex LH-20 was employed to separate individual flavan-3-ols from the semi-purified PA extract. SCPC separation was operated three times, and the same fractions were combined to enrich compounds 3-9. After separation, each fraction was analyzed by RP-HPLC/MS. HPLC analysis of each obtained

pure compound can be seen in **Fig. 6**. The affinity of PAs for Sephadex LH-20 was used for further fractionation. Here, the optimal separation of fraction IV was performed using aqueous acetone as eluting solvent in a gradient from 0% to 40% in increments of 5%. To improve the purity of the isolated sub-fractions, a stepwise elution with aqueous acetone as mobile phase was performed from 0% to 40% in increments of 10%. A sequential elution starting with 100 mL H_2O assigned compounds as theobromine (10, *m/z* 181.1), caffeine (11, *m/z* 195.1) and (-)-epicatechin (12, *m/z* 290.9) in fraction I. The eluate was collected and freeze dried immediately. Among the requested bioactive monomeric flavan-3-ol, also theobromine and caffeine could be detected in higher amounts. A further fractionation with 10% aqueous acetone, the phenolic compounds (+)-catechin (13, *m/z* 290.9) and PA dimer B2 (14, *m/z* 578.7) were obtained. Anterior impurities of theobromine and caffeine could be eliminated through sufficient aqueous extraction. Further on, the elution with 20 % aqueous acetone gained PA trimer C1 (15, *m/z* 866.5) and an unknown PA dimer (16, *m/z* 578.7). The major peak of the last elution step with 40% aqueous acetone was identified as PA tetramer A2 (17, *m/z* 1156.0). 40 mg of the 'PA fraction' obtained a yield of 1.4 mg of fraction I, 5.6 mg of fraction II, 2 mg of fraction III, and 2 mg of fraction IV.

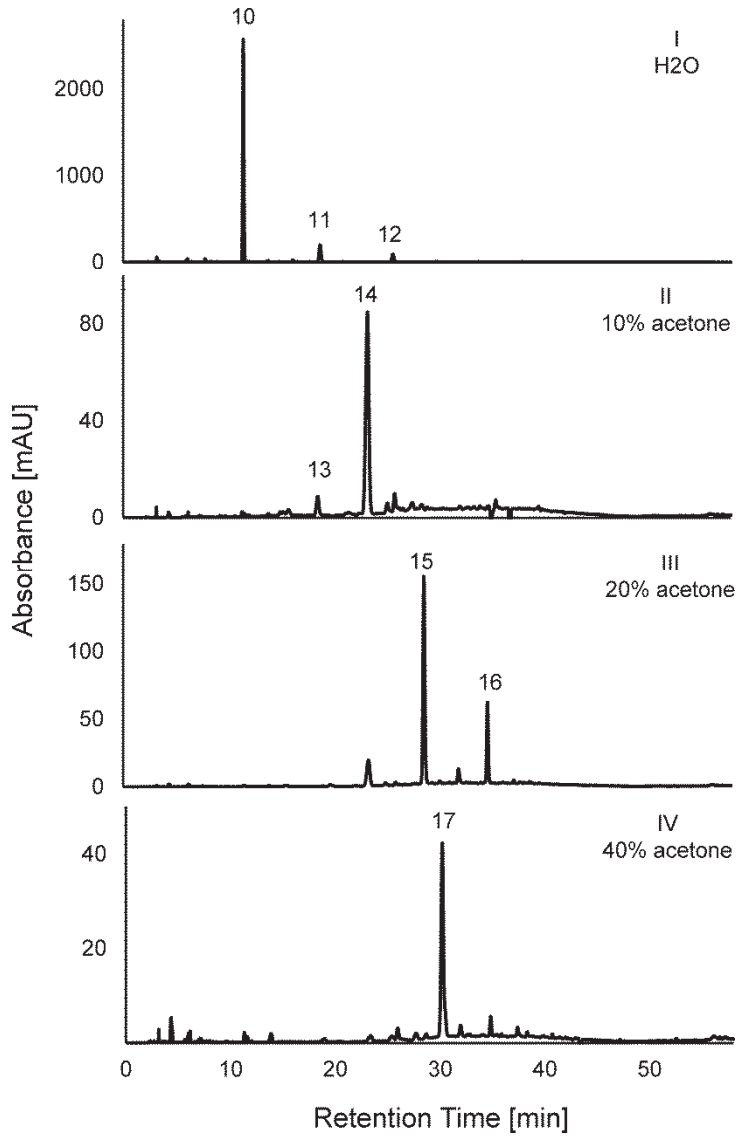

Figure 6 Purification of the main individual PAs through gel permeation chromatography on Sephadex LH-20. Peaks numbered with arabic numbers are in the order of increasing retention time and correspond to the compounds explained in the text.

The intermediate elution steps with 15% (3.5 mg), 25% (1 mg), 30% (2 mg), 35% (1 mg) aqueous acetone leads to an insufficient separation of targeted PA compounds. As a consequence, those fractions were discarded. A fully desorption of the purified flavonoids could be obtained by 50% aqueous acetone as eluent. In contrast to a direct injection of the crude cocoa extract on the Sephadex LH-20 column, the solvent is not strong enough to elute all substances, especially the group of high complex brown tannins. This complex group interacted irreversible with Sephadex LH-20.

Again, a typical color pattern could be observed, as already mentioned above. The color increased correspondingly to the elution order, beginning with a light brown by 0% aqueous acetone and ending with bright red for 30% aqueous acetone.

4.1.5. Conclusion

The present study achieved the most efficient extraction of target secondary metabolites from cocoa such as flavan-3-ols using a threefold extraction with 50% aqueous acetone (v/v), a liquid-to-solid ratio of 3-1 (v/w) and an 8 min extraction time (data not shown). The preferred temperature was set to 50 °C for minimizing solvent evaporation. The polyphenol-rich supernatant was freeze dried and used for further semi-preparative isolation for the compounds of interest.

With regard to their chemical structure, plant polyphenols are quite diverse and complex, so that an optimized extraction method for each compound is difficult to achieve. In this study, extraction efficiencies were calculated as a function of the extraction solvent composition and temperature according to the total and individual PAs in a cocoa bean extract. It was found that 50% aqueous acetone was an ideal solvent composition. Moreover, using a combination of liquid-liquid and gel chromatographic separation, it was possible to isolate high oligomeric PAs on a semi-preparative scale. This method therefore describes a relatively simple and fast procedure for the fractionation of monomeric, dimeric, trimeric, and tetrameric PAs by using gel permeation chromatography on Sephadex LH-20.

The results provided an efficient methodology for the separation and further identification of bioactive components. The described method has a broad applicability and is rapid, and suitable for preparing biologically active PAs from crude plant extracts. This method can be used to obtain purified flavan-3-ols which can be further used for bioactive investigations.

Acknowledgement

We are grateful to Dr. Thomas Pfeiffer (AlphaChrom AG, Rheinfelden, Switzerland) for his valuable support in the field of liquid-liquid chromatography.

Compliance with ethical standards

There is no conflict of interest. The manuscript was approved for publication by all authors.

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4.2. Entwicklung einer NP-HPLC-online-DPPH Methode zur Bestimmung der antioxidativen Eigenschaften von kondensierten Polyphenolen im Kakao

Der Teil dieser Arbeit beschäftigte sich mit der Charakterisierung der antioxidativen Kapazität von einzelnen kondensierten Polyphenolen. In Anlehnung an die LC-online-TEAC-Methode wurde ein NP-LC System aufgebaut, welches es erlaubt, kondensierte Proanthocyanidine in ihre homologe Reihe mit PG = 1 - 10 aufzutrennen und simultan ihre antioxidative Kapazität zu messen. Hierbei erkennt man eine Zunahme der antioxidativen Kapazität mit steigendem Molekulargewicht der PA, wobei der sogenannte ‘PA Hügel‘ die höchste antioxidative Kapazität aufweist. Eine Steigerung der antioxidativen Kapazität verläuft aber nicht linear zum Molekulargewicht, sondern flacht auf Grund von sterischen Effekten ab.

Mittels semi-präparativer NP-LC konnten kondensierte PA fraktioniert und mittels RP-LC-ESI-MS näher untersucht werden. Unter dem Sammelbegriff der monomeren PA versteht man jedoch eine Ansammlung aller monomeren Polyphenole, wie (-)-Epicatechin und (+)-Catechin, gleiches gilt für dimere PA mit einer Anhäufung von Proanthocyanidin B1, B2, B3 usw.

Eigenanteil

Experimenteller Versuchsaufbau	80%
Durchführung der Versuche	80%
Durchführung und Auswertung der chemischen Analysen	80%
Verfassen des Manuskripts	70%

Food Research International (2015)

Vol. 89, Pgs. 890-900

An online-NP-HPLC-DPPH method for the determination of the antioxidant activity of condensed polyphenols in cocoa

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4.2.1. Abstract

Unroasted cocoa beans are rich in monomeric flavanols and particularly epicatechin-based proanthocyanidin oligomers, with the latter making up to 60% of the total polyphenol content. Although the antioxidant activity of cocoa polyphenols is well known, it is still a challenging analytical field, especially, when it comes to the determination of condensed polyphenols and the evaluation of their single contribution to the overall activity. Therefore, an online NP-HPLC-DPPH assay was developed to separate the homologous series of condensed polyphenols for assessing their antioxidant capacity in relation to their degree of polymerisation (DP), simultaneously. In this context, normal-phase chromatography allows separations of polyphenols based on their degree of polymerisation. This study showed that an unroasted cocoa extract contains condensed polyphenols with a DP of up to 10 monomer units. By means of the online post-column derivatisation with 2,2-diphenyl-1-picrylhydrazyl (DPPH), the antioxidant capacity of the separated condensed polyphenols was assessed. It could be shown that, with the exception of the dimers, the contribution to the total antioxidant activity decreased from monomers to decamers. However, from the single proanthocyanidins identified, nonameric and decameric proanthocyanidins were found to have the highest values for the antioxidant capacity. The degree of polymerisation associated with each molecular weight fraction was further confirmed using electrospray ionization mass spectrometry coupled with reverse-phase liquid chromatography. The online NP-HPLC-

DPPH method can be used as qualitative and quantitative analysis of condensed proanthocyanidins and the simultaneous elucidation of the biological activity of proanthocyanidins in complex mixtures.

Keywords: condensed cocoa polyphenols; degree of polymerisation; online NP-HPLC-DPPH; antioxidant capacity

4.2.2. Introduction

Polyphenols are known to be food constituents of health-beneficial nature, often hypothesized to result from their antioxidant activity. Especially flavanol-rich diets including fruits and vegetables, tea, wine as well as cocoa are thought to have a beneficial effect on, e.g., cardiovascular health (Corti, Flammer, Hollenberg, & Lüscher, 2009). With increasing demand for polyphenol-rich extracts, there is a need to develop a rapid and efficient method to fully characterize plant extracts and to understand the physiological mechanism responsible for a corresponding mode of action of the preventive effects.

With regard to cocoa, its polyphenols are stored in the pigment cells of the cotyledons in the seeds, which are the major source for food-relevant products, such as chocolate. Three groups of polyphenols have to be considered primarily: flavan-3-ols with approx. 37%, anthocyanins with approx. 4% and proanthocyanidins with approx. 58% (Wollgast & Anklam, 2000). Among the major flavan-3-ols in cocoa and cocoa products, the monomers (-)-epicatechin and (+)-catechin are making up to 10% of the total polyphenol consumption in unroasted, fermented cocoa. These monomers can form polymers, the so-called proanthocyanidins (PA). The coupling between the monomeric flavan-3-ols occurs primarily between the positions 4 and 8, but may also involve the positions 4 and 6. Proanthocyanidin dimers (e.g., B1, B2, B3, B4, B5), trimers (e.g., C1), and tetramers (e.g., cinnamtannin A2), wherein the monomers are linked by 4->8, represent the most important cocoa PA (Prior & Gu, 2005). Further structural complexity is also given by interactions with insoluble polymeric plant material such as polysaccharides or proteins (Matthews, Mila, Scalbert, & Donnelly, 1997; Rohr, Meier & Sticher, 2000; Gu *et al*., 2004; Naczk & Shahidi, 2004).

The molecular weight of oligomeric PA is expressed as their degree of polymerisation (DP) and is referred to as monomers, dimers, trimers, tetramers etc. According to Gu *et al*. (2002), they are further defined as monomers with DP = 1, oligomers with DP = 2 - 10, and polymers with DP > 10. Much higher degrees of polymerisation might also exist in cocoa beans, but would be insoluble and quite challenging to analyse. However, the portion of condensed polyphenols present in cocoa is much higher than that of monomeric polyphenols (US Department of Agriculture NDL, 2004; Neveu *et al*., 2010).

With regard to antioxidant activity, not only low molecular polyphenols have the ability to scavenge radicals, but also oligo- and polymers of higher molecular weight are thought to possess a significant bioactivity (Saura-Calixto, 2012). Moreover, they contribute significantly to the human intake of antioxidants (Dorenkott *et al*., 2014; Gu *et al*. 2002). So far, the majority of polyphenol studies, however, focused on the smaller, well extractable molecules, which can be easily detected by RP-HPLC (Bandoniene & Murkovic, 2002; Niederländer *et al*., 2008; Malherbe, de Beer, & Joubert, 2012). Consequently, most of the *in vitro* and *in vivo* studies on antioxidant activity dealt with the low molecular weight polyphenols. The question remains whether and how the antioxidant capacity varies with the degree of polymerisation. Due to the lack of reference substances, it is difficult to predict the antioxidant capacity of higher molecular polyphenols *in vitro*. None of the mentioned techniques provides information about the radical scavenging activity of the separated oligomeric PA.

It was the aim of the present study to develop an online NP-HPLC-DPPH method for assessing the radical scavenging activity of high molecular weight polyphenols. This method bases on a separation with liquid chromatography coupled online with the well-known antioxidant capacity assay using DPPH as synthetic, stable radical, scavenged by the target compounds. Thus, the measurement of the antioxidant capacities and the parallel estimation of the separated compounds are permitted. From the countless assays for determining the antioxidant activity, the DPPH assay has become a quite popular method for the analysis of the antioxidant activity of all kinds of substrates. As it was the aim to only compare between the antioxidant activities of the procyanidins, the DPPH assay has been chosen, as it allows a fast reaction with most of the phenolic compounds. The optimum composition of the reagents, reaction time and temperature which affect the sensitivity range of spectrophotometric assays have been studied for the new application technique of the online NP-HPLC-DPPH assay.

4.2.3. Material and Methods

4.2.3.1. Materials and Reagents

Polyphenol standard substances such as (-)-epicatechin and (+)-catechin were purchased from Sigma Aldrich Chemie GmbH (Buchs, Switzerland). Proanthocyanidin B2, proanthocyanidin C1, and cinnamtannin A2 were purchased from PhytoLab GmbH & Co. KG (Vestenbergsgreuth, Germany). Proanthocyanidin B3 and B4 were purchased from TransMIT GmbH (Giessen, Germany). 2,2-diphenyl-1-picrylhydrazyl (DPPH), HPLC grade acetonitrile, methanol, 2-propanol and formic acid were obtained from Sigma Aldrich Chemie

GmbH (Buchs, Switzerland). Double-distilled water (Merck & Cie KG, Schaffhausen, Switzerland), was used throughout the whole study.

4.2.3.2. Sample Preparation

Removal of lipids from the cocoa beans

Unroasted cocoa beans, from a Trinitario variety from Finca La Amistad of Costa Rica, were manually dehulled and frozen (-20°C). To minimize heat development, frozen cocoa beans were then crushed in a knife mill (A 11 basic Analytical Mill, IKA®-Werke GmbH & Co. KG, Staufen, Germany) for 30 sec. In order to remove cocoa lipids from the beans, crushed material was extracted with n-hexane at a ratio of 1:5 (w/v) for 5 minutes at 20 °C. The hexane phase was centrifuged at 2,880 *x* g for 5 minutes (Centrifuge 5810, Vaudaux-Eppendorf AG, Schönenbuch, Switzerland). This procedure of defatting was repeated 5 times until the n-hexane extract appeared colourless. The defatted material was air dried for evaporating any hexane residue. After drying, the cocoa powder was sieved and particles of an average particle size of < 100 µm were extracted further. The particle fraction was obtained using a sieve shaker (Vibratory Sieve Shaker AS 200 basic, measuring range < 100 µm, Retsch GmbH, Haan, Germany).

Extraction of the analytes

For HPLC analysis, 2 g of the sieved cocoa material were weighed into a 50 mL centrifuge vessel and extracted three times with 6 mL 50% acetone / water for 8 minutes at 50 °C. After each extraction step, the mixture was centrifuged for 5 minutes at 2,880 *x* g (Centrifuge 5810, Vaudaux-Eppendorf AG, Schönenbuch, Switzerland). The combined polyphenol-rich supernatant (liquid cocoa extract) was used for further analysis and for semi-preparative isolation of single compounds of interest.

4.2.3.3. Online NP-HPLC-DPPH assay

The online NP-HPLC-DPPH assay can be used for a rapid assessment of antioxidant compounds in complex mixtures. As already described above, oligomeric PA are eluting in the order of their increasing degree of polymerisation, when using normal phases (Adamson *et al.*, 1999, Hammerstone, Lazarus, & Schmitz, 1999; Kelm, Johnson, Robbins, Hammerstone, & Schmitz, 2006; Yanagida *et al.*, 2007). A method combining the separation

of the condensed polyphenol compounds and the simultaneous determination of their antioxidant activity will be an advantage for characterizing bioactivity. However, the greatest benefit of this method is that the contribution of a single compound to the overall activity of a mixture of antioxidants can be measured. The more rapidly the absorbance decreases of the compound, the more potent is the antioxidant activity in terms of hydrogen-donating ability (Yen & Duh, 1994).

For the online NP-HPLC-DPPH assay, a solvent gradient was used on a UV/Vis-HPLC system (Knauer GmbH, Berlin, Germany), equipped with a diol-HILIC column (3.0 mm i.D. x 150 mm, 5 µm; YMC Triart Diol-HILIC, Stagroma AG, Reinach, Switzerland) and a pre-column of the same phase. The separation was carried out under the following conditions: Column temperature 30 °C; flow rate, 0.3 mL/min; sample injection volume, 1 µL. The mobile phase consisted of acetonitrile-water-formic acid (97.9:2:0.1, v:v:v) (solvent A) and methanol-water-formic acid (97.9:2:0.1, v:v:v) (solvent B), applied in the following gradient elution order: 1% B at 1-6 min, 1-20% B at 6-11 min, 20% B at 11-18 min, 20-26% B at 18-34 min, 26% B at 34-50 min, 26-95% B at 50-55 min and 95% B at 55-63 min. Between runs there was a 9 min postrun to re-equilibrate the column. The chromatogram was recorded by means of UV/Vis-detection (UVD 2.1 S, Knauer GmbH, Berlin, Germany) at a wavelength of 275 nm.

The DPPH free radical scavenging activity of the extracts was analysed using the DPPH assay, as originally described by Blois (1958) and modified by Brand-Williams, Cuvelier, & Berset (1995). Briefly, the working solution was prepared by dissolving 24 mg DPPH in 550 mL methanol, to obtain an absorbance of 1.1 ± 0.2 units at 515 nm (Thaipong, Boonprakob, Crosby, Cisneros-Zevallos, & Hawkins Byrne, 2006).

The DPPH reagent was filtered through a 0.45 µm membrane filter and carefully degassed before use. The scavenging reaction between DPPH and the condensed polyphenols as well as the DPPH reagent were protected from light.

Following the HPLC column, tubes were connected with a “T” piece and DPPH solution was added at a flow rate of 0.3 mL/min using a separate pump. The scavenging reaction took place in a reaction coil of 4.8 m length, i.D. of 0.38 mm and a volume of 0.550 mL, at a temperature of 60 °C. The induced bleaching of the coloured reagent was detected photometrically at 515 nm and presented in the chromatograms as negative peaks. The instrumental setup is depicted in **Figure 1**. The HPLC analysis was done in triplicate for each sample. The standard substances consists of (-)-epicatechin, proanthocyanidin dimer B2, trimer C1, and cinnamtannin A2 which are commercial available and differs in their condensation pattern.

4.2.3.4. Quantification of oligomeric PA using online NP-HPLC-DPPH assay

Quantification of individual oligomeric PA and determination of their antioxidant activity in unroasted liquid cocoa extract was done using external standard calibration.

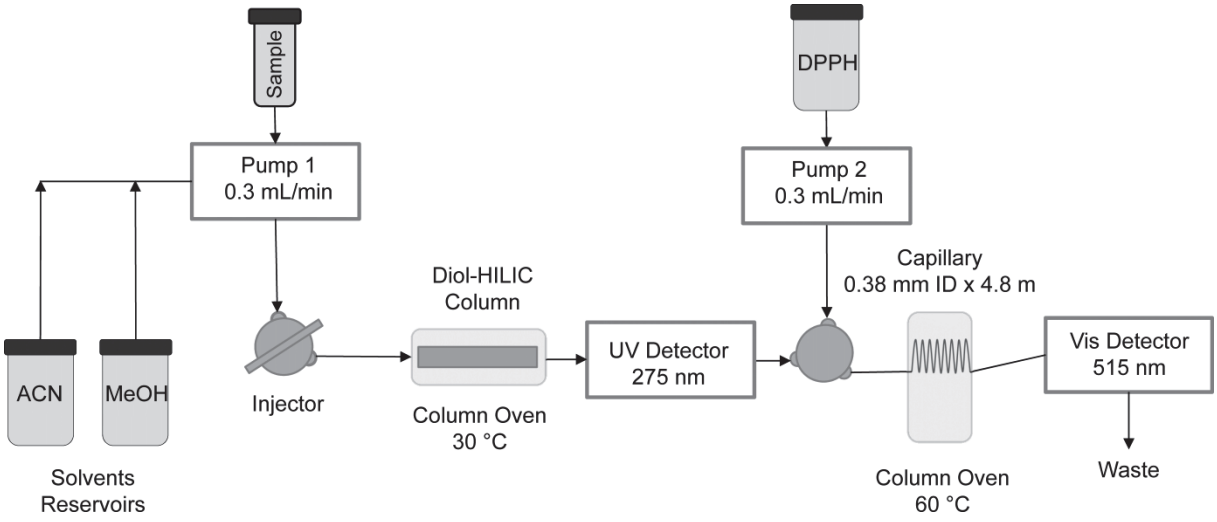

Figure 1 Instrumental setup of the online post-column NP-HPLC-DPPH methodology.

Stock solutions containing the standard substances were therefore prepared and diluted to appropriate concentrations with the same solvent. Briefly, the calibration was achieved using the standard substances (-)-epicatechin as monomer, proanthocyanidin B2 as dimer, proanthocyanidin C1 as trimer and cinnamtannin A2 as tetramer in a concentration range from c = 0.05 g/L to 1 g/L. Quantification was based on flat baseline integration as recommended by Gu *et al*. (2002). The calibrated curves were generated for each oligomeric class using a quadratic fit of area sum versus concentration. The method optimization was performed using standard substances and afterwards applied to liquid cocoa extracts.

The results of the PA content and their antioxidant activity are expressed for DP 1 as milligrams of (-)-epicatechin per gram non-fatty dry matter, for DP 2 as mg of procyanidin dimer B2, for DP 3 as mg trimer C1 and for DP ≥ 4 as mg cinnamtannin A2 per gram non-fatty dry matter due to the lack of standard substances.

The limits of detection and quantification were determined at a signal-to-noise ratio of about 3.0. Interday variability was chosen to validate the method and to specify the developed assay. Therefore, the mixed standard solution was analysed for three replicates within one day and analysed in duplicate weekly for a month. A linear dependence of negative peak area on concentration of the reference antioxidants was observed.

4.2.3.5. DPPH cuvette test

The different antioxidant kinetic behaviour of oligomeric PA was assayed photometrically and the results were compared with those of the online NP-HPLC-DPPH assay. It is a giving need for a new developed assay to make it compatible with an already given method of antioxidant activity measurement like the DPPH cuvette test.

Braude, Brook, & Lindstead (1954) discovered the hydrogen transfer mechanism between the stable free radical DPPH and antioxidant compounds. As it is described by Brand-Williams, Cuvelier, & Berset (1995) and some modifications introduced by Thaipong, Boonprakob, Crosby, Cisneros-Zevallos, & Hawkins Byrne (2006), the working solution was prepared by dissolving 24 mg DPPH in 500 mL methanol and left to react in the dark for 24 h at room temperature to obtain an absorbance of 1.1 ± 0.2 units at 515 nm. A composite standard was prepared using commercially available (-)-epicatechin. As this is the main flavonoid compound, it was used as a representative for oligomeric PA in the preliminary tests, although its detection response factor differs from PA which should be kept in mind. Stock solutions were made at the following concentration range: 0.05, 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 g/L, whereby 150 µL standard solution was allowed to react with 2,850 µL of the DPPH working solution. To determine the reaction kinetics, the DPPH radical quenching of (-)-epicatechin was measured at 25 °C and 60 °C. The absorbance was noted after an incubation time of 2 and 5 minutes at 515 nm with methanol as blank and an UV/Vis spectrophotometer (Genesys^{TM} 10S, Thermo Fisher Scientific AG, Reinach, Switzerland).

4.2.3.6. Isolation of oligomeric PA using semi-preparative NP-HPLC

As most of the PA beyond a DP of 4 are not commercially available, isolation and purification of individual oligomeric PA was required for the validation of the method.

A semi-preparative separation of the PA was achieved using a diol-HILIC column with 20.0 mm i.D. x 150 mm, 5 µm particle size (YMC-Actus Triart Diol-HILIC, Stagroma AG, Reinach, Switzerland). The separation was carried out under following conditions: The flow rate was set to 1.9 mL/min, and the column temperature was 30 °C. As described above, the mobile phase consisted of a mixture of acetonitrile-water-formic acid (97.9:2:0.1, v:v:v) (solvent A) and methanol-water-formic acid (97.9:2:0.1, v:v:v) (solvent B), applied in the following gradient elution order: 1% B at 1-8 min, 1-20% B at 8-22 min, 20% B at 22-45 min, 20-25% B at 45-55 min, 25% B at 55-80 min, 25-95% B at 80-85 min and 95% B at 85-100 min to wash remaining residues off the column. The detector wavelength was set to 275 nm. The semi-preparative NP-HPLC analysis was performed on the same HPLC system as

described above for the analytical method. Sample injection was done using a 200 µL injection loop.

For semi-preparative injection, the liquid cocoa extract was freeze-dried to remove the extraction solvent entirely. The freeze-dried extract was named 'PA cocoa extract' (PACE). To prevent the HPLC system from precipitation of solids, 100 mg of PACE was re-dissolved in 400 µL 40% isopropanol, suspended using an ultrasonic bath, and filtered through a 0.45 µm cellulose syringe filter prior to injection.

Fractions of appropriate volumes were collected manually in 10 mL amber glass vials for a couple of minutes following the chromatographic the peak shape visually.

The content of suitable fractions (F1: from 21-22 min; F2: from 34-35 min; F3: from 39-40; F4: from 43-45; F5: from 48-50 min; F6: from 55-57 min and F7: from 62-64 min) were pooled, evaporated to dryness under nitrogen with a sample concentrator (Portmann Instruments AG, Biel-Benken, Switzerland), subsequently dissolved in 100 µL in methanol and analysed by RP-HPLC-ESI/MS.

4.2.3.7. Characterization of oligomeric PA using RP-HPLC-ESI/MS

For a satisfyingly determination of the PA, several techniques had to be combined. With an offline two dimensional diol-HILIC normal phase *x* reversed phase liquid chromatography, the analysis of PA can be effectively performed. In the first dimension, oligomeric PA can be separated according to their molecular weight and the second dimension offers a potent analytical technique of untargeted characterization of individual compounds. An advantage of this method is given by the fact that, although RP-HPLC separates flavan-3-ols monomers, dimers, and trimers without difficulties, there are obviously limitations for higher oligomers. In a crude matrix the oligomers are co-eluting up to a DP ≥ 4 as a large unresolved peak.

Identification of individual compounds up to tetramers [(+)-catechin, (-)-epicatechin, proanthocyanidin dimer B2, B3, B4, proanthocyanidin trimer C1, and proanthocyanidin tetramer A2] was confirmed by RP-HPLC-ESI/MS. This was performed on an Agilent 1200 series liquid chromatography and quadrupole mass spectrometer with electrospray ionization interface (LC/MS 6120, G6100 series, Agilent Technologies AG, Waldbronn, Germany). The liquid cocoa extract was analysed using a gradient mixture of water-formic acid (99.9:0.1, v:v) (solvent A) and acetonitrile-water-formic acid (94.9:5:0.1, v:v:v) (solvent B). A 2.1 x 150 mm Eclipse XDB-C18 (5 µm) column (Agilent Technologies AG, Waldbronn, Germany) was used. The separation was affected using a linear gradient at 30 °C with a flow of 0.7 mL/min as follows: 1% B at 0-1.5 min, 1-5% B at 1.5-5 min, 5-10% B at 5-12 min, 10% B at 12-18 min, 10-15% B at 18-25 min, 15-20% B at 25-32 min, 20-25% B at 32-37 min, 25-30% B at

37-43 min, 30% at 43-46 min, 30-40% at 46-50 min, 40-50% at 50-52 min, 50-70% B at 52-55 min, 70% B at 55-56 min and 70-10% B at 56-58 min. The re-equilibration time was 8 min.

For ESI/MS analysis, the positive capillary voltage was set at 4,000 V and the negative at 3,000 V. The drying gas temperature was 350 °C and the drying gas flow 12 mL/min. The samples were analysed using a full scan from 100 - 2,000 *m/z* in positive ionization mode. The comparison of retention times and characteristic fragmentation patterns was done using the aforementioned standard substances.

4.2.4. Results and Discussion

4.2.4.1. Online NP-HPLC-DPPH analysis of standard PA

So far, various research groups have used diverse protocols for the investigation of the antioxidant activity of countless plant based products. The DPPH radical is widely used for measuring the efficiency of antioxidants, because of its radical stability, the sensitivity, and the technical simplicity of the assay execution (Huang, Ou, & Prior, 2005). It has become a quite popular method for the analysis of the antioxidant activity of phenolic compounds, as it allows a fast reaction with most of the phenolic compounds. Further assays (providing different antioxidant mechanisms) have not been applied, as it was the aim to only compare between the different cocoa fractions.

The idea behind the online NP-HPLC-DPPH method was to adapt the traditional DPPH cuvette test to HPLC conditions, whereby HPLC allows a full characterisation of individual condensed PA in complex mixtures in one single run under the same conditions. For both approaches, the online NP-HPLC-DPPH derivatisation and the optimized analysis in a photometer, the reaction conditions have been adjusted according to the individual reaction kinetics. For an improved reproducibility, the radical scavenging reaction of oligomeric PA with DPPH was optimized in terms of temperature and time. The antioxidant kinetic behaviour was evaluated photometrically and the results were adapted to the online NP-HPLC-DPPH methodology. A linear relationship over a concentration range from 0.05 g/L to 1 g/L was observed at a temperature of 60 °C, when using a reaction time of 2 minutes (or a corresponding length of the reaction capillary, resp.). The HPLC-PTFE reaction coil was therefore thermostated at 60 °C and the reaction time was kept short (1 minute).

The UV and the DPPH chromatograms of the oligomeric standard PA are shown in **Figure 2.** Peaks with retention times at 4.4, 5.2, 8.7, 18.9, and 22.2 min in the UV chromatogram and the DPPH chromatogram are (-)-epicatechin, (-)-epigallocatechin, PA dimer B2, trimer C1, and cinnamtannin A2.

The reaction was optimized with regard to intensity and shape of the peak signals resulting from the DPPH radical quenching. When applying a non-standardized procedure, the DPPH radical reaction mixture can cause side reactions such as polymerisation of catechins and *ortho*-quinones to higher molecular weight oligomers, or adducts between the oxidized form of catechin and DPPH radical (Osman, 2011).

The small peak with the retention time of 5.2 min in the UV chromatogram was identified as (-)-epigallocatechin by RP-HPLC-ESI/MS. Although it has a small UV absorption, its antioxidant activity is much higher than that of (-)-epicatechin. It could be proven that the commercially available reference substance (-)-epicatechin has a slight contamination of less than 0.05% of (-)-epigallocatechin, whereby the antioxidant activity is approx. 30% higher. Previous studies of antioxidant activity supported the role of specific structural components as requisites for radical scavenging (Nanjo, Goto, Seto, Suzuki, Sakai, & Hara, 1996; Nanjo, Mori, Goto, & Hara, 1999). They also confirmed the assertion that the presence of an *ortho*-hydroxyl group in the B ring is indispensable for the radical scavenging effect. Those results showed the DPPH scavenging ability of tea catechins whereby (-)-epigallocatechin and (-)-epigallocatechin gallate were stronger than (+)-catechin, (-)-epicatechin, and (-)-epicatechin gallate. The concentration required to give a 50% decrease of the signal intensity of DPPH radical was 2.5 µM for (-)-epicatechin and 1.7 µM for (-)-epigallocatechin.

It should be kept in mind that contaminations like this or any other potentially antioxidant substances cannot be adequately considered in spectrophotometric tests.

4.2.4.2. Online NP-HPLC-DPPH analysis of a liquid cocoa extract

The method developed can be used as fast identification of bioactives in extracts without the need of a complex sample preparation. **Figure 3** shows combined UV (positive peaks) and DPPH radical-quenching (negative peaks) chromatograms. There are ten major UV- and antioxidant-active compounds in cocoa beans extract which show a significant radical-quenching capacity. From comparison with the standard substances (-)-epicatechin monomer, proanthocyanidin B2 dimer, C1 trimer, and cinnamtannin A2 tetramer, the peaks with the retention time at 4.8, 10.1, 22.9, 30.7, 34.7, 38.2, 41.2, 44.3, 46.4, and 48.9 min in the UV chromatogram, and DPPH chromatogram were identified as (-)-epicatechin monomer, dimer, trimer, tetramer, pentamer, hexamer, heptamer, octamer, nonamer, and decamer, respectively. The identification is based on the retention time with standard substances, the UV spectra and MS fragmentation pattern. Obviously, there is no antioxidant capacity detected for the solvent peak (acetone) with a retention time at 3.2 or for the caffeine or theobromine peaks with a retention time at 4.1 and 5.4 min.

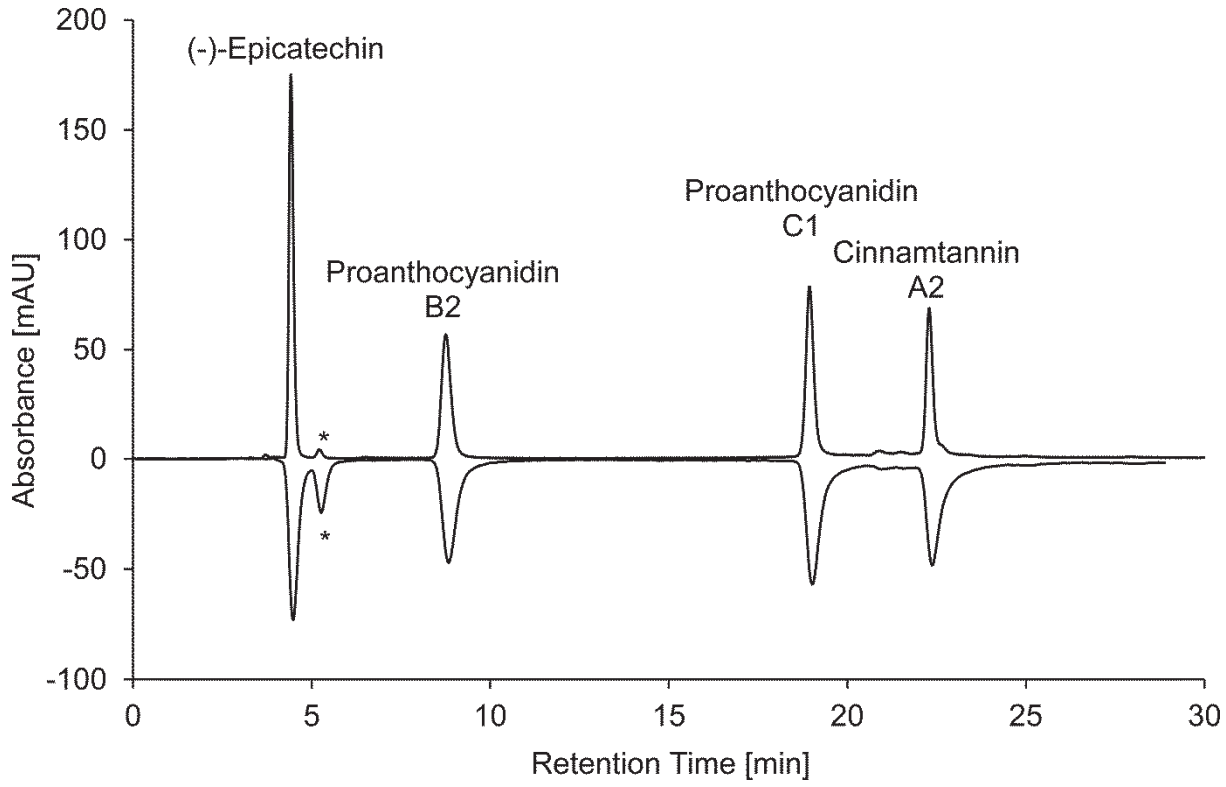

Figure 2 Correlation of standard substances of oligomeric PA from unroasted cocoa beans extracts using online screening with antioxidant capacity. The polyphenols have a concentration of 1 g/L. The asterisk indicates the contamination of the (-)-epicatechin reference sample.

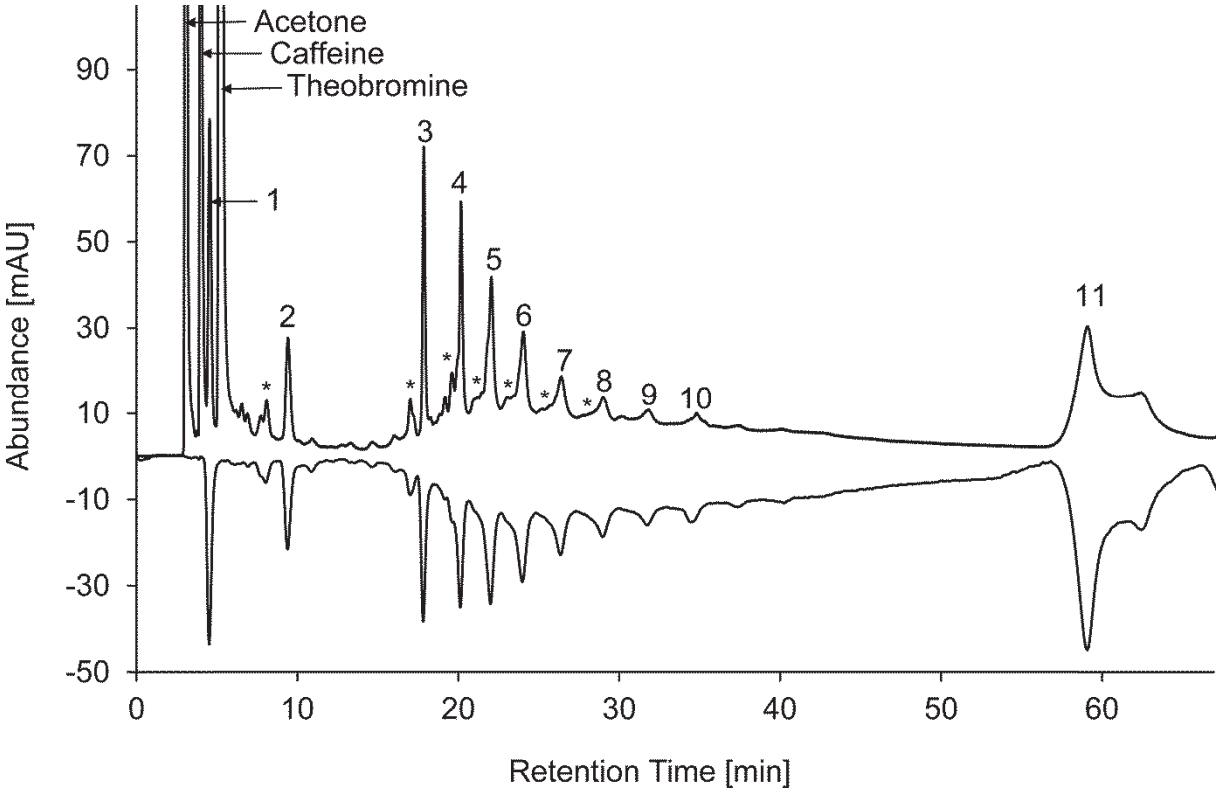

Figure 3 Bioactive screening of condensed polyphenols from unroasted cocoa beans extract using online screening NP-HPLC-DPPH (injection volume: 1µ; UV wavelength: positive 275 nm, negative 515 nm); Identified PA: epicatechin monomer (1), dimers (2), trimers (3), tetramers (4), pentamers (5), hexamers (6), heptamers (7), octamers (8), nonamers (9), decamers (10), and an unresolved "proanthocyanidin (PA) hump" (11). The asterisks are indications for glycoside bound proanthocyanidin oligomers.

The content of identified PA in the unroasted liquid cocoa extract is shown in **Table 1**. Levels of PA were expressed as epicatechin equivalents per g fat free dry mass. This form of calculation makes it easier to compare the results also with other studies. Except for dimers, the proanthocyanidin content and the antioxidant activity decrease from monomers to decamers in accordance with the descriptions of Ioannone, Di Mattia, De Gregorio, Sergi, Serafini, & Sacchetti (2015), Bordiga, Locatelli, Travaglia, Coïsson, Mazza, & Arlorio (2015), and Di Mattia *et al*. (2013). Although, monomers were the most abundant polyphenols (with regard to their concentration), the high antioxidant activities were also found for some other fractions (e.g., trimers to pentamers) (**Table 1**).

Besides that, contents of proanthocyanidins can be expressed at hand of corresponding standard compounds. E.g., for dimers as mg proanthocyanidin B2 equivalents, for trimers as mg proanthocyanidin C1 equivalents and for fractions bigger or equal than tetramers as mg cinnamtannin A2 equivalents. The evaluation indicates that PA tetramers were the most abundant compounds in this extract with a high antioxidant activity (**Table 1**)

In general, the content and composition of PA in cocoa beans differs due to genetic, geographic origin, and environmental factors. Most of the work in literature reported a cascading decrease along monomers to decamers. Nevertheless, in this work unfermented, raw cocoa beans had a relatively high abundance of trimers and pentamers when calculating through external standards. However, data obtained in this work are not significantly different when taken the statistical variability of PA into account as well as generally natural fluctuation in cocoa beans. Further studies need to be done concerning different varieties, and regions of productions. Also Kim & Keeney (1984) observed a wide range of (-)-epicatechin from 21.89 - 43.27 mg/g non-fatty dry matter among cocoa beans of varying genetic origin.

In addition, the antioxidant capacity was determined as the ratio of the antioxidant activity per content of the single proanthocyanidin fractions (**Table 1**). This provides information on the effectiveness of PA to scavenge DPPH radicals (Vizzotto, Cisneros-Zevallos, & Byrne, 2007; Jacobo-Velázquez & Cisneros-Zevallos, 2009). A high value implies that this compound has a high relevance in the total antioxidant activity of the sample. Nevertheless, although trimeric PA show the highest antioxidant activity, it has a comparatively moderate capacity. The highest value for the capacities was found for nonamers and decamers (**Table 1**). From the chemical point of view, this seems to be kind of reasonable, as those large molecules provide more structural features for interacting with radicals.

This approach has proven that oligomeric PA are efficient DPPH radical scavengers despite their high molecular weight and potential steric hindrance. According to Arteel & Sies (1999), long chain PA are better scavengers than short chain PA, particular tetramer was found efficient against oxidation and nitration reaction. Ramljak *et al*. (2005) claimed for pentameric cocoa proanthocyanidin even a growth inhibition effect of human breast cancer cells.

In an offline approach by Rösch, Bergmann, Knorr, & Kroh (2003), a quite similar order was found for the antioxidant activity of oligomeric PA from sea buckthorn (*Hippophae rhamnoides*). PA tetramers showed hereby the highest antioxidant activity. Lotito *et al*. (2000) reported that monomers, dimers, and trimers were the most effective antioxidants in the aqueous phase, while PA of higher molecular weight were more effective in the lipid phase. These results also underline the observation described by Lu & Yeap Foo (2000) who characterised apple polyphenols. They found trimers and tetramers to be more efficient free radical scavenger than proanthocyanidin B2 and (-)-epicatechin. But they also claimed that this trend of antioxidant activity do not extend to higher molecular PA. The present study demonstrates that to a point the increasing PA enhances the effectiveness against radicals. But with an increase of molecular weight, the in antioxidant activity leads to a slight, non-linear reduction for polyphenols with DP > 8. According to Lu & Yeap Foo (2000), the slight

increase of the antioxidant activity could be explained by the increasing crowding and therefore the less availability of the hydrogen donating effect.

A further aspect to consider is the separation of PA by polymerisation degree when using normal phases, whereby different PA with the same molecular weight are often determined as one fraction. However, separation of single PA only succeeds with reversed phases, so far.

Table 1. Quantification of oligomeric PA and their antioxidant activity in an unroasted liquid cocoa extract obtained by online NP-HPLC-DPPH. PA content and its antioxidant activity are expressed as epicatechin equivalents (using epicatechin as standard; ECE) or as proanthocyanidin equivalents (using cinnamtannin A2 as standard; PAE). Antioxidant capacity is defined in this study as the ratio of antioxidant activity per soluble PA content. Values represent value mean, with n = 3 (± standard deviation).

Peak No.	Degree of Polymerisation	PA Content [mg ECE/g]	Antioxidant Activity [mg ECE/g]	Antioxidant Capacity [for ECE]	PA Content [mg PAE/g]	Antioxidant Activity [mg PAE/g]	Antioxidant Capacity [for PAE]
1	DP 1	5.02 (± 0.53)	3.43 (± 0.48)	0.68 (± 0.04)	5.02 (± 0.53)	1.86 (± 0.48)	0.37 (± 0.06)
2	DP 2	2.50 (± 0.32)	2.64 (± 0.45)	1.05 (± 0.09)	3.58 (± 0.45)	1.41 (± 0.43)	0.39 (± 0.08)
3	DP 3	4.19 (± 0.54)	3.68 (± 0.39)	0.88 (± 0.05)	5.55 (± 0.69)	2.09 (± 0.49)	0.37 (± 0.05)
4	DP 4	3.97 (± 0.48)	3.25 (± 0.31)	0.81 (± 0.07)	6.81 (± 0.81) *	2.15 (± 0.49) *	0.31 (± 0.05) *
5	DP 5	4.11 (± 0.46)	3.25 (± 0.32)	0.79 (± 0.10)	7.04 (± 0.77) *	2.16 (± 0.50) *	0.30 (± 0.07) *
6	DP 6	3.03 (± 0.55)	2.72 (± 0.47)	0.90 (± 0.13)	5.26 (± 0.92) *	1.64 (± 0.45) *	0.31 (± 0.06) *
7	DP 7	2.00 (± 0.35)	2.24 (± 0.35)	1.13 (± 0.14)	3.54 (± 0.59) *	1.18 (± 0.34) *	0.33 (± 0.08) *
8	DP 8	1.39 (± 0.30)	1.79 (± 0.20)	1.31 (± 0.23)	2.53 (± 0.50) *	2.02 (± 0.19) *	0.29 (± 0.07) *
9	DP 9	0.55 (± 0.19)	1.61 (± 0.32)	3.22 (± 0.21)	1.13 (± 0.31) *	0.56 (± 0.40) *	0.56 (± 0.67) *
10	DP 10	0.56 (± 0.13)	1.27 (± 0.11)	2.39 (± 0.21)	1.15 (± 0.22) *	0.24 (± 0.10) *	0.23 (± 0.14) *
11	‘PA hump’	22.52 (± 3.80)	17.07 (± 2.02)	0.76 (± 0.06)	37.65 (± 6.31) *	15.56 (± 1.96) *	0.42 (± 0.03) *

* Values are expressed as cinnamtannin A2 equivalents

As it is obvious from **Figure 3**, structures larger than decamers elute as an unresolved group of peaks at the end of the chromatogram which corresponds to the majority of the material present. So far, all traditional separation methods failed to resolve this 'proanthocyanidin (PA) hump'. This has to do with the fact that the resolution of a NP-HPLC stationary phase under HILIC conditions is not sufficient to separate such complex samples (Kalili & Villiers, 2009). There is much evidence suggesting that a chromatographic separation of larger polymers is not possible due to their complexity. Polymeric PA with a higher molecular mass cannot be determined with NP-HPLC (Lazarus, Hammerstone, Adamson, & Schmitz, 2001).

However, the cocoa 'proanthocyanidin hump' shows a powerful antioxidant capacity that, beyond all doubt, represents more or less the majority of the sample material. Although it is difficult to characterize the PA hump completely, it is necessary to compare the PA hump in interaction with other compounds and their antioxidant activity. With an antioxidant activity of approx. 20 mg per gram fat free dry matter (expressed as epicatechin equivalents), the PA hump is as powerful as all other PA together. However, with regard to the antioxidant capacity of this fraction, the effectiveness to neutralize DPPH radicals can be considered as comparatively low (**Table 1**).

Unfortunately, only a few studies showed an unadorned chromatogram including the PA hump (e.g., Guyot, Marnet, & Drilleau, 2001, Gu *et al*., 2002, Esatbeyoglu, Wray, & Winterhalter, 2015). However, some researcher also observed analogous unresolved humps in their chromatograms when analysing different plant extracts. Kuhnert (2010) as well as Kuhnert, Dairpoosh, Yassin, Golon, & Jaiswal (2013) highlighted similar observations in the chromatographic behaviour when describing the so-called "thearubigin hump" in the analysis of black tea. Although the molecular weight of the thearubigins ranges from 1,000 to 40,000 (Yao *et al*., 2006), Sinha & Ghaskadbi (2013) recently presumed strong antioxidant properties of black tea thearubigins. Here again, although thearubigins are present in large amounts in black tea, the information on formation and structure of this heterogeneous polymer is very limited.

In the present study, it could be shown that the unroasted cocoa extract contains a high amount of extractable condensed polyphenols which can be separated on a normal phase column whereby their antioxidant capacity can be assessed by online post-column derivatisation.

Besides the homologous series of oligomeric PA, other UV-active flavanol moieties can be seen on a HILIC-column. The poor separation between each oligomer peak suggests that PACE is quite inhomogeneous including different structures showing a high complexity in its PA subunits and the linkages between them (Yanagida *et al*., 2007; Valls, Millán, Pilar Martí, Borràs, & Arola, 2009). Besides linear PA also large, branched molecules can be present.

Another to date unsatisfyingly investigated phenomenon when using a HILIC column is the appearance of smaller unresolved UV-absorbing peaks. They merged into big peaks with shoulders. In the chromatogram they are marked with an asterisk (**Figure 3**). Similar effects were showing during the NP-HPLC analyses of grapes or wine, indicating the presence of different mono-galloylated, galloylated and non-galloylated PA (Nunes, Gómez-Cordovés, Bartolomé, Hong, & Mitchell, 2006). Callemien & Collin (2008) isolated dimers and trimers from lager beer extract by normal phase column and obtained, using thioacidolysis, that catechin forms the main terminal unit, whereas both catechin and gallocatechin are major constituents of the extension unit. For cocoa, it can be hypothesized that such smaller unresolved peaks are PA linked either to *C*- or *O*-glucose, -arabinose, -galactose (Hatano *et al*., 2002).

4.2.4.3. Quantification of oligomeric PA using the online NP-HPLC-DPPH assay

In this study, oligomeric PA were quantified using an external calibration for UV-NP-HPLC analysis with a diol-HILIC column. For each analyte, peak areas from chromatogram were plotted against the concentration of the stock solution of known concentrations. As expected, PA show a positive correlation between the concentration against UV-absorption. The calibration curves were as follows: (-)-epicatechin, y = 1618.8x + 11.922 (R^2 = 0.9994, 0.05-1.0 g/L), proanthocyanidin B2, y = 1154.5x + 3.04 (R^2 = 0.9995, 0.05-1.0 g/L), proanthocyanidin C1, y = y = 1266.7x - 16.46 (R^2 = 0.9999, 0.05-1.0 g/L) and cinnamtannin A2, y = 973.69x – 11.756 (R^2 = 0.9999, 0.05-1.0 g/L).

The DPPH radical solution is a deep violet chromogen and any quenching of the radical results in a loss of color, getting yellowish and is indicated by signal intensity decrease and represented by a negative peak in the chromatogram. The radical scavenging properties are detected at 515 nm. The DPPH scavenging activity of oligomeric PA were calculated from the working calibration curves: (-)-epicatechin, y = 1233.7x + 109.36 (R^2 = 0.9836, 0.05-1.0 g/L), proanthocyanidin B2, y = 1306.6x + 51.282 (R^2 = 0.9923, 0.05-1.0 g/L), proanthocyanidin C1, y = 1507.5x + 46.693 (R^2 = 0.9927, 0.05-1.0 g/L), and cinnamtannin A2, y = 1271.6x + 34.386 (R^2 = 0.9938, 0.05-1.0 g/L). The activities tested with the online NP-HPLC-DPPH assay decreased in following order: proanthocyanidin C1 > proanthocyanidin B2 > cinnamtannin A2 > (-)-epicatechin.

For the validation of the online NP-HPLC-DPPH assay, the limits of detection (LOD) and the limits of quantification (LOQ) of the PA compounds and their antioxidant capacity were calculated based on the standard deviation of y intercept of the regression against the slope of the calibration curve. However, the UV absorption is comparatively sensitive (LOD = 7-21 µg/mL) as the DPPH reaction (LOD = 6-27 µg/mL) (**Table 2**).

As depicted from the calibration curves the PA content is highly correlated with the antioxidant capacity (R^2 > 0.99). This suggests that PA accounts for a major portion of the antioxidant activity in unroasted cocoa beans. This could also be an indicator for potential biological activity.

The common limitation of the analysis is the weak UV absorption of higher oligomeric PA due to the wider chromatogram peaks. Furthermore, the response for UV-absorbing chromophores decreases in relation to the degree of polymerisation. This approach can be explained by the higher molecular weight and effects of steric hindrance.

It can be further discussed if measurement for condensed polyphenols might be determined at lower wavelengths such as 205 nm, where they give better response at low concentration. Lee & Ong (2000) analyzed sixteen types of tea ingredients at 205 nm and obtained a higher sensitivity with a LOD ≈ 0.05 µg/mL.

Some methodologies are also using fluorescence detectors to improve sensitivity and selectivity for the analysis of catechins (Arts & Hollman, 1998; Lazarus, Adamson, Hammerstone, & Schmitz, 1999). Typical concentrations for fluorescence measurements are in the range 5-16 µg/mL (Hemingway & Karchesy, 1989). Hellström & Mattila (2008) achieved LOD values for extractable PA varying from 1.0-15 µg/mL for a concentration level from 20-2,000 µg/mL.

Table 2. LOD and LOQ of standard substances in the online NP-HPLC-DPPH assay determined at a concentration range 50 µg/mL to 400 µg/mL.

Compound	UV-detection at 275 nm		Vis-detection at 515 nm	
	LOD [µg/mL]	LOQ [µg/mL]	LOD [µg/mL]	LOQ [µg/mL]
(-)-Epicatechin	7	22	27	83
Procyanidin B2	12	38	6	19
Procyanidin C1	17	54	11	33
Cinnamtannin A2	21	64	16	49

4.2.4.4. Quantification of oligomeric PA using the traditional photometric assay

To evaluate whether both methods can be compared, it was important to quantify oligomeric PA using also the traditional photometric assay. To determine the reaction kinetics, the DPPH radical quenching of (-)-epicatechin was measured at 25 °C and 60 °C. The absorbance at 515 nm was noted after an incubation time of 2 and 5 minutes using a UV-visible spectrophotometer with methanol as blank. The absorption measured at 60 °C

with an incubation time of 2 min showed the most linear response over the whole range of concentrations from 0.05 g/L to 1.0 g/L.

Accordingly, the four main condensed cocoa polyphenols were investigated under the optimized conditions. The working calibration curves are representing a polynomial of the second degree: (-)-epicatechin, $y = 0.5817x^2-1.3405x+0.9578$ ($R^2 = 0.9997$, 0.05-1.0 g/L), proanthocyanidin B2, $y = 0.224x^2-0.7919x+0.934$ ($R^2 = 0.9994$, 0.05-1.0 g/L), proanthocyanidin C1, $y = 0.2277x^2-0.7261+0.9289$ ($R^2 = 0.9986$, 0.05-1.0 g/L) and cinnamtannin A2, $y = 0.1855x^2-0.6405x+0.9331$ ($R^2 = 0.9996$, 0.05-1.0 g/L).

The resulting dose-response curves showed a decrease in DPPH-scavenging activity in the following order: cinnamtannin A2 > proanthocyanidin C1 > proanthocyanidin B2 > (-)-epicatechin (**Figure 4**).

The comparatively higher oligomers had a higher antioxidant activity than the monomers. Among PA, tetramers were the most potent while epicatechin was the least active. The antioxidant activity increases depending on DP. The important structural feature for DPPH scavenging is the *ortho*-hydroxyl group in the B-ring(s), the additional presence of a 3-OH group(s), and the abundant 4->8 linkage between the subunits (Heim, Tagliaferro, & Bobilya, 2002).

4.2.4.5. Comparison of the traditional photometric assay versus the online NP-HPLC-DPPH assay

The results of the traditional photometric DPPH assay and the online NP-HPLC-DPPH assay can be expressed and calculated as (-)-epicatechin equivalents for the first four homologous polyphenols. Here again, the four main standard substances (-)-epicatechin, procyanidins B2, C1, and A2 were used for the calculation and have been indicated as representatives of DP 1, DP 2, DP 3 and DP 4 (**Figure 5**).

Proanthocyanidin B2 (DP 2), when expressed as (-)-epicatechin units/equivalents, shows a slightly higher antioxidant activity than (-)-epicatechin itself. With regard to the traditional photometric assay, this trend extends up to the tetramers: Cinnamtannin A2 has a more effective radical scavenging activity than the monomeric (-)-epicatechin.

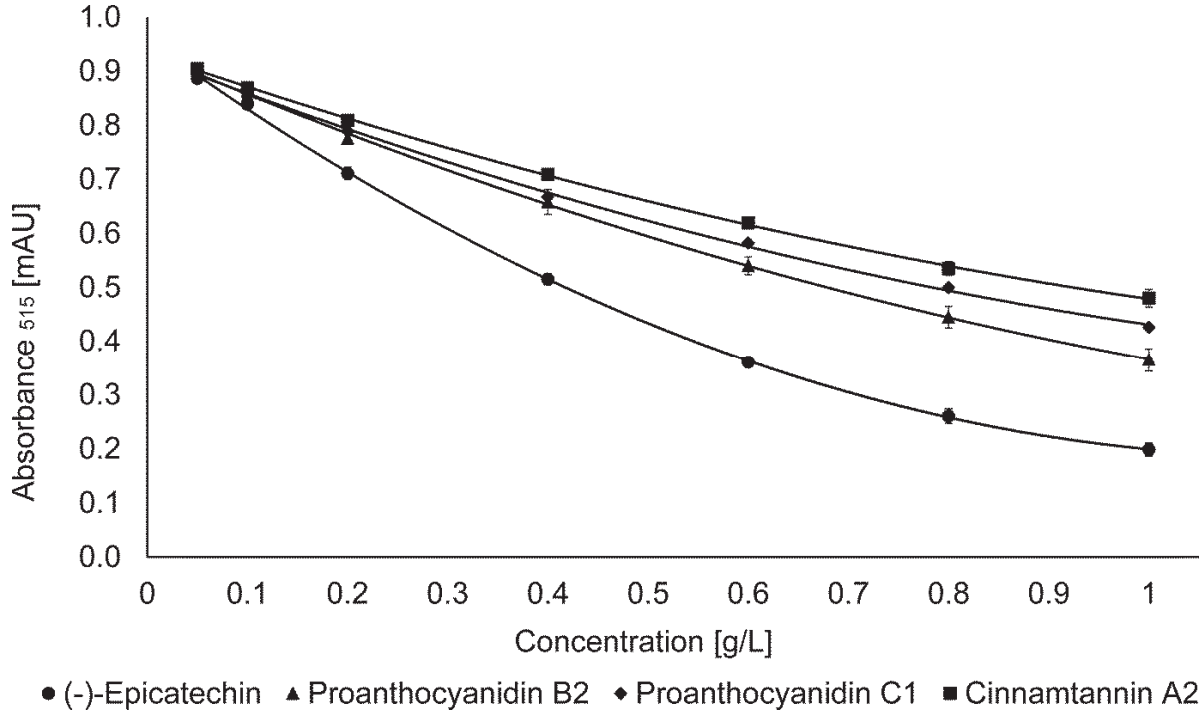

Figure 4 Dose-response curves obtained by the DPPH cuvette assay for (-)-epicatechin, proanthocyanidin B2, C1 and cinnamtannin A2 at concentrations from 0.05 g/L to 1.0 g/L measured at 515 nm and 2 minutes at 60 °C incubation. Each value represents the mean values and the standard deviations from three determinations.

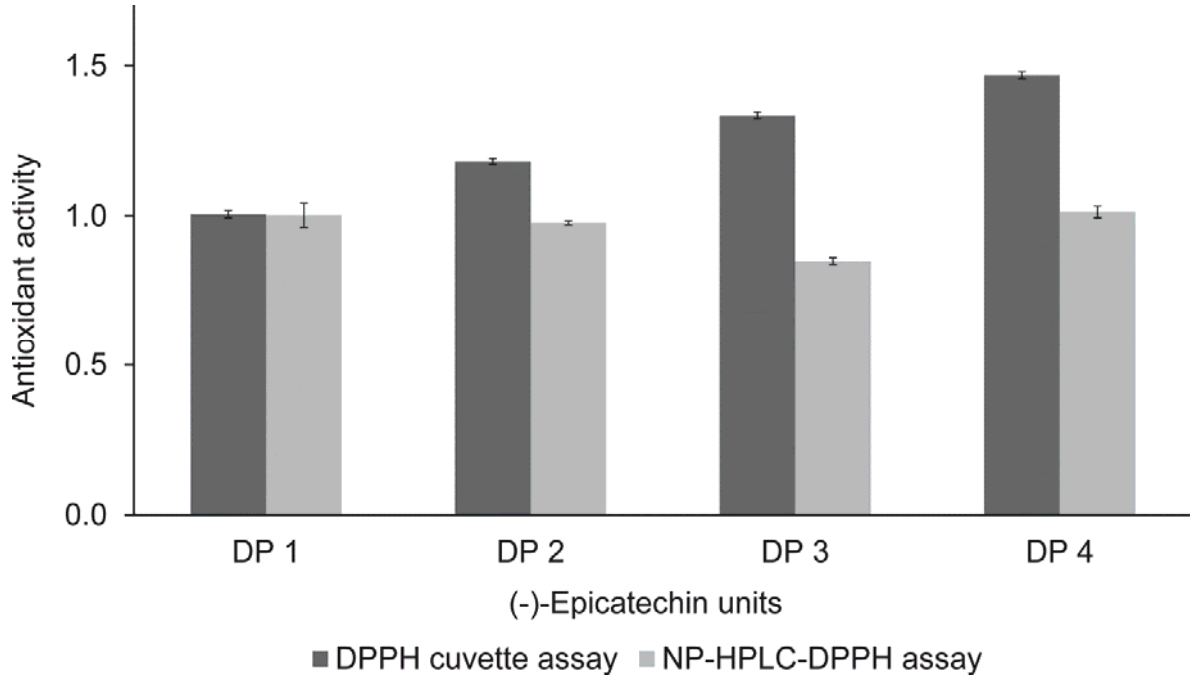

Figure 5 Comparison of DPPH scavenging activity of PA between the DPPH cuvette assay and the online NP-HPLC-DPPH assay. The results are expressed as (-)-epicatechin equivalents. The error bars are the result of a triple determination.

Hence, the results of the both assays cannot be compared satisfyingly. The antioxidant activity determined with the online NP-HPLC-DPPH assay is diminished in relation to the traditional photometric assay. One explanation for that phenomenon could be the hydrogen-bond accepting ability of the HPLC solvents methanol and acetonitrile as described by Jabbari & Moallem (2015). An increase of the organic solvent influences the radical scavenging activity of flavonoids. Presumably, they undergo interactions with DPPH due to solute-solvent effects. Also Sharma & Bhat (2009) recommended for a better sensitivity and a high range of accuracy, the preparation of the DPPH solution in methanol without any further additives.

Recent studies showed that the antioxidant activity is closely related to the molecular structure of the polyphenol and the solute-solvent present (Jabarri, Mir, Kanaani, & Ajloo, 2014). Also a lower pH in the mobile phase can lead to a dramatic loss of sensitivity and to a decrease in absorbance of the DPPH radical (Malherbe, de Beer, & Joubert, 2012).

The direct comparison of the DPPH cuvette assay with the NP-HPLC-DPPH assay leads to the conclusion that each assay has its particular advantage and disadvantage. The method ultimately chosen will depend on speed of analysis, accuracy required, sensitivity required and the determination of single compounds in complex matrixes. The DPPH cuvette assay describes a simple high throughput colorimetric assay which is reliable and generates reproducible measurements, expressed as total antioxidant capacity (TAC). The disadvantage, on the other hand, is in the analysis of complex matrixes. The online HPLC-DPPH assay gives more information about the composition of the mixture as well as a rapid assessment of the antioxidant capacity of every single compound.

However, the use of an internal standard is pretty common when using RP-HPLC. Antioxidant capacities of phenolic substances in plant extracts are usually quantified with an internal standard such as trolox (Riehle, Vollmer, & Rohn, 2013). Data are normally presented as trolox equivalent antioxidant capacity (TEAC) which is expressed as mg trolox equivalents per g sample. The resulting values are usually comparatively higher than the real amount of polyphenols. This has to do with the fact that trolox has a higher antioxidant activity than polyphenols usually have. For example, according to Arts, Haenen, Voss, & Bast, chrysin has a relatively high TEAC value while its antioxidant activity in other assays is relatively low. In the present case, the quantification using an internal standard on a diol-HILIC column proved to be rather difficult. Neither known cocoa polyphenols nor typical standards such as trolox or vitamin C could be used as internal standard due to adverse co-eluting with other known substances. For this reason, trolox was deliberately refrained as internal standard.

4.2.4.6. Isolation of oligomeric PA using semi-preparative NP-HPLC

Additional investigation of isolated oligomeric PA was needed for several reasons: Primarily to understand the contribution of each single PA to the total antioxidant activity of an isolated fraction. Furthermore, increasing the scale of the NP-HPLC chromatography is of particular interest regarding the preparation of self-made analytical standards in considerable amounts for substances that are difficult to obtain commercially.

A separation of the unroasted PA cocoa extract with a semi-preparative NP-HPLC column is illustrated in **Figure 6,** whereby PA up to hexamers can be observed in a 60 min run. Fractions were numbered F1 - F6, respectively in their order of elution from the diol-HILIC column. Despite column overload, fractions were clearly separated from each other. The colour of the fractions increased from colourless for F1, upon light pink for F4 and to deep red for F7. Colour intensification results from condensation of an increased number of monomeric units. Monomers such as (-)-epicatechin are known as white powder, whereas dimers such as proanthocyanidin B2 are coloured white to beige. This trend continues towards polymeric tannins known as brownish powders.

Although the analysis of the liquid cocoa extract on the analytical column gives fractions with up to decamers, it is surprising that on the semi-preparative column only monomers to hexamers could be separated although obviously material was eluting from the column (between 60 and 90 min) (**Figure 6**). A proposed rationale could be the different retention mechanism for larger diol-HILIC columns and there insufficient separation with increasing molecular weight (DP). According to the VAN DEEMTER equation the efficiency diminishes in relation to the column width and results in peak broadening.

An important element of this study was the right sample preparation and dissolution of the PACE for semi-preparative purpose. Since the knowledge of oligomeric PA is still incomplete, the conditions have to be optimised to ensure an efficient extraction and injection, so that no information is lost. On the other hand, to prevent the system from precipitation most of the researchers (Kelm, Johnson, Robbins, Hammerstone, & Schmitz, 2006) dissolve their extract in the HPLC mobile phase whereby some of them additionally dissolved it in ethanol before injected onto the column. Because of its limited solution power this study purposely decided not to dissolve the PACE in the HPLC solvent. To prevent the HPLC system from precipitation of solids, the ‘PA cocoa extract’ was re-dissolved in 40% isopropanol, suspended using an ultrasonic bath, and filtered through a 0.45 µm cellulose syringe filter prior to injection so that no resinous material was seen. Similar to Kelm, Johnson, Robbins, Hammerstone, & Schmitz (2006) and their observation on unfermented cocoa PA, in the present study only monomers to heptamers could be separated clearly.

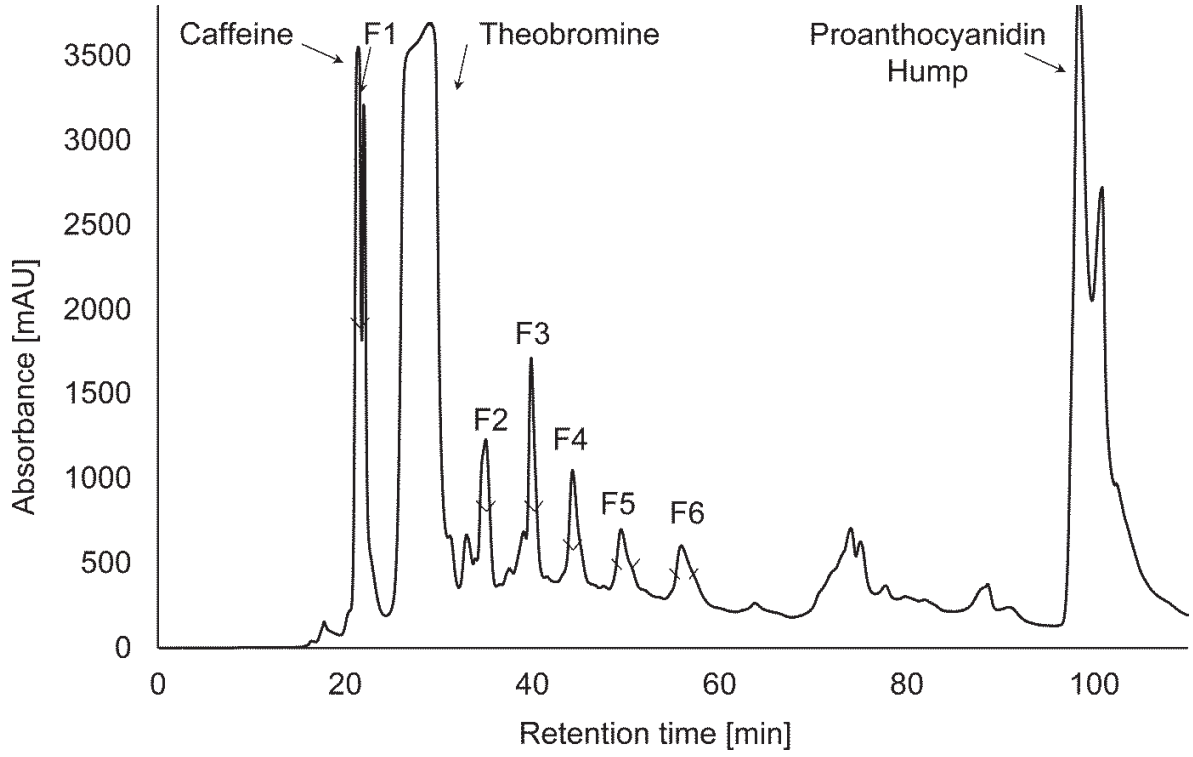

Figure 6 Preparative diol-HILIC profile of 50 mg unroasted cocoa powder extract injected onto the column (injection volume: 200 µL). The fractions are labelled F1-F7 according to their DP. The black bar in the middle of each peak reflects the manually collected fractions.

PACE could be prevented from precipitation by using the new method of complete dissolution. Various injection methods to increase the injection load for right sample preparation are currently under investigation.

A major benefit of the method described is the isolation of single compounds in high purity, whereby their identities were deduced by RP-HPLC-ESI/MS.

4.2.4.7. Identification of oligomeric PA using RP-HPLC-ESI/MS

The compounds F1-F6 of the 'PA cocoa extract' (PACE) were isolated by semi-preparative HPLC using UV-Vis detection. As this technique cannot provide compound identification or structural information the optimized RP-HPLC method was coupled to electrospray ionization to complete the characterization of PACE.

Identification was based on retention time and mass spectra summarized in **Table 3**. This table also includes the experimental *m/z*, the fragmentation of fission products and the molecular formulas.

The six successive eluted fractions were measured in positive ionization TIC mode $[M+H]^+$. For an increased sensitivity, each fraction is shown in the extraction ion mode (**Figure 7**). Briefly, the first fraction is shown by measuring at *m/z* 291, the second fraction at

m/z 579, the third fraction at *m/z* 867, etc. A total of six oligomeric PA and the two alkaloids theobromine and caffeine were identified using RP-HPLC-ESI/MS.

As mentioned above, several PA can elute as a cluster of peaks of isomers on a normal phase column. The separation is not sensitive enough to difference PA of the same degree of polymerization. Detailed structure can be just determined by RP-HPLC.

Figure 7a shows an extracted ion chromatogram (EIC) of $[M+H]^+$ = 291 for fraction F1 which contains two peaks at t_R = 18.8 min and 26.1 min and were assigned as (+)-catechin and (-)-epicatechin after comparison with the retention time and fragmentation pattern of commercially available standards. (-)-Epicatechin has a much higher absolute intensity than (+)-catechin. The third peak with $[M+H]^+$ = 291 at t_R = 34.7 min is a pseudomolecular ion coming from procyanidin B2. All things considered, the antioxidant activity of the monomeric fraction in **Figure 7a** is contributed equally by (-)-epicatechin and (+)-catechin.

Figure 7b is an EI-chromatogram of $[M+H]^+$ = 579 for fraction F2 with proanthocyanidin B3 at t_R = 17.4 min, PA B4 at t_R = 22.1 min and PA B2 at t_R = 23.5 min. Furthermore there are characteristic signals for dimers at t_R = 25.5 min, 31.4 min and 35.1 min whereby they remain unidentified due to a lack of standard substances. So altogether, there are at least six PA dimers contributing to the antioxidant activity of the dimeric fraction.

Figure 7c is an EIC of $[M+H]^+$ = 867 with proanthocyanidin C1 at t_R = 28.9 min. In addition there are three more non-identified trimers with t_R = 23.4 min, 30.8 min, and 35.2 min. Altogether, four PA trimers contribute to the antioxidant activity of the trimeric fraction.

Figure 7d is an EIC of $[M+H]^+$ = 1,155 with cinnamtannin A2 at t_R = 30.5 min. Furthermore, there are three more non-identified tetramers with t_R = 25.7 min, 31.8 min, and 37.9 min. Due to the lack of standard substances there is no clear classification of PA besides that possible. Altogether, four PA tetramers contribute to the antioxidant activity of the tetrameric fraction.

Figure 7e is an EIC with the mass-to-charge ratio *m/z* 1,444 with clear peaks at t_R = 32.1 min, and t_R = 37.8 min. Altogether, two PA pentamers contribute to the antioxidant activity of the pentameric fraction.

Figure 7f is an EIC with the mass-to-charge ratio *m/z* 1,732 with clear peaks at t_R = 33.5 min and a smaller one at t_R = 35.6 min, providing information that a minimum of two PA hexamers contribute to the antioxidant activity of the hexameric fraction.

Behind every fraction there is a couple of isobaric, sometimes isomeric PA which altogether contributes to the overall antioxidant capacity.

Table 3. RP-HPLC-ESI/MS determination of oligomeric proanthocyanidins in unroasted cocoa beans extract. Only single charged ions for F1 - F7 could be detected in the positive ion mode.

Compound	Molecular formula	t_R [min]	Peak	Molecular Ion $[M+H]^+$	Major Fragments $[M+H]^+$
Monomer	$C_{15}H_{14}O_6$	4.3	F1	291.1	139.0; 165.1
Dimer	$C_{30}H_{26}O_{12}$	8.1	F2	579.2	291.1
Trimer	$C_{45}H_{38}O_{18}$	16.2	F3	867.2	579.2; 1155.5
Tetramer	$C_{60}H_{50}O_{24}$	18.5	F4	1155.3	n.d.
Pentamer	$C_{75}H_{62}O_{30}$	20.1	F5	1443.3	579.2; 867.0
Hexamer	$C_{90}H_{74}O_{36}$	21.8	F6	1731.4	579.9; 867.0; 1155.3

n.d. not detected

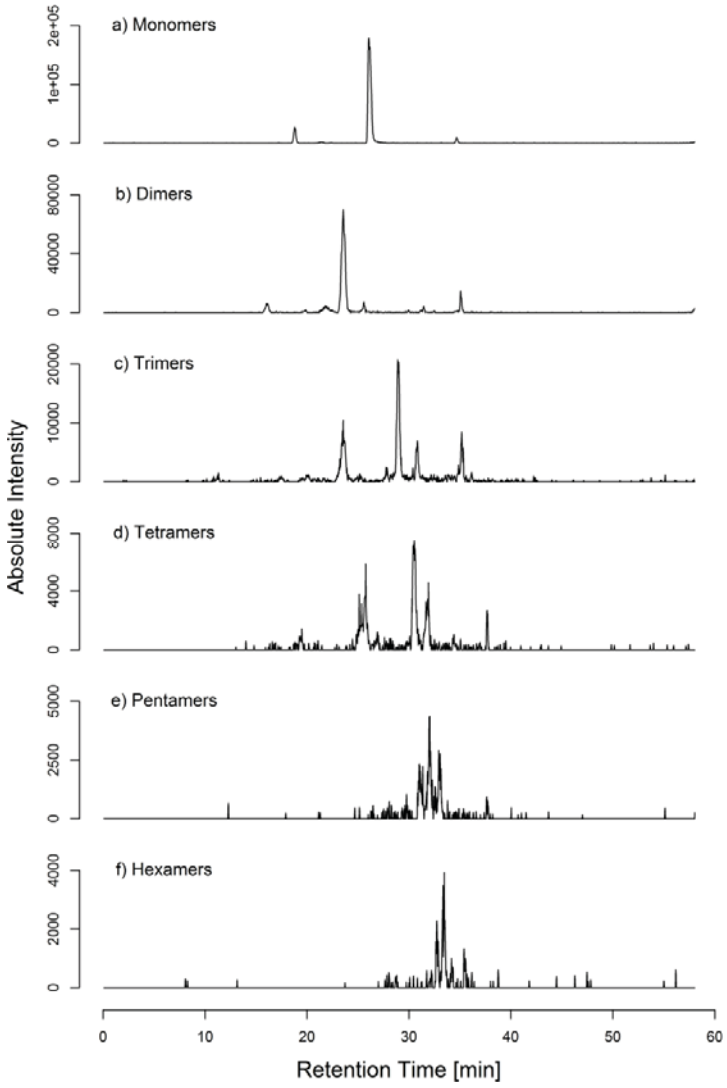

Figure 7a-f Extracted Ion Chromatograms (EIC) obtained from the analysis of the unroasted cocoa extract using RP-HPLC/MS.

However, the efficiency of the monomeric fraction and the corresponding chromatographic resolution is much higher than for the dimeric, trimeric, tetrameric fractions, etc. Motilva, Serra, & Macià (2013) observed a decrease of the absolute intensity during each fraction of cocoa flavanols and the reduction of the signal-to-noise ratio. This can be explained by the poor extraction yield of the semi-preparative NP-HPLC within each fraction. The insufficient resolution can be avoided by an efficient sample preparation after semi-preparative isolation by decreasing matrix suppression.

4.2.5. Conclusions

The method described can be used for a rapid evaluation of condensed antioxidant components in complex matrices such as plant or food extracts. For chromatographic separation of mono- and oligomeric PA, a standard procedure using a NP-HPLC system was established. To simultaneously assess the antioxidant capacities of PA, the traditional photometric assay was adapted and coupled online to a HPLC system. The well-known antioxidant assay using 2,2-diphenyl-1-picrylhydrazyl (DPPH) was chosen, because of its easy and rapid way to determine the antioxidant activity and its reactivity of its stable free radical (Prior, Wu, & Schaich, 2005).

The DPPH concentration was optimized and the effect of reaction time, temperature, and stability of the antioxidant compound during the assay (reaction time < 1 min) was characterized. The results suggest that this method can be used for the simultaneous determination of condensed polyphenols according to their DP as single antioxidant capacities. Several antioxidants were identified in the cocoa extract whereby the results indicate that the radical scavenging activity of the homologous series of condensed PA is increasing with increasing DP. Nonameric and decameric PA fractions showed the highest antioxidant capacity.

Besides that, it should be generally questioned whether it is useful to evaluate the PA amount as epicatechin equivalents (ECE) or as PA equivalents (PAE). When calculating as ECE, the values appeared more significant. The evaluation as PAE, however, makes the values appear more equal, but compounds used for the calculation are chemically closer related to the substance that is quantified. Despite to that, it should be also taken into account that the UV activity of oligomeric PA is decreasing with increasing DP. This can be seen in the calibration curve whereby the slope of the straight line drops from (-)-epicatechin to cinnamtannin A2.

Furthermore, it can be discussed whether the newly developed assay can be improved in terms of detection limits for higher polymeric PA with DP > 10. Also, the standard deviation for higher PA could be minimized maybe by using a fluorescence detector.

The online NP-HPLC-DPPH assay can also be considered as a promising technique for quality control in the chocolate manufacturing process whereby the question arises whether the antioxidant capacity of individual oligomeric PA changes throughout the process.

However, the most promising substances, because of their high potential antioxidant activity, still seem to be the unresolved. Further, higher condensed polyphenols occur at the end of the NP-HPLC chromatogram ('PA hump') providing future challenges for their analysis and characterization.

To develop a complete understanding of the biological activity of cocoa, additional structure determination is important: Research on structure elucidation can be done by isolation of oligomeric substances and their further depolymerisation reaction in presence of nucleophiles such benzyl mercaptan (thiolysis) (Matthews *et al*., 1997; Ramirez-Coronel, Marnet, Kumar Kolli, Roussos, Guyot, & Augur 2004; Callemien, Guyot, & Collin, 2008), phloroglucinol (phloroglucinolysis) (Lorrain, Ky, Pechamat, & Teissedre, 2013), or enzymatic treatments of the extract (Mandalari *et al*., 2006).

Acknowledgments

Markus Kneubühl helped with the experimental design. Carlo Weber supported sample preparation. Petra Huber participated in the data interpretation. Special thanks go to Ansgar Schlüter and Franco Spescha for providing all samples.

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4.3. Bestimmung von oligomeren Proanthocyanidinen und deren antioxidativer Aktivität bei unterschiedlichen Schokoladenproduktions-schritten mittels NP-HPLC-online-DPPH

Mittels NP-HPLC-online-DPPH konnte der Einfluss unterschiedlicher Verarbeitungsschritte auf die Veränderung oligomere Proanthocyanidine über die gesamte Wertschöpfungskette nachverfolgt werden. Im Zuge dieser Arbeit wurde das Proanthocyanidinprofil von (1) frischen Kakaobohnen, über (2) fermentierten und getrockneten Kakaobohnen, zu (3) gerösteten Kakaobohnen, (4) 1 h conchierter Kakaomasse, (5) 4 h conchierter Kakaomasse, bis hin zur (6) fertigen Schokolade angeschaut. Den isolierten oligomeren PA wurde ebenso ihre antioxidative Kapazität gegenübergestellt. Neben der Bestimmung einzelner oligomerer PA wurde ebenso die Auswirkung der unterschiedlichen Verarbeitungsstufen auf kondensierte PA mit einem PG > 13 quantitifiziert.

Zur Beurteilung stand der durchgeführte Schokoladenherstellungsprozess und sein Einfluss auf oligomere PA und deren antioxidative Kapazität. Anhand der erhaltenen Resultate können Rückschlüsse auf künftige Prozessschritte besser verstanden werden, um auch instabile wertgebenden phenolische Substanzen über die gesamte Verarbeitung zu bewahren.

Eigenanteil

Experimenteller Versuchsaufbau	70%
Durchführung der Versuche	70%
Durchführung und Auswertung der chemischen Analysen	70%
Verfassen des Manuskripts	70%

Food Chemistry (2017)

Vol. 214, Pgs. 523-532

Determination of oligomeric proanthocyanidins and their antioxidant activity from different chocolate manufacturing stages using the NP-HPLC-online-DPPH methodology

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4.3.1. Abstract

Cocoa beans are a well-known source of antioxidant polyphenols. Especially individual oligomeric proanthocyanidins demonstrated a significant contribution to the total antioxidant activity of cocoa compared to monomeric compounds. An NP-HPLC-online-DPPH assay was developed for separating the homologous series of oligomeric proanthocyanidins and the simultaneous assessment of their antioxidant capacity in relation to the degree of polymerization (DP).

The present study describes the influence of the different stages of a lab-scale chocolate manufacturing process on the content of oligomeric proanthocyanidins and their antioxidant capacity. The sum of the total proanthocyanidin content (∑ DP1 - DP13) decreased from 30 mg epicatechin equivalents per gram non-fat dry matter in raw fresh cocoa beans to 5 mg epicatechin equivalents per gram in the final chocolate. The antioxidant activity decreased accordingly from 25 mg epicatechin equivalents per gram non-fat dry matter in raw fresh cocoa beans to 4 mg/g in the final chocolate product.

Keywords: Chocolate manufacturing process; Oligomeric proanthocyanidins; Antioxidant activity

4.3.2. Introduction

Cocoa and its most prominent finishing product chocolate are known as luxury food providing a pleasantly astringent taste and a typical sensual cocoa flavor. Being an important economical crop, cocoa beans are predominantly used for the chocolate industry. In the last few years, the health benefits of chocolate for human nutrition became more and more evident. The main active substances, primarily the alkaloids theobromine and caffeine, are known for their stimulating effect. Polyphenols from cocoa beans or chocolate products have been reported to provide even anti-inflammatory effects. Especially in Switzerland, 'the land of chocolate' with many small manufactories, there is a huge interest in a high quality product with an enriched content of polyphenols. Therefore, gaining a deeper understanding of cocoa beans and their polyphenolic content, respectively of the transformations of the composition during the cocoa manufacturing process, is important, as different processes lead to divergent amounts of polyphenols with their corresponding antioxidant capacity.

Previous studies dealing with changes during the chocolate manufacturing process focused on flavan-3-ols, especially (-)-epicatechin and (+)-catechin and other low molecular weight polyphenols, which can be easily assessed using RP-HPLC. However, data about detailed information on oligomeric proanthocyanidins (PAs) and their changes during manufacturing processes are generally rare. Over the past five decades, researchers have focused intensively on monomeric cocoa polyphenols, although only approx. 5-10% of the total polyphenols account for monomers, while oligomers and polymers account for more than 90% (Andres-Lacueva *et al*., 2008). Counet, Callemien, & Collin (2006) claimed for PAs found in commercial products such as grape, wine, olive, and dark chocolate a distribution of monomers and dimers of up to 71% of the total PA fraction from DP1-DP6.

PA are shown to be highly effective inhibitors of radical chain reactions. In cocoa, they consist of oligomers or polymers of (+)-catechin and/or (-)-epicatechin units. The size of PAs is specified by their degree of polymerization (DP). For evaluating the distribution of the PAs, the use of NP-HPLC seems to be preferable as analytical methodology (Hammerstone *et al*., 1999). As presented recently, the antioxidant capacity of condensed cocoa polyphenols can be measured simultaneously using a NP-HPLC-online-DPPH approach (Pedan, Fischer, & Rohn, 2015a). The question now arises how PAs and their antioxidant activity are altered during different chocolate manufacturing stages. During this process, there are many determinants for influencing the PAs. The main steps are described in the following:

Cocoa beans are natural products and their composition is highly depending on genotype and ecophysiological factors (e.g., temperature, radiation, plant nutrition). Initially, genetic variation can cause a 4-fold difference in the flavanols content of fresh cocoa beans (Clapperton *et al*., 1994). Kim and Keeney (1984) observed (-)-epicatechin concentrations ranging from 21.9-43.3 mg/g non-fat dry matter among freshly harvested cocoa beans of

varied genetic origin. De Brito, García, & Amâncio (2002) observed a variation in the total polyphenol content from 12 to 18% of non-fat dry matter in fermented and dried cocoa beans whereby 60% were PAs of which the half were dimeric structures.

Beside genetic variations, a huge effect on polyphenol composition is the fermentation which is carried out in the countries of origin. In fact, after cutting the ripe cocoa pods from the trees, the beans are removed from the pod and transferred with the pulp to boxes/sacks/piles for fermentation lasting 5 to 7 days (Aikpokpodion & Dongo, 2010). Micro-organisms and endogenous enzymes lead to a degradation of the pulp that surrounds the fresh beans and limit the development of pathogenic microorganisms. During the fermentation, polyphenols undergo an enzymatic transformation by polyphenol oxidase(s).

(-)-Epicatechin polymerizes with (+)-catechin to form brown high molecular weight polymers (Albertini *et al*., 2015). Investigations of fermented cocoa beans from different origins showed a 6-fold variation of the (-)-epicatechin content. The (-)-epicatechin concentration ranges from 2.7 mg/g of dry defatted samples from Jamaica to 16.5 mg/g from Costa Rica (Kim & Keeney, 1984). Kealey *et al*. (2001) showed a 3- to 5-fold decrease for pentameric PAs during fermentation ranging from 4.5-6.5 mg/g defatted sample at the beginning to 0.5 - 2 mg/g at the end of the fermentation. A diffusion of polyphenols compounds through the cotyledons and irreversible or reversible binding due to complexation with proteins or polysaccharides take place (Forsyth, Quesnel, Roberts, 1958).

During the drying process beans are placed in shallow trays. The oxidation reaction that began with the fermentation now continues during drying. Hereby, polyphenol-oxidases catalyze the enzymatic browning accompanied by non-enzymatic browning, both leading to an *o*-quinone polymerization and reactions with other constituents such as proteins and polysaccharides (De Brito, Pezoa García, Gallão, Cortelazzo, Fevereiro & Braga, 2000). During these steps, the amount of polymeric polyphenols is reduced leading to a corresponding reduction of astringency (Niemenak, Rohsius, Elwers, Omokolo Ndoumou, Lieberei, 2006). During drying, the moisture content drops from 60% to a residual humidity below 8%, saving the cocoa bean from mold infestations and inactivating enzymes (Afoakwa, Kongor, Takrama, Simpson Budu, & Mensah-Brown 2013). Once the beans are dried, they are weighed and packed into sacks for transporting them to the chocolate production sites.

Until this step, cocoa beans are not really going through a standardized process. The three main steps of harvesting, fermentation, and drying heavily vary in the countries of origin, whereby a fluctuation in polyphenols content is enormous depending on the different parameters. However, the subsequent chocolate processing stages in the countries of manufacture are strictly controlled and highly reproducible. Hereby, cocoa beans are sterilized, broken, roasted, and ground to cocoa liquor which can be used directly for preparing chocolate products.

The present study aimed at monitoring the amount of PAs during the different chocolate manufacturing process steps. Correspondingly, the antioxidant activity of the intermediate products was analyzed as well. Here, the recently introduced NP-HPLC-online-DPPH methodology was used (Pedan, Fischer, & Rohn, 2015b). All steps from opening the fresh cocoa pods to fermentation, drying, roasting, conching, and finishing of the chocolate bars were done in a lab-scale.

4.3.3. Material and Methods

(-)-Epicatechin, 2,2-diphenyl-1-picrylhydrazyl (DPPH), HPLC grade water, acetonitrile, methanol, and formic acid were obtained from Sigma Aldrich Chemie GmbH (Buchs, Switzerland). Polyphenol oligomers such as proanthocyanidin B2, proanthocyanidin C1, and cinnamtannin A2 were purchased from PhytoLab GmbH & Co. KG (Vestenbergsgreuth, Germany).

4.3.3.1. Cocoa Samples

Obviously, the concentration of all polyphenols can vary depending on the source of the beans, the processing conditions, and the chocolate manufacturing process stages. To evaluate the effect of the manufacturing process on the PA composition of cocoa and the resulting products, one single batch of 5 kg fresh cocoa beans was studied across the different processing stages. Cocoa was a Trinitario variety and fruits were flown within five days after harvesting at ‘Finca La Amistad’ in Costa Rica to Switzerland. It has to be assumed that during transportation no cooling was applied to the cocoa fruits. A schematic summary of the processing steps involved is shown in **Fig. 1**. Roughly, the first step in this study was to break up the cocoa fruit and to remove the hulls from the seeds by clamping the cocoa fruits in a vice on a workbench and opening the hull with an ax. The tegument was removed and the seeds covered in their sweet mucilaginous pulp were further deposited for fermentation and drying. Standard fermentation conditions were applied under controlled temperature, whereby fruit pulp was degraded by yeast and bacteria. The fruit material was placed in perforated trays at 40 °C for two days and another four days at 50 °C in a pressure tank (Eureka, Tecnogen S.r.l., Pergine Valsugana, Italy). During the fermentation, the mucilagious pulp could not drain away. Microorganism growth was not controlled. After six days of fermentation, the liquid medium was discarded and the cocoa beans were carefully rinsed with water. Following the fermentation process, cocoa seeds with a residual water content of 60% were dried and equilibrated in an vacuum drying chamber (Model VD 53, Binder GmbH, Tuttlingen, Germany) for 7 days at a moderate temperature of 40-45 °C to

reduce the water content to 3%. In the following, dried cocoa nibs were broken using a cocoa bean breaker (Type ESRIO, Disco Drives (King's Lynn) Ltd, Norfolk, United Kingdom) and winnowed to separate the broken nibs from the shells (Cocoa Winnower Large 240-1-50, Capco Test Equipment, Suffolk, United Kingdom). Cocoa nibs were further ground by a corundum stone mill resulting in cocoa mass (MK 180, FrymaKoruma AG, Rheinfelden, Switzerland). Fine ground cocoa mass was roasted in a stirred glass vessel (ESCO-Labor Mixer Type EL 1, Esco-Labor AG, Riehen, Switzerland). One batch of about 1 kg was heated up to 120 °C and held for 2 minutes before cooling. To obtain an average particle size of approx. 20 µm, cocoa mass and sugar were ground subsequently using a three-roll mill (SDY-200, Bühler AG, Uzwil, Switzerland). For the conching process, solid ingredients such as sucrose and lecithin were mixed with the cocoa mass to finally develop the typical chocolate flavor and smooth texture (Frisse Elk'olino conche, Bühler AG, Uzwil, Switzerland). To ensure mild processing conditions, the conching process consisted of a 12 h step at a maximum temperature of 45 °C, which is also important to evaporate acetic acid. Samples were taken after 1 h and 4 h. Finally, the chocolate was tempered at 45 °C (ChocoMa 6T 12, Pitec AG, Oberriet, Switzerland), cooled down to 28 °C, and warmed up again to 32 °C to achieve a shiny and glossy surface. Finally, the molded chocolate was left to harden for a few hours in thermostated cabinet at 17 °C (TS 606-G/4-i, WTW Wissenschaftlich-Technische Werkstätten GmbH, Weilheim, Germany) and stored at 12 °C.

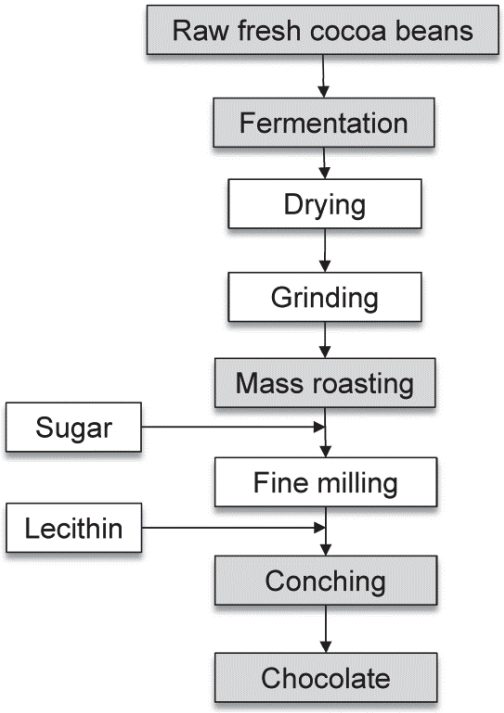

Figure 1 Schematic chocolate manufacturing process as applied in this study.

Changes in the PA profile were recorded in a model process and may not be fully in line with real-life crop or industrial scale processing. Nevertheless, one batch was processed and aliquots of about 50 g were sampled from each processing step. Samples were collected from (1) raw fresh cocoa beans, (2) fermented, dried cocoa beans (3) roasted cocoa mass, (4) 1 h conched cocoa mass, (5) 4 h conched cocoa mass, and (5) molded chocolate bars. Samples were stored immediately at -20 °C until analysis.

4.3.3.2. Sample Preparation

All samples were treated in the same way, whereby the sample preparation was designed to obtain an enriched fraction of polyphenol compounds and methylxanthines according to Pedan, Fischer, & Rohn (2016). The resulting cocoa extract was used for further HPLC analysis.

4.3.3.3. NP-HPLC-online-DPPH analyses of oligomeric PAs

HPLC separations were performed on a Knauer Smartline system (Knauer GmbH, Berlin, Germany) as described previously (Pedan, Fischer, & Rohn, 2015a). The LC system was coupled with post column derivatisation using DPPH in order to analyze the antioxidant capacity simultaneously. Chromatograms were recorded by means of UV/Vis-detection (UVD 2.1 S, Knauer GmbH, Berlin, Germany) at a wavelength of 275 nm for determination of the phenolic compound profile. The radical scavenging activity was detected photometrically at 515 nm with a second UV/Vis-detector (Smartline UV/Vis-Detektor 2520, Knauer GmbH, Berlin, Germany) for estimation of the contribution of the single phenolic substances to the antioxidant capacity. Data were acquired and processed using ClarityChrom software package (Version 3.0.5.505).

PAs were quantified through an external calibration using (-)-epicatechin for monomeric PAs, proanthocyanidin B2 for dimeric PAs, proanthocyanidin C1 for trimeric PAs, and cinnamtannin A2 for PAs with DP ≥ 4 on the one side for comparability, all of the detected PAs were quantified as (-)-epicatechin equivalent on the other side. Definition of this approach is as described in Pedan, Fischer, and Rohn (2015a). However, a positive correlation could be detected between the PA concentrations against UV-absorption. Hereby, the PA content was represented by a positive peak and the DPPH signal by a negative peak in the chromatogram. The HPLC analysis was done in triplicate for each sample.

4.3.4. Results and Discussion

PAs are eluting in their order of polymerization (DP1 -> DP2 -> DP3 etc.) when using normal phase chromatography. The NP-HPLC-online-DPPH methodology was already proven to be useful for a rapid assessment of antioxidant compounds in complex mixtures (Pedan, Fischer, & Rohn, 2015b). A method combining the separation of the condensed polyphenol compounds and the simultaneous assessment of their antioxidant capacity is an advantage for characterizing the bioactivity/reactivity of bioactive compounds. However, the major benefit of this method is that the contribution of a single compound to the overall activity of a mixture of antioxidants can be measured (**Fig. 2**). The method stating therefore both, the antioxidant activity as a general outcome of the result and the antioxidant capacity as a quantitative evaluation of single PAs investigated. Definition of this terminology is as described in Rohn and Kroh (2005).

During HPLC analysis, attention must be paid to a proper storage of the samples, as the extracted PAs might not be stable in 50% aqueous acetone and could interact with each other. As a consequence, the samples were prepared freshly directly before HPLC analysis. Otherwise, unknown interferences can appear for especially the octamers and nonamers, as shown in the UV-chromatogram in **Fig. 2**. However, polyphenols are also susceptible to polyphenol-oxidase reactions and consequently to enzymatic browning. Samples can be stabilized by addition of inorganic halides such as sodium chloride, sodium fluoride or a bivalent salt like calcium chloride, which has proved to have an even more considerable effect on the inhibitory behaviour of polyphenol oxidase (Le Bourvellec & Renard, 2012).

4.3.4.1. Effect of the chocolate manufacturing process on the content and profile of PAs

Little is known about changes of PAs during a complete chocolate manufacturing process. Food composition data of cocoa beans are often reported for selected processed food products (Gu *et al*., 2004), but rarely referring to one continuous process. To completely understand the chocolate manufacturing process, it is important to take a closer look not only at low molecular polyphenols, but also at the quantitatively larger group – the PAs. The aim of the present work was to investigate the profile of PAs over six different stages of chocolate manufacturing. The stages represent the most pronounced steps in chocolate finishing, from raw over fermented to roasted beans, to conching and finishing. Data on changes of the content of PAs during the chocolate manufacturing process are difficult to access. However, studies done by Cooper, Campos-Giménez, Jiménez Alvarez, Nagy, Donovan, & Williamson (2007) indicated that (-)-epicatechin concentration can help to predict the content of polyphenols such as PAs B2, C1, and even the total polyphenol content.

The results obtained using the NP-HPLC-online-DPPH method are presented in **Table 1** and **Table 2**. The quantitative data and the data on the respective antioxidant activity demonstrate the changes of the different PAs and the corresponding changes of the antioxidant activity across the mentioned manufacturing stages. In general, PAs and their antioxidant capacity are expressed as monomeric (-)-epicatechin.

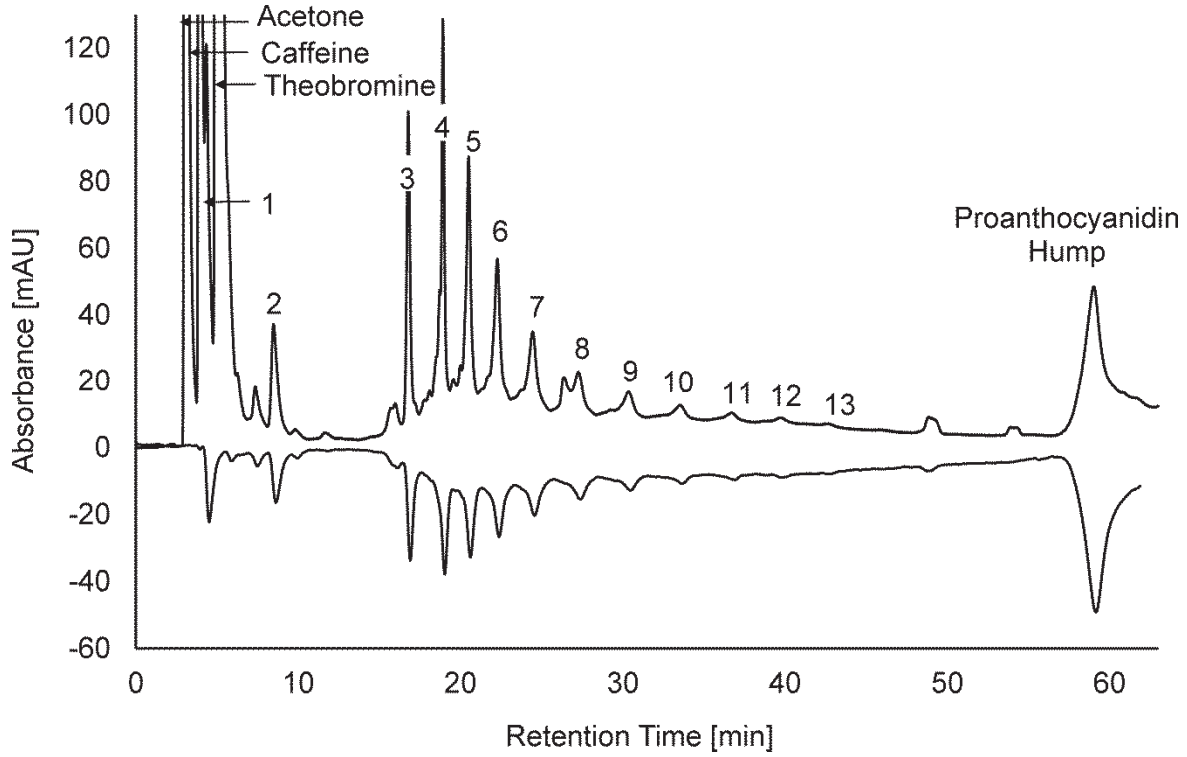

Figure 2 Chromatogram of the oligomeric PAs from unroasted cocoa beans extract and their antioxidant activity using the NP-HPLC-online-DPPH (positive peaks at 280 nm, negative peaks at 515 nm). Identified PAs: epicatechin monomer (1), dimers (2), trimers (3), tetramers (4), pentamers (5), hexamers (6), heptamers (7), octamers (8), nonamers (9), decamers (10), undecamers (11), dodecamers (12), tridecamers (13), and an unresolved 'PA hump'.

Therefore, the results were expressed as (-)-epicatechin equivalents (ECE). The total PA content is reported as the total sum of the fractions ∑ DP1-DP13 for each processing step.

Fermentation process

In the present study, the initial acidity of the pulp (pH 6.6) resulting from the presence of citric acid, together with low oxygen levels, remained almost constant for three days, before it declined over the next six days to a final pH value of 5.5. First cocoa flavor precursors were noticed. Hereby, the initial total PA content (∑ DP1 - DP13) of the raw cocoa bean seeds was 29.98 mg ECE/g non-fat dry matter which corresponds to an antioxidant capacity of 25.08 mg ECE/g. Furthermore, the amount of individual PAs detected in cocoa bean extract

decreased from DP1 to DP13, whereby the monomeric fraction DP1 was more concentrated than polyphenols with DP>1. However, according to Counet & Collin (2003), DP1 shows a higher extraction yield than higher molecular weight PAs, whereby the yield of higher oligomeric PAs can be increased due to optimized extraction solvents. In the present study, the unresolved HPLC signal identified as 'PA hump' in the chromatogram (**Fig. 2**) represents PAs with DP>13, and/or other higher molecular PAs with different molecular structure. In this study, the so called 'PA hump' was responsible for up to 23.3 mg ECE/g. The sum of PAs from DP1-DP13 is approx. the same as that of the 'PA hump', illustrating the necessity to not neglect its overall quantitative and antioxidant contribution.

In the present study, the total PA content (∑ DP1-DP13) decreased only slightly to 26.7 mg ECE/g during the fermentation, which is about 11% less compared to the initial value (**Table 1**). The profile of PAs changed significantly and monomers appeared even more concentrated than the oligomers in the raw samples. In particularly, the amount of monomeric PAs increased to 8.78 mg ECE/g which corresponds to an increase of 60%. In parallel, the degree of polymerization decreased to a verifiable content of DP11. At the same time, the content of the compounds under the 'PA hump' doubled to 54.1 mg ECE/g.

Generally, during the fermentation, microorganisms and endogenous enzymes lead to a degradation of the pulp that surrounds the fresh beans and limits the development of pathogenic microorganisms. Flavor precursors are formed and polyphenols undergo an enzymatic transformation by polyphenoloxidases. In detail, polyphenols diffuse from the vacuoles through the cotyledons and interact covalently as well as non-covalently with proteins or polysaccharides (Forsyth, Quesnel, & Roberts, 1958; De Brito, García, & Amâncio, 2002). Following the enzymatic oxidation, (-)-epicatechin polymerizes with further (-)-epicatechin or (+)-catechin units to form high molecular weight polymers (Albertini *et al*., 2015). Afoakwa, Kongor, Takrama, Simpson Budu, & Mensah-Brown (2013) observed through Folin-Ciocalteu assay a decrease of the total polyphenol content ranging from 169.1 mg/g to 148.8 mg/g for a 3 day fermentation and a further decrease to 119.4 mg/g in 7 days and to 83.5 mg/g after 10 days. Forsyth (1952) stated a loss of the total polyphenols by 24% after 60 h of fermentation, and 58% after 8 days. A similar percentage was found by De Brito, Pezoa García, Gallão, Cortelazzo, Fevereiro, & Braga (2000) during their investigation whereby the total phenolic content slightly decreased from 231 ± 5 mg/g non-fat dry matter at time 0 h to 213 ± 5 mg/g after 72 h. Aikpokpodion & Dongo (2010) observed a more pronounced decrease of the polyphenol content from day 0 with 161.1 mg/g to 60.1 mg/g at day 6. A predominance of (-)-epicatechin was observed for some cocoa cultivars during fermentation with an initial value of 3.88 ± 0.20 mg/g at timepoint 0 h towards a doubling of the value to 7.25 ± 1.07 mg/g after 72 h (Cruz, Leite, Soares, & Bispo, 2015). By extending the fermentation time, the increase of epimerization products was more pronounced. In the

present study, the strong increase of the monomers could be explained as result of the decrease of DP11, DP12, and DP13. Cruz, Leite, Soares, & Bispo (2015) reported also a sharp decrease of polyphenolic compounds of about 70% and especially, a loss of (-)-epicatechin of up to 90%. Also Camu, De Winter, Addo, Takrama, Bernaert, and De Vuyst (2008) described a linear decrease of (-)-epicatechin due to diffusion, polyphenol oxidation, and condensation started from 11 mg/g at the beginning of fermentation to a more than 70% loss after 144 h of fermentation. In general, the decrease in the amount of polyphenols is accompanied by enzymatic and non-enzymatic browning resulting in quite complex polymers. The increase of the 'PA hump' can be an indicator for this polymerization. PAs with a DP>13 and further UV-active polymers (e.g., complex browning products) may co-elute as a big hump at the end of the chromatographic run. Afoakwa, Kongor, Takrama, Simpson Budu, & Mensah-Brown (2013) suggested that during the fermentation, polyphenols are released from their storage cell organelles, and become substrates for enzymes leading to oxidation and polymerization reactions. Further condensation with amino acids, proteins and flavonoids leads to high molecular weight fractions.

Table 1 Oligomeric proanthocyanidins and their antioxidant activity (AA) obtained by NP-HPLC-online-DPPH from different chocolate manufacturing stages. The values are expressed as mg (-)-epicatechin equivalents per gram of the non-fat dry matter. PA content and its antioxidant activity are expressed as (-)-epicatechin equivalents (using (-)-epicatechin as standard, ECE). Values represent value mean, with n = 3 (± standard deviation). The bold entries refer to further explanations in the text. The asterisks indicate a single determination.

Degree of Polymerization	Raw Cocoa Beans		Fermented Cocoa Beans		Roasted Cocoa Mass		1 h Conching		4 h Conching		Chocolate	
	PA [mg/g]	AA [mg/g]	PA [mg/g]	AA [mg/g]	PA [mg/g]	AA [mg/g]	PA [mg/g]	AA [mg/g]	PA [mg/g]	AA [mg/g]	PA [mg/g]	AA [mg/g]
DP 1	**5.26 ± 0.55**	3.81± 0.75	**8.78 ± 0.28**	6.84 ± 0.71	**4.21 ± 0.15**	2.84 ± 0.47	**2.59 ± 0.75**	3.20 ± 1.07	**2.70 ± 0.02**	1.82 ± 0.39	**1.77 ± 0.46**	1.49 ± 0.52
DP 2	2.65 ± 0.33	2.59 ± 0.70	4.05 ± 0.14	4.22 ± 0.36	1.68 ± 0.03	1.34 ± 0.39	1.06 ± 0.22	1.50 ± 0.59	1.02 ± 0.03	0.82 ± 0.13	0.84 ± 0.21	0.80 ± 0.05
DP 3	**4.39 ± 0.56**	4.20 ± 0.92	4.15 ± 0.09	4.66 ± 0.20	1.78 ± 0.02	1.74 ± 0.42	1.18 ± 0.28	1.72 ± 0.57	1.11 ± 0.03	0.93 ± 0.04	0.95 ± 0.27	0.90 ± 0.06
DP 4	**4.17 ± 0.50**	3.53 ± 0.95	2.99 ± 0.31	3.09 ± 0.06	1.40 ± 0.03	1.02 ± 0.16	0.85 ± 0.20	1.06 ± 0.53	0.72 ± 0.03	0.51 ± 0.15	0.60 ± 0.14	0.50 ± 0.16
DP 5	**4.31 ± 0.48**	3.54 ± 0.97	2.64 ± 0.12	2.27 ± 0.18	1.06 ± 0.02	0.69 ± 0.20	0.66 ± 0.17	0.86 ± 0.42	0.63 ± 0.05	0.27 ± 0.22	0.56 ± 0.17	0.37 ± 0.13
DP 6	3.20 ± 0.57	2.71 ± 0.72	1.22 ± 0.04	1.68 ± 0.13	0.62 ± 0.02	0.35 ± 0.29	0.50 ± 0.23	0.52 ± 0.32	0.45 ± 0.18	0.14 ± 0.10	0.34 ± 0.12	0.19 ± 0.10
DP 7	2.14 ± 0.37	1.98 ± 0.54	1.21 ± 0.14	0.87 ± 0.11	0.47 ± 0.06	0.12 ± 0.05	0.28 ± 0.05	0.21 ± 0.18	0.23 ± 0.00	0.03 ± 0.03	0.22 ± 0.03	0.07 ± 0.02
DP 8	1.51 ± 0.31	1.27 ± 0.31	0.85 ± 0.14	0.49 ± 0.01	0.40 ± 0.04	0.07 ± 0.11	0.20 ± 0.02	0.12 ± 0.15	0.20 ± 0.01	0.07*	0.16 ± 0.06	0.02*
DP 9	0.64 ± 0.19	0.99 ± 0.95	0.47 ± 0.06	0.31 ± 0.13	0.25 ± 0.01	-	0.13 ± 0.03	0.11*	0.14 ± 0.02	-	0.13 ± 0.03	-
DP 10	0.65 ± 0.14	0.47 ± 0.16	0.23*	-	0.14 ± 0.02	-	0.08 ± 0.02	-	0.10 ± 0.02	-	0.08 ± 0.01	-
DP 11	0.52 ± 0.10	-	0.36*	-	0.07 ± 0.06	-	0.03 ± 0.05	-	0.07 ± 0.06	-	0.09*	-
DP 12	0.26 ± 0.11	-	0.49*	-	-	-	-	-	-	-	-	-
DP 13	0.27 ± 0.09	-	-	-	-	-	-	-	-	-	-	-
PA Hump	**23.33 ± 3.92**	24.96 ± 3.13	**54.12 ± 3.47**	77.27 ± 3.92	29.49 ± 5.79	68.31 ± 4.17	20.31 ± 3.91	44.93 ± 2.61	16.99 ± 4.47	27.57 ± 9.94	19.68 ± 4.57	24.95 ± 4.56
∑ (1-13)	**29.98 ± 3.98**	25.08 ± 6.56	**26.72 ± 0.28**	24.43 ± 4.71	**12.08 ± 0.23**	8.16 ± 2.08	7.57 ± 1.84	9.30 ± 3.82	7.36 ± 0.09	4.60 ± 1.02	5.74 ± 1.56	4.34 ± 1.06

Table 2 Oligomeric PA and their AA obtained by NP-HPLC-online-DPPH from different chocolate manufacturing stages. The values are expressed as mg proanthocyanidin equivalents per gram of the non-fat dry matter. PA content and PA equivalents expressed as mean values with n = 3 (± standard deviation). The bold entries refer to further explanations in the text.

Degree of Polymerization	Raw Cocoa Beans		Fermented Cocoa Beans		Roasted Cocoa Mass		1 h Conching		4 h Conching		Chocolate	
	PA [mg/g]	AA [mg/g]	PA [mg/g]	AA [mg/g]	PA [mg/g]	AA [mg/g]	PA [mg/g]	AA [mg/g]	PA [mg/g]	AA [mg/g]	PA [mg/g]	AA [mg/g]
DP 1	**5.02 ± 0.53**	1.86 ± 0.48	**8.43 ± 0.27**	3.81 ± 1.36	4.01 ± 0.14	1.24 ± 0.30	**2.46 ± 1.03**	1.64 ± 0.69	**2.57 ± 0.02**	0.75 ± 0.25	**1.66 ± 0.45**	0.53 ± 0.34
DP 2	3.58 ± 0.45	1.41 ± 0.43	5.47 ± 0.20	2.40 ± 0.60	2.25 ± 0.04	0.65 ± 0.24	1.42 ± 0.43	0.79 ± 0.36	1.36 ± 0.04	0.38 ± 0.08	1.13 ± 0.29	0.37 ± 0.03
DP 3	5.55 ± 0.69	2.09 ± 0.49	5.24 ± 0.12	2.34 ± 0.27	2.31 ± 0.02	0.80 ± 0.22	1.53 ± 0.49	0.82 ± 0.30	1.45 ± 0.04	0.41 ± 0.02	1.25 ± 0.33	0.39 ± 0.03
DP 4	6.81 ± 0.81	2.15 ± 0.59	4.91 ± 0.50	1.88 ± 0.10	2.36 ± 0.05	0.58 ± 0.10	1.44 ± 0.46	0.63 ± 0.33	1.23 ± 0.04	0.28 ± 0.09	1.03 ± 0.23	0.27 ± 0.10
DP 5	**7.04 ± 0.77**	2.16 ± 0.60	**4.35 ± 0.19**	1.37 ± 0.29	1.81 ± 0.03	0.37 ± 0.12	1.14 ± 0.40	0.49 ± 0.26	1.08 ± 0.08	0.13 ± 0.14	0.97 ± 0.27	0.19 ± 0.08
DP 6	5.26 ± 0.92	1.64 ± 0.45	2.07 ± 0.06	0.99 ± 0.22	1.10 ± 0.03	0.16 ± 0.18	0.87 ± 0.54	0.29 ± 0.20	0.79 ± 0.29	0.05 ± 0.06	0.63 ± 0.20	0.08 ± 0.06
DP 7	3.54 ± 0.59	1.18 ± 0.34	2.05 ± 0.23	0.49 ± 0.18	0.86 ± 0.10	0.02 ± 0.03	0.52 ± 0.11	0.15 ± 0.06	0.45 ± 0.00	-	0.43 ± 0.05	-
DP 8	2.53 ± 0.50	2.02 ± 0.19	1.47 ± 0.23	0.25 ± 0.01	0.74 ± 0.07	0.27 ± 0.07	0.39 ± 0.05	0.10*	0.39 ± 0.02	-	0.33 ± 0.09	-
DP 9	1.13 ± 0.31	0.56 ± 0.60	0.85 ± 0.09	0.14 ± 0.21	0.50 ± 0.02	0.11 ± 0.05	0.28 ± 0.07	-	0.30 ± 0.04	-	0.28 ± 0.06	-
DP 10	1.15 ± 0.22	0.24 ± 0.10	-	-	0.33 ± 0.04	-	0.21 ± 0.05	-	0.23 ± 0.04	-	0.20 ± 0.02	-
DP 11	0.94 ± 0.17	-	-	-	0.22 ± 0.10	-	0.22 ± 0.12	-	0.24*	-	0.21*	-
DP 12	1.02 ± 0.17	-	-	-	-	-	-	-	-	-	-	-
DP 13	0.54 ± 0.14	-	-	-	-	-	-	-	-	-	-	-
PA Hump	**37.65 ± 6.31**	15.56 ± 1.96	87.19 ± 5.59	48.29 ± 2.46	47.55 ± 9.31	42.69 ± 2.61	32.75 ± 6.30	28.07 ± 1.63	27.41 ± 7.19	17.21 ± 6.22	31.75 ± 7.35	15.57 ± 2.85
∑ (1-13)	**43.59 ± 5.79**	14.03 ± 4.02	35.52 ± 0.72	13.67 ± 2.91	16.45 ± 0.20	3.84 ± 1.23	10.50 ± 2.37	4.92 ± 2.29	10.08 ± 0.16	2.00 ± 0.60	8.12 ± 2.10	1.84 ± 0.64

Drying process

During the drying process beans were placed in shallow trays. The oxidation reaction that began with the fermentation continues any further during drying. As a result, the amount of polyphenols further decreased, accompanied by a reduction in astringency. During the drying process, the moisture content dropped significantly saving the cocoa bean from mold infestations. According to Albertini *et al*. (2015), the (-)-epicatechin content is reduced to approximately 75% at the end of the fermentation and drying process. Results obtained in the present study confirmed the high impact of fermentation and drying on the PA content. Being the first processing steps which contribute directly to the typical cocoa flavor, attention should be paid to these two steps to obtain high polyphenol content chocolate.

Roasting process

Additional loss of the polyphenol content is described to occur during roasting (Kealey *et al*., 2001). Preferably, oligomeric PAs were lost during roasting process whereby in the present study the total PA content (∑ DP1 - DP13) continued falling to 12.08 mg ECE/g. Despite the high level of monomeric PAs found in the fermented cocoa beans, only 4.21 mg ECE/g were detected in the roasted cocoa beans but the remaining amount can be considered high. However, degradation also occurred for the 'PA hump' with 45% to 29.5 mg ECE/g. No DP12/13 polymers were found in the roasted cocoa beans (**Fig. 3a**, **3b**).

Roasting has a high impact on cocoa flavor resulting from the MAILLARD reaction and STRECKER degradation. High temperatures and low moistures are pre-requisites for the MAILLARD reaction. Conventional roasting temperature varies from 130 to 150 °C and lasts from 15 to 45 min, whereby in the present study roasting conditions were kept to a minimum on physical treatment. Traditional manufacturers either use whole-bean roasting or nib roasting, although cocoa-mass roasting is much more precise concerning heat transfer. As the cocoa-mass is more homogenous and degradation of valuable substances through longer heat impact can be avoided. Kothe, Zimmermann, & Galensa (2013) analyzed the stability of phenolic compounds during roasting and observed a 40% loss of flavanols during roasting temperatures beyond 120 °C and duration of 30 min. In detail, the sum of flavanols ranged from 7.75 mg/g in unroasted cocoa beans to 4.77 mg/g in roasted cocoa beans. Furthermore, they observed a loss of the primary monomers (-)-epicatechin ranged from 4.77 ± 0.08 mg/g to 3.35 ± 0.2 mg/g, as well as the PA dimers B2 ranged from 2.03 ± 0.02 mg/g to 1.33 ± 0.04 mg/g. A diminution was also confirmed for higher PAs. Furthermore, they found evidence for an epimerization of flavanol monomers and dimers as a function of temperature, whereby (-)-epicatechin decreased substantially with growing temperature. On the other

hand, as already mentioned above, (+)-catechin can increase due to heat-induced epimerization.

However, studies done by Ioannone, Di Mattia, De Gregorio, Sergi, Serafini, & Sacchetti (2015) focused on the determination of monomeric and oligomeric PAs and the devolution of the total antioxidant activity when using different roasting conditions. In that study, a total PA content (∑ DP1-DP10) of 13.1 mg/g non-fat dry matter after six minutes at 125 °C has been determined, whereby a decrease from DP1 with 4.22 mg/g to DP10 with 0.12 mg/g was observed. Furthermore, the group observed a general reduction with a more cascade shape declination from DP1 to DP10.

In a further recent study, Gültekin-Özgüven, Berktaş, & Özçelik (2016) demonstrated under similar roasting temperatures a lower content of monomeric (-)-epicatechin compared to the present study with 1.97 ± 0.16 mg/g, a dimeric B2 content with 1.11 ± 0.12 mg/g and the trimeric PA C1 content with 0.26 ± 0.02 mg/g. However, no information about roasting periods was given. The same study also described a total phenolic content of 8.79±0.30 mg catechin equivalents per gram when using the FOLIN-CIOCALTEU assay. When using similar roasting conditions, the group of Payne, Hurst, Miller, Rank, & Stuart (2010) found a total content of monomers in fermented cocoa beans ranging from 0.78 - 1.02 mg/g and an (-)-epicatechin content of 0.46 - 0.50 mg/g at 120 °C. Also here, no further information about roasting duration was provided.

Conching process

In the present study, cocoa nibs were firstly thoroughly separated from the outer shell before packed into the grinder. The longer the nibs are ground, the smoother the chocolate appears during consumption. Generally, conching periods can vary from a few hours to a few days. An adequate conching time is for the most Western European chocolatiers at least 72 hours while American chocolatiers conch their chocolate for only 18-20 hours (Alberts & Cidell, 2006). One of the main aims of the conching process is to remove unwanted acids such as acetic, propionic, and butyric acid. In the present study, samples were taken after 1 h and 4 h of conching, whereby the total PA content (∑ DP1-DP13) remained stable with from 7.57 to 7.36 mg ECE/g. A monomeric fraction of 2.59 mg ECE/g after 1 h conching and a monomeric fraction of 2.70 mg ECE/g after 4h conching were obtained. The ‘PA hump’ with 20.3 mg ECE/g declined after 1 h and further decreased slightly to 16.9 mg ECE/g after 4 h.

Contrary to Di Mattia, Martuscelli, Sacchetti, Beheydt, Mastrocola, & Pittia (2014), who did not find compounds with DP10 during a short time conching process, the present study observed more complex PAs with a DP10/11. With regard to conching parameters, they furthermore observed a slight increase of the total PA content from the initial raw chocolate mass to the conched product ranged from 8.03 ± 0.13 mg ECE/g before conching to 8.42 ±

0.08 mg ECE/g after the conching process. In accordance to the study done by Mazor Jolic, Redovnikovic, Markovic, Sipusic, & Delonga (2011), the total PA content of cocoa samples changes from 9.6 mg/g for cocoa beans to 7.7 mg/g for roasted cocoa beans to 5.6 mg/g for cocoa liquor when determined by using BATE-SMITH assay.

Chocolate

In the present study, after a 12 h conching time and a short tempering process, the chocolate bars have a total PA content (∑ DP1 - DP13) of 5.74 mg ECE /g calculated on the basis of fat-free cocoa mass which is approx. one fifth of the initial total PA content. The monomeric PA fraction decreased just slightly to 1.77 mg ECE/g per gram non-fat dry matter. The additional conching process seems to affect in a significant way the content of monomeric PAs. Nevertheless, the 'PA hump' remained stable with 19.68 mg ECE/g. As obvious from **Table 1**, the PA content correlates with the antioxidant activity. The contribution of ∑ DP1-DP6 to the total antioxidant activity is about 80% for the raw cocoa beans and increased to a contribution of 98% for the final chocolate. The contribution of these PAs slowly increased during chocolate manufacturing process. However, this result is quite contrary to the reports of Counet & Collin (2003) claiming for ∑ DP1 - DP6 a contribution of only 40% to the total antioxidant activity.

A similar distribution of PAs and antioxidant activity was found in commercial chocolate bars. Studies done by Gu, House, Wu, Ou, & Prior (2006) compared different chocolate products from major brands and determined a total PA content ranging from 8.5 to 19.8 mg/g. When using non-specific colorimetric assays (e.g., p-dimethylaminocinnamaldehyde) for the determination of the total PA content, Payne, Hurst, Stuart, Ou, Fan, Ji, & Kou, 2010 observed an amount of 7 mg/g for chocolate sample. Cooper *et al*. (2007) reported for 68 different chocolates an (-)-epicatechin content ranged from 0.071 - 1.942 mg/g, a PA dimer B2 content in the range of 0.041 - 1.174 mg/g, a PA trimer C1 in the range of not detectable - 0.905 mg/g, and PA tetramer A2 in the range of not detectable - 0.387 mg/g. Furthermore they observed that (-)-epicatechin accounted for a mean percentage of 39.0% of the total PA content. Counet, Callemien, & Collin (2006) found a total PA content (∑ DP1 - DP6) of 85.4 mg/g in a dark chocolate extract, a DP1 of 42.9 mg/g and a DP2 of 14.6 mg/g.

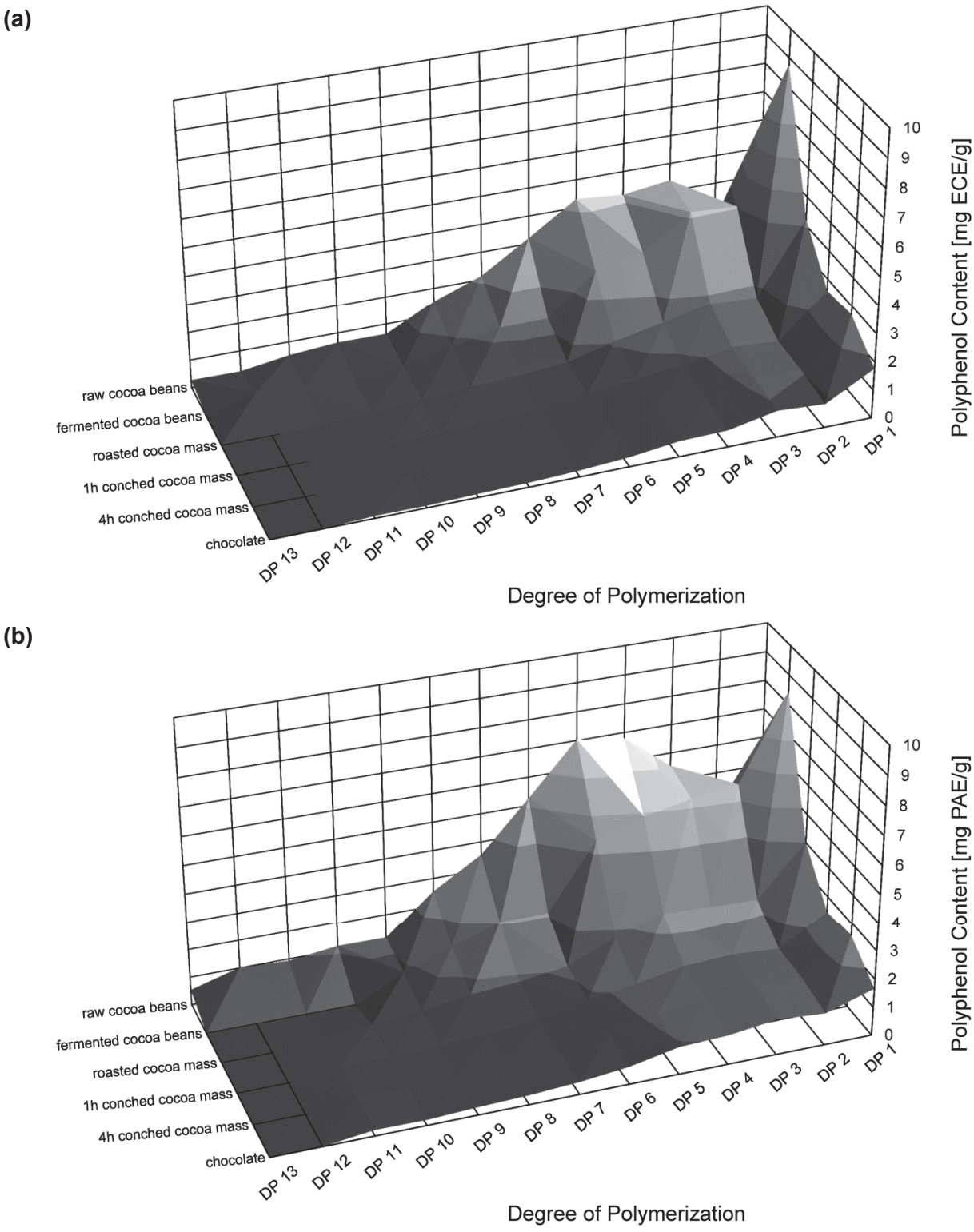

Figure 3 (a) Oligomeric proanthocyanidin profile of the model chocolate manufacturing depending on the different stages of the chocolate manufacturing process and the degree of polymerization expressed as (-)-epicatechin equivalent. **(b)** Oligomeric proanthocyanidin profile of the model chocolate manufacturing and the degree of polymerization expressed as proanthocyanidin equivalent.

Data from the present study cannot be directly compared with data found in other publications. Differences in the methodology of the phenolic compound extraction generate a wide range of data. Study done by Patras, Milev, Vrancken, & Kuhnert (2014) performed extraction with 70% aqueous methanol at room temperature overnight. Hammerstone *et al*. (1999) determined that 70% acetone in water, followed by two additional extractions with 70% methanol in water are most effective in extracting higher oligomeric cocoa PAs. Camu, De Winter, Addo, Takrama, Bernaert, & De Vuyst (2008) used boiled water for phenolic compound extraction. Besides the different extraction methods, varying origins or processing parameters make a direct comparison even more difficult.

Furthermore, suggestions from other studies (Cooper *et al*., 2007) to use the (-)-epicatechin content for quantification of oligomeric procyanidins cannot be mentioned from our point of view. A linear relationship among (-)-epicatechin and condensed polyphenols, as described there, would lead to assume that these molecules were affected the same way during cocoa processing. According to our results (**Table 1**), the relationship among PA monomers and PA dimer B2, trimers or tetramers is not a linear correlation. For an accurate quantification, still NP-HPLC analysis must be done on the basis of higher molecular PAs as standard substances.

Correlation of concentration and antioxidant capacity

Attention must be paid when comparing the content of individual PAs with their corresponding antioxidant capacity (**Table 1**, **Table 2**). The values for the 'PA hump' of roasted cocoa mass and for four hours conching (**Table 1**) can be exemplarily explained: It is evident that the concentrations do not correspond linearly with the measured antioxidant capacities. A probable reason could be that the individual peaks contain more than one component with different antioxidant capacities, and that changes in this profile take place during processing. The insufficient separation into groups of similar molecular weight rather than individual components is due to the specific separation mode of the normal phase chromatography (Pedan, Fischer, & Rohn, 2015a).

4.3.4.2. Oligomeric proanthocyanidins expressed as proanthocyanidin equivalents

Traditionally, (-)-epicatechin or gallic acid are used as standards for the estimation of the total phenolic content of cocoa and related products (Hii, Law, Suzannah, Misnawi, & Cloke, 2009). At equal concentrations, (-)-epicatechin has a higher response to UV radiation than (+)-catechin (McMurrough & McDowell, 1978). Among PAs, the monomeric (-)-epicatechin has the highest UV/Vis response followed by proanthocyanidin dimer B2, trimer C1 while the

more complex tetramer A2 is the least UV-active compound (Pedan, Fischer, & Rohn, 2015a). Consequently, using (-)-epicatechin as standard underestimates the amount of oligomeric PAs in a sample. To minimize the problem of using less suitable standards, it is proposed to calculate the content of PAs expressed at hand of closely related standard compounds, e.g., for monomers as mg (-)-epicatechin, for dimers as proanthocyanidin B2 equivalents, for trimers as proanthocyanidin C1 equivalents and for fractions more complex or equal than tetramers as mg cinnamtannin A2 equivalents, as long as the corresponding standards are not available.

Most of the studies reported in the literature showed cascading, diminished levels of the polyphenol content from monomers to decamers. In this study, the calculation at hand of related standards provided evidence that raw cocoa beans possess a high amount of pentameric PAs with 7.1 mg cinnamtannin A2 equivalents/g compared to pentameric PAs with 4.3 mg ECE/g when calculated as (-)-epicatechin (**Table 2**). During the chocolate manufacturing process, the levels additionally decrease, whereby already after fermentation the pentameric PAs decreased significantly to 4.35 mg cinnamtannin A2 equivalents/g and more monomeric PAs are formed. There is a more than 1.5-fold difference in the response between the PAs expressed as (-)-epicatechin equivalent or expressed as related standard compounds. Furthermore, this kind of evaluation indicates that pentameric PAs are the most abundant compounds in raw cocoa beans with the highest antioxidant capacity.

As presented in **Fig. 3a**, the oligomeric PA profile of the model chocolate manufacturing expressed as (-)-epicatechin equivalent is more shaped as a cascade with a decrease from monomeric to oligomeric PAs. In contrast, **Fig. 3b** shows the oligomeric PA profile of the model chocolate manufacturing and the degree of polymerization expressed as proanthocyanidin equivalents, whereby the profile occurs more accented to tetrameric up to heptameric PAs.

The complexity of oligomeric PAs and an appropriate evaluation of analytical methods are also discussed by Schofield, Mbugua, & Pell (2001). They analyzed the relationship between the tannin concentration and the absorbance of three main standard compounds delphinidin, cyanidin, and quebracho at 550nm using the butanol/HCl assay. They showed a 30-fold difference in response between the UV-reactive delphinidin and the least UV-reactive quebracho. As a result, tannin concentration is underestimated when quebracho is used as standard for tannin-containing plants.

4.3.5. Conclusion

This study contributes to understand the influence of the different chocolate manufacturing stages on the content and composition of oligomeric PAs and the antioxidant activity as well as provides information about stages which are important for loses of PAs.

The highest content of phenolic compounds can be obtained for raw unfermented cocoa beans. During the chocolate manufacturing process, the total PA content decreased continuously, whereby the final chocolate contained only about 20 % of the initial PA content. In the present study, the degree of polymerization also changed from a maximum of DP13 to DP11. The present study could confirm the main impact on the PA profile caused by fermentation resulted in a loss of the total PA content (∑ DP1 - DP13) of approx. 11 % and a declination of the total PA content (∑ DP1 - DP13) during roasting resulted of approx. 60 %. In comparison with other literature, this study helps to understand the influence of time/temperature combination, which is often used in order to modulate and increase the functional properties of some foods. For a general understanding, more emphasis should be paid to the 'PA hump' and along with it oligomeric PAs with DP >1 3. This study clearly showed that the 'PA hump' increased during the fermentation process and dropped during roasting, whereby higher oligomeric PAs of higher molecular weight are formed during fermentation and decreased during roasting.

For the quantitative determination of the PA amounts, it should be considered that a calculation as epicatechin equivalents (ECE) leads to other results than the calculation as PA equivalents (PAE) up to DP4. Through calculation as ECE, the PA profile of raw cocoa beans appears to cascade down with DP1 > DP2 > DP3 etc. In contrast, when calculating the PA as PAE, the PA profile appears to have a peak at DP5 > DP4 > DP3 etc.

Finally, the major extractable PAs in cocoa could be determined using NP-HPLC-online-DPPH. A positive relationship was shown between DPPH scavenging activity and oligomeric PAs of higher molecular weight. Despite the complexity of cocoa polyphenols, a large number of novel cocoa flavonoids have already been identified recently (e.g., Patras, Milev, Vrancken, & Kuhnert, 2014). But further efforts need to be directed towards the analysis of polymeric PAs and the investigation of the 'PA hump'.

Acknowledgments

We thank Esther Kokabi for providing the manufactured chocolate samples.

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5. Weiterführende Ergebnisse und zusammenfassende Diskussionen

In diesem Teil der Arbeit werden die einzelnen Manuskripte in der Tiefe diskutiert und zueinander in Beziehung gestellt. Darüber hinaus werden einzelne bisher unerwähnte Ergebnisse vorgestellt, die ein umfassenderes Verständnis der Probenbehandlung oder Methodenetablierung ergeben sollen.

5.1. Probenvorbereitung

Der qualitativen und quantitativen Analytik geht eine Probenvorbereitung voraus. Effektive und reproduzierbare Extraktionsverfahren tragen zu einem umfassenden Verständnis bei und erhöhen die Möglichkeit einer zielführenden Strukturaufklärung. Verschiedene Extraktionsparameter wurden in dieser Arbeit berücksichtigt und optimiert, indem physikalische Kenngrössen wie Extraktionslösemittel, -temperatur und Anzahl der Extraktionszyklen auf die zu untersuchende Matrix abgestimmt wurden. Ziel ist es unter schonenden Extraktionsbedingungen maximale Extraktionsausbeute zu generieren.

Eine Einteilung phenolischer Substanzklassen wurde durch ihre jeweilige unterschiedliche Extrahierbarkeit berücksichtigt. Hierbei wurden einerseits phenolische Substanzen eingeteilt in extrahierbare Polyphenole (EPP), extrahierbare Proanthocyanidine (EPA) und nicht extrahierbare Proanthocyanidine (NEPA). Die unterschiedlichen Messmethoden wurden in den jeweiligen Kapiteln diskutiert (**Abbildung 21**).

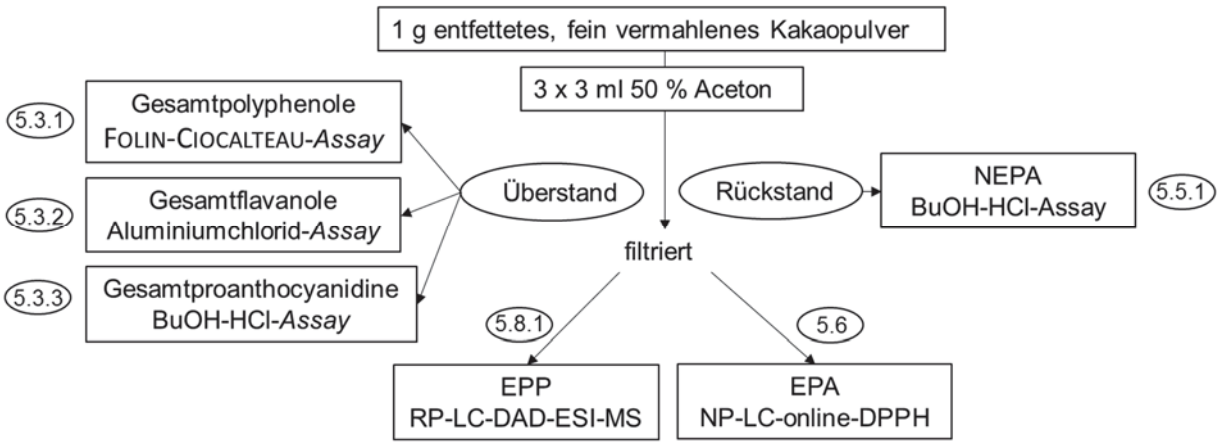

Abbildung 21: Vorgehensweisen zur Bestimmung von EPP, EPA und NEPA.

Die einzelnen Extraktionsparameter wurden einerseits mittels kolorimetrischen Messmethoden optimiert (Kapitel 5.3.1; 5.3.2; 5.3.3; 5.5.1) und andererseits mittels LC-DAD Messungen bestätigt (Kapitel 5.8.1; 5.6).

5.2. Probenaufarbeitung

Bei allen Schritten der Probenaufarbeitung wurde auf fermentierte, getrocknete und debakterisierte Kakaobohnen aus Costa Rica zurückgegriffen (s. Anhang). Diese besitzen den Vorteil, dass auf Grund der enzymatischen Inhibierung sowohl durch die Trocknung als auch Debakterisierung, Polyphenole keine weiteren Veränderungen durch Enzymaktivitäten während der Probenaufarbeitung erfahren. Somit wurde hierbei von einer nativen Polyphenolzusammensetzung ausgegangen. Änderungen im Polyphenolprofil wurden folglich auf den jeweiligen Aufarbeitungsschritt zurückgeführt.

5.2.1. Gefriertrocknung

Um den Kakaobohnen Restwasser zu entziehen, wurden sie im ersten Schritt auf -25°C tiefgekühlt und anschliessend unter Vakuum bei 1 mbar für 12 h gefriergetrocknet. Während des Vorgangs wird Wasser aus der Matrix über den Weg der Sublimation entzogen, wobei die Zellstruktur durch Spannung und Druck aufgeschlossen wird. Durch die darauffolgende Desorption werden gebundene Wassermoleküle entfernt, was chemische Reaktionen im Produkt stoppt, die biologische Aktivität hemmt und es in Folge dessen zur Denaturierung der Proteinkonformation kommt (Rindler, 2000), sowie einer damit verbundenen zwischenzeitlichen Inaktivierung der Enzymaktivität. Die Zerstörung der Zellstruktur kann eine anschließende Extraktion erleichtern.

5.2.2. Entfettung

Daneben wird das Extraktionsverhalten der Analyten von der Partikelgröße beeinflusst. Je feiner eine Probe vermahlen wird, desto grösser ist die Extraktionsausbeute. Dieser Parameter wird jedoch häufig in wissenschaftlichen Publikationen übersehen (Canini *et al*. 2007). Um eine möglichst vollständige Freisetzung wertgebender Inhaltsstoffe aus der Kakaobohne zu gewährleisten, muss diese schonend und einheitlich fein vermahlen werden. In dieser Arbeit, wurde die Vermahlung mittels einer Analysenmühle getätigt (A11 basic, IKA®-Werke GmbH & Co. KG, Staufen, Deutschland), wobei die Kakaobohnen, aus Schutz vor Überhitzung, nur 10 sec vermahlen wurden.

Die vorgetrocknete und fein vermahlene Probe wurde im weiteren Schritt mittels Kaltextraktion entfettet. Hierfür wurde ca. 15 g der vermahlenen Probe in ein 50 mL Zentrifugenröhrchen eingewogen und mit n-Hexan im Verhältnis 1:5 (w/v) für 3 Minuten mittels einem Labor-Tisch-Schüttler bei einer Schütteldrehzahl von 600 U/min (Thermomixer MHR 23, Ditabis AG, Pforzheim, Germany) extrahiert. Die Suspension wurde bei 2.880 *x* g für 1 Minute abzentrifugiert (Zentrifuge 5810, Vaudaux-Eppendorf AG, Schönenbuch, Schweiz) und die überstehende n-Hexan Phase abdekantiert. Dieser Vorgang der Entfettung wurde mindestens 5-mal wiederholt, variiert aber je nach Fettgehalt der Probe. Im Detail ist hierbei die Änderung im Farbverlauf nach jedem Entfettungsschritt sichtbar. Durch die klassische Fettextraktion nach Soxhlet konnte eine Korrelation zwischen der Entfettung und dem Farbverlauf festgestellt werden. Für diese Arbeit wurde die Entfettung als vollständig betrachtet, wenn das Filtrat nach dem Abdekantieren eine klare Farbe annahm. Auf die Fettextraktion mittels klassischer Soxhlet-Apparatur wurde verzichtet, da die Benutzung der Pilzheizhaube eine Überhitzung des Extraktionsraumes hervorruft, welches sich negativ auf die Veränderung von hitzelabilen Polyphenolen auswirken könnte. Nach dem Entfetten der Kakaoprobe wurde diese abermals für 8 h gefriergetrocknet und schlussendlich nochmals mittels Analysenmühle fein vermahlen. Eine schematische Darstellung der Probenaufarbeitung stellt die **Abbildung 22** dar.

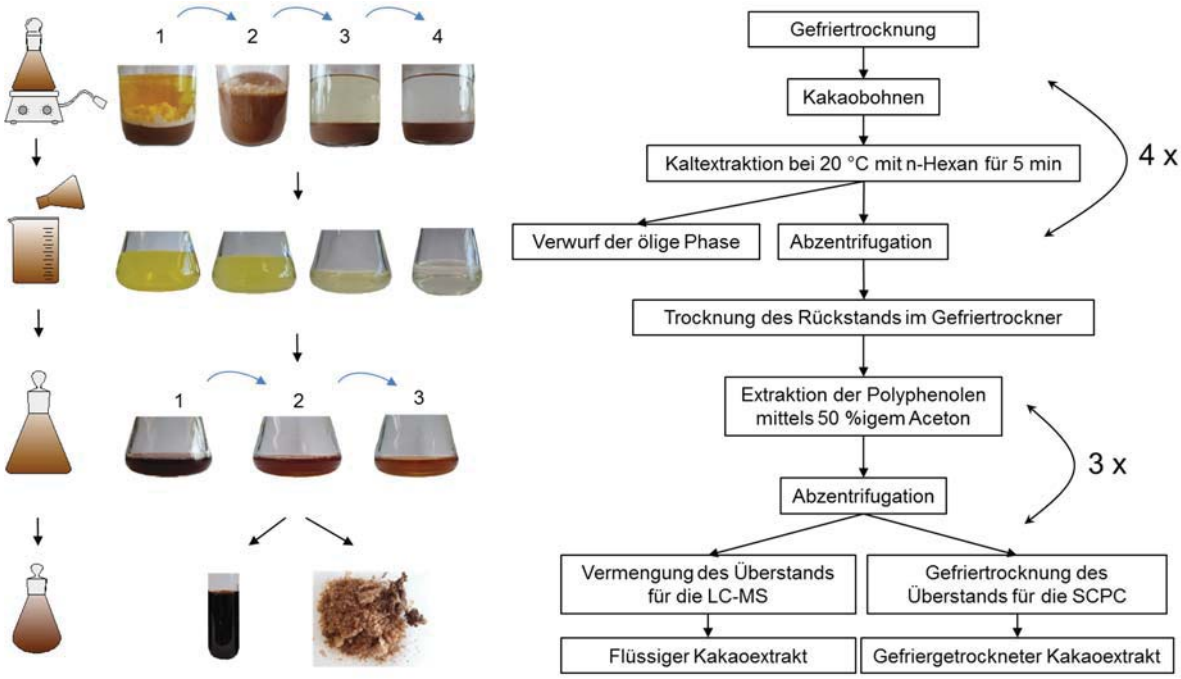

Abbildung 22: Schematische Darstellung der Probenvorbereitung.

Eine Partikelgrössenbestimmung wurde mittels einer Analysensiebmaschine durchgeführt (Vibrationssiebmaschine AS 200 control, Retsch GmbH, Haan, Deutschland), wobei nur Partikel < 100 µm für weitere Analysezwecke verwendet wurden (DIN ISO 3310-1, Ø 203 mm, Retsch GmbH, Haan, Deutschland).

Als geeignetes Lösemittel zur Extraktion von Polyphenole hat sich das 50%ige Aceton-Wasser-Gemisch herausgestellt. In den nachfolgenden Kapiteln wurde seine Fähigkeit unterschiedliche phenolische Substanzklassen zu extrahieren näher untersucht (Kapitel 5.4.1). In Kürze: 1 g der entfetteten und trockenen Probe wurden mit 3 mL 50%igem Aceton-Wasser-Gemisch dreimal extrahiert, die Suspension wurde abzentrifugiert und der polyphenolreiche Überstand abdekantiert. Der vermengte Überstand wurde als *flüssiger Kakaoextrakt* bezeichnet und entweder für weitere photometrische Analysen bereitgestellt oder für die LC-MS Analytik weiterverwendet. Für die SCPC Trennung wurde der Überstand gefriergetrocknet und als *gefriergetrockneter Kakaoextrakt* bereitgestellt. Sowohl photometrische als auch LC-DAD-ESI-MS Messungen wurden mittels Dreifachbestimmung abgesichert.

5.3. Kolorimetrische Verfahren zur Bestimmung löslicher Polyphenole

5.3.1. Gesamtpolyphenolbestimmung mittels FOLIN-CIOCALTEAU-*Assay*

Der Gesamtpolyphenolgehalt wird häufig als mg (-)-Epicatechinäquivalente wiedergegeben (Cooper *et al*., 2008; Elwers, 2008) oder mg Gallussäureäquivalente (Albertini *et al*., 2015) pro g entfetteter Kakaotrockenmasse. Daneben benutzen Autoren auch beispielsweise Ferulasäure (Othman *et al*. 2007) oder (+)-Catechin (Carrillo *et al*. 2013) als Referenzsubstanz zur Berechnung des Gesamtpolyphenolgehaltes.

Auf der Suche nach adäquaten Referenzsubstanzen für die Bestimmung des Gesamtpolyphenolgehaltes, lassen sich in wissenschaftlichen Arbeiten unterschiedliche Polyphenole finden. Um den Einfluss der Referenzsubstanzen auf die Berechnung des Gesamtpolypenolgehaltes zu verdeutlichen, wurde zur besseren Übersicht mittels unterschiedlichen phenolischen Substanzen Kalibrationsgeraden angefertigt (**Tabelle 10**). Die phenolischen Substanzen wurden auf Grund ihrer Häufigkeit der Nennung in wissenschaftlicher Literatur ausgewählt. Durch die Berechnung erkennt man, welchen Einfluss Referenzsubstanzen auf das Ergebnis des Gesamtpolyphenolgehaltes besitzen. Ausgehend von der linearen Gleichung $y = m \cdot x + n$ ist für die angebliche Extraktionsstärke die Steigung m verantwortlich. Je kleiner die Steigung m ist, desto grösser ist der angebliche

Gesamtpolyphenolgehalt. Das resultierende Ergebnis spiegelt demnach eine näherungsweise Bestimmung des tatsächlichen Gehalts an phenolischen Substanzen im Extrakt wider.

Tabelle 10: Kalibrationsgeraden verschiedener Referenzsubstanzen, die zur Berechnung des Gesamtpolyphenolgehaltes herangezogen werden können.

Substanzen	Kalibrationsgerade	Regressionskoeffizient	Konzentration [g/L]
(–)-Epicatechin	y = 18,821x + 0,0357	R^2 = 0,998	0,005 - 0,05
Gallussäure	y = 17,062x + 0,0268	R^2 = 0,9975	0,005 - 0,05
(+)-Catechin	y = 16,001x - 0,0025	R^2 = 0,9993	0,005 - 0,05
Chlorogensäure	y = 10,954x - 0,0255	R^2 = 0,999	0,005 - 0,05

Wie zu erkennen ist, ändert sich der Gesamtpolyphenolgehalt je nachdem, welcher Standard als Referenz benutzt wird. Hat ein Kakaoextrakt ein Extinktionswert von E = 0,604 AU, so ergibt sich, mittels der Formel im Anhang, durch die Berechnung über (-)-Epicatechin-, Gallussäure-, (+)-Catechin- oder Chlorogensäureäquivalente ein Gesamtpolyphenolgehalt von je 60,4 mg/g bezogen auf (-)-Epicatechin, 67,6 mg/g bezogen auf Gallussäure, 75,8 mg/g bezogen auf (+)-Catechin und der höchste Wert mit 114,9 mg/g bezogen Chlorogensäure. Obwohl der gleiche Extinktionswert erhalten wurde, kann über unterschiedlichen Referenzsubstanzen unterschiedliche Werte generiert werden. Die Berechnungsformel kann aus dem Anhang entnommen werden.

Andere kolorimetrische *Assays* basieren auf dem gleichen Prinzip. Ein direkter Vergleich von Messwerten aus verschiedenen wissenschaftlichen Publikationen ist durch die Verwendung von unterschiedlichen Referenzsubstanzen nur schwer herzustellen. So erhielten Belitz, Grosch & Schieberle (2007) für Kakaopulver einen Gesamtpolyphenolgehalt von 84 mg/g Gallussäureäquivalente und berechnet als Epicatechinäquivalente einen Gehalt von 77 mg/g. Die Dominanz von (-)-Epicatechin im Kakao macht es unabdingbar diese als Referenzsubstanz zur Bestimmung des Gesamtpolyphenolgehaltes heranzuziehen, im Gegensatz zur Gallussäure, die keine Rolle als Inhaltsstoff im Kakao hat. Ein mg/g (-)-Epicatechin entspricht ca. 1,1 mg/g Gallussäure.

5.3.2. Gesamtflavonoidbestimmung mittels Aluminiumchlorid-*Assay*

Auch beim Aluminiumchlorid-*Assay* ergab sich mittels verschiedenen Extraktionslösemitteln das gleiche Profil zur Extraktionsausbeute wie beim oben beschriebenen FOLIN-CIOCALTEU-*Assay*. Auch hier erreichte man mit 50%igem wässrigen Aceton die größte Ausbeute an Flavonoiden. Als kakaospezifische Polyphenole wurden die monomeren Polyphenole aus

der Substanzklasse der Flavan-3-ole, Flavanone, Flavone, Flavanole und Anthocyanidine verstanden, welche mittels diesem *Assay* quantifiziert werden konnten.

Als Referenzsubstanz zur Berechnung des Flavonoidgehaltes dient auch hier (-)-Epicatechin. Die gleiche phenolische Substanz wie schon bei FOLIN-CIOCALTEU wurde als gut befunden, erleichtert einen direkten Vergleich der Ergebnisse zwischen den beiden *Assays*.

Eine andere häufig angewandte Methode zur kolorimetrischen Flavonoidbestimmung ist der sogenannte Vanillin-HCl-*Assay* (Wollgast, 2005), wobei hier Faktoren, wie Zeit und Temperatur eine Abhängigkeit der Farbreaktion auf die Konzentrationsbestimmung besitzen. Daneben spielt der Einfluss von Wasser eine wichtige Rolle, indem mit steigendem Wassergehalt die Intensität des Farbkomplexes abnimmt. Aus diesen Gründen wurde auf den Einsatz dieses *Assays* verzichtet.

5.3.3. Proanthocyanidinbestimmung mittels Butanol-HCl-*Assay*

Generell wird der Butanol-HCl-*Assay* zur quantitativen Bestimmen von kondensierte PA im Pflanzenmaterial herangezogen. Trotzdem kann es je nach Matrix zu Interferenzen mit dem Probenmaterial kommen und damit zu strukturellen Änderungen des resultierenden Farbkomplexes (Schofield *et al*., 2001). So fällt die Farbintensität der Quebrachotannine weicher aus, als die der Hirsetannine (Hemingway & Karchesy, 1989). Durch die zusätzliche OH-Gruppe am C5-Atom des A-Rings im Quebracho, erhöht sich die Säurestabilität auf die Interflavanbindungen, wodurch es zu einer Reduktion der Farbintensität kommt (Giner-Chavez *et al*., 1997). Eine weitere Schwachstelle in der Methodik ist, dass auf Grund der unterschiedlichen Bindungsstellen und den unterschiedlichen Substituenten am A- oder B-Ring, es zu einem unterschiedlichen Farbverlauf kommen kann (Watermann & Mole, 1994). Desweiteren werden höher kondensierte Polyphenole nicht proportional in ihre Monomere gespalten, wodurch hier wiederrum das Absorptionsspektrum nicht linear verläuft (Rohr, 1999).

In dieser Arbeit wurde zwischen der Bestimmung der EPA im extrahierbaren Überstand und der Bestimmung von NEPA im verbleibenden Rückstand des Probenmaterials unterschieden. Die Quantifizierung der EPA spiegelt somit den Proanthocyanidingehalt in der Probe wider, wobei die Quantifizierung der NEPA auf die Bestimmung von höhermolekularen kondensierten Polyphenolen zielt. Beide Bestimmungsmethoden wurden mittels Butanol-HCl-*Assay* durchgeführt. Auf Grund des unpolaren Lösungsmittels n-Butanol als

Extraktionslösungsmittel können sich höhermolekulare hydrophobere PA besser in dem Lösemittel lösen. Die Quantifizierung der PA wird in einigen wissenschaftlichen Arbeiten mit Hilfe des Lambert-Beer'schen Gesetzes über den molaren Extinktionskoeffizienten berechnet. Rösch *et al*. (2003) haben zum Thema Sanddorn den PA-Gehalt mittels molaren Extinktionskoeffizienten über Cyanidin berechnet, wobei das Ergebnis als Cyanidinäquivalente angegeben wird. Wegen Mangel an käuflichen erwerbbaren oligomeren PA mit PG > 5, gestaltet es sich schwierig, eine adäquate Aussage über den PA-Gehalt mittels kongruenten Referenzsubtanzen zu machen.

Bereits 1978 wurde in der Arbeit von McMurrough & McDowell ein höherer Responsefaktor von (-)-Epicatechin mit 4-Dimethylaminocinnamaldehyde (DMAC) ermittelt, als bei (+)-Catechin mit DMAC, was auf einen unterschiedlichen Extinktionskoeffizienten hindeutet. Studien von Payne *et al*. (2010) haben den Gesamtproanthocyanidingehalt in verschiedenen Schokoladen mittels DMAC bestimmt und wiesen ebenfalls auf die Schwierigkeit bei der Berechnung über monomere oder oligomere Flavan-3-ole hin. Ihrer Studie nach würde die Berechnung über (+)-Catechin oder (-)-Epicatechin als Referenzsubstanz den Gesamtflavonoidgehalt in der Probe unterschätzen, wobei sie eher oligomere PA für die Quantifizierung empfehlen würden.

5.3.4. Vergleich der kolorimetrischen *Assays* untereinander

Die beschriebenen *Assays* können auf Grund ihrer Spezifikationen unterschiedliche Substanzklassen bestimmen. Eine Quantifizierung basiert auf einer schnellen Einschätzung der Konzentration, bezogen auf den Gesamtpolyphenol-, Flavonoid- und Proanthocyanidingehalt. Allen gleich ist die Berechnung eingehender Einzelphenole als Summenparameter, wobei sich das in Form einer Referenzsubstanz als Bezugspunkt ausdrückt und somit das Endergebnis als Äquivalente angegeben wird. Die drei beschriebenen *Assays* wurden nach ihrer Lösemittelunempfindlichkeit ausgewählt, genauso wie nach ihrer linearen Regression im breiten Konzentrationsbereich. Unterschieden werden sie darüber hinaus auch bezüglich ihrer Nachweisgrenze (**Tabelle 11**).

Die mit Abstand populärste Methode zur kolorimetrischen Bestimmung vom Gesamtpolyphenolgehalt ist der FOLIN-CIOCALTEU-*Assay*, wobei dieser nicht nur phenolische Komponenten misst, sondern auch die gesamte antioxidative Kapazität der Probe bestimmt. Darüber hinaus sind der größere Zeitbedarf und die Störanfälligkeit gegenüber reduzierenden Substanzen ein weiterer Nachteil. Die Empfindlichkeit der Methode ist um ein 4-faches geringer als die der anderen *Assays*. Aluminiumchlorid-*Assay* besitzt gegenüber

dem FOLIN-CIOCALTEU-*Assay* eine höhere Nachweisgrenze. Denoch ist der Aluminiumchlorid-*Assay* unempfindlicher gegenüber Störkomponenten.

Tabelle 11: Verwendete kolorimetrischen *Assays* kategorisiert nach ihrer Spezifikation.

Methode	Empfindlichkeits bereich [µg/mL]	LOD [µg/mL]	Vorteil	Nachteil
FOLIN-CIOCALTEU-*Assay*	5-50	2,35 ECÄ	Häufig angewandt, Einfach und relativ genau	Störung durch andere reduzierende Substanzen wie Zucker
Aluminiumchlorid - *Assay*	20-200	6,30 ECÄ	Nicht störanfällig, Spezifisch auf Flavonoide	zeitabhängig
Butanol-HCl-*Assay*	20-200	3,35 CyE	Nicht störanfällig Spezifisch auf kondensierte PA	zeitabhängig

Ein Nachteil der sauren Butanolyse besteht in der Zerstörung der Primärstruktur von oligomeren PA. Studien von Makkar *et al*. (1999) geben außerdem an, dass trotz des sauren Aufschlusses, immer noch ein hoher Anteil an kondensierten Tanninen im Rückstand verbleibt.

Der mittels Aceton-Wasser-Gemisch gewonnene *flüssige Kakaoextrakt* wurde mittels unterschiedlichen *Assays* vermessen und die Farbverläufe einander gegenübergestellt. Mit dem bloßen Auge erkennt man bereits die unterschiedlichen Farbverläufe und insbesondere die Dunkelfärbung um 50 - 70% herum (**Abbildung 23**). Rein optisch kann somit ebenfalls gezeigt werden, dass die drei unterschiedlichen phenolischen Substanzklassen eher durch ein 50 - 70%iges Aceton-Wasser-Gemisch extrahiert werden können.

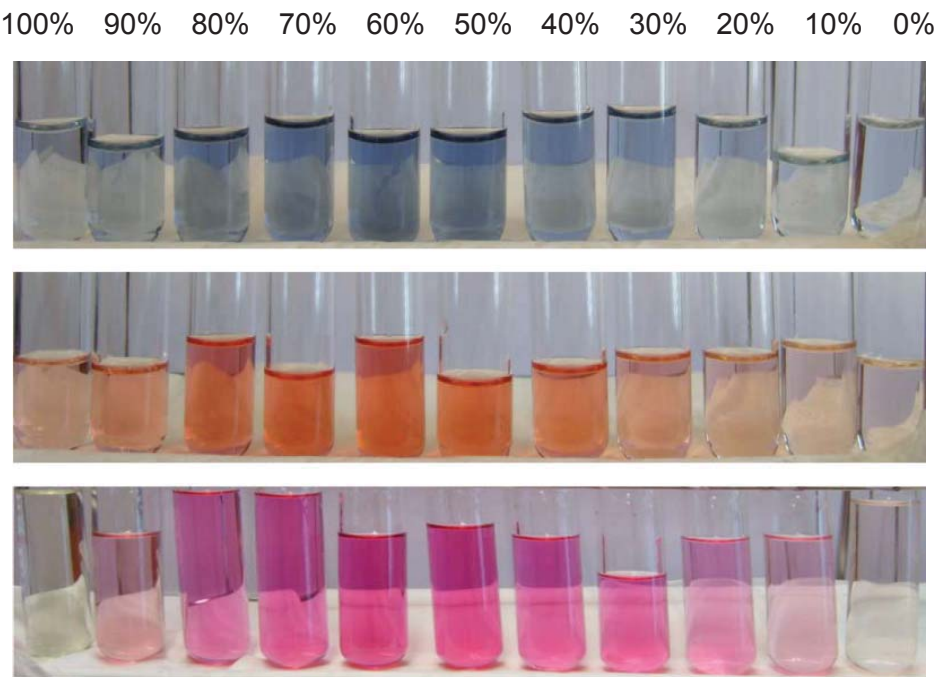

Abbildung 23: Vermessung des *flüssigen Kakaoextraktes* mittels unterschiedlichen kolorimetrischen Assays. Der angegebene Prozentsatz gibt das verwendete Aceton-Wasser-Extraktionsmittelgemisch wieder.

5.4. Einfluss ausgewählter Parameter auf die Extrahierbarkeit

Als potentielle Einflussfaktoren auf die Extrahierbarkeit von phenolischen Substanzen wurden Extraktionstemperatur, -kinetik und -zyklenanzahl ausgewählt.

5.4.1. Einfluss des Extraktionslösemittels

In der Literatur finden sich verschiedene Extraktionsmethoden, selten validiert in Bezug auf Extraktionslösemittel. So extrahieren Gu *et al*. (2013) entfettetes Kakaopulver für eine Stunde in kochendem Wasser. Cooper *et al*. (2008) benutzten ein Gemisch aus Aceton/Wasser/Ameisensäure (70/28/2, v/v/v) zur Extraktion phenolischer Komponenten aus der Kakaomatrix. Hauptsächlich werden polare Lösungsmittel wie Methanol, Ethanol verwendet, auch um durch Modifikation der Enzymstruktur über Oberflächeneffekte, oder Störung der Hydrathülle, die Enzymaktivität zu inhibieren. Die Inaktivierung der Enzymaktivität kann eine Oxidation verhindern und damit den Abbau der Polyphenole hemmen. Studien von Bonvehi & Coll (1997) und Waterhouse *et al*. (1996) greifen für die Extraktion auf 70%- oder 95%iges Methanol zurück, wobei diese Lösemittel häufig angewandt werden, um eine Sterilisation oder Inaktivierung von Mikroorganismen oder

Enzymen vorzubeugen. Einige Arbeiten gehen bei den Extraktionsversuchen vom reinen Lösemittel aus und greifen anschließend das Geeignetste auf, um daraus wiederum die bestgeeignete wässrige alkoholische Extraktionslösung zu generieren (Wollseifen, 2009). Ausgehend von dieser Herangehensweise würde man in dieser Arbeit dann fälschlicherweise von Methanol wässrige Auszüge generieren, da 100%iges Methanol ein stärkeres Extraktionsvermögen besitzt als 100%iges Aceton.

Neben dem kolorimetrischen FOLIN-CIOCALTEU-*Assay* erfolgte parallel dazu eine Auswertung der Extrakte mittels LC-DAD-ESI-MS. Die erhaltenen Chromatogramme weisen hierbei ähnliche Tendenzen zur Extraktionsausbeute auf, wie die ermittelten Werte nach FOLIN-CIOCALTEU. Durch eine rein wässrige Extraktion wurden die beiden Alkaloide Theobromin und Koffein aus der Probenmatrix extrahiert und ein geringer Teil des (-)-Epicatechins. Je höher nun der Anteil an Aceton im Extraktionslösemittel wurde desto höher war auch der Anteil an mitextrahierten Alkaloiden und ebenso der nieder- und höhermolekularen Polyphenolen (**Abbildung 24**). Laut Escribano-Bailón & Santos-Buelga (2003) eignet sich besonders Aceton zur Spaltung der Polyphenol-Protein-Komplexe. Da nun Kakao etwa 11 - 12% Protein enthält (Belitz *et al*., 2008), kann der erhöhte Extraktionsgehalt unter anderem durch die Spaltung dieser Komplexe erklärt werden.

Es zeigt sich im Chromatogramm, dass mit 100%igem Aceton ausschließlich Theobromin mit einem geringen Anteil an Koffein aus der Kakaomatrix extrahiert wurde. Die meisten phenolischen Substanzen konnten mit 50%igen wässrigen Aceton extrahiert werden. Neben dem Erscheinen von individuellen Polyphenolen im Chromatogramm, wird ebenfalls der sogenannte ‘PA Hügel‘ sichtbar, welcher bei einer Retentionszeit um 30 - 40 min eluiert. Es gibt viele Hinweise darauf, dass polymere Verbindungen auf Grund ihrer Komplexität sich nicht chromatographisch auftrennen lassen (Lazarus *et al*., 1999; Kalili & Villiers, 2009). Das Phänomen des ‘PA Hügels‘ lässt sich mit der geringen Bodenzahl und -höhe einer LC-Säule erklären, wobei mit zunehmendem Molekulargewicht eine erfolgreiche Auftrennung immer schwieriger wird.

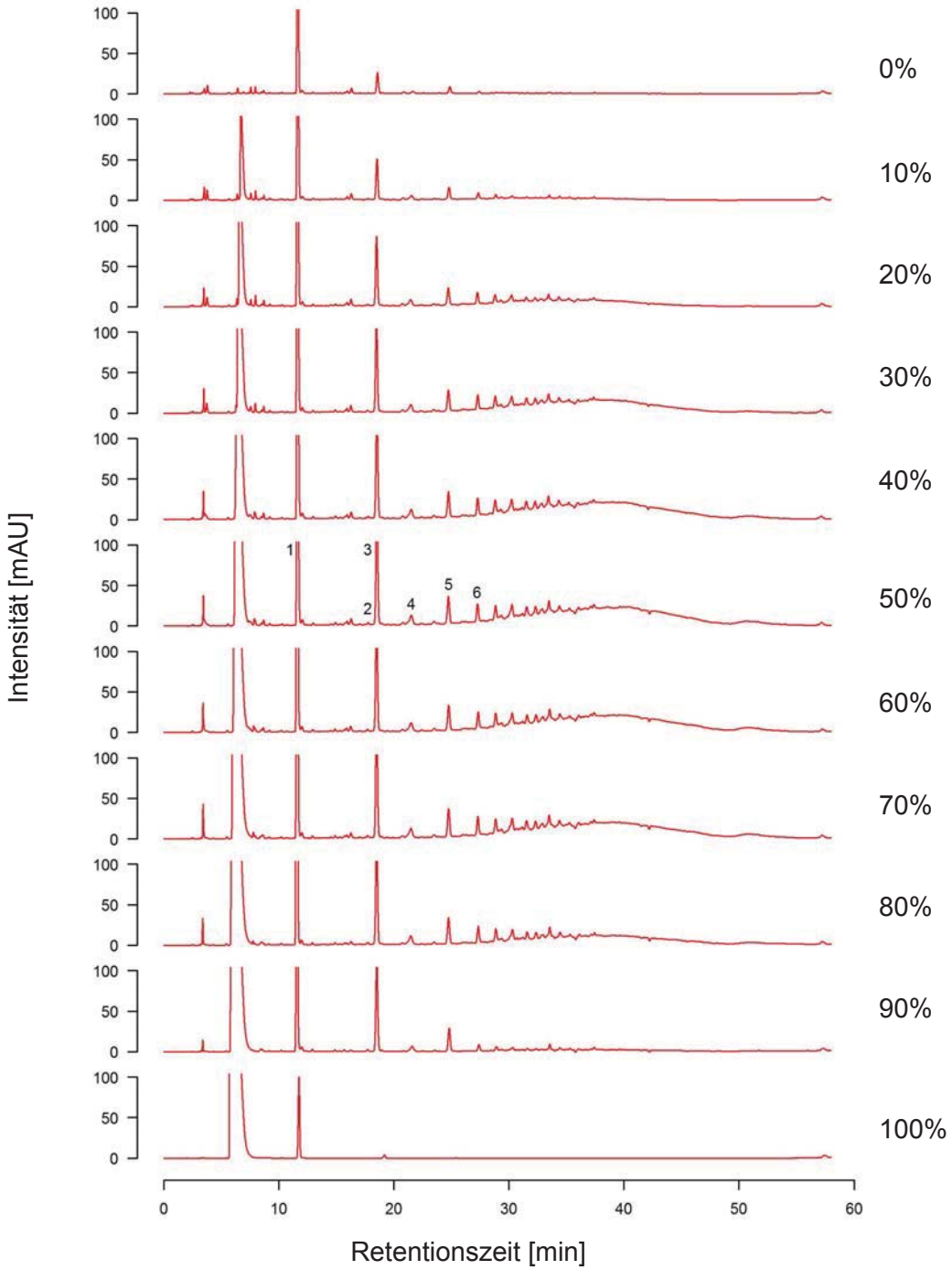

Abbildung 24: Einfluss des Aceton-Wasser-Gemisches auf die Extraktionsausbeute von phenolischen Substanzen. Identifizierte Inhaltsstoffe mit Peaknummer (1) Theobromin, (2) (+)-Catechin, (3) Koffein, (4) PA B2, (5) (-)-Epicatechin und (6) PA C1.

Für die weitere Optimierung der Probenaufarbeitung wurde mit 50%igen Aceton-Wasser-Gemisch als Extraktionslösemittel weitergearbeitet. Studien von Hammerstone und Chimel (2003) haben gezeigt, dass sowohl Aceton als auch Ethanol als die effektivsten Extraktionslösemittel für Kakao-PA gelten. Speziell für höher oligomere PA im Kakao mit

einem PG 5 - 10 ist der Effektivität im Vergleich zu anderen Lösungsmittel-Wasser-Gemischen besonders hoch. Die Widerfindungsrate vom dekameren PA stieg von 0% bei 100%igen Aceton auf über 100% bei 50%igen Aceton an. Monrad *et al*. (2010) untersuchten Traubentrester und fanden dagegen für 50%iges wässriges Ethanol das Extraktionsoptimum von PA im Vergleich zu anderen Ethanol-Wasser-Gemischen.

5.4.2. Einfluss der Extraktionstemperatur

Ein Faktor, welcher die Diffusionsgeschwindigkeit beschleunigt und damit die Extraktionsausbeute erhöhen kann, ist die Temperatur. Um den Einfluss der Extraktionstemperatur auf den Gesamtpolyphenolgehalt zu ermitteln, wurden Temperaturen von 20 °C bis 80 °C während eines Extraktionszyklus festgelegt. Hierfür wurden 2 g der Probe in ein 50 ml Zentrifugenröhrchen abgewogen und 2 mal mit 6 mL 50%igem Aceton für dreißig Minuten bei der jeweiligen Temperatur extrahiert. Die Zentrifugenröhrchen wurden in einem Labor-Tisch-Schüttler (Labor-Tisch-Schüttler HLC MKR 23, Huberlab, Schweiz) bei 700 Umdrehung/min geschüttelt. Der *flüssige Kakaoextrakt* wurde sowohl photometrisch vermessen, als auch mittels LC-DAD-ESI-MS näher charakterisiert.

Die chromatographische Auswertung des Temperaturverlaufs auf phenolische Substanzen ist in **Abbildung 25** dargestellt. Hierbei erkennt man einen leichten Anstieg vom (-)-Epicatechin von 1,5 mg/g bei 20 °C auf 1,7 mg/g bei 80 °C. Andere phenolische Substanzen zeigen ebenfalls nur einen zaghaften Anstieg bei höheren Temperaturen. So steigt das dimere PA B2 von 0,55 mg/g bei 20 °C auf 0,61 mg/g bei 80 °C an. Im Gegensatz dazu, steigt der Theobromingehalt von 11,1 mg/g bei 20 °C auf 15,2 mg/g bei 80 °C an.

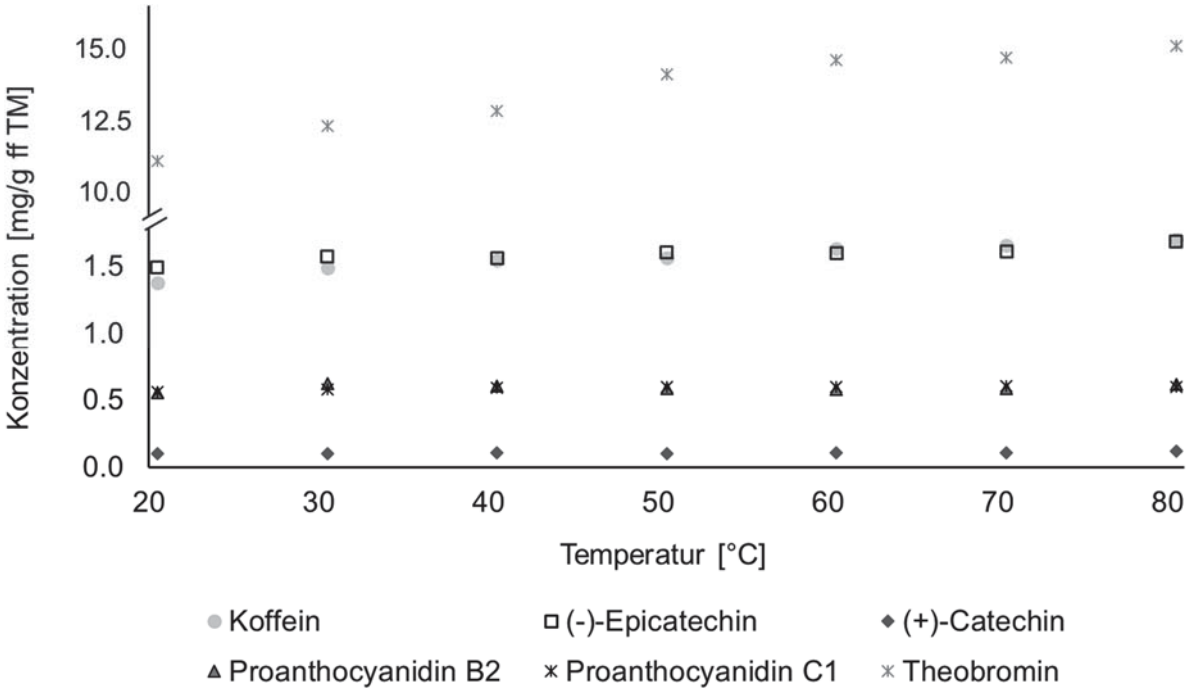

Abbildung 25: Konzentrationsänderung phenolischer Substanzen mit steigender Temperatur.

Obwohl eine Steigerung in der Extraktionsausbeute erreicht werden konnte, wurde für weitere Versuche eine Temperatur nicht höher als die des Acetonsiedepunktes gewählt, welche bei 56°C liegt. Somit konnte sichergestellt werden, dass für die betrachtete Analytgruppe ein gleichmäßiges und reproduzierbares Ergebnis erzielt werden konnte.

Bei natürlichen Rohstoffen wie *Camellia sinensis* werden Polyphenole bei Temperaturen von 70 °C extrahiert (Anesini *et al*. 2008) oder aber die Angabe der Temperatur wird unspezifisch als kochendes Wasserbad angegeben (Wang & Ruan, 2009). Palma *et al*. (2001) haben die Stabilität von phenolischen Verbindungen bei Temperaturen von 40 °C bis 150 °C untersucht. Es zeigte sich, dass bei Temperaturen von über 100°C die Zersetzung von phenolischen Verbindungen um 10 % ansteigt. Daneben ergab die gleiche Studie, dass bei Temperaturen von 50 °C und 100 °C eine gute Extraktionsausbeute generiert werden kann. Bei den Studien von Kothe *et al*. (2013) wurden die beiden Monomere (-)-Epicatechin und (+)-Catechin während des Röstprozesses untersucht und dabei einen Verlust von (-)-Epicatechin und den Proanthocyanidinen B1, B2 und B5 erst ab einer Temperatur von 100°C festgestellt.

5.4.3. Extraktionszeit und Extraktionszyklen

Um eine annäherend vollständige Extraktionausbeute sicherzustellen, wurde der Einfluss der Extraktionszeit auf den Gesamtpolyphenolgehalt gemessen. In **Abbildung 26** erkennt man bereits eine Sättigung des Extraktionslösemittels ab 8 Minuten. Eine längere Extraktionszeit führt daher nicht zur Steigerung des Extraktgehaltes.

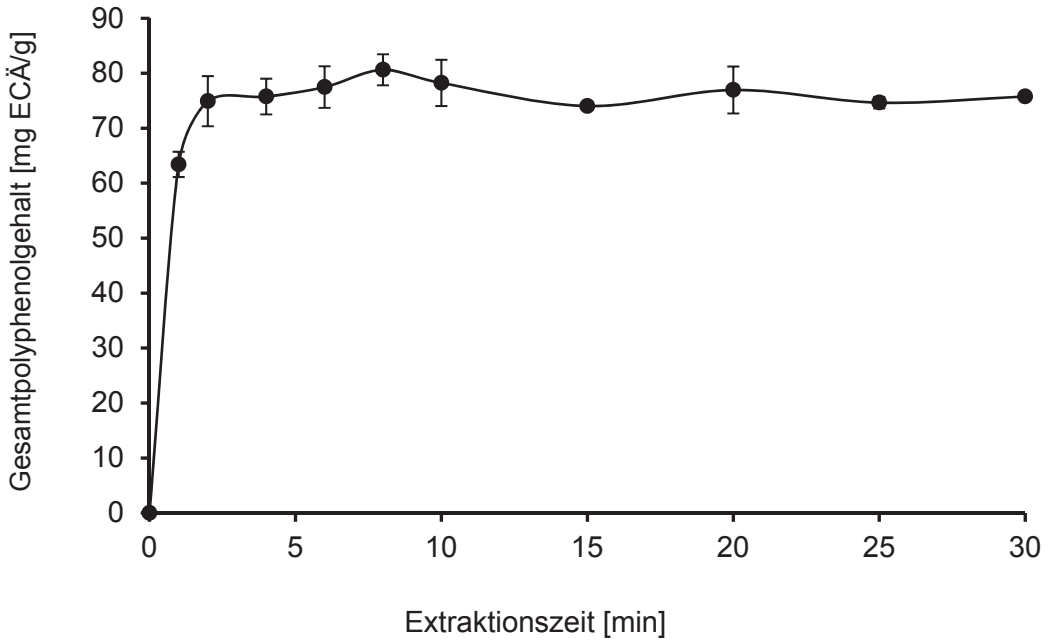

Abbildung 26: Bestimmung der Extraktionszeit anhand von ungerösteten Kakaobohnen bei 50 °C mit 50%igem Aceton. Die Fehlerbalken stellen die Standardabweichung mit einer Stichprobe von n = 3 dar.

Daneben wurde der Einfluss der Extraktionszyklen auf die Extraktionsausbeute ermittelt (**Abbildung 27**). Bezogen auf die Summe der durchgeführten Extraktionsschritte, ist erkennbar, dass im ersten Extraktionszyklus nur 52% der Gesamtpolyphenole extrahiert wurden. Für eine optimale Extraktionszyklenzahl müsste deswegen mindestens dreimal extrahiert werden, um eine annäherend vollständige Extraktionsausbeute von ca. 90% zu erhalten.

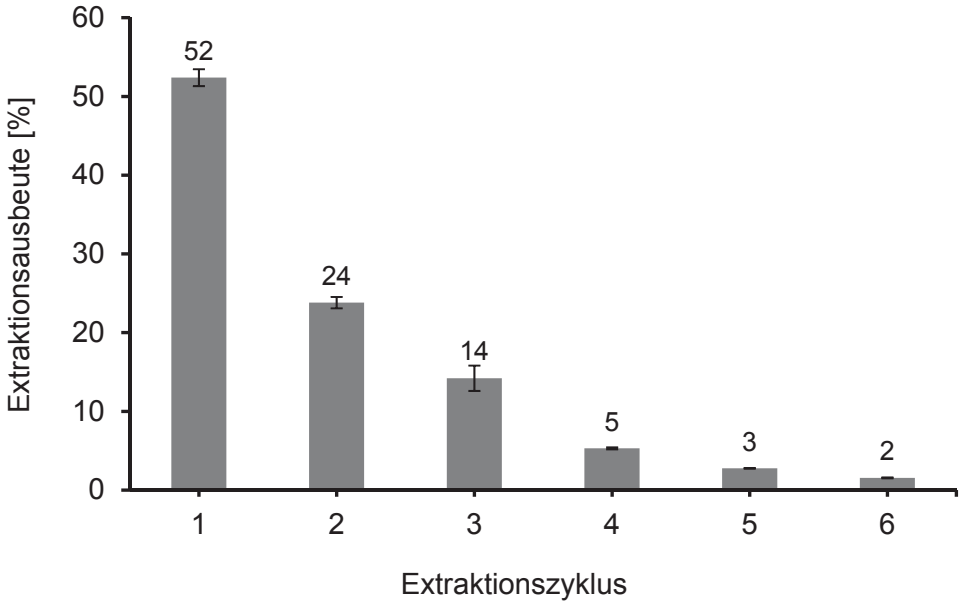

Abbildung 27: Extraktionsausbeute gemessen als Gesamtpolyphenolgehalt. Die Fehlerbalken stellen die Standardabweichung mit einer Stichprobe von n = 3 dar.

Zusammenfassend werden in **Tabelle 12** die optimalen Extraktionsbedingungen aufgezeigt. Auf 1 g Probe wurden 3 mL 50%iges Aceton gegeben und bei 50 °C für 8 min dreistufig extrahiert. Die Überstande wurden miteinander vermengt und auf extrahierbare Polyphenole (EPP) oder extrahierbare Proanthocyanidine (EPA) hin analysiert. Das Extraktionsvolumen und die damit verbundene Lösungsmittelmenge wurden so gering wie nötig gehalten, um für nachfolgende SCPC Probenaufarbeitungen den organischen Lösemittelanteil minimal zu halten.

Tabelle 12: Optimale Extraktionsparameter.

Extraktionsparameter	**Einstellung**
Extraktionsmittel	50% Aceton
Anzahl der Extraktionszyklen	3
Extraktionsdauer	8 min
Extraktionstemperatur	50 °C
Probe-zu-Lösungsmittel-Verhältnis	1 g auf 3 mL

5.5. Kolorimetrische Verfahren zur Bestimmung unlöslicher Polyphenole

Kakaopolyphenole setzen sich zusammen aus löslichen Polphenolen, die mit einem Alkohol-Wassergemisch aus der Probe herausextrahiert werden und unlöslichen kondensierten Polyphenolen oder Polyphenolen, im Komplex mit Proteinen und Polysacchariden, die nicht mittels organischer Lösemittel herausgelöst werden können. Durch RP-HPLC Analysen werden hauptsächlich Informationen über lösliche Proanthocyanidine, mit einem PG von 30,

generiert. Ein Grossteil der Proanthocyanidine bleibt jedoch im Rückstand zurück. Da sie gleichwohl von Bedeutung im humanen Metabolismus sind, ist deren Analytik von grosser Bedeutung. Vor allem in Kakaobohnen und ihrem Endprodukt Schokolade werden Gehalte von 2,3 mg/g bis zu 94 mg/g Procyanidinäquivalente gemessen (USDA, 2004).

Einfache kleinmolekulare PA sind im Pflanzengewebe in geringerer Menge vorhanden als höhermolekulare PA (Czochanska *et al*., 1980, Foo & Porter, 1980). Durch die geringe Löslichkeit von höhermolekularen PA in gängigen Lösemitteln, bleibt bei der Extraktion ein großer Teil im Rückstand, so dass die Quantifizierung der unlöslichen Proanthocyanidine einen zusätzlichen Reaktionsschritt benötigten. Die Erhitzung im sauren Milieu führt zu einer Depolymerisierung von Proanthocyanidinen, die an Polysaccharide oder Proteine gebunden sein können. Die Butanolyse stammt ursprünglich von Swain und Hillis (1959) und wurde anschließend von Bate-Smith (1973) modifiziert und von anderen übernommen (s. Reed *et al*. 1982, Porter *et al*. 1986). Hierbei werden NEPAs durch Depolymerisierung im sauren, organischen Milieu gemessen. Durch die säurekatalysierte Spaltung der Interflavan-Bindung werden PA in die Flavyliumione Anthocyanidin und in Xanthylium-Ion gespalten (**Abbildung 18**), wobei neben den niedermolekularen PA auch höhermolekulare PA und PA, die mit Proteinen und Polysacchariden verbunden sind, gespalten werden (Zurita *et al*. 2012). Über unterschiedliche Referenzsubstanzen können Kalibrationsgeraden erstellt und durch diese der quantitative Anteil der NEPA berechnet werden. In **Tabelle 13** wurden für drei gängige phenolische Referenzsubstanzen der geeignete Konzentrationsbereich für das BuOH-HCl-*Assay* bestimmt und die Kalibrationsgeraden ermittelt.

Tabelle 13: Phenolische Referenzsubstanzen und ihre Kalibrierungen mittels BuOH-HCl-*Assay*

Substanzen	Konzentrations bereich [g/L]	Kalibrationsgerade	Regressions-koeffizient	LOD-Wert [µg/mL]
Cyanidin	0,01 - 0,07	y = 13,137x - 0,009	R^2 = 0,9999	0,014
PA B2	0,02 - 0,16	y = 3,745x + 0,0519	R^2 = 0,9986	0,020
Gerbsäure	4 - 40	y = 0,019x + 0,0484	R^2 = 0,9947	0,020

Um den Einfluss der Temperatur auf die Extrahierbarkeit der NEPA verfolgen zu können, wurden ungeröstete Kakaobohnen mittels 50%igem Aceton für 8 Minuten bei jeweils unterschiedlichen Temperaturen von 20 - 140 °C erschöpfend extrahiert, was einem sechsstufigen Extraktionszyklus entspricht. Der Überstand wurde verworfen und der Rückstand für 5 h gefriergetrocknet. Anschliessend wurde der Gehalt an NEPA mittels BuOH-HCl-*Assay* bestimmt. Aus der Auftragung der NEPA gegen die verwendete Extraktionstemperatur erkennt man eine annäherend U-förmige Kurve (**Abbildung 28)**,

wobei ein deutlicher Abfall zu höheren Temperaturen von 20 - 100 °C zu erkennen ist mit einem Minimum bei 100 °C. Ab 100 °C steigt der Gehalt an NEPA im Rückstand wieder an.

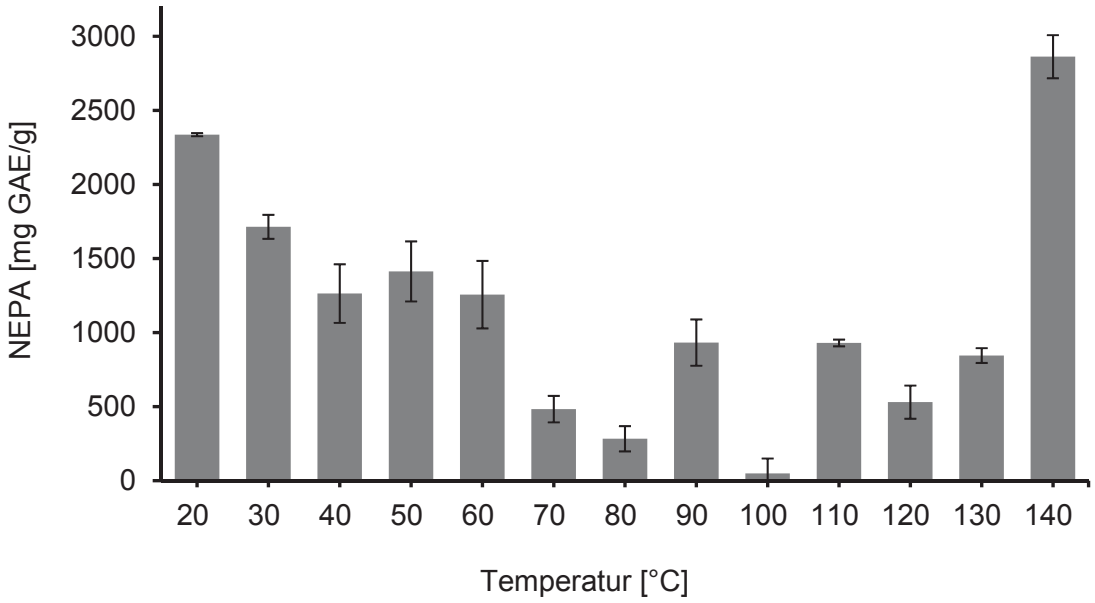

Abbildung 28: Extraktion der NEPA aus dem Rückstand als Funktion der Temperatur. Die Fehlerbalken stellen die Standardabweichung mit n = 2 dar. Die Ergebnisse wurden als mg Gallussäureäquivalente pro Gramm fettfreie Trockenmasse dargestellt.

Es findet bei der temperaturabhängigen Extraktion von 20 - 100 °C ein Abbau der NEPA statt, wobei um 100 °C ein Minimum erreicht wurde. Im Gegensatz dazu konnte eine Zunahme an EPA zu höheren Temperaturen hin beobachtet werden (Publikation 1, Abbildung 3). Eine Aussage, ob es zu Umwandlungsreaktionen zwischen NEPA und EPA kommt, kann hierbei jedoch nicht getätigt werden. Eventuell lassen sich hierbei Rückschlüsse auf eine Polymerisierung ziehen. Im Gegensatz dazu wurde bei den EPA (Publikation 1, Abbildung 3) ein tendenzieller Abfall bei diesen Temperaturen beobachtet.

5.6. NP-LC-online-DPPH Methodenvalidierung

In Rahmen dieser Arbeit wurde die NP-LC-online-DPPH Methode betreffs ihrer analytischen Eignung auf qualitative und quantitative Bestimmung von oligomeren Proanthocyanidinen hin überprüft. Hierfür wurden verschiedene Beurteilungsgrößen herangezogen, wie die Verwendung des Nachsäulenderivatisierungsreagenzes und bezüglich Reaktionszeit und -temperatur optimiert. Die verwendeten Geräteparameter des NP-LC-online-DPPH Systems sind im Anhang aufgeführt.

Die verwendete NP-HPLC-online-DPPH Methode stützt sich auf kolorimetrische Voruntersuchungen aus Kapitel 2.6. Ausgehend von der Methode nach Blois (1958) und weiter entwickelt von Brand-Williams *et al*. (1995), wurde eine DPPH-Stammlösung mit 24 mg DPPH in 550 mL Methanol hergestellt, wobei der Extinktionswert bei 1,1 ± 0,2 AU liegen soll. Wie aus der Voruntersuchung hervorgeht, baut die frisch hergestellte DPPH Lösung in den ersten 24 h ihre dunkel violette Farbe ab, wobei es im Detail zu einer Absorptionsabnahme um ca. 20% kommt. In den darauffolgenden 130 h wurde dagegen keine weitere Abnahme beobachtet. In **Abbildung 29** wurde die Absorptionsabnahme des DPPH-Radikals als Funktion der Zeit bei dem kolorimetrischen Versuchsaufbau dargestellt. Für die NP-HPLC-online-DPPH Methode wurde somit vor Versuchsbeginn die DPPH Lösung jeweils für 24 h im Dunklen bei Raumtemperatur gelagert, eher sie für die *online*-Messung verwendet wurde. Speziell für längere NP-LC-online-DPPH Sequenzen kann somit auf eine konstante Derivatisierungslösung gesetzt werden.

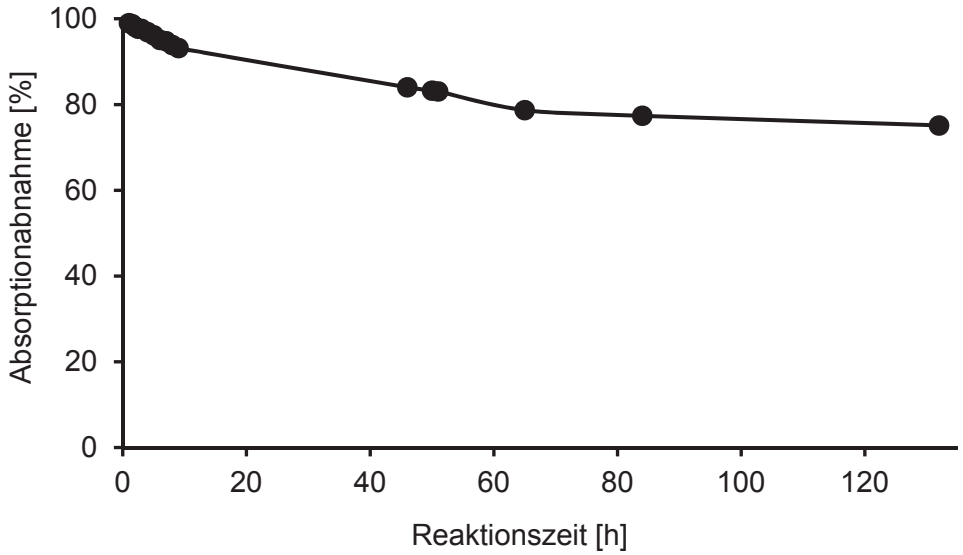

Abbildung 29: DPPH Absorptionsabnahme über die Zeit. Die Standardabweichung stellt das Ergebnis aus n = 2 Messungen dar.

Innerhalb des NP-LC-online-DPPH Systems ist die Auswahl der Reaktionskapillare entscheidend, da sie einen Einfluss auf die Reaktion und damit das Ergebnis hat. Im Speziellen ist laut Raudonis *et al*. (2010) die Veränderung der Länge, des Innendurchmessers und der Flussrate massgebend. Die optimalen Parameter wurden mittels kolorimetrischer DPPH-*Assay* entwickelt und auf den NP-LC-online-DPPH *Assay* übertragen. Die Länge der Reaktionskapillare wurde auf eine Reaktionszeit von ca. 2 min angepasst. Das Hochsetzen der Reaktionstemperatur auf 60 °C gewährleistet eine schnellere Reaktionskinetik. Für eine optimale Durchmischung der DPPH Lösung mit dem Analyten in der Reaktionskapillare wurde die Flussrate der DPPH Dosierpumpe mit der NP-LC-Pumpe gleichgesetzt.

5.6.1. Kalibrationsstrategie

Bei der Kalibrationsstrategie wurde die Verwendung eines internen oder externen Standards auf Eignung und Anwendbarkeit auf das beschriebene System hin überprüft. Bei der Verwendung eines internen Standards kam es zu Interferenzen mit anderen phenolischen Substanzen im Chromatogram, wobei alle im Anhang aufgelistet phenolischen Substanzen in Betracht gezogen wurden. Für die Quantifizierung oligomerer PA wurde auf die Berechnung mittels externen Kalibrationsgeraden zurückgegriffen. Hierfür wurden kommerzielle Referenzsubstanzen wie (-)-Epicatechin, Proanthocyanidin B2, Proanthocyanidin C1 und Cinnamtannin A2 mit einer Konzentration von 1 mg/mL hergestellt. Die Kalibrationsgeraden wurden im Konzentrationsbereich von 0,05 g/L bis 1 g/L angefertigt und die Extinktionen sowohl im UV- als auch im Vis-Bereich aufgenommen.

Die Ermittlung der Bestimmungsgrenze (LOQ = *Limit of Quantification*) sowie der Nachweisgrenze (LOD = *Limit of Detection*) wurden für das System erhoben. Für die Bestimmung des LOD-Wertes wurde das Signal/Rausch-Verhältnis von 1:3 und für die Bestimmung des LOQ-Wertes ein Signal/Rausch-Verhältnis von 1:10 definiert (Kromidas und Kuss, 2008). Die Berechnung der LOD and LOQ Werte erfolgte nach der Kalibriermethode mit linearer Regression in einem Konzentrationsbereich von 50 µg/mL bis 400 g/L. Mit der NP-LC-online-DPPH Methode konnte im UV-Bereich (-)-Epicatechin mit einer Nachweisgrenze von LOD = 7 mg/L nachgewiesen werden, PA B2 mit einem LOD von 12 mg/L, PA C1 mit einem LOD von 17 mg/L und Cinnamtannin A2 mit einem LOD von 21 mg/L. Hierbei erkennt man einen leichten Anstieg der Nachweisgrenze zu höherem PG hin. Dies könnte als Anzeichen gewertet werden, daß die UV-Aktivität der PA zu höheren PGs sinkt und somit hochmolekulare kondensierte PA mittels UV-Detektion nur schlechter detektiert werden. Ähnliches wurde für die Bestimmung der Nachweisgrenze bei der Vis-

Detektion ermittelt. Hier wurde für (-)-Epicatechin ein LOD von 27 mg/L nachgewiesen, für PA B2 ein LOD von 6 mg/L, für PA C1 ein LOD von 11 mg/L und für Cinnamtannin A2 ein LOD von 16 mg/L. Eine prinzipielle Abnahme der Nachweisgrenze zu höheren PG konnte für den Vis-Bereich nicht bestätigt werden.

Daneben deuten Studien von Esatbeyoglu (2010) darauf hin, dass Oligomere eine niedrige Absorption haben und dadurch bedingt kleine Peakhöhen, was einen fälschlicherweise niedrigeren Gehalt vortäuschen kann. In dieser Arbeit erfolgt die quantitative Bestimmung einzelner oligomerer PA im *flüssigen Kakaoextrakt* einerseits über (-)-Epicatechin als Bezugsgrösse, wobei die Ergebnisse als (-)-Epicatechinäquivalente angegeben wurden. Diese Berechung hat den Vorteil, dass alle oligomeren PA ungleich ihrer Molmassen auf eine einheitliche Bezugsgrösse normiert wurden. Andererseits nimmt die UV-Aktivität mit steigender Kettenlänge ab. Somit wird mittels einheitlicher Gleichstellung die UV-Aktivität von oligomerer PA nicht mitberücksichtigt. Somit wurde bei der Berechnung über (-)-Epicatechinäquivalente der Anteil polymerer PA mit PG > 13 um ca. 60% unterschätzt Der Anteil oligomeren PA hingegen wurde sogar um ca. 70% unterschätzt (Publikation 3, Tabelle 2, 3).

Nebst dessen, wurde in dieser Arbeit zur Quantifizierung oligomeren PA strukturell ähnliche Referenzsubstanzen herangezogen, wobei monomere PA über (-)-Epicatechin bestimmt wurden, dimere PA über das PA B2, trimere PA über das PA C1 und Tetramere und PA mit einem PG ≥ 4 über das Cinnamtannin A2. Diese Berechnungsmethoden erlaubten eine quantitative Annäherung an realistische Mengenangaben.

5.6.2. Bestimmung der antioxidativen Kapazität von oligomeren Proanthocyanidinen

Für die Bestimmung der antioxidativen Kapazität von oligomeren PA werden meistens *offline* Methoden verwendet, wobei mittels eines chromatographischen Trennsystems zuerst PA fraktioniert und isoliert werden und anschließend die antioxidative Kapazität gemessen wird. Ein *offline*-System schränkt die Betrachtung isolierter phenolischer Substanzen und ihrer Wechselwirkungen untereinander oder mit anderen Lebensmittelinhaltsstoffen ein. Über die antioxidative Kapazität von oligomeren PA zeigt sich, dass sie teilweise niedriger und teilweise höher als ihre monomeren Verbindungen sind. Lotito *et al*. (2000) postulierte für monomere, dimere und trimere PA eine höhere antioxidative Kapazität im wässrigen Milieu, für höher molekulare PA dagegen zeigt sich eine erhöhte antioxidative Kapazität im lipophilen Milieu. Studien von Lu und Yeap Foo (2000) zeigten für trimere und tetramere PA eine höhere antioxidative Kapazität als für PA B2 und (-)-Epicatechin. Die teils recht großen

Unterschiede werden von den Autoren einerseits auf die stereochemischen Unterschiede, andererseits auf die verwendete Messmethode zurückgeführt.

In dieser Arbeit konnte gezeigt werden, dass oligomere PA speziell das Nonamer und Decamer, bezogen auf ECÄ eine hohe antioxidative Kapazität, besitzen. Trotz ihres hohen PG und ihrer Stereochemie sind sie in der Lage über Dihydroxygruppen Radikale effizient abzufangen. Demgegenüber wies der ‘PA Hügel‘ mit den polymeren PA eine eher geringere antioxidative Kapazität auf. Mit einem PA-Gehalt von 23,3 mg ECÄ/g in der frischen Kakaobohne und somit einem Anteil an EPA von rund ca. 40% trägt der ‘PA Hügel‘ dagegen nur mit ca. 6% zur antioxidativen Kapazität bei (vgl. Publikation 3, Tabelle 1). Damit bestätigt sich auch die Vermutung, dass die antioxidative Kapazität nicht mit höherem Molekulargewicht steigt, sondern dass sie im Laufe der homologen Reihe stagniert.

5.6.3. Vergleich der ermittelten Proanthocyanidingehalte mit Literaturangaben

Nur wenig ist über den Einfluss des Schokoladenherstellungsprozesses auf qualitative oder quantitative Aussagen zu oligomeren PA bekannt. Im Fokus standen vermehrt Änderungen des Gesamtpolyphenolprofils als Summenparameter oder Änderungen einzelner monomerer phenolischer Substanzen. In dieser Arbeit wurden sechs Stadien des Schokoladenherstellungsprozesses und deren Einfluss auf oligomere und polymere Proanthocyanidine untersucht (Publikation 3, Tabelle 1).

Ein Abgleich mit der Literatur bestätigte die stete Abnahme der Summe der oligomeren PA ∑ (PG1 - PG13) im Laufe des Prozesses. Angefangen von der frischen Kakaobohne mit einem Anteil an ∑ (PG1 - PG13) von ca. 30,0 ± 4,0 mg ECÄ/g sinkt der Anteil von ∑ (PG1 - PG13) auf ca. 26,7 ± 0,3 mg ECÄ/g während der Fermentation (vgl. Publikation 3, Tabelle 1). Ein ähnlicher Prozentsatz wurde von De Brito *et al*. (2000) während ihrer Untersuchungen bezüglich des Gesamtpolyphenolgehaltes festgestellt, wobei der Gehalt leicht von 231 ± 5 mg/g fettfreier Trockenmasse zum Zeitpunkt 0 h auf 213 ± 5 mg/g nach 72 h sank. Aikpokpodion & Dongo (2010) detektierte dagegen einen deutlicheren Abfall des Polyphenolgehaltes von 161.1 mg/g an Tag 0 auf 60.1 mg/g an Tag 6. Während der Fermentation bilden sich wegen der Polyphenoloxidasen höher molekulare PA, die in dieser Methode als ‘PA Hügel‘ detektiert wurden. Deren Anteil widerrum stieg, von 23,3 ± 3,9 mg ECÄ/g auf das doppelte von 54,1 ± 3,5 mg ECÄ/g an.

Einen deutlich höheren Einfluss auf oligomere PA besitzt der Röstprozess. Durch thermisches Einwirken wurde in dieser Arbeit der Anteil an PA von ∑ (PG1 - PG13) auf 12,1

mg ECÄ/g drastisch reduziert, was eine Abnahme von oligomeren PA von ca. 65% bedeutet. Studien von Oracz *et al*. (2015) beschrieben den Impakt auf individuelle Flavan-3-ole während des Röstvorgangs (110, 120, 135, and 150°C) und bestätigten die Abnahme von (-)-Epicatechin, PA B2, C1 und Quercetin Gucosid, wobei es gleichzeitig zu einer Zunahme von (+)-Catechin and Quercetin (Aglykon) kam. Beim Rösten wird die Fähigkeit von Polyphenolen Interaktionen mit Proteinen einzugehen gemindert, wodurch es zur Abnahme der Adstringenz kommt (Misnawi *et al*., 2005). Vorzugsweise setzen Schokoladenhersteller wegen der leichten Handhabung entweder auf Bohnen- oder Nibröstung. Bedingt durch die Größe kommt es innerhalb der ganzen Kakaobohne jedoch zu einer ungleichen Aufheizrate, wobei somit die Kerntemperatur nicht an allen Stellen gleich ist. Die Beeinflussung des Gesamtpolyphenolgehaltes findet über die Temperatur-Zeit-Kombination statt. Demnach führen auch relativ niedrige Temperaturen wie in dieser Arbeit von ca 60 °C zu Verlusten von sowohl oligomeren PA ∑ (PG1 - PG13) als auch polymeren PA mit PG > 13. Oracz und Nebesny (2016) analysierten fünf verschiedene Kakaosorten und deren Verlauf der antioxidative Aktivität während des Röstvorgangs. Hierbei wurde bestätigt, dass die antioxidative Kapazität bei rohen Kakaobohnen am größten war. Außerdem konnte gezeigt werden, dass die antioxidative Kapazität mit steigender Rösttemperatur für alle Kakaosorten sank. Der Gesamtpolyphenolgehalt der Trinitario Varietät aus Venezuela stieg leicht von 167 mg GAE/g bei der rohen Kakaobohne auf 169 mg GAE/g bei 110 °C und sank kontinuierlich auf 164 mg GAE/g bei 120 °C und 147 mg GAE/g bei 150 °C. Insgesamt konnte in dieser Arbeit gezeigt werden, dass durch die thermische Aufarbeitung während des Röstprozesses der Gehalt an oligomeren PA ∑ (PG1 - PG13) als auch polymeren PA um ca. 50% reduziert wurde.

Beim nachfolgenden Conchieren konnte der Einfluss der Oxidation auf oligomere und polymere PA näher untersucht werden. Während des Conchiervorgangs wurde die Schokoladenmasse unter Zugabe von Lecithin und Zucker kontinuierlich gerührt, belüftet und geknetet. Während der ersten Stunde wurden ca. 30% der oligomeren PA ∑ (PG1 - PG13) und polymeren PA mit PG > 13 abgebaut. Innerhalb von 4 h, an denen die Schokoladenmasse dem Luftsauerstoff ausgesetzt war, konnte ein fortschreitender signifikanter Abbau bei polymeren PA mit PG > 13 nachgewiesen werden, jedoch kein signifikanter bei oligomeren PA ∑ (PG1 - PG13) ($p > 0,05$). In einer kürzlich veröffentlichten Studie von Gültekin-Özgüven *et al*. (2016) konnte dagegen kein unmittelbarer Effekt auf den Abbau vom Gesamtpolyphenol- oder Gesamtflavanoidgehalt feststellen werden. Di Mattia *et al*. (2014) ermittelten für lange Conchierzeiten (12 h bei 60 °C) einen geringen Abbau des Gesamtprocyanidingehalts, wohingegen bei kürzeren Conchierzeiten und höheren Temperaturen (6 h bei 90 °C und 1 h bei 60 °C) eine leichte Erhöhung des

Gesamtprocyanidingehalt festgestellt werden konnte. Im Allgemeinen ist festzuhalten, daß eine höhere Conchiertemperatur und niedrigere Conchierzeiten eher dem Abbau von Proanthocyanidinen entgegenwirken.

Schokolade war noch nie nur eine Süssigkeit, sondern wurde seit jeher als Genussmittel verzehrt. So unterschiedlich seine Herstellung ist, so unterschiedlich sind auch seine Gehalte an wertgebenen Inhaltsstoffen. Todorovic *et al*. (2015) untersuchten zwölf kommerzielle, serbische Schokoladen auf deren Gesamtpolyphenolgehalt und ermittelt einen Anteil von 2,03 bis 33,86 mg GAE/g Probe. Der Gesamtproanthocyanidingehalt ermittelt mittels BuOH-HCl-*Assay* ergab einen Wert von 0,49 bis 7,39 mg CyÄ/g Probe. Studien von Miller *et al*. (2006) ergaben bei der Untersuchung von 19 US-amerikanischen Schokoladen einen Gesamtpolyphenolgehalt von 3,66 bis 60,22 mg GAE/g Probe und einen Gesamtproanthocyanidingehalt ermittelt mittels HPLC von 0,37 bis 23,71 mg/g. In dieser Arbeit wurde nach einem 12 h Conchierprozess die Schokoladenmasse temperiert und in Tafeln abgepackt. Hierbei konnte immer noch ein hoher Anteil an oligomeren PA ∑ (PG1 - PG13) von 5,74 mg ECÄ/g Probe und ein polymerer PA-Anteil von 19,68 mg ECÄ/g Probe ermittelt werden. Im Gegensatz zur Studie von Di Mattia *et al*. (2014) konnte auch bei höheren Conchierzeiten und niedrigeren Temperaturen ein leicht erhöhter Anstieg an polymeren PA mit PG > 13 beobachtet werden. Eine signifikante Aussage konnte jedoch auch hier nicht getätigt werden.

Im Allgemeinen ist festzuhalten, dass schon bei der rohen Kakaobohne als Ausgangsprodukt zur Schokoladenherstellung viel Wert auf die Auswahl gelegt werden muss. Wertgebende Inhaltsstoffe sind nicht nur von einem intelligenten Verarbeitungsprozess, sondern auch von Anbau-, Ernte- und Fermentationsbedingungen abhängig. Dafür sollte nicht nur in den jeweiligen Verarbeitungsländern, sondern auch in den Ursprungsländern, ein rückverfolgbarer Arbeitsverlauf stattfinden, um so optimale Bedingungen für den Erhalt von bioaktiven Inhaltsstoffen zu schaffen. Hier könnte die etablierte NP-HPLC-online-DPPH als vielversprechende Methode zur Qualitätskontrolle und zum Prozessmonitoring eingesetzt werden.

Trotzdem konnte in dieser Studie keine qualitative Aussage über den Verbleib der abgebauten Polyphenole während des Schokoladenherstellungsprozesses getätigt werden. Zwar erkennt man einen kontinuierlichen Abfall der PA über die gesamte Wertschöpfungskette, einen Anhaltspunkt zum Verbleib oder der jeweiligen Umwandlung konnte jedoch nicht gefunden werden.

5.7. Semipräparative Isolierung kondensierter Polyphenole

Eine Kopplung zwischen der zentrifugalen Gegenstromverteilungschromatographie und der Gelpermeationschromatographie mittels Sephadex LH-20 wurde erarbeitet, um phenolische Substanzklassen mittels SCPC zu fraktionieren und weiter mittels Gelpermeationschromatographie in Einzelkomponenten voneinander zu trennen.

5.7.1. Isolierung eines proanthocyanidinreichen Kakaoextrakts mittels SCPC

Für die semipräparative Auftrennung wurden ungeröstete Kakaobohnen nach **Tabelle 13** extrahiert und gefriergetrocknet. Umgerechnet erhielt man für 1 g fettfreies Kakaopulver einen *gefriergetrockneten Kakaoextrakt* von 0,280 g ± 0,004 g (n = 10). Für die SCPC-Trennung wurden demnach ca. 0,7 g des *gefriergetrockneten Kakaoextraktes* in 8 mL 40%igem 2-Propanol gelöst und in das SCPC System injiziert. 40%iges 2-Propanol stellte sich unter allen Alkohol-Wasser-Gemischen als effektivstes Lösungsmittel heraus, wobei der Extrakt hier vollständig ohne Verklumpung in Lösung übergeht.

Wie bereits in der Einleitung erwähnt, ist für eine effektive SCPC Trennung die Wahl eines geeigneten Lösungsmittelsystems entscheidend. Hier gilt der Satz „Gleiches löst sich im Gleichen“. In dem in der Einleitung beschriebenen *descending* Modus eluiert demzufolge zuerst die wässrige Phase mit den darin enthaltenen hydrophilen phenolischen Substanzen, wobei nach dem Umschalten zum *ascending* Modus eher unpolare phenolische Substanzen von der SCPC-Säule eluieren. Das gewählte Lösungsmittelsystem ergab folgende Farbreihenfolge, angefangen mit einem kräftigen braunen Eluat (Fraktion I), welches über Zeit sich in ein rötliches (Fraktion II) zu einem durchsichtigen Eluat (Fraktion III) veränderte (**Abbildung 30**). Nach dem Umschalten in den *ascending* Modus wurden sogleich ein roter Farbumschlag beobachtet, welcher der Fraktion IV zugeordnet wurde.

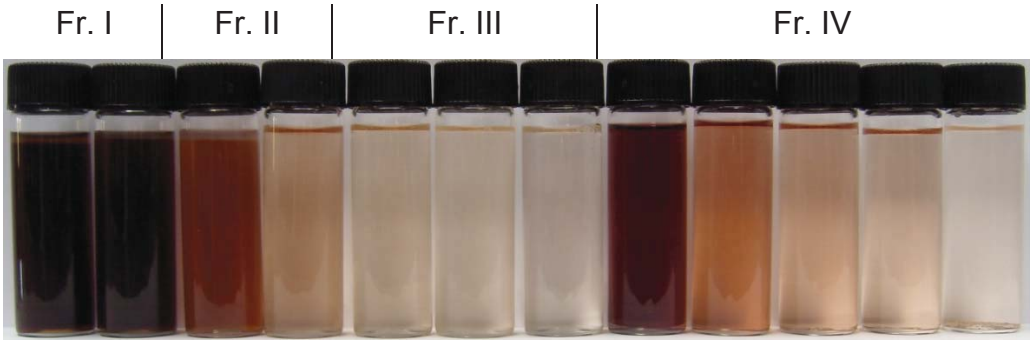

Abbildung 30: Farbverlauf der SCPC-Fraktionierung.

Die gelösten Ananlyten in Fraktion I konnten mittels LC-DAD-ESI-MS nicht aufgeklärt werden. Im Detail war hier im Chromatogramm ein Anstieg in der Basislinie zwischen 24 min bis 46 min zu erkennen, was auf den ‘PA Hügel‘ hindeutet. Eine mögliche Erklärung zur Farbgebung wurde in der Anwesenheit der braunen Phlobaphene gesehen. Fincke & Fincke (1965) beschreiben die Bildung von monomeren Gerbstoffvorstufen wie Catechin und Anthocyane zu wasserlöslischen farblosen Gerbstoffen während der Fermentation, die weiter über Umlagerungsreaktionen in wasserunlösliche, braune Phlobaphene übergehen. Trotz der kondensierten Phlobaphene und ihrer damit verbundenen Hydrophobizität lassen sie sich in der wässrigen Phase des Lösungsmittelsystems lösen. Hierbei spielt das azeotrope Lösungsmittelgemisch aus Ethylacetat und Wasser eine Rolle. In 100 mL Wasser lassen sich 8 mL Ethylacetat lösen, was zu einer Verschiebung in der Lösemittelpolarität führt, wodurch vermeintlich hydrophobe phenolische Substanzen einen guten Verteilungskoeffizienten in diesem Lösungsmittelsystem aufweisen. Im gleichen Elutionsmodus wurden die beiden Alkaloide Theobromin (Fraktion II) und Koffein (Fraktion III) getrennt.

Mit dem Umschalten zum *ascending* Modus wurden die Ethylacetat löslichen Proanthocyanidine eluiert. Ethylacetat als Extraktionslösungsmittel für PA wird häufig in wissenschaftlichen Arbeiten verwendet. So wurden monomere, oligomere und polymere PA aus Weintraubenextrakt von Shuting *et al*. (2014) mittels Säulenchromatographie voneinander getrennt, wobei zuerst phenolische Säuren mittels Phosphatpuffer eluiert wurden, gefolgt von oligomeren PA mit Ethylacetat und polymere PA mit Methanol. Auch Dumon (1990) fand Ethylacetat und Aceton-Wasser-Gemische als geeignete flüssig-flüssig-Systeme zur Extraktion von PA. Liu & White (2012) benutzten zur Extraktion von PA aus Weintraubenkernen ebenfalls ein Gemisch aus Ethylacetat-Wasser, wobei Ethylacetat alleine sehr wohl PA extrahieren kann sich, jedoch durch seine lange Reaktionszeit als unpraktisch erwies. Auch wurde beschrieben, dass Ethylacetat alleine nicht in der Lage sei, durch die Zellwand der Weintraubenkerne zu permeieren und deswegen der Zusatz von Wasser die Permeabilität und dadurch die Reaktionsrate steigert.

Studien von Esatbeyoglu *et al*. (2015) zeigen die Möglichkeit einer Isolierung von dimeren, trimeren, tetrameren und pentameren PA aus ungerösteten Kakaobohnen mittels HSCCC mittels dem Lösungsmittelsystem Tert-Butylmethlyether/n-Butanol/Wasser (4.3:0.7:5, v/v/v) auf. Eine direkte Übertragung vom HSCCC-System auf das SCPC-System kann jedoch nicht getätigt werden.

5.7.2. Isolierung individueller Proanthocyanidine mittels Gelpermeationschromatographie

Die PA-haltige Fraktion IV wurde weiter mittels Gelpermeationschromatographie auf Sephadex LH-20 aufgetrennt. Hierbei wurde die PA-haltige Fraktion IV ebenfalls in 40%igem 2-Propanol gelöst und auf die Säule injeziert, wobei PA in einer stufenförmigen Elutionssequenz in Einzelkomponenten isoliert wurden.

Für die Fraktionierung von oligomeren PA aus pflanzlichen Extrakten wurde bevorzugt auf Sephadex LH-20 als Sorptionsmittel zurückgegriffen. So fraktionierte Delcour *et al*. (1981) mittels Gelpermeationschromatographie auf Sephadex LH-20 monomere, dimere, trimere und oligomere PA aus Bier. Hierbei wurde in einer ersten Elutionsfolge aus 500 mL 60 %igem Aceton monomere PA gewonnen, wobei in einer zweiten Folge mit weiteren 650 mL 60%igem Aceton trimere und oligomere PA eluiert wurden. Lee (2013) griff in seinen Untersuchungen zur Aufreinigung von Proanthocyanidinen aus Cranberry Extrakten (*Vaccinium macrocarpon* Ait.) ebenfalls auf Sephadex LH-20 zurück. Hier wurde in einer Eluationssequenz zuerst mit Wasser gespült, anschließend mit 25%igem Methanol und darauffolgend mit 70%igem Aceton, wobei in diesem Schritt die PA-haltige Fraktion gesammelt wurde.

Aus Vorversuchen zeigte sich, dass Änderungen der Flussrate, eine Verschiebung in der Elution der oligomeren PA zur Folge haben. Deswegen wurde die Gelpermeationschromatographie mit Sephadex LH-20 in Bezug auf die Flussrate hin optimiert. Für ein *up-scaling* müsste man deswegen das chromatographische System auf die Flussrate hin optimieren. Dennoch, wurde im Gegensatz zu anderen Sorbentien für Sephadex LH-20 die besten Resultate bezüglich Absorption und Desorption festgestellt. Mittels 60%igem Aceton konnte sowohl die Säule regeneriert werden als auch die braunen Pigmente von der Säule schlussendlich eluiert werden (Delcour *et al*., 1981). Das Zurückbleiben von braunen Pigmenten auf der Säule deutet auf eine unvollständige Desorption von höher kondensierten PA hin, welche irreversibel mit der Festphase adsorbieren.

5.8. Detektion monomerer Polyphenole mittels LC-DAD-ESI-MS

5.8.1. Detektion monomerer Polyphenole mittels LC-DAD

Eine optimierte Probenvorbereitung garantiert qualitative und quantitative Aussagen über einzelne Stufen bei der Schokoladenverarbeitung, wodurch zielgerichtete Änderungen im Prozess punktuell vorgenommen werden und dadurch ein hochwertiges Endprodukt erzielt werden kann. Um chemische Veränderungen der Polyphenole im Extrakt durch Lagerung auszuschließen, wurden die Extrakte zeitnah sowohl photometrisch als auch chromatographisch, untersucht. Die Auswertung der Chromatogramme erfolgt mittels Referenzsubstanzen über ihre spezifische Retentionszeit und die jeweiligen Massenspektren.

In dieser Arbeit werden Alkaloide vollständigkeitshalber mit aufgenommen, wobei deren charakteristisches Spektrum bei einer Wellenlänge von 275 nm gemessen wurde. Beim DA-Detektor wurden demzufolge Wellenlängen von 275 nm für Flavanole und Alkaloide, 360 nm für Flavonole und 520 nm für Anthocyane eingestellt, um parallel unterschiedliche phenolische Substanzklassen zu detektieren (Mena *et al*. 2012). Die charakteristischen Wellenlängenspektren sind in **Abbildung 31** dargestellt.

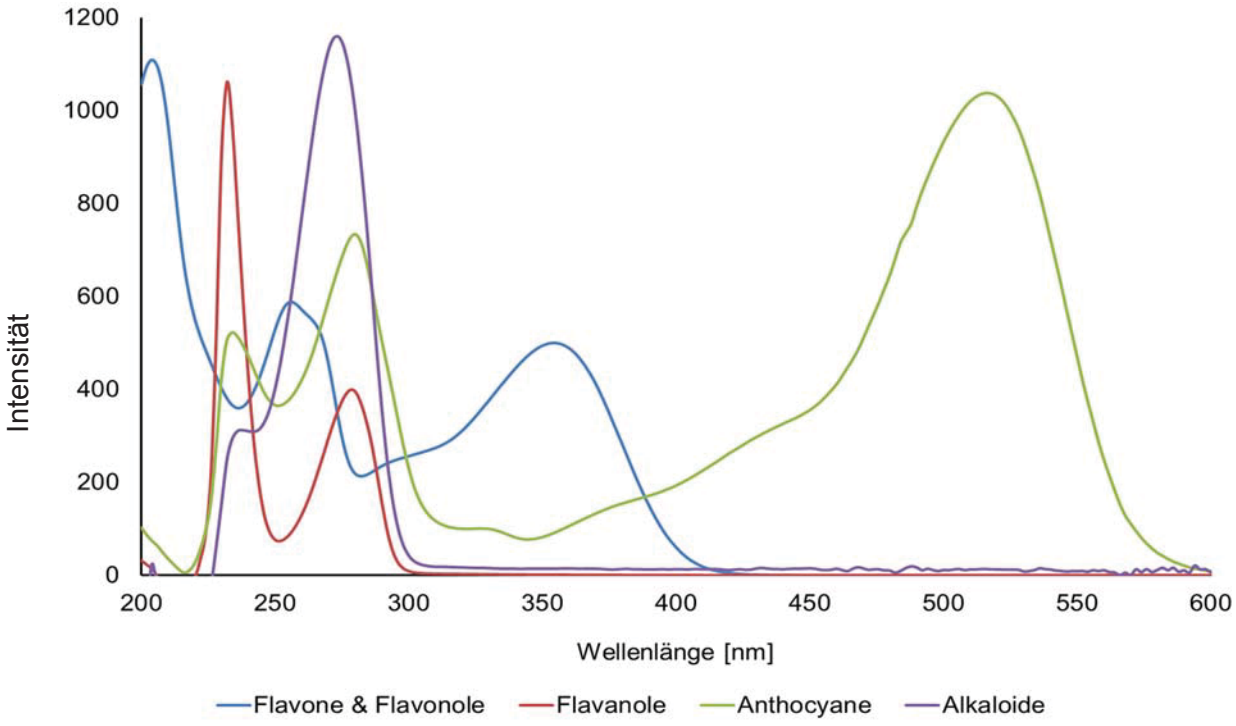

Abbildung 31: UV-Referenzspektren einiger Polyphenolklassen gelöst in Methanol. Das charakteristische Absorptionsmaximum ist die jeweilige Schulter mit der Wellenlänge in nm.

Auf Grund dieser spektroskopischen Eigenschaft kann qualitativ mit Hilfe des DA-Detektors eine Klassifizierung stattfinden. Ob ein Molekül jedoch als Aglykon oder glykosidisch gebunden vorliegt, kann hier nicht über UV/Vis-Spektroskopie aufgeklärt werden. Phenolcarbonsäuren besitzen keine zusätzlichen UV absorbierenden Gruppen. Der DA-Detektor kann über einen breiten Wellenlängenbereich UV/Vis-Strahlung aussenden, die der vorhandene Probenanalyt abschwächt und das Strahlungssignal wird über mehrere Photodioden selektiv aufgenommen. Diese Art der Analytik bietet die Möglichkeit sowohl über Referenzsubstanzen und ihrer Retentionszeit von der Trennsäule, ihres UV/Vis-Spektrums, als auch über die spezifische Massenfragmentierung abzusichern.

5.8.2. Detektion monomerer Polyphenole mittels ESI-MS

In dieser Arbeit können Moleküle < 2.000 Dalton (Da) mittels Elektrosprayionisation-Massenspektrometrie (ESI-MS) als protonierte Molekülionen detektiert werden. Bei Überblickmessungen im Fullscan-Modus werden somit alle Massen zwischen *m/z* = 100 - 2.000 Da aufgenommen. (-)-Epicatechin mit der Summenformel $C_{15}H_{14}O_6$ besitzt eine nominelle Masse MW = 290,29 Da und erhält im positiven Modus des Massenspektrums das Quasimolekülion mit $[M+H]^+$ = 291,09 Da. Neben dem Quasimolekülion $[M+H]^+$ existieren im MS-Spektrum charakteristische Massenfragmente, die zur Identifikation herangezogen wurden. Eine der wichtigsten Umlagerungsreaktionen bei monomeren Flavan-3-olen ist die Retro-Diels-Alder Fragmentierung (Gracey and Barker, 1976). Hierbei kommt es zu einer Spaltung des Pyran-Rings an Position O-C2 und C3-C4, wobei die Fragmente des A-Rings mit *m/z* 139 und des B-Rings mit *m/z* 165 entstehen (Miketova *et al*., 1998). Das Fragmentationsmuster ist in der **Abbildung 32** dargestellt. Mit einer relativen Intensität von 100%, hatte das Quasimolekülion *m/z* 291 das stärkste Signal und ist damit der Basispeak im MS-Spektrum. Auf die Fragmente *m/z* 139 fielen ~30% und für *m/z* 165 ~10% relative Intensität ab.

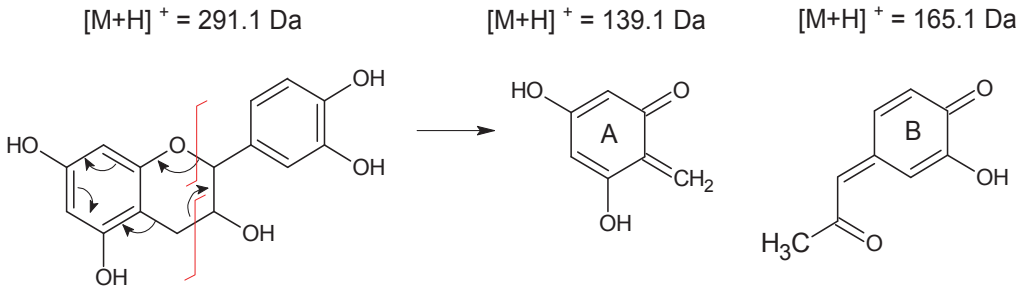

Abbildung 32: Postulierte ESI-MS Fragmentierung einer Retro-Diels-Alder-Reaktion.

Daneben wird im MS-Spektrum ein Signal bei *m/z* 123 detektiert, welches durch eine Spaltung des Pyran Rings zwischen der Position C2-C3 entsteht (**Abbildung 33**). Die relative Intensität für das entstehende Fragment besteht ebenfalls bei ~10%.

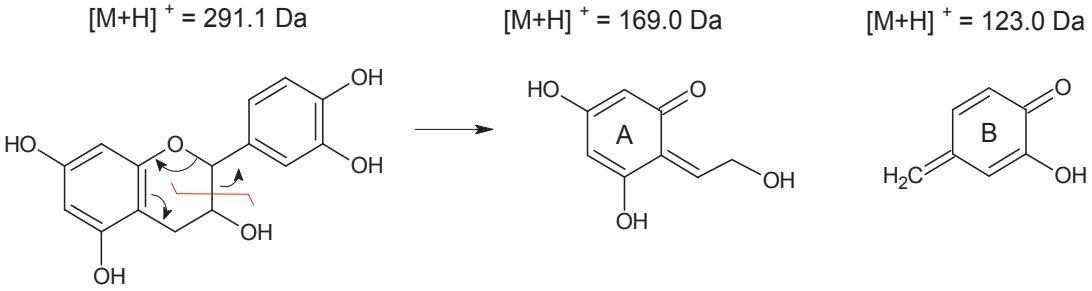

Abbildung 33: Heterozyklische Ringspaltung des (-)-Epicatechins.

Neben dem Quasimolekülion $[M+H]^+$ treten im Massenspektrum andere pseudomolekulare Ionen auf, die je nach Verunreinigung der verwendeten mobilen Phase, der eingesetzten Puffer oder Additve, die bei der Aufbewahrung der Proben, im Massenspektrum als Addukte erscheinen. Diese Interferenzen treten gehäuft im positiven ESI-Modus auf (**Tabelle 14**).

Tabelle 14: Massenspektroskopische Interferenzen und Artefakte

Ion	m/z Masse-zu-Ladung Verhältnis	Ursprung
$[M+H]^+$	$[M+H]^+$	Positives Quasimolekülion
$[M+2H]^{2+}$	$[M+2]^{2+}$	Heterodimere bei Gemischen
$[M+Na]^+$	$[M+23]^+$	Verunreinigung durch Gläser
$[M+K]^+$	$[M+39]^+$	Verunreinigung durch Gläser
$[M+NH_4]^+$	$[M+18]^+$	Komplexbildung mit Ammoniumacetat
$[2M+NH_4]^+$	$[2M+18]^+$	Komplexbildung mit Ammoniumacetat bei höheren Konzentrationen
2 $[M+H]^+$	2 $[M+1]^+$	Dimer bei höheren Konzentrationen
$[M+X]^+$	$[M+...]^+$	X = Kation des Lösungsmittels oder des Puffers
$[M+H+L]^+$	$[M+1+...]^+$	Addukte des Lösungsmittels

Im MS-Spektrum des (-)-Epicatechins erkennt man weitere Interferenzen, wie die Anlagerung des Erdalkalimetalls Natrium zu einem $[M+Na]^+$ Quasimolekülion mit *m/z* 313. Dies deutet auf eine starke Komplexbildung des Favan-3-ols mit Metallen hin. Studien von Jungbluth (2000), Yasuda (2012) bestätigen die Wechselwirkung von Polyphenolen mit Metall wie im Falle von Fe^{2+}, Fe^{3+}, Cu^{2+}, Al^{3+}. Daneben wurde im Massenspektrum des (-)-Epicatechins ein Quasimolekülion mit *m/z* 603,1 detektiert, was auf ein Dimer und einer Anlagerung mit Natrium zurückzuführen ist (**Abbildung 34**).

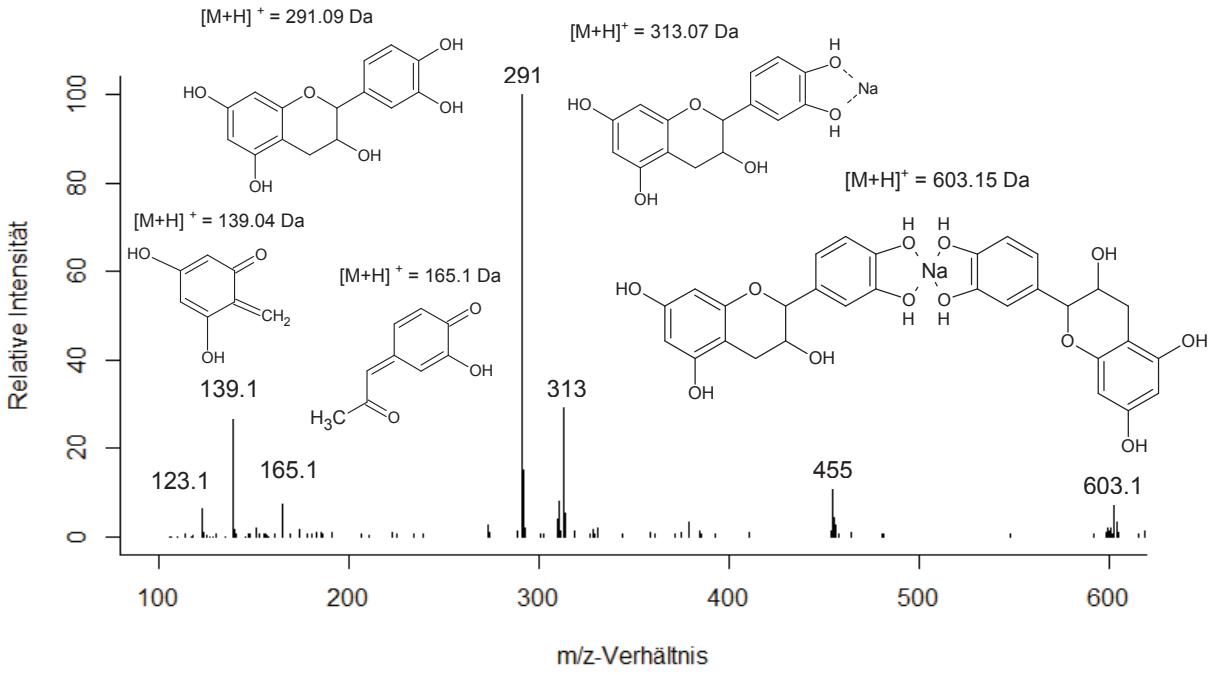

Abbildung 34: Fragmentationsmuster des (-)-Epicatechins.

Desweiteren konnten im positiven Ionenmodus beim dimeren PA B2 die Spaltprodukte Chinonmethide mit *m/z* 289 und dem Fragment einer Flavan-3-ol-Einheit mit *m/z* 291 detektiert werden, die aus einer Spaltung der Interflavan Bindung stammt (**Abbildung 33**). Die Fragmentierung erfolgt an der Interflavan Bindung zwischen der C4-C8 Verknüpfung.

Darüber hinaus wurde die Anwesenheit von tetrameren PA in der Kakaobohne bestätigt. Exemplarisch sei das ESI-MS-Fragmentationsmuster für das Cinnamtannin A2 in **Abbildung 35** dargestellt. Wegen ihrer strukturellen Zusammensetzung aus (-)-Epicatechineinheiten über C4-C8 Verbindungen, haben oligomere PA im Kakaoextrakt ein einfaches Peakprofil. Ausgehend vom Mutterion $[M+H]^+$ mit *m/z* 1155,1, wurde eine schrittweise Abspaltung der (-)-Epicatechineinheiten an C4-C8 Position postuliert mit den Fragmentionen *m/z* 867,0, *m/z* 577,2 und selten *m/z* 291,1. Das um 2 Da geringere Fragmention *m/z* 577,2 bildet sich durch die Ausbildung der Doppelbindung an den oberen Moleküleinheiten. Gleicher Reaktionsmechanismus entstand durch die Interflavanspaltung beim Chinonmethide mit *m/z* 289,1, wobei diese beiden Spaltprodukte den jeweils oberen und unteren Fragmentionen entsprechen. Daneben wurde ein Fragmention mit *m/z* 1731,4 detektiert, welches als hexameres PA identifiziert werden konnte.

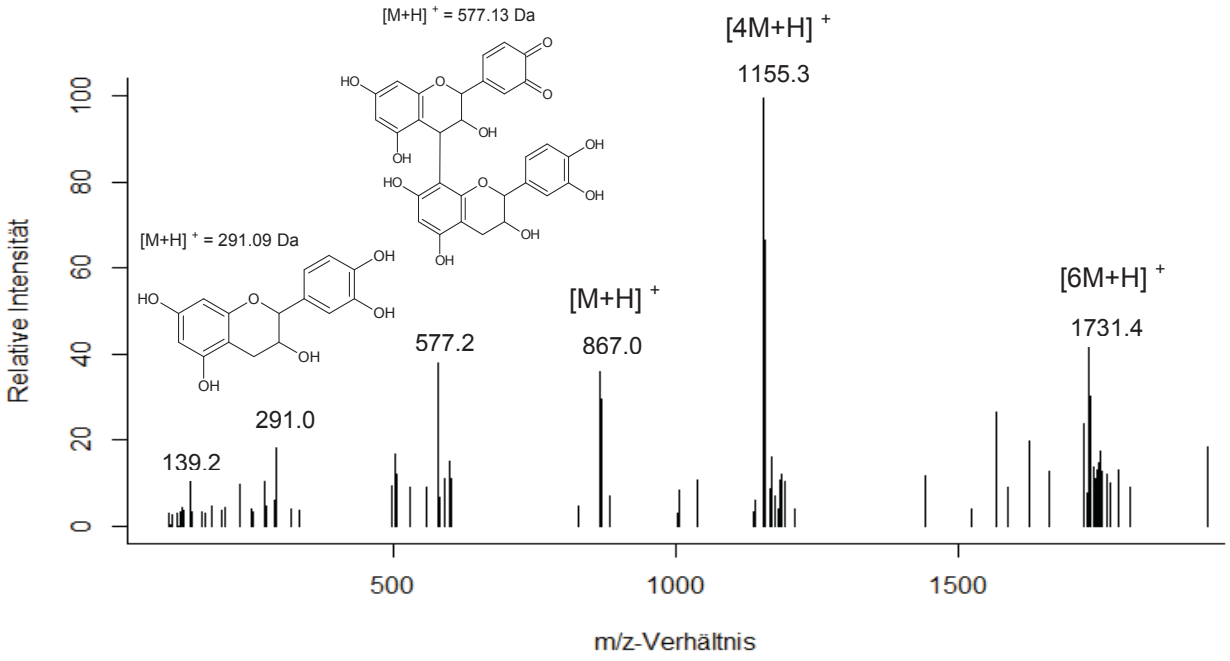

Abbildung 35: Postulierte ESI-MS Fragmentierung von Cinnamtannin A2 (angelehnt an Calderón *et al*., 2010).

Daneben wurden im Chromatogram bei 360 nm die Quercetinglykoside wie Quercetin-3-*O*-glucosid, Quercetin-3-*O*-arabinosid, sowie das Aglykon Quercetin als messbare Grössen ausfindig gemacht. Alle drei Substanzen weisen das typische Fragmentationsmuster auf, wobei jeweils das Molekülion *m/z* 303,0 auf das Aglykon Quercetin mit der jeweiligen Abspaltung des Zuckermoleküls hinweist (**Abbildung 36**). Neben dem Quasimolekülion $[M+H]^+$ wurde der, mit einem Na-Molekülion verbundene Quercetinglykosid Komplex $[M+Na]^+$ *m/z* 487,1 detektiert. Weiterhin konnte das Molekülion *m/z* 951,2 detektiert werden, welches auf einen dimeren Komplex verbunden über das Na-Molekül hindeuten könnte.

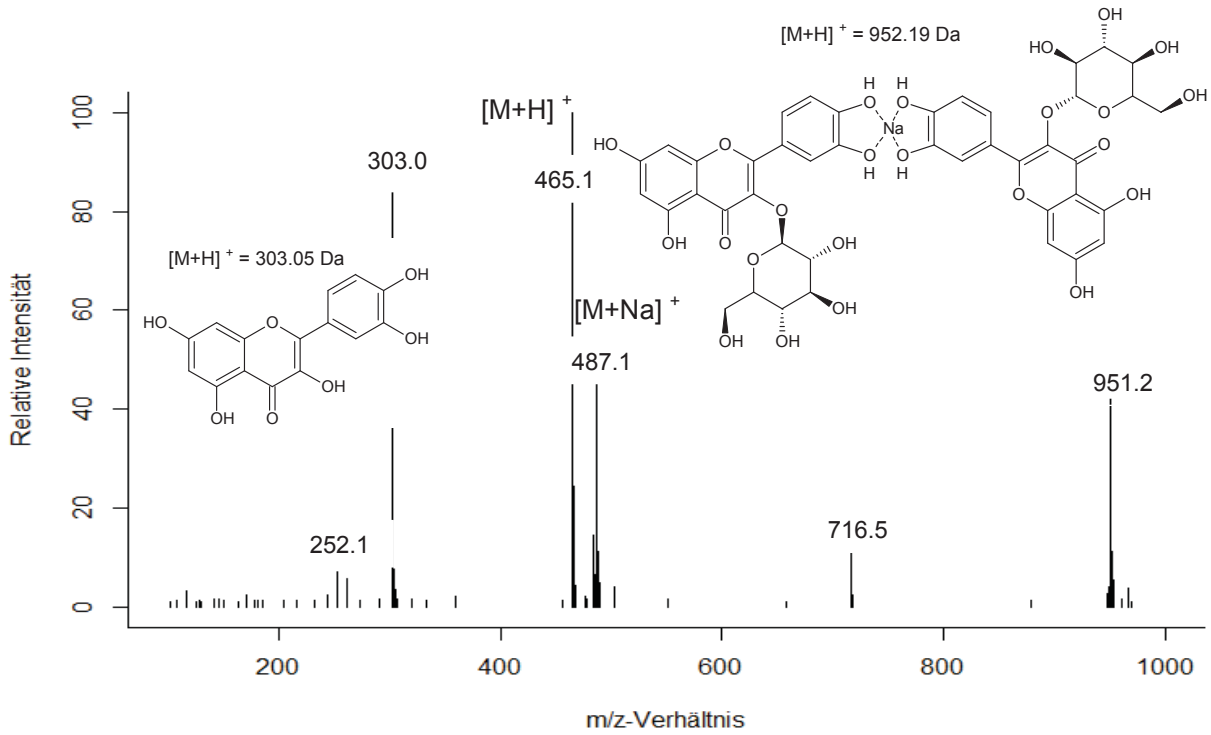

Abbildung 36: Postulierte ESI-MS Fragmentierung von Quercetin-3-*O*-glucosid.

Arbeiten von Andrés-Lacueva *et al*. (2008) oder Ortega *et al*. (2008) konnten Zuckerverbindungen wie Quercetinglucuronid und -rutinosid detektieren. Die in dieser Arbeit durchgeführten Analysen konnte jedoch keine Hinweise auf die entsprechenden Molekülionen $[M+H]^+$ finden.

Trotzdem konnten die Anthocyane in den unfermentierten Kakaobohnen durch das MS-Spektrum eindeutig zugeordnet werden. Neben dem jeweiligen Mutterion mit der glykosidischen Bindung $[M+H]^+$ konnte jeweils das Aglykon Ion mit *m/z* 287 detektiert werden (Giusti *et al*., 1999).

Eine schnelle Zuordnung von phenolischen Substanzen erfolgt in erster Linie über den Abgleich mit Referenzsubstanzen, den dazugehörigen Retentionszeiten und ihren Massenspektren mit den charakteristischen Fragmentierungsmustern. Eine tabellarische Auflistung der pseudomolekularen Quasimolekülionen und ihrer Retentionszeiten und Fragmentierungen erleichtert eine schnelle die Zuordnung im Chromatogramm (**Tabelle 15**). Für weitere kakaospezifische phenolische Substanzen konnten auf Grund der geringen Konzentration keine Molekülionen $[M+H]^+$ im untersuchten Probenmaterial detektiert werden.

Tabelle 15: Identifizierte phenolische Substanzen im *flüssigen Kakaoextrakt*. Mit Quasimolekülion $[M+H]^+$, Retentionszeit R_t und charakteristischen Massenfragmenten.

Polyphenole	Formel	R_t [min]	MW [g/mol]	*m/z* $[M+H]^+$	Charakteristische Fragmente
Alkaloide					
Koffein	$C_8H_{10}N_4O_2$	19,1	194,19	195,0	-
Theobromin	$C_7H_8N_4O_2$	11,9	180,16	181,0	-
Flavan-3-ole					
(+)-Catechin	$C_{15}H_{14}O_6$	17,7	290,26	291,0	$[M+Na]^+$ 314,0 $[-H_2O]^+$ 273,0 $[C_7H_6O_3]^+$ 139,1 $[C_9H_5O_3]^+$ 165,1
(-)-Epicatechin	$C_{15}H_{14}O_6$	25,1	290,26	291,0	$[M+Na]^+$ 314,0 $[-H_2O]^+$ 273,0 $[C_7H_6O_3]^+$ 139,1 $[C_9H_5O_3]^+$ 165,1
Proanthocyanidine					
Procyanidin A2	$C_{30}H_{22}O_{12}$	36,2	576,50	577,0	Spurenbereich
Procyanidin B2	$C_{30}H_{26}O_{12}$	21,7	578,53	579,1	$[C_{15}H_{14}O_6]^+$ 291 [M+H-152 = 427-18 -> m/z 409]
Procyanidin B3	$C_{30}H_{26}O_{12}$	16,6	578,53	579,1	s. PA B2
Procyanidin C1	$C_{45}H_{38}O_{18}$	27,7	866,79	867,1	$[C_{15}H_{12}O_6]^+$ 289,0 $[C_{30}H_{24}O_{12}]^+$ 577,2
Cinnamtannin A2	$C_{60}H_{50}O_{24}$	30,7	1155,04	1155,3	$[C_{15}H_{14}O_6]^+$ 291
Anthocyane					
Cyanidin-3-*O*-arabinosid	$C_{20}H_{19}O_{10}$	26,1	419,36	420,11	$[C_{15}H_{11}O_6]$ 287
Cyanidin-3-*O*-galactosid	$C_{21}H_{21}O_{11}$	22,6	449,38	450,12	$[C_{15}H_{11}O_6]$ 287
Flavonol					
Quercetin	$C_{15}H_{10}O_7$	48,4	302,24	303,05	$[M+H-H_2O-CO]^+$ 257 $[M+H-H_2O]^+$ 285 $[M+H-2CO-H_2O]^+$ 229
Quercetin-3-*O*-arabinosid	$C_{20}H_{18}O_{11}$	37,9	434,35	435,09	$[C_{15}H_{10}O_7]^+$ 303,0
Quercetin-3-*O*-glucosid	$C_{21}H_{20}O_{12}$	36,9	464,38	465,10	$[C_{15}H_{10}O_7]^+$ 303,0
Flavone					
Apigenin-6-*C*-glucosid	$C_{21}H_20O_{10}$	34,2	432,38	433,11	Spurenbereich
Apigenin-8-*C*-glucosid	$C_{21}H_{20}O_{10}$	34,1	432,38	433,11	Spurenbereich
Luteolin	$C_{15}H_{10}O_6$	46,5	286,24	287,06	Spurenbereich

In **Abbildung 37** ist exemplarisch ein LC-DAD Chromatogramm eines *flüssigen Kakaoextraktes* bei 280 nm abgebildet. Da Flavonole ihr charakteristisches Absorbtionsmaximum bei 360 nm haben, wurden sie in diesem Chromatogramm als negativer Peak dargestellt. Gleiches gilt für die Anthocyane Cyanidin-3-*O*-galactosid und

Cyanidin-3-*O*-arabinosid, die auf Grund ihrer Struktur ein charakteristisches Absorptionsmaximum bei 520 nm haben und deswegen in dieser Abbildung vom Chromatogramm nicht erscheinen. In den Untersuchungen konnte nur bei unfermentierten Kakaobohnen die literaturbasierten Anthocyane Cyanidin-3-*O*-galactosid oder Cyanidin-3-*O*-arabinosid detektiert werden (Forsyth & Quesnel, 1957). Im Laufe des Fermentationsprozesses, spätestens aber nach drei Tagen, wurden sie abgebaut und konnten nicht mehr nachgewiesen werden. Andere in der Literatur angegebenen Anthocyane wurden nicht detektiert. Die in dieser Arbeit verwendete RP-Säule wird nicht zur quantitativen Messung von Anthocyanen empfohlen, da ihr Retentionsmechnanismus für die Trennung dieser phenolischen Substanzklassen nicht optimal geeignet ist.

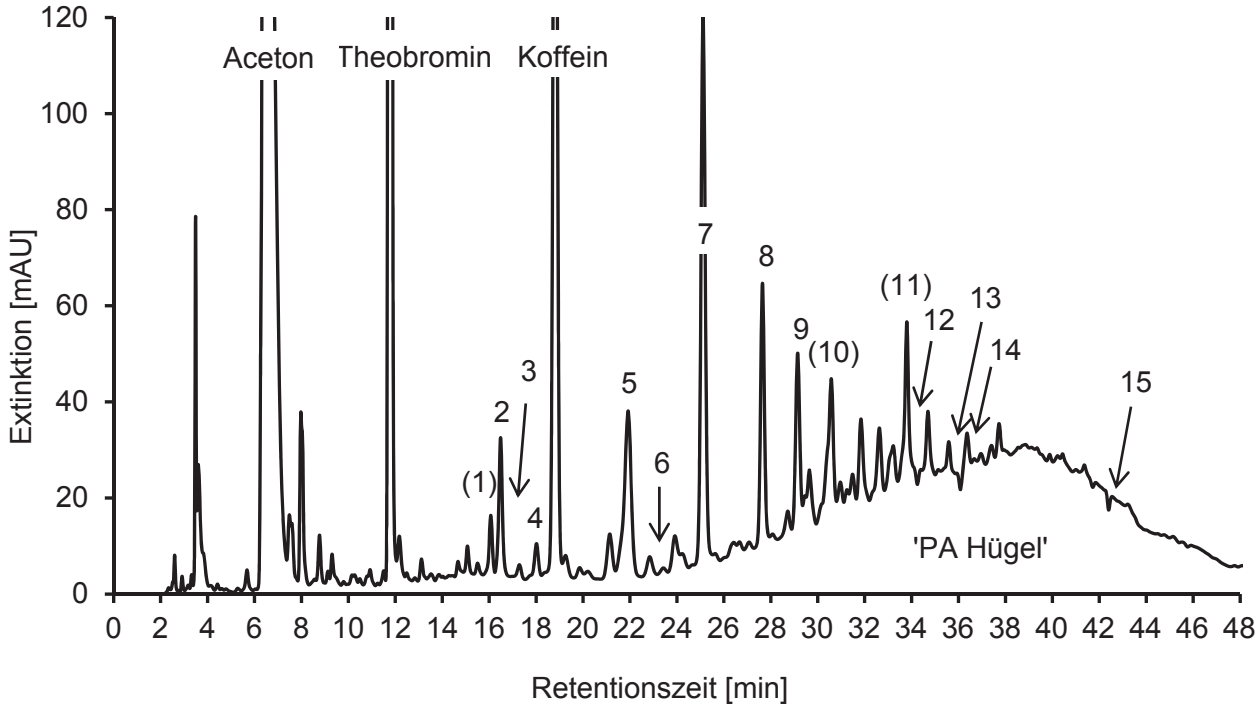

Abbildung 37: LC-DAD-ESI-MS Chromatogramm eines ungerösteten Kakaoextraktes. Injektionsvolumen: 1 µL, Wellenlänge: 280 nm.

Unter Berücksichtigung der Ergebnisse aus **Tabelle 16** konnten 12 phenolische Substanzen anhand des Abgleiches mit Referenzsubstanzen und den dazugehörigen Massenspektren eindeutig zugeordnet werden. Bei den drei in Klammern gesetzten Verbindungen, konnte anhand der fehlenden Referenzsubstanzen keine eindeutige Identifizierung vollzogen werden. Anhand des charakteristischen Mutterions $[M+H]^+$ und des Abgleiches mit Literaturdaten bezüglich der Fragmentierungsmuster wurden diese Phenole trotzdem in die Tabelle mit aufgenommen.

Tabelle 16: Identifizierte phenolische Substanzen im Kakaoextrakt.

Peak Nummer	Substanz	UV-DAD λ_{max} [nm]	*m/z* $[M+H]^+$	MS Fragmente *m/z*
(1)	n. I.	280	*m/z* 433	-
2	Procyanidin B3	280	*m/z* 579,1	291; 427; 886,9
3	Cyanidin-3-*O*-galactosid	520	*m/z* 450,1	200,4
4	(+)-Catechin	280	*m/z* 291,0	139,1; 165,1
5	Proanthocyanidin B2	280	*m/z* 579,1	291; 427; 886,9
6	Cyanidin-3-*O*-arabinosid	520	*m/z* 420,1	595,2
7	(-)-Epicatechin	280	*m/z* 291,0	139,1; 165,1
8	Proanthocyanidin C1	280	*m/z* 867,2	577,9; 1153,9; 1752,6
9	Cinnamtannin A2	280	*m/z* 1155,3	291; 886,9
(10)	Pentamer	280	*m/z* 1443,5	123,0; 290,9
(11)	Dimer	280	*m/z* 579,1	291; 886,9
12	Quercetin-3-*O*-glucosid	320	*m/z* 465,10	303,0
13	Quercetin-3-*O*-arabinosid	320	*m/z* 435,1	303,0
14	Procyanidin A2	280	*m/z* 577,2	-
15	Quercetin	320	*m/z* 303,1	-

n. I. = nicht Identifiziert

Für die Quantifizierung phenolischer Substanzen im *flüssigen Kakaoextrakt* wurden zunächst Stammlösungen kommerziell erworbener Referenzsubstanzen mit einer Konzentration von 1 mg/mL hergestellt. Die im Anhang aufgeführten Referenzsubstanzen wurden hierzu in Methanol, bzw. die Alkaloide, Flavonole, Flavone, Flavanone in DMSO, gelöst und die jeweilige chemische Reinheit der Substanzen für die Berechnung der Konzentration berücksichtigt. Weiterhin wurden für die Referenzsubstanzen im Anhang angegebene Kalibrationsreihen hergestellt. Als untere Grenze der Kalibrationsreihe wurde der LOQ-Wert der jeweiligen Standardsubstanz gewählt. Die Ermittlung des LOD-Wertes erfolgte nach Kromidas (2008) auf Basis des Grundrauschens von sechs Methanolmessungen (Blindwert). Anhand von externen Kalibrierungen wurde für die Flavan-3-ole (+)-Catechin, (-)-Epicatechin, die Dimere PA B1, B2, dem Trimer PA C1, sowie den zwei Alkaloiden Theobromin und Koffein, eine Kalibrationsgerade mit n > 10 angefertigt. Die Kalibrationsgrenzen für die quantitative Bestimmung betrugen zwischen 0,03 - 1 g/L. Die Geradengleichungen sind im Anhang dargestellt.

6. Schlussfolgerung

Theobroma cacao Linné enthält eine Vielzahl phenolischer Verbindungen, angefangen von einfachen monomeren Flavonoiden bis hin zu komplexen kondensierten Proanthocyanidinen (PA). Eine qualitative und quantiative Bestimmung erfolgt in erster Linie meist über kolorimetrische Methoden. Die jeweiligen Bestimmungsmethoden müssen für das jeweilige Probenmaterial adaptiert und gegebenfalls optimiert werden. Um eine ubiquitäre Vergleichbarkeit zu gewährleisten, muss sowohl die Bestimmungsmethode als auch das resultierende Ergebnis einheitlich angegeben werden. Dieses hängt zwar im wesentlichen vom jeweiligen Polyphenolprofil ab, im konkreten Fall bedeutet das für kolorimetrische Messungen im Forschungsbereich Kakao, dass eine Angabe zum Gesamtpolyphenogehalt über (-)-Epicatechin- und/oder Gallussäureäquivalente sinnvoller wäre.

Mittels geeigneter Extraktionsverfahren, lässt sich auf schonende Weise ein Maximum an phenolischen Verbindungen aus der Kakaomatrix extrahieren. Hierfür wurden gängige Extraktionslösemittel ausprobiert und ihre Optimierung, bezogen auf Extraktionstemperatur, -zeit, -zyklus, vorgenommen. Der gewonnene Kakaoextrakt beinhaltet eine originäre Zusammensetzung an phenolischen Komponenten, die mittels LC-, HPTLC- oder SCPC Messungen detektiert werden können.

Die Ergebnisse dieser Arbeit tragen einerseits dazu bei, größere Mengen phenolischer Referenzsubstanzen zu generieren, um beispielsweise Bioaktivitätstests als auch humanphysiologische Messungen machen zu können. Andererseits können isolierte PA mehr zum Verständnis der antioxidativen Aktivität von oligomeren und polymeren Polyphenolen beitragen. Ratsam ist es aber auch, die bis dato semipräparative Trenntechnik weiterhin auszubauen, um eine größere Menge an Referenzsubstanzen zu erhalten. Ein *up-scaling* der Methode müsste sowohl bei der SCPC, als auch bei der nachfolgenden Gelpermeationschromatographie ansetzen.

Mittels NP-HPLC-online-DPPH konnte eine Methode etabliert werden, die es erlaubt sowohl einzelne oligomere PA zu detektieren als auch simultan ihre antioxidative Kapazität zu vermessen. Somit können mittels einer Messung Rückschlüsse sowohl auf das Polyphenolprofil der Probe als auch auf das antioxidative Potenzial gezogen werden. Neben einzelnen isolierten PA Peaks ist im Chromatogramm auch der sogenannte ‘PA Hügel‘ detektierbar, aus dem sich Informationen bezüglich hochkondensierter polymerer Proanthocyanidine ableiten. Nur wenige wissenschaftliche Arbeiten berücksichtigen die Veränderung beim ‘PA Hügel‘, wobei die Ergebnisse dieser Arbeit eindeutig eine hohe

antioxidative Kapazität für diesen belegen. Bei der NP-HPLC-online-DPPH Messung muss jedoch berücksichtigt werden, dass die Aussagen sich auf das jeweilige monomere, dimere, trimere PA etc. beziehen. Unter einem monomeren PA-Peak versteht man jedoch die Summe aller monomeren Stereoisomeren, gleiches gilt für die darauffolgenden oligomeren Peaks, die als Summenparameter des jeweiligen Stereoisomeren eines Polymerisationsgrades verstanden werden müssen.

Bei Proben aus dem Schokoladenherstellungsprozess konnten Auswirkungen auf das Polyphenolspektrum der einzelne Verarbeitunsgstufen heruntergebrochen werden. Das Ergebnis aus den Untersuchungen bestätigt die Literaturdaten. Der Gesamt-PA-Gehalt (∑ PG1 - PG13) sinkt im Laufe der Schokoladenherstellung, wobei das Endprodukt Schokolade rund ein sechstel der Polyphenolkonzentration der frischen Kakaobohne besitzt. Auch die Zunahme des ‘PA Hügels‘ und damit verbunden die Bildung höher kondensierter PA wurde im Laufe der Fermentation sichtbar. Der Zerfall ist eine Folge der erhöhten Temperatur bei der Röstung.

Schokolade ist heute weit aus mehr als nur Genussmittel. Das Verständnis von antioxidativ wirksamen Inhaltsttoffen verstärkt das Umdenken der Manufakturen zum mehr nachhaltigen und schonenderem Umgang mit Lebensmitteln. Durch eine lückenlose Rückverfolgung von wertgebenden Inhaltsstoffen von der Kakaobohne über die gesamte Wertschöpfungskette bis hin zur Schokolade, können physikalische Einflüsse auf die Zusammensetzung dokumentiert und negative Einflüsse eliminiert werden. Auch in den Anbauländern lohnt sich die Optimierung der jeweiligen Verarbeitungsstufen. Ein intelligentes Prozessdesign könnte die native und damit antioxidativ wirksamere Zusammensetzung von primären und sekundären Kakaoinhaltsstoffen über die gesamten Schokoladenprozessstufen sicherstellen.

Das am Anfang der Arbeit genannte Postulat, dass „kein zweites Mal die Natur solche Fülle der wertvollsten Nährstoffe auf einem so kleinen Raum zusammengedrängt hat, wie gerade bei der Kakaobohne“ (Alexander von Humboldt), mag demnach tatsächlich stimmen. Mit ihrer Fülle an antioxidativ wirksamen phenolischen Substanzen, der großen Menge an stimulierenden Alkaloiden, den höchwertigen Fetten, gilt die Kakaobohne als eine der wichtigsten Konsumgüter aus Kulturpflanzne. Diese bioaktiven Inhaltsstoffe sind vermutlich für die zahlreichen beschriebenen Wirkungen von *Theobroma cacao* Linné verantwortlich. Ein positiver Beitrag gegenüber Membranschäden, unkontrollierter Zellteilung, Oxidationsstress könnte durch eine Aufnahme von Kakaopolyphenolen geleistet werden (Bearden, *et al*. 2000; Ramljak *et al*. 2005). Denoch müssen weiterführende

Bioaktivitätstests mit PA-reichen Kakaoextrakten und/oder isolierten oligomeren PA durchgeführt werden, um zu einem besseren Verständnis der Wirkung beizutragen.

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8. Anhang

Das in dieser Arbeit verwendete Probenmaterial gehört zur Kakaosorte Trinitario und wurde auf der ‚Finca La Amistad' in Costa Rica geerntet. Der Genotyp ist nicht bekannt.

8.1. Laborgeräte

Die in dieser Arbeit verwendeten Geräte sind in der folgenden Tabelle aufgeführt. Die chromatographischen Apparaturen werden in den nachfolgenden Abschnitten behandelt.

Gerät	Modell	Hersteller
Analysenmühle	A 11 basic	IKA® Werke GmbH & Co. KG, Staufen im Breisgau, Deutschland
Analysenwaagen	AT261 Delta Range	Mettler-Toledo GmbH, Greifensee, Schweiz
	AB104-S/FACT	
	XA503S	
Gefriertrockner	ALPHA 2-4 LC plus	Martin Christ Gefriertrocknungsanlagen GmbH, Osterode, Deutschland
Labor-Tisch-Schüttler	HLC MHR 13	Huberlab AG, Aesch, Schweiz
Magnetrührer	RTC basic safety control	IKA® Werke GmbH & Co. KG, Staufen im Breisgau, Deutschland
pH-Meter	S220 SevenCompact™	Mettler-Toledo GmbH, Greifensee, Schweiz
Pipetten	Xplorer® plus (Volumina: 10-100 µL; 20-200 µL und 100-1000 µL)	Vaudaux-Eppendorf AG, Schönenbuch, Schweiz
Probenkonzentrator	PIAG Blockthermostat, PI-TB-EVAPOR-v.01	Portmann Instruments AG, Biel-Benken, Schweiz
Siebmaschine	AS 200	Retsch GmbH & Co. KG, Haan, Deutschland
Ultraschallbad	HB 10	IKA® Werke GmbH & Co. KG, Staufen im Breisgau, Deutschland
UV/Vis-Spektrometer	Genesys 10-S	Thermo Fisher Scientific AG, Reinach, Schweiz
Vortex-Mixer	IP 40 Lab Dancer	IKA® Werke GmbH & Co. KG, Staufen im Breisgau, Deutschland
Wasserbad mit Kontrollmodul	Thermo Haake C10-V26	Thermo Fisher Scientific AG, Reinach, Schweiz
Zentrifuge	5810	Vaudaux-Eppendorf AG, Schönenbuch, Schweiz

8.2. Chromatographische Laborgeräte

LC-DAD-ESI/MS

Degasser:	1260 HiP Degasser G4225A, Agilent Technologies (Waldbronn)
Binäre Pumpe:	1260 Bin Pump, G1312B, Agilent Technologies (Waldbronn)
Injektor:	1260 ALS, G1329B, Agilent Technologies (Waldbronn)
Säulenofen:	1290 TCC, G1316C, Agilent Technologies (Waldbronn)
DAD Detektor:	1260 DAD, G4212B, Agilent Technologies (Waldbronn)
Massenspektrometer:	6120 Quadrupole LC/MS, Agilent Technologies (Waldbronn)
Software:	LC/MSD ChemStation, Rev. B. 04.03-SP1 (87), Agilent Technologies (Waldbronn)

NP-LC-online-DPPH

Pumpe I:	Smartline Pumpe 1050, Knauer GmbH (Berlin) Smartline Manager 5050, Knauer GmbH (Berlin)
Pumpe II:	Smartline Pumpe 100, Knauer GmbH (Berlin)
Autosampler:	Optimas Typ 820, Spark (Emmen, Holland)
Probenschleife:	10 µL Peek Tubing, 1/16” × 0.38 mm ID
Säule:	YMC Triart Diol-HILIC Säule (3.0 mm i.D. × 150 mm, 5 µm), (Stagroma AG, Reinach, Switzerland) mit Vorsäule
Derivatisierung:	4.8 m Peek Tubing, 1/16“ × 0.38mm ID, BGB Analytik AG, (Boeckten, Schweiz)
Säulenofen I:	SFD 12560, Knauer GmbH (Berlin)
Säulenofen II:	Jetstream 2 Plus, Flowspek AG, (Basel, Schweiz)
Detektor I:	Azura Detektor UVD 2.1S, Knauer GmbH (Berlin)
Detektor II:	Smartline UV Detektor 2500 mit Halogenlampe, Knauer GmbH (Berlin)
Software:	ClarityChrom version 3.0.5.505, Knauer (Berlin)

Präparative NP-HPLC

Pumpe I:	Smartline Pumpe 1050, Knauer GmbH (Berlin) Smartline Manager 5050, Knauer GmbH (Berlin)
Autosampler:	Optimas Typ 820, Spark (Emmen, Holland)
Probenschleife:	200 µL Peek Tubing, 1/16” x 0.5 mm ID
Säulenofen II:	Jetstream 2 Plus, Flowspek AG, (Basel, Schweiz)
Detektor I:	Azura Detektor UVD 2.1S, Knauer GmbH (Berlin)
Software:	ClarityChrom version 3.0.5.505, Knauer (Berlin)

SCPC

Pumpe:	Rainin Dynamax Solvent Delivery System, Model SD-200, Rainin (Kalifornien, USA)
Probenschleife:	10 ml
Probenaufgabe:	Bis zu 6 g
CPC-Säule:	SCPC-250, Säulenvolumen: 250 ml, Armen Instruments (Saint-Avé, Frankreich)
Detektor:	Rainin Dynamax Absorbance Detector, Model UV-1, Rainin (Kalifornien, USA)
Fraktionensammler:	Isco Foxy 200 Fraktionensammler, Teledyne Isco (Nebraska, USA)
Software:	Star Chromatography Workstation, System Control Version 6.41, Star L0-0304 (Varian, USA)

Präparative LC mit Sephadex LH-20

Pumpe I:	Smartline Pumpe 1050, Knauer GmbH (Berlin) Smartline Manager 5050, Knauer GmbH (Berlin)
Autosampler:	Optimas Typ 820, Spark (Emmen, Holland)
Glassäule:	ECO10/120V0V (YMC Europe GmbH, Dinslaken, Germany) Sephadex LH-20 (GE Healthcare Life Sciences, Glattbrug, Schweiz)
Probenschleife:	1mL Peek Tubing, 1/16” x 0.5 mm ID
Detektor I:	Azura Detektor UVD 2.1S, Knauer GmbH (Berlin)
Software:	ClarityChrom version 3.0.5.505, Knauer (Berlin)

8.3. Materialien

8.3.1. Herstellerangaben zu den verwendeten Referenzsubstanzen

Die Referenzsubstanzen der angegebenen Hersteller wurden in HPLC Qualität erworben.

Referenzsubstanz	Hersteller	Reinheit	CAS Nr.	Artikel Nr.
Apigenin-6-*C*-glucosid	Extrasynthese	> 99%	38953-85-4	1235 S
Apigenin-8-*C*-glucosid	Extrasynthese	> 99%	3681-93-4	1232 S
(+)-Catechin	Sigma-Aldrich Chemie GmbH	> 99%	154-23-4	43412-10MG
(-)-Catechin	Sigma-Aldrich Chemie GmbH	≥ 97%	18829-70-4	C0567-5MG
(-)-Catechin gallat	PhytoLab	≥ 98%	130405-40-2	82497
Chlorogensäure	PhytoLab	> 90%	327-97-9	89175
Cinnamtannin A2	PhytoLab	> 90%	86631-38-1	83372
p-Coumarinsäure	Sigma Aldrich	> 98%	501-98-4	C9008-1G
Cyanidin-3-*O*-arabinosid	PhytoLab	> 90%	111613-04-8	89614
Cyanidin-3-*O*-galactosid	PhytoLab	> 90%	27661-36-5	89463
Cyanidin-3-*O*-Glc-Rha	PhytoLab	> 90%	18719-76-1	80577
(-)-Epicatechin	Sigma-Aldrich Chemie GmbH	> 90%	490-46-0	E1753-1G
(-)-Epicatechin gallat	Extrasynthese	≥ 97.5%	1257-08-5	0978 S
(-)-Epigallocatechin	Extrasynthese	≥ 98%	970-74-1	0979 S
Ferulasäure	Extrasynthese	≥ 90%	537-98-4	6077
Gallussäure	Extrasynthese	≥ 99%	149-91-7	4993 S
(-)-Gallocatechin	Extrasynthese	≥ 99%	3371-27-5	0973 S
(-)-Gallocatechin gallat	Extrasynthese	≥ 98%	4233-96-9	0974 S
Luteolin	Extrasynthese	> 90%	491-70-3	89245
Luteolin-7-*O*-glucosid	PhytoLab	> 90%	5373-11-5	89724
Luteolin-6-*C*-glucosid	TransMIT	> 98%	4261-42-1	L 013
Luteolin-8-*C*-glucosid	Extrasynthese	≥ 99%	28608-75-5	1054 S
Naringenin	Extrasynthese	≥ 99%	480-41-1	1128 S
Naringenin-7-*O*-glucosid	TransMIT	TLC	529-55-5	N 008
Neochlorogensäure	PhytoLab	> 95%	906-33-2	80504
Proanthocyanidin A2	Extrasynthese	≥ 99%	41743-41-3	0985 S
Proanthocyanidin B1	Extrasynthese	≥ 80%	20315-25-7	0983
Proanthocyanidin B2	PhytoLab	> 90%	29106-49-8	89553
Proanthocyanidin B3	TransMIT	> 95%	23567-23-9	P 040
Proanthocyanidin B4	TransMIT	> 90%	29106-51-2	P 041
Proanthocyanidin B6	TransMIT	> 90%	12798-58-2	P 042
Proanthocyanidin B2-3-O-gallate	TransMIT	> 90%	73086-04-1	P 044
Proanthocyanidin C1	PhytoLab	> 80%	37064-30-5	89537
Proanthocyanidin C2	TransMIT	> 90%	37064-31-6	P 045
Proanthocyanidin trimer Cat-Cat-Epi	TransMIT	> 90%	-	P 054
Protocatechusäure	HWI Analytik GMBH	> 95%	99-50-3	0393-05-90
Quercetin	Sigma-Aldrich Chemie GmbH	≥ 95%	117-39-5	Q4951-10G
Quercetin-3-*O*-arabinosid	PhytoLab	> 90%	572-30-5	80361
Quercetin-3-*O*-galactosid	PhytoLab	> 90%	482-36-0	89227
Quercetin-3-*O*-glycosid	PhytoLab	> 90%	482-35-9	89230
Quercetin-3-*O*-glucuronid	PhytoLab	> 90%	22688-79-5	80349
Quercetin-3-*O*-rutinoside	Extrasynthese	≥ 99%	153-18-4	1139 S

8.3.2. GHS-Gefahrenstoffkennzeichnung der verwendeten Referenzsubstanzen

GHS-Gefahrenstoffkennzeichnung der in dieser Arbeit verwendeten Referenzsubstanzen

Referenzsubstanzen	**GHS-Symbol**	**H-Sätze**	**P-Sätze**
Apigenin-6-*C*-glucosid		-	262-260
Apigenin-8-*C*-glucosid	-	-	262-260
(+)-Catechin		315-319-335	260-337+313-305+351+338-280
(-)-Catechin		315-319-335	-
(-)-Catechin-3-gallat		315-319-335	260-337+313-305+351+338-280
(-)-Chinasäure	-	262-260	-
Cinnamtannin A2	-	-	-
Chlorogensäure	-	262-260	-
p-Coumarinsäure		315-319-335	337+313-305+351+338-280-260
Cyanidin-3-*O*-arabinosid	-	262-260	-
Cyanidin-3-*O*-galactosid	-	262-260	-
(-)-Epicatechin		315-319-335	260-337+313-305+351+338-280
(-)-Epicatechingallat	-	262-260	-
(-)-Epigallocatechin		315-319-335	-
Ferulasäure		315-319-335	337+313-305+351+338-280-260
Gallussäure	-	262-260	-
(-)-Gallocatechin		315-319-335	
(-)-Gallocatechingallat		302-315-319-335	337+313-305+351+338-280-260
Kaffeesäure		351-361d-335-315-319	337+313-305+351+338-280-260
Luteolin		315-319-335	260-337+313-305+351+338-280
Luteolin-7-*O*-glucosid	-	262-260	-
Luteolin-6-*C*-glucosid	-	262-260	-
Luteolin-8-*C*-glucosid	-	262-260	-
Naringenin		315-319-335	260-337+313-305+351+338-280
Naringenin-7-*O*-glucosid	-	262-260	-
Neochlorogensäure	-	262-260	-
Proanthocyanidin A2	-	262-260	-
Proanthocyanidin B1, B2, B3, B4, C1	-	262-260	-
Protocatechusäure		315-319-335	337+313-305+351+338-280-260

Referenzsubstanzen	GHS-Symbol	H-Sätze	P-Sätze
Quercetin		301	301+310
Quercetin-3-*O*-arabinosid	-	-	-
Quercetin-3-*O*-galactosid	-	262-260	-
Quercetin-3-*O*-glucosid	Dieser Stoff ist gemäß der Verordnung (EG) Nr. 1272/2008 nicht als gefährlich eingestuft		
Quercetin-3-*O*-glucuronid	-	262-260	-
Quercetin-3-*O*-rutinosid		315-319-335	260-337+313-305+351+338-280

8.3.3. GHS-Gefahrenstoffkennzeichnung der verwendeten Chemikalien

GHS-Gefahrenstoffkennzeichnung der in dieser Arbeit verwendeten Chemikalien

Substanz	CAS-Nr.	GHS-Symbol	H-Sätze	P-Sätze	Hersteller
Aceton (ACS Grade)	67-64-1		225-319-336	210-261-305+351+338	Sigma-Aldrich Chemie GmbH
Acetonitril (LC-MS Grade)	75-05-8		225, 302+312+332, 319	210, 305+351+338, 403+235	Sigma-Aldrich Chemie GmbH
Aluminiumchlorid	12125-02-9		302-319	305+351+338	Sigma-Aldrich Chemie GmbH
Ameisensäure	64-18-6		226, 314	260, 280, 301+330+331, 305+351+338	Sigma-Aldrich Chemie GmbH
Ammoniak (25%)	1336-21-6		314, 335, 400	273, 280, 301+330 +331, 304+340, 305+351+338	Sigma-Aldrich Chemie GmbH
Ammoniumeisen (III)-sulfat	10138-04-2		315-319	280, 305+351+338, 302+352, 321, 362, 332+313	Sigma-Aldrich Chemie GmbH
Ammoniumsulfat (≥99%)	7783-20-2	Dieser Stoff ist gemäß der Verordnung (EG) Nr. 1272/2008 nicht als gefährlich eingestuft.			Sigma-Aldrich Chemie GmbH
1-Butanol	71-36-3		226, 302, 318, 315, 335, 336	280, 302+352, 305+351+338, 313	Sigma-Aldrich Chemie GmbH
2-Butanol	78-92-2		226, 319, 335+336	201, 304+340, 305+351+338	Sigma-Aldrich Chemie GmbH
1-Propanol	71-23-8		225-318-336	210, 233, 280, 305+351+338, 313	Carl Roth GmbH & Co KG
2-Propanol	67-63-0		225, 319, 336	210, 233, 305+351+338	Sigma-Aldrich Chemie GmbH
DMSO (anhydrous)	67-68-5		335	305+351+338	Sigma-Aldrich Chemie GmbH
DPPH	1898-66-4		317, 334	261, 280, 342+331	Sigma-Aldrich Chemie GmbH

Substanz	CAS-Nr.	GHS-Symbol	H-Sätze	P-Sätze	Hersteller
Essigsäureethyl-ester	141-78-6		225-319-336	210, 240, 305+351+338	Carl Roth GmbH & Co KG
Ethanol (96%)	64-17-5		225	210, 233, 241, 243	Sigma-Aldrich Chemie GmbH
FOLIN-CIOCALTEU Reagenz	–		314	280-305+ 351+338, 310	Merck KGaA
Hexan	110-54-3		225, 361, 304, 373, 315, 336, 411	210, 240, 273, 301+310, 331, 302+352, 403+235	Carl Roth GmbH & Co KG
Natriumcarbonat	497-19-8		319	260, 305+351+338	VWR International GmbH
Natriumnitrit	7632-00-0		272-301-400	273, 309+311	Sigma-Aldrich Chemie GmbH
Methanol	67-56-1		225, 301+311 +331, 370	210, 280, 302+352, 403+235	VWR International GmbH
Salzsäure (37%)	7647-01-0		314, 335	261, 280, 305, 351, 338, 310	VWR International GmbH
Schwefelsäure (>95%)	7664-93-9		314	280, 305+ 351+338, 310	Sigma-Aldrich Chemie GmbH
Wasser (LC-MS Grade)	7732-18-5	Dieser Stoff ist gemäß der Verordnung (EG) Nr. 1272/2008 nicht als gefährlich eingestuft.			Carl Roth GmbH & Co KG

8.4. Formeln und Berechnungen

Berechnung des Gesamtpolyphenolgehaltes mittels FOLIN-CIOCALTEU-*Assay*

Die Bestimmung des Gesamtpolyphenolgehaltes erfolgte unter der Verwendung einer externen Epicatechinkalibrierung. Der Gesamtpolyphenolgehalt wurde in mg Epicatechinäquivalente (ECA) pro 1 g fettfreier Kakaotrockenmasse mittels folgender Gleichung berechnet:

$$C_{Phenol} = \frac{\left(\left(E_{\mathrm{Pr}orbe} - n\right) \cdot V_{Ex} \cdot V_{Kakao} \cdot VF\right)}{m \cdot m_{\mathrm{Pr}obe} \cdot V_{Aliquot} \cdot V_{\mathrm{Pr}} \cdot 1000}$$

c_{Phenol}	Gesamtpolyphenolgehalt [mg ECA/g fettfreier Kakaotrockenmasse]
E_{Probe}	Extinktion der Probe bei λ = 750 nm
n	y-Achsenabschnitt
V_{Ex}	Extraktionsvolumen (0,018 L)
VF	Verdünnungsfaktor
m	Steigung der Regressionsgeraden
m_{Probe}	Einwaage der Probe [g]
$V_{aliquot}$	Volumen des Aliquots innerhalb der Probenaufarbeitung
1000	Umrechnungsfaktor: mg auf g

Kalibrationsgerade für die Bestimmung des Gesamtphenolgehaltes mittels FOLIN-CIOCALTEU-*Assay*

Konz. [g/L]	Extinktion [AU] (-)-Epicatechin	Extinktion [AU] (+)-Catechin	Extinktion [AU] Chlorogensäure	Extinktion [AU] Gallussäure
0,005	0,113	0,071	-	-
0,010	0,221	0,151	0,081	0,183
0,015	0,323	0,241	0,135	0,282
0,020	0,423	0,325	0,193	0,368
0,025	0,516	0,405	0,254	0,465
0,030	0,608	0,472	0,308	0,543
0,035	0,681	0,567	0,359	0,626
0,040	0,805	0,638	0,414	0,716
0,045	0,886	0,713	0,471	0,801
0,050	0,957	0,792	0,51	0,896
	y = 18.821x + 0.0357 R^2 = 0.998	y = 16.001x - 0.0025 R^2 = 0.9993	y = 10.954x - 0.0255 R^2 = 0.999	y = 17.062x + 0.0268 R^2 = 0.9975

Kalibrationsgerade für die Bestimmung von Flavonoiden mittels Aluminiumchlorid-*Assay*

Konz. [g/L]	Extinktion [AU] (-)-Epicatechin
0,025	0,11
0,05	0,207
0,075	0,308
0,1	0,412
0,125	0,502
0,15	0,595
0,175	0,71
0,2	0,806
0,225	0,922
y = 4.0213x + 0.0053 R^2 = 0.9993	

Kalibrationsgerade für die Bestimmung von kondensierten Proanthocyanidinen mittels BuOH-HCl-*Assay*

Konz. [g/L]	Extinktion [AU] Cyanidin Chlorid	Konz. [mg/L]	Extinktion [AU] PA B2	Konz. [mg/L]	Extinktion [AU] Gerbsäure
0,01	0,13	0,02	0,104	4	0,118
0,02	0,24	0,04	0,222	5	0,133
0,03	0,394	0,06	0,298	6	0,169
0,04	0,538	0,08	0,383	7	0,184
0,05	0,642	0,11	0,462	8	0,217
0,06	(0,712)	0,16	0,629	9	0,235
0,07	0,921	-	-	10	0,254
-	-	-	-	20	0,422
-	-	-	-	30	0,632
-	-	-	-	40	0,834
y = 13,137x - 0,009 R^2 = 0,9982		y = 3,7455x + 0,0519 R^2 = 0,9905		y = 0,0195x + 0,0484 R^2 = 0,9982	

Kalibrationsgerade für die Bestimmung der antioxidativen Kapazität mittels DPPH-*Assay*

Konz. [g/L]	Extinktion [AU] (-)-Epicatechin	Extinktion [AU] PA B2	Extinktion [AU] PA C1	Extinktion [AU] Cinnamtannin A2
0,05	0,886	0,902	0,902	0,905
0,1	0,839	0,854	0,853	0,869
0,2	0,711	0,776	0,789	0,809
0,4	0,515	0,658	0,667	0,709
0,6	0,360	0,540	0,582	0,619
0,8	0,260	0,445	0,499	0,535
1,0	0,198	0,365	0,426	0,480
	$y = 0.5817x^2 - 1.3405x + 0.9578$ R^2 = 0.9997	$y = 0.224x^2 - 0.7919x + 0.934$ R^2 = 0.9994	$y = 0.2277x^2 - 0.7261x + 0.9289$ R^2 = 0.9986	$y = 0.1855x^2 - 0.6405x + 0.9331$ R^2 = 0.9996

Berechnung der Nachweis- und Bestimmungsgrenze vom NP-HPLC-online-DPPH System

Für die Berechnung der Nachweis- (LOD: *Limit of Detection*) und Bestimmungsgrenzen (LOQ: *Limit of Quantification*) wurden Mittelwert und Standardabweichung des Grundrauschens aus zehn Messpunkten aus vier NP-HPLC-online-DPPH Messungen eines Methanolblindwertes mittels folgender Gleichungen berechnet:

Die Nachweisgrenze ist die kleinste Konzentration des Analyten in der Probe, die qualitativ noch erfasst werden kann. Die Ermittlung läuft auf Basis des Grundrauschens

$$LOD = \frac{3 \cdot c_{Std}}{H_{Std}}$$

Bei der Bestimmungsgrenze handelt es sich um die kleinste Konzentration eines Analyten in der Probe, die unter Erfüllung der Anforderungen an eine entsprechende Präzision und Richtigkeit, quantitativ ermittelt werden kann. (Wellmitz *et al*., 2005)

$$LOQ = \frac{10 \cdot c_{Std}}{H_{Std}}$$

c_{Std} Konzentration Standardsubstanz [g/L]

H_{Std} Peakhöhe Standardsubstanz [mAU]

Berechnung der antioxidativen Kapazität mittels NP-LC-online-DPPH

Für die Quantifizierung polyphenolischer Substanzen mittels NP-LC-online-DPPH wurden nachfolgende Kalibriergeraden eingesetzt mit n = 4:

Kalibrationsgerade für die Bestimmung Polyphenole (280 nm)

Konz. [g/L]	E [AU] EC	Konz. [g/L]	E [AU] PA B2	Konz. [g/L]	Extinktion [AU] PA C1	Konz. [g/L]	Extinktion [AU] Cinnamtannin A2
0,053	89,87	0,048	55,84	0,050	59,09	0,050	41,77
0,106	181,67	0,097	109,46	0,100	114,73	0,100	86,21
0,211	359,49	0,193	222,96	0,200	241,83	0,200	180,34
0,423	733,81	0,386	463,93	0,399	511,74	0,400	391,02
0,634	1055,76	0,579	666,48	0,599	742,26	0,600	582,42
0,845	1369,22	0,772	861,81	0,798	966,92	0,800	772,47
1,057	1710,32	0,966	1076,31	0,998	1228,55	1,000	973,14
y = 1637,3x R² = 0,9987		y = 1128x R² = 0,9986		y = 1228,3x R² = 0,9988		y = 968,93x R² = 0,9995	

Kalibrationsgerade für die Bestimmung der antioxidativen Aktivität (515 nm)

Konz. [g/L]	E [AU] EC	Konz. [g/L]	E [AU] PA B2	Konz. [g/L]	E [AU] PA C1	Konz. [g/L]	E [AU] Cinnamtannin A2
0,053	105,86	0,048	75,92	0,050	86,14	0,050	58,20
0,106	210,40	0,097	150,84	0,100	184,78	0,100	148,84
0,211	388,27	0,193	312,98	0,200	360,60	0,200	290,18
0,423	714,56	0,386	620,93	0,399	708,51	0,400	602,36
0,634	953,58	0,579	850,47	0,599	967,62	0,600	824,00
0,845	1161,13	0,772	1045,44	0,798	1207,05	0,800	1060,36
1,057	1337,29	0,966	1276,21	0,998	1419,98	1,000	1262,20
y = 1381x R² = 0,9616		y = 1382,2x R² = 0,9871		y = 1514,7x R² = 0,9829		y = 1320,5x R² = 0,9915	

8.5. Kalibrationsgeraden

Für die Quantifizierung polyphenolischer Substanzen mittels LC-DAD-ESI-MS wurden nachfolgende Kalibriergeraden eingesetzt mit n = 3:

Theobromin	y = 5945x + 9,0707				R² = 1			
Konzentration [g/L]	0,050	0,100	0,201	0,402	0,603	0,804	1,005	2,009
Peakfläche [mAU×s]	305	612	1209	2383	3611	4783	5971	11958

Koffein	y = 5134,2x + 1,9838				R² = 1			
Konzentration [g/L]	0,050	0,101	0,201	0,402	0,604	0,805	1,006	2,012
Peakfläche [mAU×s]	258	519	1037	2052	3125	4134	5159	10332

(-)-Epicatechin	y = 899,01x + 42,944					R² = 0,9986			
Konzentration [g/L]	0,054	0,108	0,217	0,437	0,650	0,867	1,083	2,167	3,250
Peakfläche [mAU×s]	45	111	229	452	658	895	1006	1979	2950

(+)-Catechin	y = 838,77x + 36,233					R² = 0,9986			
Konzentration [g/L]	0,048	0,096	0,191	0,383	0,574	0,765	0,957	1,913	2,870
Peakfläche [mAU×s]	36	91	189	368	548	737	831	1640	2426

Procyanidin A2	y = 929,45x + 25,457					R² = 0,9986			
Konzentration [g/L]	0,027	0,053	0,106	0,212	0,318	0,424	0,530	1,060	1,590
Peakfläche [mAU×s]	25	58	120	235	330	456	516	1015	1489

Procyanidin B1	y = 681,02x + 19,729					R² = 0,9986			
Konzentration [g/L]	0,032	0,065	0,129	0,259	0,388	0,517	0,647	1,293	1,940
Peakfläche [mAU×s]	20	51	104	201	300	406	456	897	1333

Procyanidin B2	y = 861,32x + 4,4753					R² = 0,9991			
Konzentration [g/L]	0,065	0,129	0,258	0,516	0,774	1,032	1,290	2,580	3,870
Peakfläche [mAU×s]	44	113	235	460	687	941	1041	2236	3339

Procyanidin C1	y = 816,97x + 23,229					R² = 0,9982			
Konzentration [g/L]	0,028	0,056	0,113	0,225	0,338	0,451	0,563	1,127	1,690
Peakfläche [mAU×s]	23	53	111	216	314	432	479	931	1400

Cinnamtannin A2	y = 1432x + 89,409			R² = 0,9728		
Konzentration [g/L]	0,102	0,204	0,408	0,612	0,816	1,02
Peakfläche [mAU×s]	169,87	394,31	669,03	1043,15	1361,06	1426,89

Epicatechingallat	y = 937,98x + 23,657			R² = 0,937		
Konzentration [g/L]	0,058	0,115	0,208	0,345	0,460	0,575
Peakfläche [mAU×s]	55	121	219	385	524	490

(-)-Epigallocatechin	y = 1331,4x - 24,403			R² = 0,9974	
Konzentration [g/L]	0,103	0,205	0,410	0,615	0,820
Peakfläche [mAU×s]	111,42	270,81	492,24	788,41	1081,05

(-)-Gallocatechin	y = 1776x - 13,343			R² = 0,9988	
Konzentration [g/L]	0,095	0,191	0,381	0,572	0,763
Peakfläche [mAU×s]	151,24	341,00	660,71	978,24	1357,59

(-)-Catechingallat	y = 1776x – 13,343			R² = 0,9988	
Konzentration [g/L]	0,095	0,191	0,381	0,572	0,763
Peakfläche [mAU×s]	151,24	341,00	660,71	978,24	1357,59

Quercetin	y = 5390,5x + 30,17			R² = 0,9995		
Konzentration [g/L]	0,022	0,054	0,108	0,217	0,435	0,652
Peakfläche [mAU×s]	123,61	319,43	623,76	1203,2	2423,9	3509,9

Quercetin-3-*O*-arabinosid	y = 3349,3x + 33,997			R² = 0,9986		
Konzentration [g/L]	0,020	0,049	0,098	0,195	0,390	0,585
Peakfläche [mAU×s]	73,97	189,03	369,59	702,93	1382,12	1960,13

Apigenin-8-*C*-glucosid	y = 2961,4x - 1,4896			R² = 0,9994	
Konzentration [g/L]	0,053	0,106	0,160	0,213	0,319
Peakfläche [mAU×s]	162,65	312,10	468,87	617,18	950,94

Luteolin	y = 5671,1x - 7.9265			R² = 0,9994	
Konzentration [g/L]	0,052	0,104	0,156	0,207	0,311
Peakfläche [mAU×s]	299,00	578,47	874,14	1152,31	1774,79

Cyanidin-3-*O*-arabinosid	y = 5379x			R^2 = 0.9981	
Konzentration [g/L]	0,0455	0,091	0,182	0,273	0,364
Peakfläche [mAU×s]	-	445,4	1000,4	1464,9	1960,9

8.6. Publikationen

Im Folgenden werden die drei Publikationen aufgeführt, wie sie in den jeweiligen Fachzeitschriften veröffentlicht wurden.

Anal Bioanal Chem (2016) 408:5905–5914
DOI 10.1007/s00216-016-9705-7

RESEARCH PAPER

Extraction of cocoa proanthocyanidins and their fractionation by sequential centrifugal partition chromatography and gel permeation chromatography

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Received: 29 March 2016 / Revised: 4 June 2016 / Accepted: 8 June 2016 / Published online: 18 June 2016

Abstract Cocoa beans contain secondary metabolites ranging from simple alkaloids to complex polyphenols with most of them believed to possess significant health benefits. The increasing interest in these health effects has prompted the need to develop techniques for their extraction, fractionation, separation, and analysis. This work provides an update on analytical procedures with a focus on establishing a gentle extraction technique. Cocoa beans were finely ground to an average particle size of <100 μm, defatted at 20 °C using *n*-hexane, and extracted three times with 50 % aqueous acetone at 50 °C. Determination of the total phenolic content was done using the Folin-Ciocalteu assay, the concentration of individual polyphenols was analyzed by electrospray ionization high performance liquid chromatography-mass spectrometry (ESI-HPLC/MS). Fractions of bioactive compounds were separated by combining sequential centrifugal partition chromatography (SCPC) and gel permeation column chromatography using Sephadex LH-20. For SCPC, a two-phase solvent system consisting of ethyl acetate/*n*-butanol/water (4:1:5, *v*/*v*/*v*) was successfully applied for the separation of theobromine, caffeine, and representatives of the two main phenolic compound classes flavan-3-ols and flavonols. Gel permeation chromatography on Sephadex LH-20 using a stepwise elution sequence with aqueous acetone has been shown for effectively separating individual flavan-3-ols. Separation was obtained for (−)-epicatechin, proanthocyanidin dimer B2, trimer C1, and tetramer cinnamtannin A2. The purity of alkaloids and phenolic compounds was determined by HPLC analysis and their chemical identity was confirmed by mass spectrometry.

Keywords Cocoa proanthocyanidins · Sequential centrifugal partition chromatography · Gel permeation chromatography · Total phenolic content

Introduction

The frequently reported relationship between plant phenolic compounds, often referred to as polyphenols, and health has accelerated the research into these bioactive compounds. Promisingly, proanthocyanidins (PAs) in plant food are investigated because of their reported antioxidant, antiinflammatory, and antimicrobial activity. Especially, PAs from cocoa (*Theobroma cacao* L.) are assumed to possess health benefits [e.g., 1]. In general, cocoa beans contain compounds ranging from simple alkaloids such as theobromine and caffeine to complex polyphenols, which can be classified into the main groups of phenolic acids (hydroxybenzoic and hydroxycinnamic acids), and the quite large group of flavonoids comprising flavan-3-ols (catechin, epicatechin), anthocyanins (cyanidins), flavonols (quercetin), flavones (luteolin), and flavanones (naringenin). Especially higher oligomeric PAs, resulting from a condensation of flavan-3-ols, are assumed to have a higher antioxidant capacity than the monomers or lower oligomeric PAs. Despite the high scientific interest in compounds like cocoa PAs, the majority of them are either not commercially available or are very expensive.

Parts of this work have been presented at Euroanalysis 2015 in Bordeaux, France.

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It is generally consent that at the moment it is impossible to develop an extraction method suitable for all bioactive compounds from plant materials. Nevertheless, a technique that generates significant amounts of purified compounds is essential for analytical purposes or bioavailability and related bioactivity studies. Accordingly, one objective of this work was to evaluate the highest content of total phenols obtained with different extraction conditions. Folin-Ciocalteu method was used for the quantification of the total phenolic content. The proximate composition of the total flavonoid content and the total PA content were also determined photometrically.

In order to further fractionate complex phenolic extracts, preparative techniques in various modes are frequently used to separate bioactive compounds. Separation of PAs in cocoa according to their molecular size has already been reported for normal phase HPLC [2]. Preparative purifying processes for catechins and PAs on reversed phase HPLC of root bark have been also described [3], as well as size exclusion chromatography for PAs obtained from various plant bodies including cocoa beans [4]. In the case of phenolic compounds, solid-liquid chromatography suffers from major drawbacks. The recovery rate and the obtained amounts are much less than the other separation mechanisms. To overcome these problems, separation using liquid-liquid chromatography has attracted researches as a more efficient approach. Shibusawaa, Yanagidaa, Isozakia, Shindoa, and Ito [5] separated apple PAs with regard to degree of polymerization (DP) using *high speed countercurrent chromatography* (HSCCC). Liquid-liquid chromatographic techniques such as HSCCC or *sequential centrifugal partition chromatography* (SCPC) are covering an important niche among the chromatographic methods applied for the isolation of secondary plant metabolites. A significant advantage can be seen in the avoidance of irreversible adsorption interactions on solid stationary phases, the limited risk of sample denaturation, the possibility for a total sample recovery, a comparatively large load capacity, and the possibility to perform separations on a semi-preparative scale [6]. SCPC is a relatively new continuous cyclic liquid-liquid chromatographic separation technology [7]. In contrast to CCC, CPC chromatography enables a fast chromatographic run and an almost unlimited upscaling.

A study by Esatbeyoglu, Wray, and Winterhalter [8] showed the possibility of isolating dimeric, trimeric, tetrameric, and pentameric PAs from unroasted cocoa beans using HSCCC with *tert*-butylmethlyether/*n*-butanol/water (4.3:0.7:5, *v*/*v*/*v*) as solvent system. Further, different modes of operation do however not permit a simple transfer of a HSCCC solvent system for isolation of cocoa polyphenols using to a SCPC separation. A complete separation of the main cocoa compounds using SCPC has not been described, yet. Furthermore, as the separation efficiency of liquid-liquid chromatographic systems is typically not sufficient for achieving a complete separation of complex samples in one single chromatographic run, the combination of SCPC with other methods such as preparative column chromatography is necessary because of their complementary properties.

Combinations of HSCCC and gel permeation chromatography using Sephadex LH-20 have already been shown to be an efficient separation technique. In a study by Yang, Li, and Wan [9], the main individual tannins from black tea were purified, whereby theaflavin, theaflavin-3-gallate, theaflavin-3-gallate, and theaflavin-3,3-digallate were obtained in a separation process using a combination of HSCCC and a chromatographic separation with Sephadex LH-20. Also, Zhou, Liang, Zhang, Zhao, Guo, and Shi [10] obtained a purification of glucosidase inhibitors from *Polygonatum odoratum* by HSCCC and a following separation with Sephadex LH-20. A study done by Cheel, Minceva, Urajová, Aslam, Hrouzek, and Kopecký [11] used also preparative CPC separation followed by gel permeation chromatography to obtain on 50 mg of crude soil cyanobacteria extract a yield of 3.5 mg Aeruginosin-865, with a purity over 95 % as determined by HPLC.

Therefore, the aim of the present study was first to find an efficient extraction method for cocoa PAs. For this purpose, various extraction solvents were studied and extraction conditions such as temperature, solid-liquid ratio, and extraction time were optimized. The second objective was to establish a new method for the separation and purification of cocoa bean extracts using sequential centrifugal partition chromatography combined with gel permeation chromatography. Critical parameters such as the two-phase solvent system and the sample load for the SCPC separation were optimized. The chemical structures of the purified compounds obtained were confirmed by mass spectrometry.

Materials and methods

Materials

The phenolic reference substances (−)-epicatechin and (+)-catechin were purchased from Sigma Aldrich Chemie GmbH (Buchs, Switzerland). Proanthocyanidin B2, proanthocyanidin C1, and cinnamtannin A2 were purchased from PhytoLab GmbH & Co. KG (Vestenbergsgreuth, Germany). Proanthocyanidin B3 and B4 were purchased from TransMIT GmbH (Giessen, Germany). Further reference substances such as theobromine, caffeine, (−)-epicatechin, (+)-catechin, proanthocyanidin B1, quercetin, quercetin-3-*O*-arabinoside, and quercetin-3-*O*-glycoside were obtained from Extrasynthese (Lyon, France). Anhydrous sodium carbonate, aluminum chloride hexahydrate, *n*-butanol, Folin-Ciocalteu reagent, sodium hydroxide, and sodium nitrite were purchased from Sigma-Aldrich (Buchs, Switzerland). HPLC grade acetonitrile, water, ethanol, acetone, methanol, 1-propanol,

2-propanol, and formic acid were obtained from Sigma Aldrich Chemie GmbH (Buchs, Switzerland). Double-distilled water (Merck & Cie KG, Schaffhausen, Switzerland) was used throughout the whole study. Column chromatography was carried out with Sephadex LH-20 gel (GE Healthcare Bio-Sciences AB, Glattbrugg, Switzerland).

Sample preparation

Dried and fermented cocoa beans were from a genuine Trinitario variety grown and harvested in Ghana. Optimization of sample preparation was performed as described previously [12]. Primarily, frozen cocoa beans were freeze dried (Alpha 2-4 LDplus, Martin Christ, Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany), manually dehulled, and crushed in a knife mill (A 11 basic Analytical Mill, IKA ®-Werke GmbH & Co. KG, Staufen, Germany) for approx. 10 s to minimize heat development. In order to remove lipids, cocoa beans were extracted five times with the fivefold volume (*v*/*w*) of *n*-hexane for 5 min at 20 °C. The hexane phase was removed by centrifugation at 2880×*g* for 1 min (Centrifuge 5810, Vaudaux-Eppendorf AG, Schönenbuch, Switzerland). This procedure of defatting and grinding was done four times until the *n*-hexane phase appeared colorless. After defatting, cocoa bean powder with an average particle size of <100 μm was further used for the extraction of target compounds. For the analysis of the total phenolic content, 2 g of defatted and dry cocoa powdered samples was weighed into a 50-mL centrifuge vessel and extracted three times with 6 mL of aqueous acetone mixture for 8 min at 50 °C (Thermomixer MHR 23, Ditabis AG, Pforzheim, Germany). After each extraction step, the mixture was centrifuged and the combined supernatant was considered as *liquid cocoa extract* and directly used for spectrophotometric assays, or freeze dried for SCPC analysis and named *freeze dried cocoa extract*.

Solvent and temperature optimization

A range of solvents were tested for their efficacy in extracting target compounds from ground cocoa bean extract, including methanol, ethanol, 1-propanol, 2-propanol, and acetone, and their respective mixtures with water. Thirteen extraction temperatures (20–140 °C) were evaluated for their effect on the extraction of valuable cocoa compounds.

Generally, 2 g of cocoa bean powder was extracted three times with 6 mL of solvent/water mixtures for 8 min in a 50-mL centrifuge tube at different temperatures using a benchtop shaker (Thermomixer MHR 23, Ditabis AG, Pforzheim, Germany) at 800 rpm. The liquid cocoa extract obtained was analyzed for the total phenolic content using the photometric Folin-Ciocalteu assay, and for individual components using RP-HPLC/MS. Subsequently, individual compounds of the maintained liquid cocoa extracts were measured by RP-HPLC/MS. All extraction experiments were done in triplicate.

Determination of the total phenolic content (TPC) using Folin-Ciocalteu assay

The photometric method for determining the total phenolic content is used frequently in food analysis and is applicable to polar solvents. However, there are other reducing substances present, such as sugars or proteins, which are also able to react with the Folin-Ciocalteu reagent [13]. The total phenolic content (TPC) of cocoa beans was determined according to Blois [14] with some minor modifications: For *liquid cocoa extract* suspected of having high reactivity, the following dilutions were made: 1:10, 1:100. Following, a 1-mL aliquot of the *liquid cocoa extract* was mixed with 1 mL of Folin-Ciocalteu reagent (2 N reagent diluted 1:3 with dest. H_2O); 2 mL distilled water was added and the sample incubated for 3 min at room temperature. Thereafter, 2 mL of anhydrous sodium carbonate solution (20 % Na_2CO_3, *w*/*v*) was added. The solution was kept for 2 h at room temperature for color formation, and the absorption of the blue colored sample was measured at 750 nm using an UV/Vis spectrophotometer (Genesys™ 10S, Thermo Fisher Scientific AG, Reinach, Switzerland) against a blank sample containing the same reagents and 1 mL distilled water. (−)-Epicatechin was used as a calibration standard and final results were expressed as milligrams of (−)-epicatechin equivalent per gram of non-fatty dry matter (mg ECE/g). The calibration curve was linear from 5 to 50 mg/L with a linear regression line of $y = 18.821x + 0.0357$ and $R^2 = 0.998$.

Determination of the total flavonoid content (TFC) using the aluminum chloride assay

Besides the determination of the TPC, the total flavonoid content (TFC) of the cocoa extract gives an indication of the phenolic composition of cocoa beans and the yield of the different extraction procedures. The determination of the TFC was done photometrically according to the method described by Zzaman, Bhat, and Yang [15] using the same extract as described before: for *liquid cocoa extract* suspected of having high reactivity, the following dilutions were made: 1:10, 1:100. Further on, an aliquot (1 mL) of the *liquid cocoa extract* or the standard solution was added to a test tube containing 4 mL distilled water. Afterwards, 0.3 mL sodium nitrite solution (5 % $NaNO_2$, *w*/*v*) was added and the sample was incubated for 6 min at room temperature. Aluminum trichloride solution (0.3 mL; 10 % $AlCl_3$, *w*/*v*) were added and the sample incubated for another 6 min. Two milliliters of a 1 M sodium hydroxide solution (4 % NaOH, *w*/*v*) was added and the sample was filled up to 10 mL using distilled water. After 15 min, the absorption of the pink solution was

measured at 510 nm. A linear calibration line was obtained using (−)-epicatechin at concentration range of 25 to 225 mg/L with a linear regression line $y = 4.1072x - 0.0025$ and $R^2 = 0.998$, whereby the total flavonoid content was expressed in milligrams of (−)-epicatechin equivalent per gram non-fatty dry matter (mg ECE/g).

Determination of the total proanthocyanidin content (TPAC) using the acid butanol assay

The acid butanol assay is widely used to determine the total proanthocyanidin content (TPAC) in food. The method by Porter, Hrstich, and Chan [16] and a modified one by Pérez-Jiménez, Arranz, and Saura-Calixto [17] were used during this study. For *liquid cocoa extract* suspected of having high reactivity, the following dilutions were made: 1:5, 1:10. Further on, in a screw cap tube, 6 mL of acid butanol (5 mL 37 % HCl plus 95 mL *n*-butanol) reagent was added to 1 mL aliquot of the *liquid cocoa extract*. As a catalyst, 1 mL of a 2 % solution of ammonium iron(III)sulfate in 2 N HCl (2 % $NH_4Fe(SO_4)_2 \cdot 12H_2O$) was added. The tube was sealed and incubated at 95 °C for 50 min using a benchtop shaker, followed by an immediate cool down with cold water. Afterwards, the absorption of the pink color was measured photometrically at 550 nm against a blank. The proanthocyanidin content was calculated using cyanidin at concentration range of 10 to 70 mg/L using the following equation: $y = 13.137x - 0.009$ and $R^2 = 0.9982$. The proanthocyanidin content was expressed in milligram of cyanidin equivalent per gram non-fatty dry matter (mg CyE/g).

Sequential centrifugal partition chromatography (SCPC)

The separation was carried out on a semi-preparative SCPC liquid-liquid partition chromatography system (Armen Instruments, Saint-Avé, France) with a total volume of 250 mL. A HPLC pump (Pump Model SD-300, Rainin Instrument Co. Inc., Oakland, CA, USA) was used to deliver the mobile phase, in ascending and descending mode, respectively. The maximum rotational speed used was 3000 rpm and a maximum back pressure of 60 bar. The effluent was continuously monitored at 275 nm using an UV detector (UV1, Rainin Instrument Co. Inc. Oakland, CA, USA). A fraction collector (Foxy 200, Teledyne Isco, Lincoln, NE, USA) was connected to the detector outlet. A manual injection valve (10 mL Rheodyne loop) allowed injections ranging up to 6 g. The choice of the biphasic solvent system and the elution mode are crucial for successful liquid-liquid chromatographic separations. The biphasic solvent system ethyl acetate/butanol/water with 4:1:5 (*v*/*v*/*v*) was prepared by mixing defined portions of ethyl acetate, butanol, and water and degassing vigorously. After degassing, the solvent was equilibrated at room temperature for 30 min. In the meantime, the freeze dried cocoa extract (700 mg) was dissolved in 2 mL 40 % aqueous 2-propanol supported by the use of an ultrasonic bath for 5 min at room temperature. The sample solution was then partitioned between 4 mL each of the mobile and the stationary phase and filtered through a 5.0-μm syringe filter (Nylon 66, BGB Analytik AG, Boeckten, Switzerland). Afterwards, sample solution was injected into the SCPC column through the manual injection valve. The chromatogram was continuously monitored and fractions were collected for a period of 5 min each with the fraction collector.

For the SCPC fractionation, the column was initially filled with the stationary phase (organic phase, upper phase) in the *descending* mode at a flow rate of 1.5 mL/min and a revolution speed of 500 rpm. The mobile phase (aqueous phase, lower phase) was then pumped at a revolution speed of 1900 rpm until an equilibrium was established after 90 mL dead volume. After the separation of the first major peaks in the chromatogram was achieved, the column mode was turned to *ascending* after 220 min, in order to recover the components that were not eluted under these conditions. The preparative separation of individual substance classes was carried out in a single run. The peak fractions (I–IV) were collected automatically, correspondingly to the chromatogram obtained and subjected to further HPLC-MS analyses.

Gel permeation chromatography

As applying a single separation technique typically is not sufficient to obtain individual components covering a wide range of polarities, a combination of chromatographic methods needs to be performed. Therefore, semi-preparative SCPC was selected as first and most important separation technique for its lack of a solid stationary phase that can lead to irreversible adsorption of phenolic components (e.g., on reversed phase chromatographic material) and a complete sample recovery [18]. The second purification step applied for isolating fractions or even single compounds of high purity was done on a lipophilic Sephadex LH-20 gel. Traditional column chromatography has been already proven for being suitable of separating a number of phenolic compounds. Especially, alkylated crosslinked dextran Sephadex LH-20 is suitable for a separation of polyphenolic polymers when using aqueous acetone as eluent [19, 20].

In the present study, a glass column 10 × 120 mm with a volume of 6 mL (ECO10/120V0V, YMC Europe GmbH, Dinslaken, Germany) was packed with 1 g of Sephadex LH-20. For purification, 40 mg of 'PA fraction' was dissolved in 100 mL 40 % aqueous 2-propanol and directly subjected to Sephadex LH-20. Saturation and equilibration were done with pure water before adding the sample. The elution was performed by stepwise elution, starting with 100 mL defined solvent at a moderate flow rate of 0.6 mL/min. The effluents were collected in 100 mL volumetric flasks. Subsequently, the

elution step was performed starting with 100 mL dest. H_2O as mobile phase, continuing with 100 mL 10 % aqueous acetone, and further with 100 mL 15 % aqueous acetone etc. until 100 mL 50 % aqueous acetone. The fractions containing target compounds were freeze dried and dissolved in 40 % aqueous 2-propanol for RP-HPLC/MS analysis. Regeneration of the column was done with 40 % aqueous 2-propanol for at least twofold column volume before exposing again to sample.

Determination of individual cocoa compounds using RP-HPLC/MS analyses

Samples were analyzed with a liquid chromatograph coupled to a quadrupole mass spectrometer with electrospray ionization interface (LC/MS 6120, Agilent Technologies AG, Waldbronn, Germany). The fractions were analyzed in ESI-MS as total ion chromatogram in the positive mode *m*/*z* 100–2000. Detailed conditions of the HPLC system and gradient elution are given in Pedan, Fischer, and Rohn [12]. All data obtained was processed with LC/MSD ChemStation software version Rev. B.04.03-SP1 (Agilent Technologies AG, Waldbronn, Germany).

Results and discussion

Effect of extraction solvents on cocoa polyphenols

As already mentioned, cocoa extract is rich in monomeric and oligomeric PAs. The yield of PAs varies with the two main extraction parameters: type of solvent and extraction temperature. Initially, the efficiency of the various extraction solvents was investigated at room temperature. The results of the TPC are summarized in Fig. 1, whereby the different extraction yields of PAs are shown in relation to the type of solvent used. The yield of extracted PAs increases within the homologous series of alcohols such as methanol, ethanol, and 1-propanol and is influenced by a decreasing polarity. It can also be pointed out that extraction yield is more intense with increasing chain length of the solvent and a decrease of the amount of water in the extraction solvent. The results show that the mixture of 50 % aqueous acetone was the most efficient in extracting phenolic compounds.

Besides the determination of the TPC, also the TFC and the TPAC were measured in the different aqueous acetone mixtures. Figure 2 shows the extraction yields for all three assays. Again, the 50 % aqueous acetone mixture showed the best efficiency for extracting the different polyphenol classes in cocoa. The content of extracted total phenols of an unroasted cocoa extract was 60.4 mg ECE/g, the TFC was up to 37.2 mg ECE/g, and the TPAC was up to 25.4 mg CyE/g for 50 % aqueous acetone.

In order to substantiate the colorimetric results, RP-HPLC/MS analyses of the *liquid cocoa extracts* were carried in addition. Cocoa bean compounds were identified based on retention times and mass spectra of reference compounds. In detail, it could be shown that 100 % acetone as extraction solvent selectively extracts the two alkaloids theobromine and caffeine. With increasing water content (e.g., 10 %, 90 % aqueous acetone) (+)-catechin and (−)-epicatechin, followed by PA dimer were extracted. Extraction efficiency was improved for PA trimers and tetramers with solvent concentrations between 20 and 80 % aqueous acetone and culminates with the highest extraction yield for condensed polyphenols using 50 % aqueous acetone. The obtained cocoa extracts consist of at least oligomeric PA with DP = 4. Generally, same order of solvent efficiency as shown in Fig. 1 (50 > 40 %, 60 %, etc.) was obtained when using RP-HPLC/MS analysis for identifying the individual phenolic compounds. Extraction efficiency with lower percentage of acetone/water mixture, or even water, was more selective for extracting single or low molecular weight polyphenols. On the other side, an indication for high oligomeric polyphenols can be observed through the additional peak eluted at the end of the chromatogram.

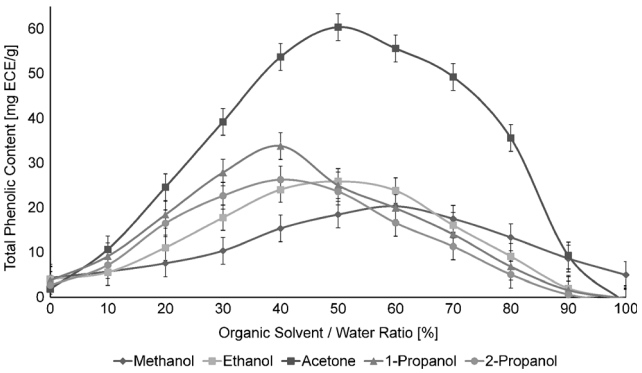

Fig. 1 The influence of extraction solvent on the total phenolic content (TPC). Values are expressed as milligram (−)-epicatechin equivalents per gram non-fat dry matter (mg ECA/g)

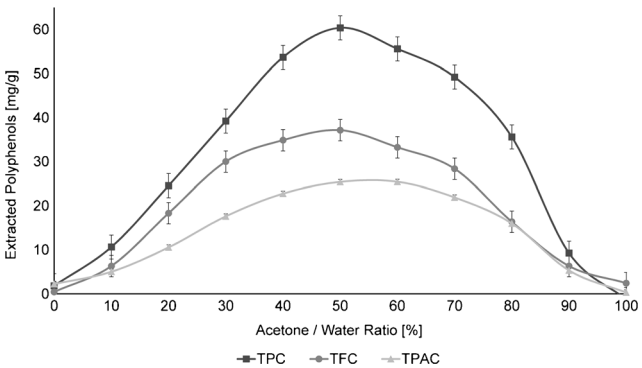

Fig. 2 The influence of 50 % aqueous acetone mixture on the total phenolic content (TPC), total flavonoid content (TFC), and total proanthocyanidin content (TPAC)

Therefore, at a combination of acetone/water (50 %), preferentially more PA oligomers are extracted (see 'PA hump').

Separation of complex mixtures typically fails when using reversed phase columns. Generally known, the efficiency of a reversed phase column for separating higher oligomeric PAs is limited due to the separation of complex polyphenols. In some cases, only peak broadening occurs, whereas in many cases, a group of higher oligomeric PAs occurs as a "hump" at the end of the chromatographic run. Tarascou et al. [21] proved that high polymerized polyphenols eluting as a hump at the end of the chromatographic profiles. Kuhnert [21] stated that a chromatographic hump is generally a consequence of a complex mixture containing a too large number of compounds to be chromatographically resolved. Similar observations in the chromatographic behavior were observed in previous studies of the so-called PA hump during the analysis of unroasted cocoa beans [12]. In case of the so-called thearubigin hump, containing condensed black tea polyphenols, major efforts have been undertaken in recent years by Kuhnert [22] to resolve this into several dozens of individual components consisting of quite similar structural subunits. It is reasonable to assume that a similar situation exists in cocoa, based on the similarity of the polyphenol (epicatechin) subunits and the proven occurrence of linearly condensed higher PAs.

In the present study, acetone-based solvents were most efficient for extracting oligomeric PAs, as compared to other extractions solvents, e.g., alcohol/water mixtures. The extraction is therefore preferably carried out with 50 % aqueous acetone. Hammerstone and Chimel [23] found acetone and ethanol being the most effective solvents in extracting higher oligomeric cocoa PAs. Especially for PAs with DP5-10, the efficiency increases significantly when comparing different water to solvent ratios, like e.g., the average percentage recovery of PA decamer increased from 0 % using 100 % acetone to more than 100 % when using 50 % aqueous acetone. Monrad, Howard, King, Srinivas, and Mauromoustakos [24] investigated red grape pomace and found 50 % aqueous ethanol to be optimal for extracting total procyanidins than other ethanol/water compositions.

Effect of temperature on the extraction of cocoa polyphenols

An extraction can be carried out at temperatures ranging from chilled solvents up to the boiling points of the solvents. Since polyphenols are heat labile components, flavon-3-ol losses occur at elevated temperatures. Further, investigations on temperature conditions for comparing changes in content and composition are mandatory. In the present study, the total phenolic content was measured by Folin-Ciocalteu extracted three times for 8 min using 50 % aqueous acetone as solvent. Hereby, the concentration of cocoa polyphenols increased significantly with increasing temperature in the range of 20–110 °C, in detail 88 mg ECE/g at 20 °C to 116 mg ECE/g at 110 °C and began to decrease again to 99 mg ECE/g at 140 °C (Fig. 3). The data obtained confirmed the significant effect of temperature on decreases or increases of TPC dependent on the applied temperature. In addition to the photometric assays, RP-HPLC/MS analyses of the *liquid cocoa extracts* from the different extraction procedures were carried out. In general, the yield of individual bioactive compounds such as (+)-catechin, (−)-epicatechin, PA dimer B2, and trimer C1 increased only slightly within the temperature range (data not shown). In contrast to another study dealing with the temperature influence [25], no degradation or loss could be observed at temperatures from 20 to 140 °C during an 8-min extraction period. However, according to the present results, the extraction temperature was kept at 50 °C to ensure a reproducible polyphenol extraction below the boiling point of acetone as part of the extraction solvent. In addition, the moderate conditions help to preserve most of the initial phenolic compounds and to obtain the genuine flavan-3-ol composition.

Studies by Kothe et al. [25] claimed for epicatechin and PA dimer B1, B2, B5 progressive changes within a temperature range from 100 to 140 °C. Temperatures above 140 °C did not have an accelerated influence on further degradation.

Hammerstone and Chimel [23] also observed a temperature effect on the recovery of oligomeric cocoa PAs and recommended an elevated extraction temperature at 50 °C with an aqueous acetone mixture. Furthermore, it was pointed out in that study that the effect of temperature is non-significant for monomeric PAs and is significantly pronounced for more complex oligomeric PAs.

As already stated above, phenolic compounds' degradation depends on roasting time and temperature. The product pattern, resulting from thermal treatment at high temperatures under non-aqueous conditions (roasting) is quite different. For some flavonol, mechanism for degradation under roasting conditions is a deglycosylation to the corresponding aglycone. The main aglycone product remains stable during further

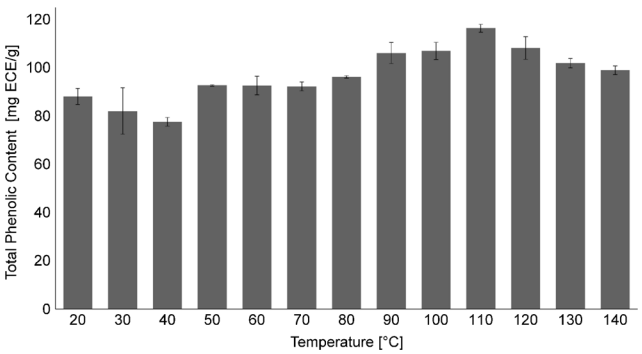

Fig. 3 Influence of temperature extraction (20–140 °C) on the total phenolic content (TPC) as determined by using 50 % aqueous acetone and 8 min of extraction

roasting. Compared to the cooking process, flavonol glycosides showed several reaction products. In this context, studies observed degradation for flavonol glycosides in aqueous solution when heating up to 100 °C, whereby glycosides showed an intensive breakdown but did not form aglycones [26, 27].

Structural elucidation of isolated PAs with RP-HPLC/MS

The chromatogram of the SCPC separation gave four fractions (Fig. 4) and the coil fraction. Separation was completed within about 2.5 h so that at least two chromatographic runs could be performed within 1 day. Fractions I and II eluted in sharp peak starting at a retention time of about 70–80 min, respectively 85–120 min. Fraction III eluted in a broad peak between 155 min followed by return to baseline at about 220 min. By switching, the elution mode fraction IV eluted within 220–250 min. Several SCPC runs were repeated under the same condition, whereby the retention times were consistent between chromatographic runs.

The following compounds were identified according to their retention time and confirmed by mass spectrometry and authentic reference compounds. PAs were detected at 280 nm. In Fig. 5, the peaks numbered with roman numerals correspond to the compounds assigned in the following text. During the separation in *descending* elution mode, polar compounds eluted earlier. The polar fraction I from the partition of crude *freeze dried cocoa extract* contained mainly a mix of hydrophilic, largely complex, mostly branched molecules. An indication might be the 'PA hump' explained earlier in the study.

For the numbered peaks, it was possible to obtain a MS signal and in some cases MS fragmentation pattern. The resulting data are shown in Table 1. Based on the experiments and knowledge, the fragmentation pattern $[M+H]^+$ of the identified compounds was summarized here as well.

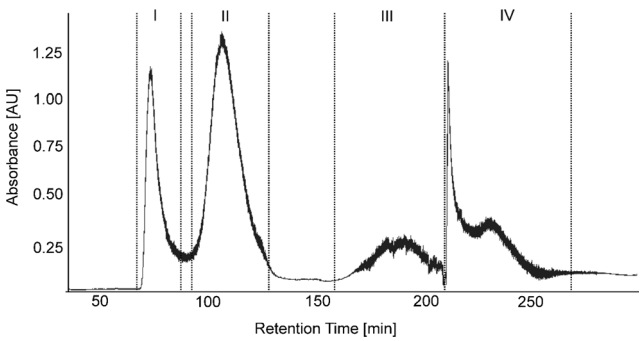

Fig. 4 SCPC chromatogram when applying 700 mg cocoa extract. SCPC conditions: solvent system, EtOAc/BuOH/H_2O 4:1:5 (*v/v/v*); stationary phase, upper phase; revolution speed, 1900 rpm; detection wavelength, 275 nm; flow rate, 1.5 mL/min. Switchover from descending to ascending after 220 min. Fr I–IV with (*I*) hydrophilic complex compounds; (*II*) theobromine; (*III*) caffeine; (*IV*) flavonols and proanthocyanidins

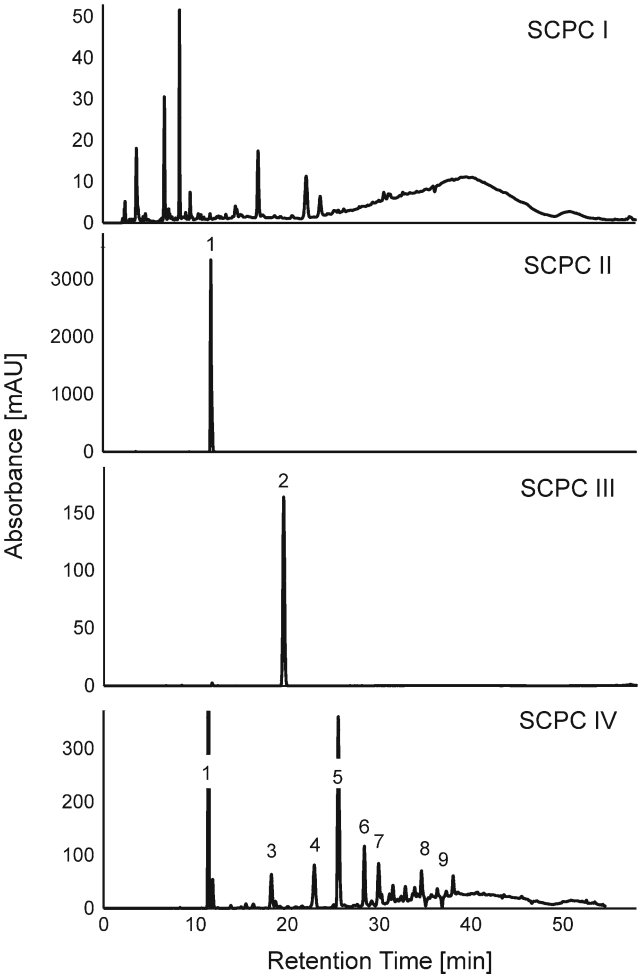

Fig. 5 HPLC chromatograms of the SCPC fractions Fr I–IV. Peaks numbered with roman numbers are in the order of increasing retention time and correspond to the compounds explained in the text. SCPC separation of fraction IV was operated three times before analyzing. (*I*) Hydrophilic complex compounds; (*II*) theobromine; (*III*) caffeine; and (*IV*) flavonols and proanthocyanidins

In detail, a major portion of polar compounds eluted preferably at the beginning of the HPLC run. Furthermore, an unresolved 'PA hump' was observed for fraction I eluting in a retention timeframe of 35–45 min. Fraction II has been identified as theobromine (1, *m/z* 181.1). Fraction III contained caffeine (2, *m/z* 195.1).

In contrast, the fraction obtained with butanol (fraction IV) contained compounds with a high structural diversity such as flavan-3-ols with up to four epicatechin subunits and flavonol aglycones, with the latter also glycosylated with arabinose, galactose, and glucose. However, the fractions were still very complex in their composition and presence of residual theobromine (1). This underlines that the partition coefficient of flavan-3-ols and flavonols like quercetin and their glycosylated products are comparatively similar, being the main reason for not finding complete separation conditions.

Table 1 RP-HPLC-ESI/MS determination of phenolic compounds in an extract of unroasted cocoa beans. Compounds with peak no. 1–17 were detected in positive ion mode

Peak no.	Compound	t_R [min]	Molecular weight [g/mol]	Molecular ion $[M+H]^+$	Major fragments $[M+H]^+$
1; 10	Theobromine	11.7	180.16	181.1	n.d.
2; 11	Caffeine	19.6	194.19	195.1	n.d.
3; 13	(+)-Catechin	18.3	290.26	291.1	139.0; 165.1
4; 14	PA B2	22.9	578.52	579.2	291.1
5; 12	(−)-Epicatechin	25.5	290.26	291.1	139.0; 165.1
6; 15	PA C1	28.4	866.77	867.2	579.2; 1155.5
7; 17	Cinnamtannin A2	30.0	1155.04	1155.3	579.2; 867.0
8	Quercetin-3-O-glucoside	34.6	464.38	465.1	303.1
9	Quercetin-3-O-arabinoside	38.1	434.35	435.1	303.0
16	Unknown PA dimer	34.8	866.77	867.2	579.2; 1155.5

n.d. not detected

In detail, fraction IV contained eight major peaks identified as target phenolic compounds (peaks 3–9). Based on the HPLC/MS analysis, they were determined as (+)-catechin (3, *m/z* 291.1), PA dimer B2 (4, *m/z* 579.1, with a dominant fragment ion *m/z* 288.9), (−)-epicatechin (5, *m/z* 291.1), PA trimer C1 (6, *m/z* 866.5), as well as PA tetramer A2 (7, *m/z* 1155.1). Further compounds were UV-active at 360 nm and therefore assigned as negative peaks in the chromatogram (peak 8, 9). They were identified as quercetin-3-*O*-glucoside (8, *m/z* 465.1, with fragment ions *m/z* 303.1) and quercetin-3-*O*-arabinoside (9, *m/z* 435.1, with fragment ions *m/z* 303.0), tentatively. However, their amount is low compared to flavan-3-ols.

Nevertheless, this method separates of about 700 mg *freeze dried cocoa extract* (I), 232 mg of hydrophilic complex compounds, (II) 88 mg theobromine, (III) 19 mg caffeine, (IV) 149 mg flavones and flavan-3-ols, and 90 mg retained in the coil.

On the basis of a high PA accumulation in fraction IV, this fraction is called 'PA fraction'. The occurrence of theobromine (1) in fraction IV is significant and due to carry-over effects in the partition cells. Nevertheless, this effect of sample carry-over is still limited compared to conventional separatory-funnel partition. Nevertheless, an additional sample clean-up is required to minimize such "contamination effects."

However, the SCPC run resulted in four fractions according to the corresponding peaks separated. A typical color pattern can be observed for the eluted fractions. Fraction I had an intensive brown color. According to Fincke and Fincke [28], the brown color typically indicates water-insoluble phlobaphene. These compounds are structurally complex (see PA hump) and are responsible for the pure brown to the violet-tinged color of the fermented cocoa bean. Moreover, it was recently shown that the color intensity increases from monomeric to polymeric PAs [12]. During cocoa fermentation, anthocyanidins are oxidized by polyphenol oxidase to quinones. The quinones can covalently react with amino acids and proteins or polymerize to form tannins. High molecular weight tannins can also interact non-covalently with proteins through hydrogen bonding, resulting as well in a brown, water-insoluble pigment that gives cocoa its characteristic brown color [29]. With increasing retention time, the color appeared more violet-like for fraction II and fraction III. Upon turning the column mode to *ascending* mode, a quick color change appeared to a bright red for fraction IV, containing the flavan-3-ols and flavonols. The occurrence of the reddish color can be explained by the presence of purple anthocyanidin pigments (e.g., cyanidin arabinoside, -galactoside) in fresh cocoa beans. Although during fermentation these pigments are mostly hydrolyzed by glycosidases, resulting in a more pale purple color [30], they still occur in the extracts of unroasted cocoa.

Isolation of PAs from the freeze dried cocoa extract obtained by gel permeation chromatographie using Sephadex LH-20

In order to compare the purification grade of the main cocoa PAs with SCPC, a further clean-up step by gel permeation chromatography on Sephadex LH-20 was employed to separate individual flavan-3-ols from the semi-purified PA extract. SCPC separation was operated three times, and the same fractions were combined to enrich compounds 3–9. After separation, each fraction was analyzed by RP-HPLC/MS. HPLC analysis of each obtained pure compound can be seen in Fig. 6. The affinity of PAs for Sephadex LH-20 was used for further fractionation. Here, the optimal separation of fraction IV was performed using aqueous acetone as eluting solvent in a gradient from 0 to 40 % in increments of 5 %. To improve the purity of the isolated sub-fractions, a stepwise elution with aqueous acetone as mobile phase was performed

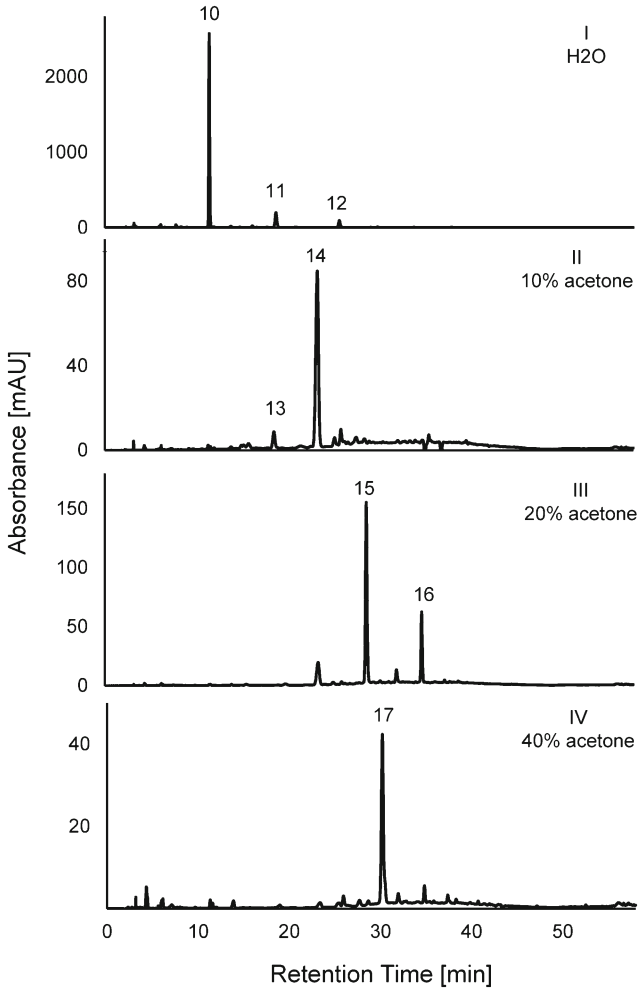

Fig. 6 Purification of the main individual PAs through gel permeation chromatography on Sephadex LH-20. Peaks numbered with roman numbers are in the order of increasing retention time and correspond to the compounds explained in the text

from 0 to 40 % in increments of 10 %. A sequential elution starting with 100 mL H_2O assigned compounds as theobromine (10, *m/z* 181.1), caffeine (11, *m/z* 195.1) and (−)-epicatechin (12, *m/z* 290.9) in fraction I. The eluate was collected and freeze dried immediately. Among the requested bioactive monomeric flavan-3-ol, also theobromine and caffeine could be detected in higher amounts. A further fractionation with 10 % aqueous acetone, the phenolic compounds (+)-catechin (13, *m/z* 290.9) and PA dimer B2 (14, *m/z* 578.7) were obtained. Anterior impurities of theobromine and caffeine could be eliminated through sufficient aqueous extraction. Further on, the elution with 20 % aqueous acetone gained PA trimer C1 (15, *m/z* 866.5) and an unknown PA dimer (16, *m/z* 578.7). The major peak of the last elution step with 40 % aqueous acetone was identified as PA tetramer A2 (17, *m/z* 1156.0). Forty milligrams of the "PA fraction" obtained a yield of 1.4 mg of fraction I, 5.6 mg of fraction II, 2 mg of fraction III, and 2 mg of fraction IV.

The intermediate elution steps with 15 % (4.5 mg), 25 % (2 mg), 30 % (3 mg), 35 % (2 mg) aqueous acetone lead to an insufficient separation of targeted PA compounds. As a consequence, those fractions were discarded. A fully desorption of the purified flavonoids could be obtained by 50 % aqueous acetone as eluent. In contrast to a direct injection of the crude cocoa extract on the Sephadex LH-20 column, the solvent is not strong enough to elute all substances, especially the group of high complex brown tannins. This complex group interacted irreversible with Sephadex LH-20.

Again, a typical color pattern could be observed, as already mentioned above. The color increased correspondingly to the elution order, beginning with a light brown by 0 % aqueous acetone and ending with bright red for 30 % aqueous acetone.

Conclusion

The present study achieved the most efficient extraction of target secondary metabolites from cocoa such as flavan-3-ols using a threefold extraction with 50 % aqueous acetone (*v*/*v*), a liquid-to-solid ratio of 1-3 (*w*/*v*), and an 8-min extraction time (data not shown). The preferred temperature was set to 50 °C for minimizing solvent evaporation. The polyphenol-rich supernatant was freeze dried and used for further semi-preparative isolation for the compounds of interest.

With regard to their chemical structure, plant polyphenols are quite diverse and complex, so that an optimized extraction method for each compound is difficult to achieve. In this study, extraction efficiencies were calculated as a function of the extraction solvent composition and temperature according to the total and individual PAs in a cocoa bean extract. It was found that 50 % aqueous acetone was an ideal solvent composition. Moreover, using a combination of liquid-liquid and gel chromatographic separation, it was possible to isolate high oligomeric PAs on a semi-preparative scale. This method therefore describes a relatively simple and fast procedure for the fractionation of monomeric, dimeric, trimeric, and tetrameric PAs by using gel permeation chromatography on Sephadex LH-20.

The results provided an efficient methodology for the separation and further identification of bioactive components. The described method has a broad applicability and is rapid, and suitable for preparing biologically active PAs from crude plant extracts. This method can be to obtain purified flavan-3-ols which can be further used for bioactive investigations.

Acknowledgments We are grateful to Dr. Thomas Pfeiffer (AlphaChrom AG, Rheinfelden, Switzerland) for his valuable support in the field of liquid-liquid chromatography.

Compliance with ethical standards

Conflict of interest There is no conflict of interest. The manuscript was approved for publication by all authors.

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Food Research International 89 (2016) 890–900

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

An online NP-HPLC-DPPH method for the determination of the antioxidant activity of condensed polyphenols in cocoa

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ARTICLE INFO

Article history:
Received 18 October 2015
Accepted 31 October 2015
Available online 9 November 2015

Keywords:
Condensed cocoa polyphenols
Degree of polymerization
Online NP-HPLC-DPPH
Antioxidant capacity

ABSTRACT

Unroasted cocoa beans are rich in monomeric flavanols and particularly epicatechin-based proanthocyanidin oligomers, with the latter making up to 60% of the total polyphenol content. Although the antioxidant activity of cocoa polyphenols is well known, it is still a challenging analytical field, especially, when it comes to the determination of condensed polyphenols and the evaluation of their single contribution to the overall activity. Therefore, an online NP-HPLC-DPPH assay was developed to separate the homologous series of condensed polyphenols for assessing their antioxidant capacity in relation to their degree of polymerization (DP), simultaneously. In this context, normal-phase chromatography allows separation of polyphenols based on their degree of polymerization. This study showed that an unroasted cocoa extract contains condensed polyphenols with a DP of up to 10 monomer units. By means of the online post-column derivatisation with 2,2-diphenyl-1-picrylhydrazyl (DPPH), the antioxidant capacity of the separated condensed polyphenols was assessed. It could be shown that, with the exception of the dimers, the contribution to the total antioxidant activity decreased from monomers to decamers. However, from the single proanthocyanidins identified, nonameric and decameric proanthocyanidins were found to have the highest values for the antioxidant capacity. The degree of polymerization associated with each molecular weight fraction was further confirmed using electrospray ionization mass spectrometry coupled with reverse-phase liquid chromatography. The online NP-HPLC-DPPH method can be used as qualitative and quantitative analysis of condensed proanthocyanidins and the simultaneous elucidation of the biological activity of proanthocyanidins in complex mixtures.

1. Introduction

Polyphenols are known to be food constituents of health-beneficial nature, often hypothesised to result from their antioxidant activity. Especially flavanol-rich diets including fruits and vegetables, tea, wine as well as cocoa are thought to have a beneficial effect on, e.g., cardiovascular health (Corti, Flammer, Hollenberg, & Lüscher, 2009). With increasing demand for polyphenol-rich extracts, there is a need to develop a rapid and efficient method to fully characterize plant extracts and to understand the physiological mechanism responsible for a corresponding mode of action of the preventive effects.

With regard to cocoa, its polyphenols are stored in the pigment cells of the cotyledons in the seeds, which are the major source for food-relevant products, such as chocolate. Three groups of polyphenols have to be considered primarily: flavan-3-ols with approx. 37%, anthocyanins with approx. 4% and proanthocyanidins with approx. 58% (Wollgast & Anklam, 2000). Among the major flavan-3-ols in cocoa and cocoa products, the monomers (−)-epicatechin and (+)-catechin are making up to 10% of the total polyphenol consumption in unroasted, fermented cocoa. These monomers can form polymers, the so-called proanthocyanidins (PA). The coupling between the monomeric flavan-3-ols occurs primarily between the positions 4 and 8, but may also involve the positions 4 and 6. Proanthocyanidin dimers (e.g., B1, B2, B3, B4, B5), trimers (e.g., C1), and tetramers (e.g., cinnamtannin A2), wherein the monomers are linked by 4 ≥ 8, represent the most important cocoa PA (Prior & Gu, 2005). Further structural complexity is also given by interactions with insoluble polymeric plant material such as polysaccharides or proteins (Gu et al., 2004; Matthews, Mila, Scalbert, & Donnelly, 1997; Naczk & Shahidi, 2004; Rohr, Meier & Sticher, 2000).

The molecular weight of oligomeric PA is expressed as their degree of polymerization (DP) and is referred to as monomers, dimers, trimers, tetramers etc. According to Gu et al. (2002), they are further defined as monomers with DP = 1, oligomers with DP = 2–10, and polymers with DP > 10. Much higher degrees of polymerization might also exist in cocoa beans, but would be insoluble and quite challenging to analyse. However, the portion of condensed polyphenols present in cocoa is much higher than that of monomeric polyphenols (Neveu et al., 2010; US Department of Agriculture NDL, 2004).

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http://dx.doi.org/10.1016/j.foodres.2015.10.030
0963-9969/

With regard to antioxidant activity, not only low molecular polyphenols have the ability to scavenge radicals, but also oligo- and polymers of higher molecular weight are thought to possess a significant bioactivity (Saura-Calixto, 2012). Moreover, they contribute significantly to the human intake of antioxidants (Dorenkott et al., 2014; Gu et al. 2002). So far, the majority of polyphenol studies, however, focused on the smaller, well extractable molecules, which can be easily detected by RP-HPLC (Bandoniene & Murkovic, 2002; Malherbe, de Beer, & Joubert, 2012; Niederländer et al., 2008). Consequently, most of the in vitro and in vivo studies on antioxidant activity dealt with the low molecular weight polyphenols. The question remains whether and how the antioxidant capacity varies with the degree of polymerization. Due to the lack of reference substances, it is difficult to predict the antioxidant capacity of higher molecular polyphenols in vitro. None of the mentioned technique provides information about the radical scavenging activity of the separated oligomeric PA.

It was the aim of the present study to develop an online NP-HPLC-DPPH method for assessing the radical scavenging activity of high molecular weight polyphenols. This method is based on a separation with liquid chromatography coupled online with the well-known antioxidant capacity assay using DPPH as synthetic, stable radical, scavenged by the target compounds. Thus, the measurement of the antioxidant capacities and the parallel estimation of the separated compounds are permitted. From the countless assays for determining the antioxidant activity, the DPPH assay has become a quite popular method for the analysis of the antioxidant activity of all kinds of substrates. As it was the aim to only compare between the antioxidant activities of the procyanidins, the DPPH assay has been chosen, as it allows a fast reaction with most of the phenolic compounds. The optimum composition of the reagents, reaction time and temperature which affect the sensitivity range of spectrophotometric assays have been studied for the new application technique of the online NP-HPLC-DPPH assay.

2. Material and methods

2.1. Materials and reagents

Polyphenol standard substances such as (−)-epicatechin and (+)-catechin were purchased from Sigma Aldrich Chemie GmbH (Buchs, Switzerland). Proanthocyanidin B2, proanthocyanidin C1, and cinnamtannin A2 were purchased from PhytoLab GmbH & Co. KG (Vestenbergsgreuth, Germany). Proanthocyanidin B3 and B4 were purchased from TransMIT GmbH (Giessen, Germany). 2,2-diphenyl-1-picrylhydrazyl (DPPH), HPLC grade acetonitrile, methanol, 2-propanol and formic acid were obtained from Sigma Aldrich Chemie GmbH (Buchs, Switzerland). Double-distilled water (Merck & Cie KG, Schaffhausen, Switzerland) was used throughout the whole study.

2.2. Sample preparation

2.2.1. Removal of lipids from the cocoa beans

Unroasted cocoa beans, from a Trinitario variety from Finca La Amistad of Costa Rica, were manually dehulled and frozen (−20 °C). To minimize heat development, frozen cocoa beans were then crushed in a knife mill (A 11 basic Analytical Mill, IKA®-Werke GmbH & Co. KG, Staufen, Germany) for 30 s. In order to remove cocoa lipids from the beans, crushed material was extracted with n-hexane at a ratio of 1:5 (*w*/*v*) for 5 min at 20 °C. The hexane phase was centrifuged at 2880 ×*g* for 5 min (Centrifuge 5810, Vaudaux-Eppendorf AG, Schönenbuch, Switzerland). This procedure of defatting was repeated 5 times until the n-hexane extract appeared colourless. The defatted material was air dried for evaporating any hexane residue. After drying, the cocoa powder was sieved and particles of an average particle size of <100 μm were extracted further. The particle fraction was obtained using a sieve shaker (Vibratory Sieve Shaker AS 200 basic, measuring range < 100 μm, Retsch GmbH, Haan, Germany).

2.2.2. Extraction of the analytes

For HPLC analysis, 2 g of the sieved cocoa material were weighed into a 50 mL centrifuge vessel and extracted three times with 6 mL 50% acetone/water for 8 min at 50 °C. After each extraction step, the mixture was centrifuged for 5 min at 2880 ×*g* (Centrifuge 5810, Vaudaux-Eppendorf AG, Schönenbuch, Switzerland). The combined polyphenol-rich supernatant (liquid cocoa extract) was used for further analysis and for semi-preparative isolation of single compounds of interest.

2.3. Online NP-HPLC-DPPH assay

The online NP-HPLC-DPPH assay can be used for a rapid assessment of antioxidant compounds in complex mixtures. As already described above, oligomeric PA are eluting in the order of their increasing degree of polymerization, when using normal phases (Adamson et al., 1999, Hammerstone, Lazarus, & Schmitz, 2000; Kelm, Johnson, Robbins, Hammerstone, & Schmitz, 2006; Yanagida et al., 2007). A method combining the separation of the condensed polyphenol compounds and the simultaneous determination of their antioxidant activity will be an advantage for characterizing bioactivity. However, the greatest benefit of this method is that the contribution of a single compound to the overall activity of a mixture of antioxidants can be measured. The more rapidly the absorbance of the compound decreases, the more potent is the antioxidant activity in terms of hydrogen-donating ability (Yen & Duh, 1994).

For the online NP-HPLC-DPPH assay, a solvent gradient was used on a UV/Vis-HPLC system (Knauer GmbH, Berlin, Germany), equipped with a diol-HILIC column (3.0 mm i.D. × 150 mm, 5 μm; YMC Triart Diol-HILIC, Stagroma AG, Reinach, Switzerland) and a pre-column of the same phase. The separation was carried out under the following conditions: Column temperature 30 °C; flow rate, 0.3 mL/min; sample injection volume, 1 μL. The mobile phase consisted of acetonitrile–water–formic acid (97.9:2:0.1, v:v:v) (solvent A) and methanol–water–formic acid (97.9:2:0.1, v:v:v) (solvent B), applied in the following gradient elution order: 1% B at 1–6 min, 1–20% B at 6–11 min, 20% B at 11–18 min, 20–26% B at 18–34 min, 26% B at 34–50 min, 26–95% B at 50–55 min and 95% B at 55–63 min. Between runs there was a 9 min postrun to re-equilibrate the column. The chromatogram was recorded by means of UV/Vis-detection (UVD 2.1S, Knauer GmbH, Berlin, Germany) at a wavelength of 275 nm.

The DPPH free radical scavenging activity of the extracts was analysed using the DPPH assay, as originally described by Blois (1958) and modified by Brand-Williams, Cuvelier, and Berset (1995). Briefly, the working solution was prepared by dissolving 24 mg DPPH in 500 mL methanol, to obtain an absorbance of 1.1 ± 0.2 units at 515 nm (Thaipong, Boonprakob, Crosby, Cisneros-Zevallos, & Hawkins Byrne, 2006).

The DPPH reagent was filtered through a 0.45 μm membrane filter and carefully degassed before use. The scavenging reaction between DPPH and the condensed polyphenols as well as the DPPH reagent were protected from light.

Following the HPLC column, tubes were connected with a “T” piece and DPPH solution was added at a flow rate of 0.3 mL/min using a separate pump. The scavenging reaction took place in a reaction coil of 4.8 m length, i.d. of 0.38 mm and a volume of 0.550 mL, at a temperature of 60 °C. The induced bleaching of the coloured reagent was detected photometrically at 515 nm in presented in the chromatograms as negative peaks. The instrumental setup is depicted in Fig. 1. The HPLC analysis was done in triplicate for each sample. The standard substances consist of (−)-epicatechin, proanthocyanidin dimer B2, trimer C1, and cinnamtannin A2 which are commercially available and differ in their condensation pattern.

2.4. Quantification of oligomeric PA using online NP-HPLC-DPPH assay

Quantification of individual oligomeric PA and determination of their antioxidant activity in unroasted liquid cocoa extract was done

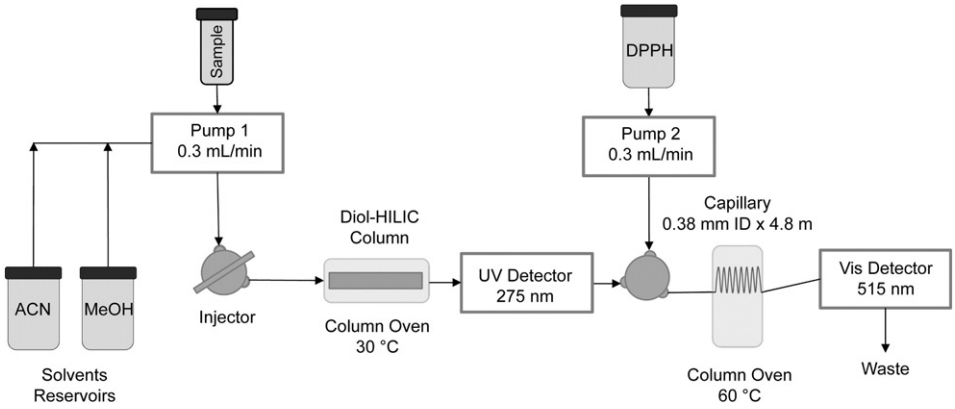

Fig. 1. Instrumental setup of the online post-column NP-HPLC-DPPH methodology.

using external standard calibration. Stock solutions containing the standard substances were therefore prepared and diluted to appropriate concentrations with the same solvent. Briefly, the calibration was achieved using the standard substances (−)-epicatechin as monomer, proanthocyanidin B2 as dimer, proanthocyanidin C1 as trimer and cinnamtannin A2 as tetramer in a concentration range from c = 0.05 g/L to 1 g/L. Quantification was based on flat baseline integration as recommended by Gu et al. (2002). The calibrated curves were generated for each oligomeric class using a quadratic fit of area sum versus concentration. The method optimization was performed using standard substances and afterwards applied to liquid cocoa extracts.

The results of the PA content and their antioxidant activity are expressed for DP 1 as milligrammes of (−)-epicatechin per gramme non-fatty dry matter, for DP 2 as mg of procyanidin dimer B2, for DP 3 as mg trimer C1 and for DP ≥ 4 as mg cinnamtannin A2 per gramme non-fatty dry matter due to the lack of standard substances.

The limits of detection and quantification were determined at a signal-to-noise ratio of about 3.0. Interday variability was chosen to validate the method and to specify the developed assay. Therefore, the mixed standard solution was analysed for three replicates within one day and analysed in duplicate weekly for a month. A linear dependence of negative peak area on concentration of the reference antioxidants was observed.

2.5. DPPH cuvette test

The different antioxidant kinetic behaviours of oligomeric PA were assayed photometrically and the results were compared with those of the online NP-HPLC-DPPH assay. It is worth comparing a new developed assay with an already given method of antioxidant activity measurement such as the DPPH cuvette test.

Braude, Brook, & Lindstead (1954) discovered the hydrogen transfer mechanism between the stable free radical DPPH and antioxidant compounds. As described by Brand-Williams, Cuvelier, and Berset (1995) and some modifications introduced by Thaipong, Boonprakob, Crosby, Cisneros-Zevallos, and Hawkins Byrne (2006), the working solution was prepared by dissolving 24 mg DPPH in 550 mL methanol and left to react in the dark for 24 h at room temperature to obtain an absorbance of 1.1 ± 0.2 units at 515 nm. A composite standard was prepared using commercially available (−)-epicatechin. As this is the main flavonoid compound, it was used as a representative for oligomeric PA in the preliminary tests, although its detection response factor differs from PA which should be kept in mind. Stock solutions were made at the following concentration range: 0.05, 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 g/L, whereby 150 μL standard solution was allowed to react with 2850 μL of the DPPH working solution. To determine the reaction kinetics, the DPPH radical quenching of (−)-epicatechin was measured at 25 °C and 60 °C. The absorbance was noted after an incubation time of 2 and 5 min at 515 nm with methanol as blank and a UV/Vis spectrophotometer (Genesys™ 10S, Thermo Fisher Scientific AG, Reinach, Switzerland).

2.6. Isolation of oligomeric PAs using semi-preparative NP-HPLC

As most of the PAs beyond a DP of 4 are not commercially available, isolation and purification of individual oligomeric PA was required for the validation of the method.

A semi-preparative separation of the PA was achieved using a diol-HILIC column with 20.0 mm i.d. × 150 mm, 5 μm particle size (YMC-Actus Triart Diol-HILIC, Stagroma AG, Reinach, Switzerland). The separation was carried out under following conditions: The flow rate was set to 1.9 mL/min, and the column temperature was 30 °C. As described above, the mobile phase consisted of a mixture of acetonitrile–water–formic acid (97.9:2:0.1, v:v:v) (solvent A) and methanol–water–formic acid (97.9:2:0.1, v:v:v) (solvent B), applied in the following gradient elution order: 1% B at 1–8 min, 1–20% B at 8–22 min, 20% B at 22–45 min, 20–25% B at 45–55 min, 25% B at 55–80 min, 25–95% B at 80–85 min and 95% B at 85–100 min to wash remaining residues off the column. The detector wavelength was set to 275 nm. The semi-preparative NP-HPLC analysis was performed on the same HPLC system as described above for the analytical method. Sample injection was done using a 200 μL injection loop.

For semi-preparative injection, the liquid cocoa extract was freeze-dried to remove the extraction solvent entirely. The freeze-dried extract was named 'PA cocoa extract' (PACE). To prevent the HPLC system from precipitation of solids, 100 mg of PACE was re-dissolved in 400 μL 40% isopropanol, suspended using an ultrasonic bath, and filtered through a 0.45 μm cellulose syringe filter prior to injection.

Fractions of appropriate volumes were collected manually in 10 mL amber glass vials for a couple of minutes following the chromatographic the peak shape visually.

The content of suitable fractions (F1: from 21 to 22 min; F2: from 34 to 35 min; F3: from 39 to 40; F4: from 43 to 45; F5: from 48 to 50 min; F6: from 55 to 57 min and F7: from 62 to 64 min) were pooled, evaporated to dryness under nitrogen with a sample concentrator (Portmann Instruments AG, Biel-Benken, Switzerland), subsequently dissolved in 100 μL in methanol and analysed by RP-HPLC-ESI/MS.

2.7. Characterization of oligomeric PA using RP-HPLC-ESI/MS

For a satisfying determination of the PA, several techniques had to be combined. With an offline two dimensional diol-HILIC normal phase × reversed phase liquid chromatography, the analysis of PA can be effectively performed. In the first dimension, oligomeric PA can be separated according to their molecular weight and the second dimension offers a potent analytical technique of untargeted characterization of individual compounds. An advantage of this method is given by the fact that,

although RP-HPLC separates flavan-3-ols monomers, dimers, and trimers without difficulty, there are obviously limitations for higher oligomers. In a crude matrix the oligomers are co-eluting up to a DP ≥ 4 as a large unresolved peak.

Identification of individual compounds up to tetramers [(+)-catechin, (−)-epicatechin, proanthocyanidin dimer B2, B3, B4, proanthocyanidin trimer C1, and proanthocyanidin tetramer A2] was confirmed by RP-HPLC-ESI/MS. This was performed on an Agilent 1200 series liquid chromatography and quadrupole mass spectrometer with electrospray ionization interface (LC/MS 6120, G6100 series, Agilent Technologies AG, Waldbronn, Germany). The liquid cocoa extract was analysed using a gradient mixture of water-formic acid (99.9:0.1, v:v) (solvent A) and acetonitrile-water-formic acid (94.9:5:0.1, v:v:v) (solvent B). A 2.1 × 150 mm Eclipse XDB-C18 (5 μm) column (Agilent Technologies AG, Waldbronn, Germany) was used. The separation was affected using a linear gradient at 30 °C with a flow of 0.7 mL/min as follows: 1% B at 0–1.5 min, 1–5% B at 1.5–5 min, 5–10% B at 5–12 min, 10% B at 12–18 min, 10–15% B at 18–25 min, 15–20% B at 25–32 min, 20–25% B at 32–37 min, 25–30% B at 37–43 min, 30% at 43–46 min, 30–40% at 46–50 min, 40–50% at 50–52 min, 50–70% B at 52–55 min, 70% B at 55–56 min and 70–10% B at 56–58 min. The re-equilibration time was 8 min.

For ESI/MS analysis, the positive capillary voltage was set at 4000 V and the negative at 3000 V. The drying gas temperature was 350 °C and the drying gas flow 12 mL/min. The samples were analysed using a full scan from 100 to 2000 *m/z* in positive ionization mode. The comparison of retention times and characteristic fragmentation patterns was done using the aforementioned standard substances.

3. Results and discussion

3.1. Online NP-HPLC-DPPH analysis of standard PA

So far, various research groups have used diverse protocols for the investigation of the antioxidant activity of countless plant based products. The DPPH radical is widely used for measuring the efficiency of antioxidants, because of its radical stability, the sensitivity, and the technical simplicity of the assay execution (Huang, Ou, & Prior, 2005). It has become a quite popular method for the analysis of the antioxidant activity of phenolic compounds, as it allows a fast reaction with most of the phenolic compounds. Further assays (providing different antioxidant mechanisms) have not been applied, as it was the aim to only compare between the different cocoa fractions.

The idea behind the online NP-HPLC-DPPH method was to adapt the traditional DPPH cuvette test to HPLC conditions, whereby HPLC allows a full characterization of individual condensed PA in complex mixtures in one single run under the same conditions. For both approaches, the online NP-HPLC-DPPH derivatisation and the optimized analysis in a photometer, the reaction conditions have been adjusted according to the individual reaction kinetics. For an improved reproducibility, the radical scavenging reaction of oligomeric PA with DPPH was optimized in terms of temperature and time. The antioxidant kinetic behaviour was evaluated photometrically and the results were adapted to the online NP-HPLC-DPPH methodology. A linear relationship over a concentration range from 0.05 g/L to 1 g/L was observed at a temperature of 60 °C, when using a reaction time of 2 min (or a corresponding length of the reaction capillary, resp.). The HPLC-PTFE reaction coil was therefore thermostated at 60 °C and the reaction time was kept short (1 min).

The UV and the DPPH chromatograms of the oligomeric standard PA are shown in Fig. 2**.** Peaks with retention times at 4.4, 5.2, 8.7, 18.9, and 22.2 min in the UV chromatogram and the DPPH chromatogram are (−)-epicatechin, (−)-epigallocatechin, PA dimer B2, trimer C1, and cinnamtannin A2.

The reaction was optimized with regard to intensity and shape of the peak signals resulting from the DPPH radical quenching. When applying a non-standardized procedure, the DPPH radical reaction mixture can cause side reactions such as polymerization of catechins and *ortho*-quinones to higher molecular weight oligomers, or adducts between the oxidized form of catechin and DPPH radical (Osman, 2011).

The small peak with the retention time of 5.2 min in the UV chromatogram was identified as (−)-epigallocatechin by RP-HPLC-ESI/MS. Although it has a small UV absorption, its antioxidant activity is much higher than that of (−)-epicatechin. It could be proven that the commercially available reference substance (−)-epicatechin has a slight contamination of less than 0.05% of (−)-epigallocatechin, whereby the antioxidant activity is approx. 30% higher. Previous studies of antioxidant activity supported the role of specific structural components as requisites for radical scavenging (Nanjo, Goto, Seto, Suzuki, Sakai, & Hara, 1996; Nanjo, Mori, Goto, & Hara, 1999). They also confirmed the assertion that the presence of an *ortho*-hydroxyl group in the B ring is indispensable for the radical scavenging effect. Those results showed the DPPH scavenging ability of tea catechins whereby (−)-epigallocatechin and (−)-epigallocatechin gallate were stronger than (+)-catechin, (−)-epicatechin, and (−)-epicatechin gallate. The concentration required to give a 50% decrease of the signal intensity of DPPH radical was 2.5 μM for (−)-epicatechin and 1.7 μM for (−)-epigallocatechin.

It should be kept in mind that contaminations like this or any other potentially antioxidant substances cannot be adequately considered in spectrophotometric tests.

3.2. Online NP-HPLC-DPPH analysis of a liquid cocoa extract

The method developed can be used as fast identification of bioactives in extracts without the need for a complex sample preparation. Fig. 3 shows combined UV (positive peaks) and DPPH radical-quenching (negative peaks) chromatograms. There are ten major UV- and antioxidant-active compounds in cocoa bean extract which show a significant radical-quenching capacity. From comparison with the standard substances (−)-epicatechin monomer, proanthocyanidin B2 dimer, C1 trimer, and cinnamtannin A2 tetramer, the peaks with the retention time at 4.8, 10.1, 22.9, 30.7, 34.7, 38.2, 41.2, 44.3, 46.4, and 48.9 min in the UV chromatogram, and DPPH chromatogram were identified as (−)-epicatechin monomer, dimer, trimer, tetramer, pentamer, hexamer, heptamer, octamer, nonamer, and decamer, respectively. The identification is based on the retention time with standard substances, the UV spectra and MS fragmentation pattern. Obviously, there is no antioxidant capacity detected for the solvent peak (acetone) with a retention time at 3.2 or for the caffeine or theobromine peaks with a retention time at 4.1 and 5.4 min.

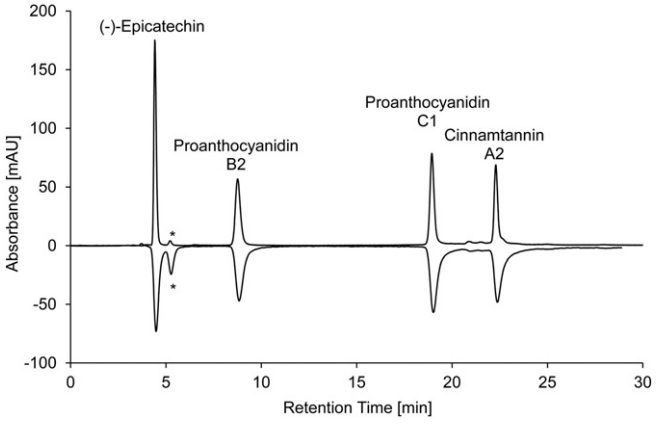

Fig. 2. Correlation of standard substances of oligomeric PA from unroasted cocoa beans extracts using online screening with antioxidant capacity. The polyphenols have a concentration of 1 g/L. The asterisk indicates the contamination of the (−)-epicatechin reference sample.

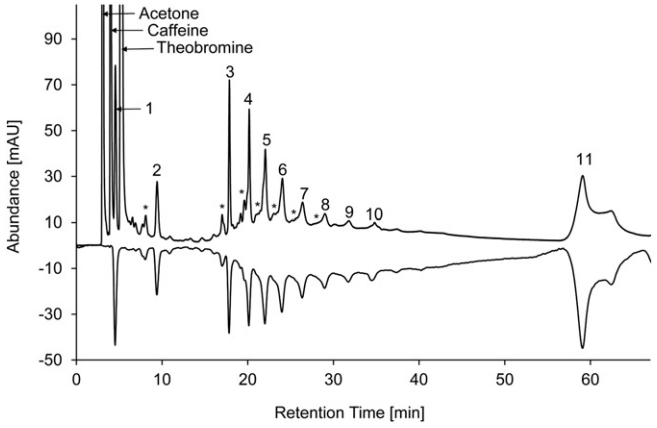

Fig. 3. Bioactive screening of condensed polyphenols from unroasted cocoa bean extract using online screening NP-HPLC-DPPH (injection volume: 1 μ; UV wavelength: positive signals at 275 nm, negative signals at 515 nm); Identified PA: epicatechin monomer (1), dimers (2), trimers (3), tetramers (4), pentamers (5), hexamers (6), heptamers (7), hexamers (8), nonamers (9), decamers (10), and an unresolved "proanthocyanidin (PA) hump" (11). The asterisks are indications for the type A proanthocyanidin in cocoa.

The content of identified PA in the unroasted liquid cocoa extract is shown in Table 1. Levels of PA were expressed as epicatechin equivalents per g fat free dry mass. This form of calculation makes it easier to compare the results also with other studies. Except for dimers, the proanthocyanidin content and the antioxidant activity decrease from monomers to decamers in accordance with the descriptions of Ioannone, Di Mattia, De Gregorio, Sergi, Serafini, and Sacchetti (2015), Bordiga, Locatelli, Travaglia, Coïsson, Mazza, and Arlorio (2015), and Di Mattia et al. (2013). Although, monomers were the most abundant polyphenols (with regard to their concentration), the high antioxidant activities were also found for some other fractions (e.g., trimers to pentamers (Table 1).

Besides that, contents of proanthocyanidins can be expressed at hand of corresponding standard compounds. E.g., for dimers as mg proanthocyanidin B2 equivalents, for trimers as mg proanthocyanidin C1 equivalents and for fractions bigger or equal than tetramers as mg cinnamtannin A2 equivalents. The evaluation indicates that PA pentamers were the most abundant compounds in this extract with a high antioxidant activity (Table 1).

In general, the content and composition of PA in cocoa beans differ due to genetic, geographic origin, and environmental factors. Most of the work in the literature reported a cascading decrease along monomers to decamers. Nevertheless, in this work unfermented, raw cocoa beans had a relatively high abundance of trimers and pentamers when calculating through external standards. However, data obtained in this work are not significantly different when taking the statistical variability of PA into account as well as generally natural fluctuation in cocoa beans. Further studies need to be done concerning different varieties, and regions of productions. Also Kim and Keeney (1984) observed a wide range of (−)-epicatechin from 21.89 to 43.27 mg/g non-fatty dry matter among cocoa beans of varying genetic origin.

In addition, the antioxidant capacity was determined as the ratio of the antioxidant activity per content of the single proanthocyanidin fractions (Table 1). This provides information on the effectiveness of PA to scavenge DPPH radicals (Jacobo-Velázquez & Cisneros-Zevallos, 2009; Vizzotto, Cisneros-Zevallos, & Byrne, 2007). A high value implies that this compound has a high relevance in the total antioxidant activity of the sample. Nevertheless, although trimeric PA shows the highest antioxidant activity, it has a comparatively moderate capacity. The highest value for the capacities was found for nonamers and decamers (Table 1). From the chemical point of view, this seems to be kind of reasonable, as those large molecules provide more structural features for interacting with radicals.

This approach has proven that oligomeric PA are efficient DPPH radical scavengers despite their high molecular weight and potential steric hindrance. According to Arteel and Sies (1999), long chain PA are better scavengers than short chain PA, particular tetramer was found efficient against oxidation and nitration reaction. Ramljak et al. (2005) claimed for pentameric cocoa proanthocyanidin even a growth inhibition effect of human breast cancer cells.

In an offline approach by Rösch, Bergmann, Knorr, and Kroh (2003), a quite similar order was found for the antioxidant activity of oligomeric PA from sea buckthorn (*Hippophae rhamnoides*). PA tetramers showed hereby the highest antioxidant activity. Lotito et al. (2000) reported that monomers, dimers, and trimers were the most effective antioxidants in the aqueous phase, while PA of higher molecular weight were more effective in the lipid phase. These results also underline the observation described by Lu and Yeap Foo (2000) who characterized apple polyphenols. They found trimers and tetramers to be more efficient free radical scavenger than proanthocyanidin B2 and (−)-epicatechin. But they also claimed that this trend of antioxidant activity do not extend to higher molecular PA. The present study demonstrates that to a point the increasing PA enhances the effectiveness against radicals. But with an increase of molecular weight, the in antioxidant activity leads to a slight, non-linear reduction for polyphenols with DP > 8. According to Lu and Yeap Foo (2000), the slight increase of the antioxidant activity could be explained by the increasing crowding and therefore the less availability of the hydrogen donating effect.

A further aspect to consider is the separation of PA by polymerization degree when using normal phases, whereby different PA with the same molecular weight are often determined as one fraction. However, separation of single PA only succeeds with reversed phases, so far.

As obvious from Fig. 3, structures larger than decamers elute as an unresolved group of peaks at the end of the chromatogram which

Table 1
Quantification of oligomeric PA and their antioxidant activity in an unroasted liquid cocoa extract obtained by online NP-HPLC-DPPH. PA content and its antioxidant activity are expressed as epicatechin equivalents (using epicatechin as standard; ECE) or as proanthocyanidin equivalents (using cinnamtannin A2 as standard; PAE). Antioxidant capacity is defined in this study as the ratio of antioxidant activity per soluble PA content. Values represent value mean, with n = 3 (±standard deviation). The bold entries refer to further explanations in the text.

Peak no.	Degree of polymerization	PA content [mg ECE/g]	Antioxidant activity [mg ECE/g]	Antioxidant capacity [for ECE]	PA content [mg PAE/g]	Antioxidant activity [mg PAE/g]	Antioxidant capacity [for PAE]
1	DP 1	**5.02 (±0.53)**	**3.43 (±0.48)**	0.68 (±0.04)	5.02 (±0.53)	1.86 (±0.48)	0.37 (±0.06)
2	DP 2	2.50 (±0.32)	2.64 (±0.45)	1.05 (±0.09)	3.58 (±0.45)	1.41 (±0.43)	0.39 (±0.08)
3	DP 3	4.19 (±0.54)	**3.68 (±0.39)**	0.88 (±0.05)	5.55 (±0.69)	2.09 (±0.49)	0.37 (±0.05)
4	DP 4	3.97 (±0.48)	**3.25 (±0.31)**	0.81 (±0.07)	6.81 (±0.81)[a]	2.15 (±0.49)[a]	0.31 (±0.05)[a]
5	DP 5	4.11 (±0.46)	**3.25 (±0.32)**	0.79 (±0.10)	**7.04 (±0.77)**[a]	**2.16 (±0.50)**[a]	0.30 (±0.07)[a]
6	DP 6	3.03 (±0.55)	2.72 (±0.47)	0.90 (±0.13)	5.26 (±0.92)[a]	1.64 (±0.45)[a]	0.31 (±0.06)[a]
7	DP 7	2.00 (±0.35)	2.24 (±0.35)	1.13 (±0.14)	3.54 (±0.59)[a]	1.18 (±0.34)[a]	0.33 (±0.08)[a]
8	DP 8	1.39 (±0.30)	1.79 (±0.20)	1.31 (±0.23)	2.53 (±0.50)[a]	2.02 (±0.19)[aa]	0.29 (±0.07)[a]
9	DP 9	0.55 (±0.19)	1.61 (±0.32)	**3.22 (±0.21)**	1.13 (±0.31)[a]	0.56 (±0.40)[a]	0.56 (±0.67)[a]
10	DP 10	0.56 (±0.13)	1.27 (±0.11)	**2.39 (±0.21)**	1.15 (±0.22)[a]	0.24 (±0.10)[a]	0.23 (±0.14)[a]
11	'PA hump'	22.52 (±3.80)	17.07 (±2.02)	0.76 (±0.06)	37.65 (±6.31)[a]	15.56 (±1.96)[a]	0.42 (±0.03)[a]

[a] Values are expressed as cinnamtannin A2 equivalents.

corresponds to the majority of the material present. So far, all traditional separation methods failed to resolve this 'proanthocyanidin (PA) hump'. This has to do with the fact that the resolution of a NP-HPLC stationary phase under HILIC conditions is not sufficient to separate such complex samples (Kalili & de Villiers, 2009). There is much evidence suggesting that a chromatographic separation of larger polymers is not possible due to their complexity. Polymeric PA with a higher molecular mass cannot be determined with NP-HPLC (Lazarus, Hammerstone, Adamson, & Schmitz, 2001).

However, the cocoa 'proanthocyanidin hump' shows a powerful antioxidant capacity that, beyond all doubt, represents more or less the majority of the sample material. Although it is difficult to characterize the PA hump completely, it is necessary to compare the PA hump in interaction with other compounds and their antioxidant activity. With an antioxidant activity of approx. 20 mg per gramme fat free dry matter (expressed as epicatechin equivalents), the PA hump is as powerful as all other PA together. However, with regard to the antioxidant capacity of this fraction, the effectiveness to neutralize DPPH radicals can be considered as comparatively low (Table 1).

Unfortunately, only a few studies showed an unadorned chromatogram including the PA hump (e.g., Esatbeyoglu, Wray, & Winterhalter, 2015, Gu et al., 2002, Guyot, Marnet, & Drilleau, 2001). However, some researcher also observed analogous unresolved humps in their chromatograms when analysing different plant extracts. Kuhnert (2010) as well as Kuhnert, Dairpoosh, Yassin, Golon, and Jaiswal (2013) highlighted similar observations in the chromatographic behaviour when describing the so-called "thearubigin hump" in the analysis of black tea. Although the molecular weight of the thearubigins ranges from 1000 to 40,000 (Yao et al. 2006), Sinha and Ghaskadbi (2013) recently presumed strong antioxidant properties of black tea thearubigins. Here again, although thearubigins are present in large amounts in black tea, the information on formation and structure of this heterogeneous polymer is very limited.

In the present study, it could be shown that the unroasted cocoa extract contains a high amount of extractable condensed polyphenols which can be separated on a normal phase column whereby their antioxidant capacity can be assessed by online post-column derivatisation.

Besides the homologous series of oligomeric PA, other UV-active flavanol moieties can be seen on a HILIC-column. The poor separation between each oligomer peak suggests that PACE is quite inhomogeneous including different structures showing a high complexity in its PA subunits and the linkages between them (Valls, Millán, Pilar Martí, Borràs, & Arola, 2009; Yanagida et al., 2007). Besides linear PA also large, branched molecules can be present. Another to date unsatisfyingly investigated phenomenon when using a HILIC column is the appearance of smaller unresolved UV-absorbing peaks. They merged into big peaks with shoulders. In the chromatogram they are marked with an asterisk (Fig. 3). Similar effects were mentioned by Pereira-Caro, Borges, Nagai, Jackson, Yokota, Crozier, and Ashihara, (2013) indicating the different interflavan bonds of proanthocyanidins as type A and type B. Besides that, NP-HPLC analyses of grapes or wine, indicat the presence of different mono-galloylated, galloylated and non-galloylated PA (Nunes, Gómez-Cordovés, Bartolomé, Hong & Mitchell, 2006). Callemien and Collin (2008) isolated dimers and trimers from lager beer extract by normal phase column and obtained, using thioacidolysis, that catechin forms the main terminal unit, whereas both catechin and gallocatechin are major constituents of the extension unit. For cocoa, it can be hypothesised that such smaller unresolved peaks are PA linked either to *C*- or *O*-glucose, -arabinose, -galactose (Hatano et al., 2002). Also Hammerstone, Lazarus, Mitchell, Rucker, and Schmitz (1999) proofed a small quantity of di-, tri-, and tetrasaccharides in unfermented cocoa extract.

3.3. Quantification of oligomeric PA using the online NP-HPLC-DPPH assay

In this study, oligomeric PA were quantified using an external calibration for UV-NP-HPLC analysis with a diol-HILIC column. For each analyte, peak areas from chromatogram were plotted against the concentration of the stock solution of known concentrations. As expected, PA show a positive correlation between the concentration against UV-absorption. The calibration curves were as follows: (−)-epicatechin, $y = 1618.8x + 11.922$ ($R^2 = 0.9994$, 0.05–1.0 g/L), proanthocyanidin B2, $y = 1154.5x + 3.04$ ($R^2 = 0.9995$, 0.05–1.0 g/L), proanthocyanidin C1, $y = 1266.7x - 16.46$ ($R^2 = 0.9999$, 0.05–1.0 g/L) and cinnamtannin A2, $y = 973.69x - 11.756$ ($R^2 = 0.9999$, 0.05–1.0 g/L).

The DPPH radical solution is a deep violet chromogen and any quenching of the radical results in a loss of colour, getting yellowish and is indicated by signal intensity decrease and represented by a negative peak in the chromatogram. The radical scavenging properties are detected at 515 nm. The DPPH scavenging activity of oligomeric PA were calculated from the working calibration curves: (−)-epicatechin, $y = 1233.7x + 109.36$ ($R^2 = 0.9836$, 0.05–1.0 g/L), proanthocyanidin B2, $y = 1306.6x + 51.282$ ($R^2 = 0.9923$, 0.05–1.0 g/L), proanthocyanidin C1, $y = 1507.5x + 46.693$ ($R^2 = 0.9927$, 0.05–1.0 g/L), and cinnamtannin A2, $y = 1271.6x + 34.386$ ($R^2 = 0.9938$, 0.05–1.0 g/L). The activities tested with the online NP-HPLC-DPPH assay decreased in following order: proanthocyanidin C1 > proanthocyanidin B2 > cinnamtannin A2 > (−)-epicatechin.

For the validation of the online NP-HPLC-DPPH assay, the limits of detection (LOD) and the limits of quantification (LOQ) of the PA compounds and their antioxidant capacity were calculated based on the standard deviation of y intercept of the regression against the slope of the calibration curve. However, the UV absorption is comparatively sensitive (LOD = 7–21 μg/mL) as the DPPH reaction (LOD = 6–27 μg/mL) (Table 2).

As depicted from the calibration curves the PA content is highly correlated with the antioxidant capacity ($R^2 > 0.99$). This suggests that PA accounts for a major portion of the antioxidant activity in unroasted cocoa beans. This could also be an indicator for potential biological activity.

The common limitation of the analysis is the weak UV absorption of higher oligomeric PA due to the wider chromatogram peaks. Furthermore, the response for UV-absorbing chromophores decreases in relation to the degree of polymerization. This approach can be explained by the higher molecular weight and effects of steric hindrance.

It can be further discussed if measurement for condensed polyphenols might be determined at lower wavelengths such as 205 nm, where they give better response at low concentration. Lee and Ong (2000) analysed sixteen types of tea ingredients at 205 nm and obtained a higher sensitivity with a LOD ≈ 0.05 μg/mL.

Some methodologies are also using fluorescence detectors to improve sensitivity and selectivity for the analysis of catechins and escpecially for proanthocyanidins (Arts & Hollman, 1998; Lazarus, Adamson, Hammerstone, & Schmitz, 1999; Robbins, Leonczak, Johnson, Li, Kwik-Uribe, Prior, & Gu, 2009). Typical concentrations for fluorescence measurements are in the range 5–16 μg/mL (Hemingway & Karchesy, 1989). Hellström and Mattila (2008) achieved LOD values for extractable PA varying from 1.0–15 μg/mL for a concentration level from 20 to 2000 μg/mL.

Table 2
LOD and LOQ of standard substances in the online NP-HPLC-DPPH assay determined at a concentration range 50 μg/mL to 400 μg/mL.

Compound	UV-detection at 275 nm		Vis-detection at 515 nm	
	LOD [μg/mL]	LOQ [μg/mL]	LOD [μg/mL]	LOQ [μg/mL]
(−)-Epicatechin	7	22	27	83
Procyanidin B2	12	38	6	19
Procyanidin C1	17	54	11	33
Cinnamtannin A2	21	64	16	49

3.4. Quantification of oligomeric PA using the traditional photometric assay

To evaluate whether both methods can be compared, it was important to quantify oligomeric PA using also the traditional photometric assay. To determine the reaction kinetics, the DPPH radical quenching of (−)-epicatechin was measured at 25 °C and 60 °C. The absorbance at 515 nm was noted after an incubation time of 2 and 5 min using a UV–visible spectrophotometer with methanol as blank. The absorption measured at 60 °C with an incubation time of 2 min showed the most linear response over the whole range of concentrations from 0.05 g/L to 1.0 g/L.

Accordingly, the four main condensed cocoa polyphenols were investigated under the optimized conditions. The working calibration curves are representing a polynomial of the second degree: (−)-epicatechin, $y = 0.5817x^2 - 1.3405x + 0.9578$ ($R^2 = 0.9997$, 0.05–1.0 g/L), proanthocyanidin B2, $y = 0.224x^2 - 0.7919x + 0.934$ ($R^2 = 0.9994$, 0.05–1.0 g/L), proanthocyanidin C1, $y = 0.2277x^2 - 0.7261 + 0.9289$ ($R^2 = 0.9986$, 0.05–1.0 g/L) and cinnamtannin A2, $y = 0.1855x^2 - 0.6405x + 0.9331$ ($R^2 = 0.9996$, 0.05–1.0 g/L).

The resulting dose–response curves showed a decrease in DPPH-scavenging activity in the following order: cinnamtannin A2 > proanthocyanidin C1 > proanthocyanidin B2 > (−)-epicatechin (Fig. 4).

The comparatively higher oligomers had a higher antioxidant activity than the monomers. Among PA, tetramers were the most potent while epicatechin was the least active. The antioxidant activity increases depending on DP. The important structural feature for DPPH scavenging is the *ortho*-hydroxyl group in the B-ring(s), the additional presence of a 3-OH group(s), and the abundant 4 ≥ 8 linkage between the subunits (Heim, Tagliaferro, & Bobilya, 2002).

3.5. Comparison of the traditional photometric assay versus the online NP-HPLC-DPPH assay

The results of the traditional photometric DPPH assay and the online NP-HPLC-DPPH assay can be expressed and calculated as (−)-epicatechin equivalents for the first four homologous polyphenols. Here again, the four main standard substances (−)-epicatechin, procyanidins B2, C1, and A2 were used for the calculation and have been indicated as representatives of DP 1, DP 2, DP 3 and DP 4 (Fig. 5).

Proanthocyanidin B2 (DP 2), when expressed as (−)-epicatechin units/equivalents, shows a slightly higher antioxidant activity than (−)-epicatechin itself. With regard to the traditional photometric assay, this trend extends up to the tetramers: Cinnamtannin A2 has a more effective radical scavenging activity than the monomeric (−)-epicatechin.

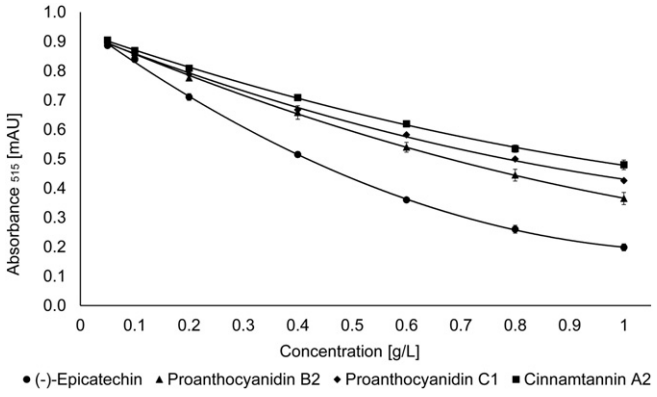

Fig. 4. Dose–response curves obtained by the DPPH cuvette assay for (−)-epicatechin, proanthocyanidin B2, C1 and cinnamtannin A2 at concentrations from 0.05 g/L to 1.0 g/L measured at 515 nm and 2 min at 60 °C incubation. Each value represents the mean values and the standard deviations from three determinations.

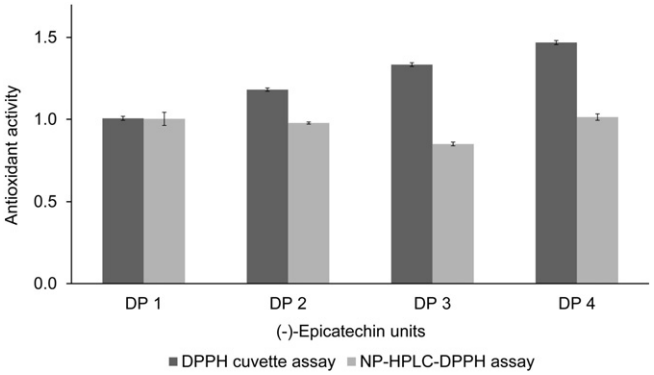

Fig. 5. Comparison of DPPH scavenging activity of PA between the DPPH cuvette assay and the online NP-HPLC-DPPH assay. The results are expressed as (−)-epicatechin equivalents. The error bars are the result of a triple determination.

Hence, the results of both assays cannot be compared satisfyingly. The antioxidant activity determined with the online NP-HPLC-DPPH assay is diminished in relation to the traditional photometric assay. One explanation for that phenomenon could be the hydrogen-bond accepting ability of the HPLC solvents methanol and acetonitrile as described by Jabbari and Moallem (2015). An increase of the organic solvent influences the radical scavenging activity of flavonoids. Presumably, they undergo interactions with DPPH due to solute–solvent effects. Also Sharma and Bhat (2009) recommended a better sensitivity and a high range of accuracy, the preparation of the DPPH solution in methanol without any further additives.

Recent studies showed that the antioxidant activity is closely related to the molecular structure of the polyphenol and the solute–solvent present (Jabbari, Mir, Kanaani, & Ajloo, 2014; Jabbari & Moallem, 2015). Also a lower pH in the mobile phase can lead to a dramatic loss of sensitivity and to a decrease in absorbance of the DPPH radical (Malherbe, de Beer, & Joubert, 2012).

The direct comparison of the DPPH cuvette assay with the NP-HPLC-DPPH assay leads to the conclusion that each assay has its particular advantage and disadvantage. The method ultimately chosen will depend on speed of analysis, accuracy required, sensitivity required and the determination of single compounds in complex matrixes. The DPPH cuvette assay describes a simple high throughput colorimetric assay which is reliable and generates reproducible measurements, expressed as total antioxidant capacity (TAC). The disadvantage, on the other hand, is in the analysis of complex matrixes. The online HPLC-DPPH assay gives more information about the composition of the mixture as well as a rapid assessment of the antioxidant capacity of every single compound.

However, the use of an internal standard is pretty common when using RP-HPLC. Antioxidant capacities of phenolic substances in plant extracts are usually quantified with an internal standard such as trolox (Riehle, Vollmer, & Rohn, 2013). Data are normally presented as trolox equivalent antioxidant capacity (TEAC) which is expressed as mg trolox equivalents per g sample. The resulting values are usually comparatively higher than the real amount of polyphenols. This has to do with the fact that trolox has a higher antioxidant activity than polyphenols usually have. For example, according to Arts, Haenen, Voss, and Bast (2004), chrysin has a relatively high TEAC value while its antioxidant activity in other assays is relatively low. In the present case, the quantification using an internal standard on a diol-HILIC column proved to be rather difficult. Neither known cocoa polyphenols nor typical standards such as trolox or vitamin C could be used as internal standard due to adverse co-eluting with other known substances. For this reason, we deliberately refrained from using trolox as internal standard.

3.6. Isolation of oligomeric PA using semi-preparative NP-HPLC

Additional investigation of isolated oligomeric PA was needed for several reasons: Primarily to understand the contribution of each single PA to the total antioxidant activity of an isolated fraction. Furthermore, increasing the scale of the NP-HPLC chromatography is of particular interest regarding the preparation of self-made analytical standards in considerable amounts for substances that are difficult to obtain commercially.

A separation of the unroasted PA cocoa extract with a semi-preparative NP-HPLC column is illustrated in Fig. 6, whereby PA up to hexamers can be observed in a 60 min run. Fractions were numbered F1–F6, respectively in their order of elution from the diol-HILIC column. Despite column overload, fractions were clearly separated from each other. The colour of the fractions increased from colourless for F1, to light pink for F4 and to deep red for F7. Colour intensification results from condensation of an increased number of monomeric units. Monomers such as (−)-epicatechin are known as white powder, whereas dimers such as proanthocyanidin B2 are coloured white to beige. This trend continues towards polymeric tannins known as brownish powders.

Although the analysis of the liquid cocoa extract on the analytical column gives fractions with up to decamers, it is surprising that on the semi-preparative column only monomers to hexamers could be separated although obviously material was eluting from the column (between 60 and 90 min) (Fig. 6). A proposed rationale could be the different retention mechanism for larger diol-HILIC columns and their insufficient separation with increasing molecular weight (DP). According to the Van Deemter equation the efficiency diminishes in relation to the column width and results in peak broadening.

An important element of this study was the right sample preparation and dissolution of the PACE for semi-preparative purpose. Since the knowledge of oligomeric PA is still incomplete, the conditions have to be optimized to ensure an efficient extraction and injection, so that no information is lost. On the other hand, to prevent the system from precipitation most of the researchers (Kelm, Johnson, Robbins, Hammerstone, & Schmitz, 2006) dissolved their extract in the HPLC mobile phase whereby some of them additionally dissolved it in ethanol before injecting it into the column. Because of its limited solution power this study purposely decided not to dissolve the PACE in the HPLC solvent. To prevent the HPLC system from precipitation of solids, the 'PA cocoa extract' was re-dissolved in 40% isopropanol, suspended using an ultrasonic bath, and filtered through a 0.45 μm cellulose syringe filter prior to injection so that no resinous material was seen. Similar to Kelm, Johnson, Robbins, Hammerstone, and Schmitz (2006) and their observation on unfermented cocoa PA, in the present study only monomers to heptamers could be separated clearly. PACE could be prevented from precipitation by using the new method of complete dissolution. Various injection methods to increase the injection load for right sample preparation are currently under investigation.

A major benefit of the method described is the isolation of single compounds in high purity, whereby their identities were deduced by RP-HPLC-ESI/MS.

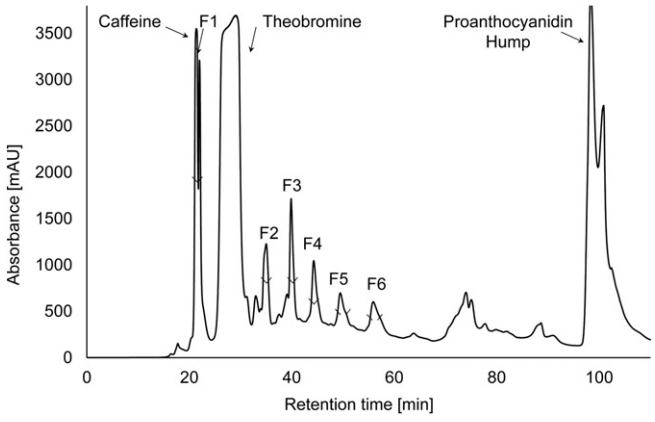

Fig. 6. Preparative diol-HILIC profile of 50 mg unroasted cocoa powder extract injected into the column (injection volume: 200 μL). The fractions are labelled F1–F7 according to their DP. The black bar in the middle of each peak reflects the manually collected fractions.

3.7. Identification of oligomeric PA using RP-HPLC-ESI/MS

The compounds F1–F6 of the 'PA cocoa extract' (PACE) were isolated by semi-preparative HPLC using UV–Vis detection. As this technique cannot provide compound identification or structural information the optimized RP-HPLC method was coupled with electrospray ionization to complete the characterization of PACE.

Identification was based on retention time and mass spectra summarized in Table 3. This table also includes the experimental *m/z*, the fragmentation of fission products and the molecular formulas.

The six successive eluted fractions were measured in positive ionization TIC mode $[M + H]^+$. For an increased sensitivity, each fraction is shown in the extraction ion mode (Fig. 7). Briefly, the first fraction is shown by measuring at *m/z* 291, the second fraction at *m/z* 579, the third fraction at *m/z* 867, etc. A total of six oligomeric PA and the two alkaloids theobromine and caffeine were identified using RP-HPLC-ESI/MS.

As mentioned above, several PA can elute as a cluster of peaks of isomers on a normal phase column. The separation is not sensitive enough to differentiate PA of the same degree of polymerization. Detailed structure can just be determined by RP-HPLC.

Fig. 7a shows an extracted ion chromatogram (EIC) of $[M + H]^+ = 291$ for fraction F1 which contains two peaks at $t_R = 18.8$ min and 26.1 min and were assigned as (+)-catechin and (−)-epicatechin after comparison with the retention time and fragmentation pattern of commercially available standards. (−)-Epicatechin has a much higher absolute intensity than (+)-catechin. The third peak with $[M + H]^+ = 291$ at $t_R = 34.7$ min is a pseudomolecular ion coming from procyanidin B2. All things considered, the antioxidant activity of the monomeric fraction in Fig. 7a is contributed equally by (−)-epicatechin and (+)-catechin.

Fig. 7b is an EI-chromatogram of $[M + H]^+ = 579$ for fraction F2 with proanthocyanidin B3 at $t_R = 17.4$ min, PA B4 at $t_R = 22.1$ min and PA B2 at $t_R = 23.5$ min. Furthermore there are characteristic signals for dimers at $t_R = 25.5$ min, 31.4 min and 35.1 min whereby they remain unidentified due to a lack of standard substances. So altogether, there are at least six PA dimers contributing to the antioxidant activity of the dimeric fraction.

Fig. 7c is an EIC of $[M + H]^+ = 867$ with proanthocyanidin C1 at $t_R = 28.9$ min. In addition there are three more non-identified trimers with $t_R = 23.4$ min, 30.8 min, and 35.2 min. Altogether, four PA trimers contribute to the antioxidant activity of the trimeric fraction.

Fig. 7d is an EIC of $[M + H]^+ = 1{,}155$ with cinnamtannin A2 at $t_R = 30.5$ min. Furthermore, there are three more non-identified tetramers with $t_R = 25.7$ min, 31.8 min, and 37.9 min. Due to the lack of standard substances there is no clear classification of PA besides that possible.

Table 3
RP-HPLC-ESI/MS determination of oligomeric proanthocyanidins in unroasted cocoa bean extract. Only single charged ions for F1–F7 could be detected in the positive ion mode.

Compound	Molecular formula	t_R [min]	Peak	Molecular ion $[M + H]^+$	Major fragments $[M + H]^+$
Monomer	$C_{15}H_{14}O_6$	4.3	F1	291.1	139.0; 165.1
Dimer	$C_{30}H_{26}O_{12}$	8.1	F2	579.2	291.1
Trimer	$C_{45}H_{38}O_{18}$	16.2	F3	867.2	579.2; 1155.5
Tetramer	$C_{60}H_{50}O_{24}$	18.5	F4	1155.3	n.d.
Pentamer	$C_{75}H_{62}O_{30}$	20.1	F5	1443.3	579.2; 867.0
Hexamer	$C_{90}H_{74}O_{36}$	21.8	F6	1731.4	579.9; 867.0; 1155.3

n.d. not detected.

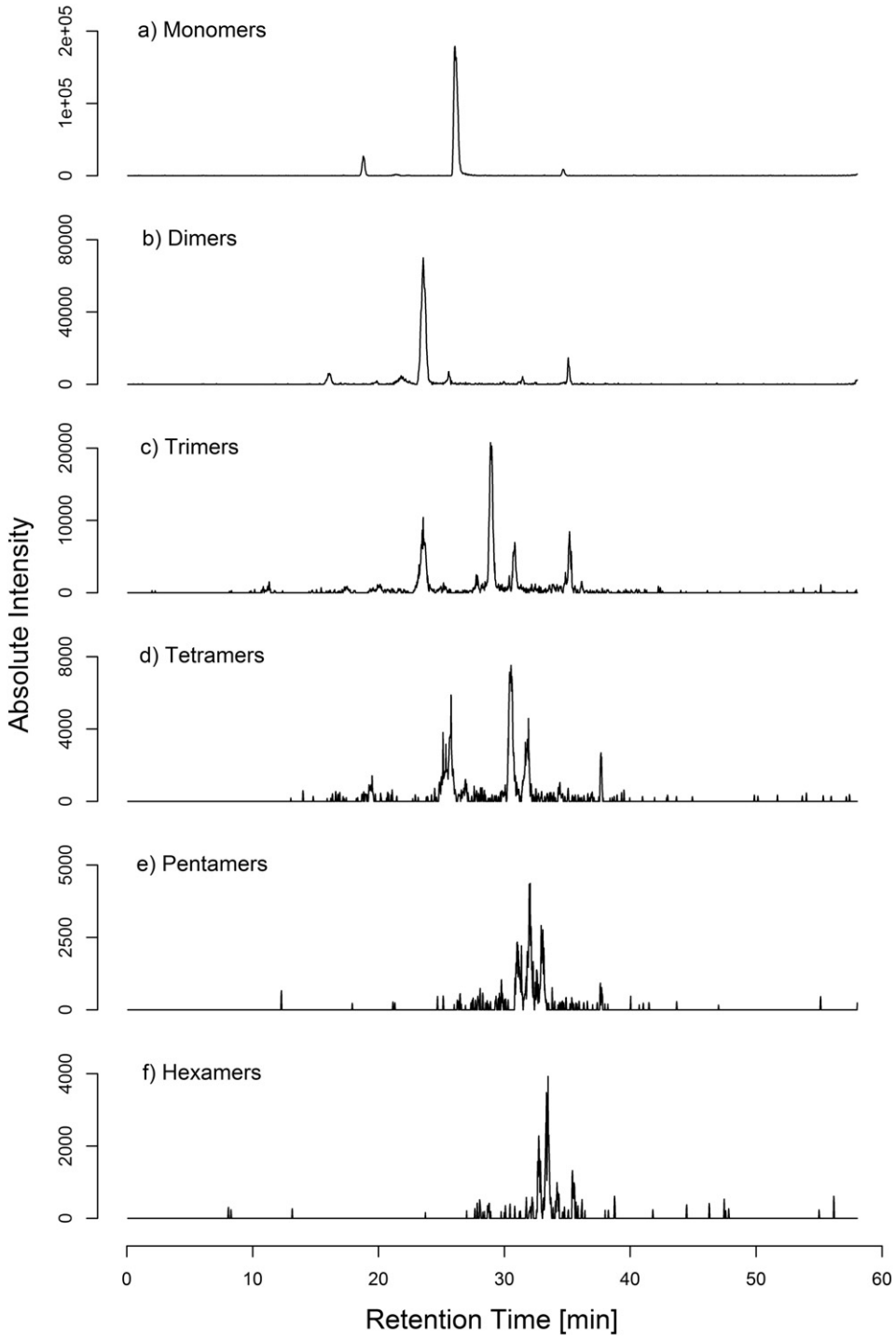

Fig. 7. a–f Extracted ion chromatograms (EIC) obtained from the analysis of the unroasted cocoa extract using RP-HPLC/MS.

Altogether, four PA tetramers contribute to the antioxidant activity of the tetrameric fraction.

Fig. 7e is an EIC with the mass-to-charge ratio *m/z* 1444 with clear peaks at t_R = 32.1 min, and t_R = 37.8 min. Altogether, two PA pentamers contribute to the antioxidant activity of the pentameric fraction.

Fig. 7f is an EIC with the mass-to-charge ratio *m/z* 1732 with clear peaks at t_R = 33.5 min and a smaller one at t_R = 35.6 min, providing information that a minimum of two PA hexamers contribute to the antioxidant activity of the hexameric fraction.

Behind every fraction there is a couple of isobaric, sometimes isomeric PA which altogether contributes to the overall antioxidant capacity.

However, the efficiency of the monomeric fraction and the corresponding chromatographic resolution is much higher than for the dimeric, trimeric, tetrameric fractions, etc. Motilva, Serra, and Macià (2013) observed a decrease of the absolute intensity during each fraction of cocoa flavanols and the reduction of the signal-to-noise ratio. This can be explained by the poor extraction yield of the semi-preparative NP-HPLC within each fraction. The insufficient resolution can be avoided by

an efficient sample preparation after semi-preparative isolation by decreasing matrix suppression.

4. Conclusions

The method described can be used for a rapid evaluation of condensed antioxidant components in complex matrices such as plant or food extracts. For chromatographic separation of mono- and oligomeric PA, a standard procedure using a NP-HPLC system was established. To simultaneously assess the antioxidant capacities of PA, the traditional photometric assay was adapted and coupled online with a HPLC system. The well-known antioxidant assay using 2,2-diphenyl-1-picrylhydrazyl (DPPH) was chosen, because of its easy and rapid way to determine the antioxidant activity and its reactivity of its stable free radical (Prior, Wu, & Schaich, 2005).

The DPPH concentration was optimized and the effect of reaction time, temperature, and stability of the antioxidant compound during the assay (reaction time < 1 min) was characterized. The results suggest that this method can be used for the simultaneous determination of condensed polyphenols according to their DP as single antioxidant capacities. Several antioxidants were identified in the cocoa extract whereby the results indicate that the radical scavenging activity of the homologous series of condensed PA is increasing with increasing DP. Nonameric and decameric PA fractions showed the highest antioxidant capacity.

Besides that, it should be generally questioned whether it is useful to evaluate the PA amount as epicatechin equivalents (ECE) or as PA equivalents (PAE). When calculating as ECE, the values appeared more significant. The evaluation as PAE, however, makes the values appear more equal, but compounds used for the calculation are chemically closer related to the substance that is quantified. Despite to that, it should be also taken into account that the UV activity of oligomeric PA is decreasing with increasing DP. This can be seen in the calibration curve whereby the slope of the straight line drops from (−)-epicatechin to cinnamtannin A2.

Furthermore, it can be discussed whether the newly developed assay can be improved in terms of detection limits for higher polymeric PA with DP > 10. Also, the standard deviation for higher PA could be minimized maybe by using a fluorescence detector.

The online NP-HPLC-DPPH assay can also be considered as a promising technique for quality control in the chocolate manufacturing process whereby the question arises whether the antioxidant capacity of individual oligomeric PA changes throughout the process.

However, the most promising substances, because of their high potential antioxidant activity, still seem to be the unresolved. Further, higher condensed polyphenols occur at the end of the NP-HPLC chromatogram ('PA hump') providing future challenges for their analysis and characterization.

To develop a complete understanding of the biological activity of cocoa, additional structure determination is important: Research on structure elucidation can be done by isolation of oligomeric substances and their further depolymerisation reaction in the presence of nucleophiles such as benzyl mercaptan (thiolysis) (Callemien, Guyot, & Collin, 2008; Matthews, Mila, Scalbert, Pollet, et al., 1997; Ramirez-Coronel, Marnet, Kumar Kolli, Roussos, Guyot, & Augur 2004), phloroglucinol (phloroglucinolysis) (Lorrain, Ky, Pechamat, & Teissedre, 2013), or enzymatic treatments of the extract (Mandalari et al., 2006).

Acknowledgments

Markus Kneubühl helped with the experimental design. Carlo Weber supported sample preparation. Petra Huber participated in the data interpretation. Special thanks go to Ansgar Schlüter and Franco Spescha for providing all samples.

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Food Chemistry 214 (2017) 523–532

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Determination of oligomeric proanthocyanidins and their antioxidant capacity from different chocolate manufacturing stages using the NP-HPLC-online-DPPH methodology

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ARTICLE INFO

Article history:
Received 2 February 2016
Received in revised form 15 June 2016
Accepted 12 July 2016
Available online 14 July 2016

Keywords:
Chocolate manufacturing process
Oligomeric proanthocyanidins
Antioxidant capacity

ABSTRACT

Cocoa beans are a well-known source of antioxidant polyphenols. Especially individual oligomeric proanthocyanidins demonstrated a significant contribution to the total antioxidant activity of cocoa compared to monomeric compounds. An NP-HPLC-online-DPPH assay was developed for separating the homologous series of oligomeric proanthocyanidins and the simultaneous assessment of their antioxidant capacity in relation to the degree of polymerization (DP).

The present study describes the influence of the different stages of a lab-scale chocolate manufacturing process on the content of oligomeric proanthocyanidins and their antioxidant capacity. The sum of the total proanthocyanidin content ($\sum$ DP1-DP13) decreased from 30 mg epicatechin equivalents per gram non-fat dry matter in raw fresh cocoa beans to 6 mg epicatechin equivalents per gram in the final chocolate. The antioxidant capacity decreased accordingly from 25 mg epicatechin equivalents per gram non-fat dry matter in raw fresh cocoa beans to 4 mg/g in the final chocolate product.

1. Introduction

Cocoa and its most prominent finishing product chocolate are known as luxury food providing a pleasantly astringent taste and a typical sensual cocoa flavor. Being an important economical crop, cocoa beans are predominantly used for the chocolate industry. In the last few years, the health benefits of chocolate for human nutrition became more and more evident. The main active substances, primarily the alkaloids theobromine and caffeine, are known for their stimulating effect. Polyphenols from cocoa beans or chocolate products have been reported to provide even anti-inflammatory effects. Especially in Switzerland, 'the land of chocolate' with many small manufactories, there is a huge interest in a high quality product with an enriched content of polyphenols. Therefore, gaining a deeper understanding of cocoa beans and their polyphenolic content, respectively of the transformations of the composition during the cocoa manufacturing process, is important, as different processes lead to divergent amounts of polyphenols with their corresponding antioxidant capacity.

Previous studies dealing with changes during the chocolate manufacturing process focused on flavan-3-ols, especially (−)-epicatechin and (+)-catechin and other low molecular weight polyphenols, which can be easily assessed using RP-HPLC. However, data about detailed information on oligomeric proanthocyanidins (PAs) and their changes during manufacturing processes are generally rare. Over the past five decades, researchers have focused intensively on monomeric cocoa polyphenols, although only approx. 5–10% of the total polyphenols account for monomers, while oligomers and polymers account for >90% (Andres-Lacueva et al., 2008). Counet, Callemien, and Collin (2006) claimed for PAs found in commercial products such as grape, wine, olive, and dark chocolate a distribution of monomers and dimers of up to 71% of the total PA fraction from DP1-DP6.

PAs are shown to be highly effective inhibitors of radical chain reactions. In cocoa, they consist of oligomers or polymers of (+)-catechin and/or (−)-epicatechin units. The size of PAs is specified by their degree of polymerization (DP). For evaluating the distribution of the PAs, the use of NP-HPLC seems to be preferable as analytical methodology (Hammerstone, Lazarus, Mitchell, Rucker, & Schmitz, 1999). As presented recently, the antioxidant capacity of condensed cocoa polyphenols can be measured simultaneously using a NP-HPLC-online-DPPH approach (Pedan, Fischer, & Rohn, 2015). The question now arises how PAs and their antioxidant activity are altered during different chocolate manufacturing

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http://dx.doi.org/10.1016/j.foodchem.2016.07.094
0308-8146/

stages. During this process, there are many determinants for influencing the PAs. The main steps are described in the following:

Cocoa beans are natural products and their composition is highly depending on genotype and ecophysiological factors (e.g., temperature, radiation, plant nutrition). Initially, genetic variation can cause a 4-fold difference in the flavanols content of fresh cocoa beans (Clapperton et al., 1994). Kim and Keeney (1984) observed (−)-epicatechin concentrations ranging from 21.9 to 43.3 mg/g non-fat dry matter among freshly harvested cocoa beans of varied genetic origin. De Brito, García, and Amâncio (2002) observed a variation in the total polyphenol content from 12% to 18% of non-fat dry matter in fermented and dried cocoa beans whereby 60% were PAs of which the half were dimeric structures.

Beside genetic variations, a huge effect on polyphenol composition is the fermentation which is carried out in the countries of origin. In fact, after cutting the ripe cocoa pods from the trees, the beans are removed from the pod and transferred with the pulp to boxes/sacks/piles for fermentation lasting 5–7 days (Aikpokpodion & Dongo, 2010). Micro-organisms and endogenous enzymes lead to a degradation of the pulp that surrounds the fresh beans and limit the development of pathogenic microorganisms. During the fermentation, polyphenols undergo an enzymatic transformation by polyphenol oxidase(s). (−)-Epicatechin polymerizes with (+)-catechin to form brown high molecular weight polymers (Albertini et al., 2015). Investigations of fermented cocoa beans from different origins showed a 6-fold variation of the (−)-epicatechin content. The (−)-epicatechin concentration ranges from 2.7 mg/g of dry defatted samples from Jamaica to 16.5 mg/g from Costa Rica (Kim & Keeney, 1984). Kealey et al. (2001) showed a 3- to 5-fold decrease for pentameric PAs during fermentation ranging from 4.5 to 6.5 mg/g defatted sample at the beginning to 0.5–2 mg/g at the end of the fermentation. A diffusion of polyphenols compounds through the cotyledon and irreversible or reversible binding due to complexation with proteins or polysaccharides take place (Forsyth, Quesnel, Roberts, 1958).

During the drying process beans are placed in shallow trays. The oxidation reaction that began with the fermentation now continues during drying. Hereby, polyphenol-oxidases catalyze the enzymatic browning accompanied by non-enzymatic browning, both leading to an *o*-quinone polymerization and reactions with other constituents such as proteins and polysaccharides (De Brito et al., 2000). During these steps, the amount of polymeric polyphenols is reduced leading to a corresponding reduction of astringency (Niemenak et al., 2006). During drying, the moisture content drops from 60% to a residual humidity below 8%, saving the cocoa bean from mold infestations and inactivating enzymes (Afoakwa, Kongor, Takrama, Simpson Budu, & Mensah-Brown, 2013). Once the beans are dried, they are weighed and packed into sacks for transporting them to the chocolate production sites.

Until this step, cocoa beans are not really going through a standardized process. The three main steps of harvesting, fermentation, and drying heavily vary in the countries of origin, whereby a fluctuation in polyphenols content is enormous depending on the different parameters. However, the subsequent chocolate processing stages in the countries of manufacture are strictly controlled and highly reproducible. Hereby, cocoa beans are sterilized, broken, roasted, and ground to cocoa liquor which can be used directly for preparing chocolate products.

The present study aimed at monitoring the amount of PAs during the different chocolate manufacturing process steps. Correspondingly, the antioxidant activity of the intermediate products was analyzed as well. Here, the recently introduced NP-HPLC-online-DPPH methodology was used (Pedan et al., 2015). All steps from opening the fresh cocoa pods to fermentation, drying, roasting, conching, and finishing of the chocolate bars were done in a lab-scale.

2. Material and methods

2.1. Materials

(−)-Epicatechin, 2,2-diphenyl-1-picrylhydrazyl (DPPH), HPLC grade water, acetonitrile, methanol, and formic acid were obtained from Sigma Aldrich Chemie GmbH (Buchs, Switzerland). Polyphenol oligomers such as proanthocyanidin B2, proanthocyanidin C1, and cinnamtannin A2 were purchased from PhytoLab GmbH & Co. KG (Vestenbergsgreuth, Germany).

2.2. Cocoa samples

Obviously, the concentration of all polyphenols can vary depending on the source of the beans, the processing conditions, and the chocolate manufacturing process stages. To evaluate the effect of the manufacturing process on the PA composition of cocoa and the resulting products, one single batch of 5 kg fresh cocoa beans was studied across the different processing stages. Cocoa was a Trinitario variety and fruits were flown within five days after harvesting at 'Finca La Amistad' in Costa Rica to Switzerland. It has to be assumed that during transportation no cooling was applied to the cocoa fruits. A schematic summary of the processing steps involved are shown in Fig. 1. Roughly, the first step in this study was to break up the cocoa fruit and to remove the hulls from the seeds by clamping the cocoa fruits in a vice on a workbench and opening the hull with an ax. The tegument was removed and the seeds covered in their sweet mucilaginous pulp were further deposited for fermentation and drying. Standard fermentation conditions were applied under controlled temperature, whereby fruit pulp degraded by yeast and bacteria and lactic and acetic acid formed during heat development. The fruit material was placed in perforated trays at 40 °C for two days and another four days at 50 °C in a pressure tank (Eureka, Tecnogen S.r.l., Pergine Valsugana, Italy). During the fermentation, the mucilagious pulp could not

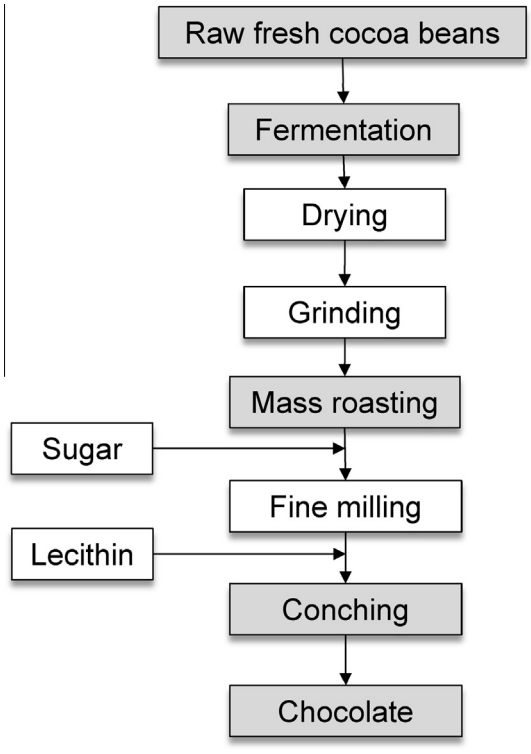

Fig. 1. Schematic chocolate manufacturing process as applied in this study.

drain away. Microorganism growth was not controlled. After six days of fermentation, the liquid media was discarded and the cocoa beans were carefully rinsed with water. Following the fermentation process, cocoa seeds with a residual water content of 6% were dried and equilibrated in an vacuum drying chamber (Model VD 53, Binder GmbH, Tuttlingen, Germany) for 7 days at a moderate temperature of 40–45 °C to reduce the water content to 3%. In the following, dried cocoa nibs were broken using a cocoa bean breaker (Type ESRIO, Disco Drives (King's Lynn) Ltd, Norfolk, United Kingdom) and winnowed to separate the broken nibs from the shells (Cocoa Winnower Large 240-1-50, Capco Test Equipment, Suffolk, United Kingdom). Cocoa nibs were further ground by a corundum stone mill resulting in cocoa mass (MK 180, Fryma-Koruma AG, Rheinfelden, Switzerland). Fine ground cocoa mass was roasted in a stirred glass vessel (ESCO-Labor Mixer Type EL 1, Esco-Labor AG, Riehen, Switzerland). One batch of about 1 kg was heated up to 120 °C and held for 2 min before cooling. To obtain an average particle size of approx. 20 μm, cocoa mass and sugar were ground subsequently using a three-roll mill (SDY-200, Bühler AG, Uzwil, Switzerland). For the conching process, solid ingredients such as sucrose and lecithin were mixed with the cocoa mass to finally develop the typical chocolate flavor and smooth texture (Frisse Elk'olino conche, Bühler AG, Uzwil, Switzerland). To ensure mild processing conditions, the conching process consisted of a 12 h step at a maximum temperature of 45 °C, which is also important to evaporate acetic acid. Samples were taken after 1 h and 4 h. Finally, the chocolate was tempered at 45 °C (ChocoMa 6T 12, Pitec AG, Oberriet, Switzerland), cooled down to 28 °C, and warmed up again to 32 °C to achieve a shiny and glossy surface. Finally, the molded chocolate was left to harden for a few hours in thermostated cabinet at 17 °C (TS 606-G/4-i, WTW Wissenschaftlich-Technische Werkstätten GmbH, Weilheim, Germany) and stored at 12 °C.

Changes in the PA profile were recorded in a model process and may not be fully in line with real-life crop or industrial scale processing. Nevertheless, one batch was processed and aliquots of about 50 g were sampled from each processing step. Samples were collected from (1) raw fresh cocoa beans, (2) fermented, dried cocoa beans (3) roasted cocoa mass, (4) 1 h conched cocoa mass, (5) 4 h conched cocoa mass, and (5) molded chocolate bars. Samples were stored immediately at −20 °C until analysis.

2.3. Sample preparation

All samples were treated in the same way, whereby the sample preparation was designed to obtain an enriched fraction of polyphenol compounds and methylxanthines according to Pedan, Fischer, and Rohn (2016). The resulting cocoa extract was used for further HPLC analysis.

2.4. NP-HPLC-online-DPPH analyses of oligomeric PAs

HPLC separations were performed on a Knauer Smartline system (Knauer GmbH, Berlin, Germany) as described previously (Pedan et al., 2015). The LC system was coupled with post column derivatisation using DPPH in order to analyze the antioxidant capacity simultaneously. Chromatograms were recorded by means of UV/Vis-detection (UVD 2.1 S, Knauer GmbH, Berlin, Germany) at a wavelength of 275 nm for determination of the phenolic compound profile. The radical scavenging activity was detected photometrically at 515 nm with a second UV/Vis-detector (Smartline UV/Vis-Detektor 2520, Knauer GmbH, Berlin, Germany) for estimation of the contribution of the single phenolic substances to the antioxidant capacity. Data were acquired and processed using ClarityChrom software package (Version 3.0.5.505).

PAs were quantified through an external calibration using (−)-epicatechin for monomeric PAs, proanthocyanidin B2 for dimeric PAs, proanthocyanidin C1 for trimeric PAs, and cinnamtannin A2 for PAs with DP ⩾ 4 on the one side. For comparability, all of the detected PAs were quantified as (−)-epicatechin equivalent on the other side. Definition of this approach is as described in Pedan et al. (2015). However, a positive correlation could be detected between the PA concentrations against UV-absorption. As well as by the PA content represents by a positive peak and the DPPH signal represents by a negative peak in the chromatogram. The HPLC analysis was done in triplicate for each sample.

3. Results and discussion

PAs are eluting in their order of polymerization (DP1 -> DP2 -> DP3 etc.) when using normal phase chromatography. The NP-HPLC-online-DPPH methodology was already proven to be useful for a rapid assessment of antioxidant compounds in complex mixtures (Pedan et al., 2015). A method combining the separation of the condensed polyphenol compounds and the simultaneous assessment of their antioxidant capacity is an advantage for characterizing the bioactivity/reactivity of bioactive compounds. However, the major benefit of this method is that the contribution of a single compound to the overall activity of a mixture of antioxidants can be measured (Fig. 2). The method stating therefore both, the antioxidant activity as a general outcome of the result and the antioxidant capacity as a quantitative evaluation of single PAs investigated. Definition of this terminology is as described in Rohn and Kroh (2005).

During HPLC analysis, attention must be paid to a proper storage of the samples, as the extracted PAs might not be stable in 50% aqueous acetone and could interact with each other. As a consequence, the samples were prepared fresh directly before HPLC analysis. Otherwise, unknown interferences can appear for especially the octamers and nonamers, as shown in the UV-chromatogram in Fig. 2. However, polyphenols are also susceptible to polyphenol oxidase reactions and consequently to enzymatic browning. Samples can be stabilized by addition of inorganic halides such as sodium chloride, sodium fluoride or a bivalent salt like calcium chloride, which has proved to have an even more considerable effect on the inhibitory behaviour of polyphenoloxidase (Le Bourvellec & Renard, 2012).

3.1. Effect of the chocolate manufacturing process on the content and profile of PAs

Little is known about changes of PAs during a complete chocolate manufacturing process. Food composition data of cocoa beans are often reported for selected processed food products (Gu et al., 2004), but rarely referring to one continuous process. To completely understand the chocolate manufacturing process, it is important to take a closer look not only at low molecular polyphenols, but also at the quantitatively larger group – the PAs. The aim of the present work was to investigate the profile of PAs over six different stages of chocolate manufacturing. The stages represent the most pronounced steps in chocolate finishing, from raw over fermented to roasted beans, to conching and finishing. Data on changes of the content of PAs during the chocolate manufacturing process are difficult to access. However, studies done by Cooper et al. (2007) indicated that (−)-epicatechin concentration can help to predict the content of polyphenols such as PAs B2, C1, and even the total polyphenol content.

The results obtained using the NP-HPLC-online-DPPH method are presented in Tables 1 and 2. The quantitative data and the data

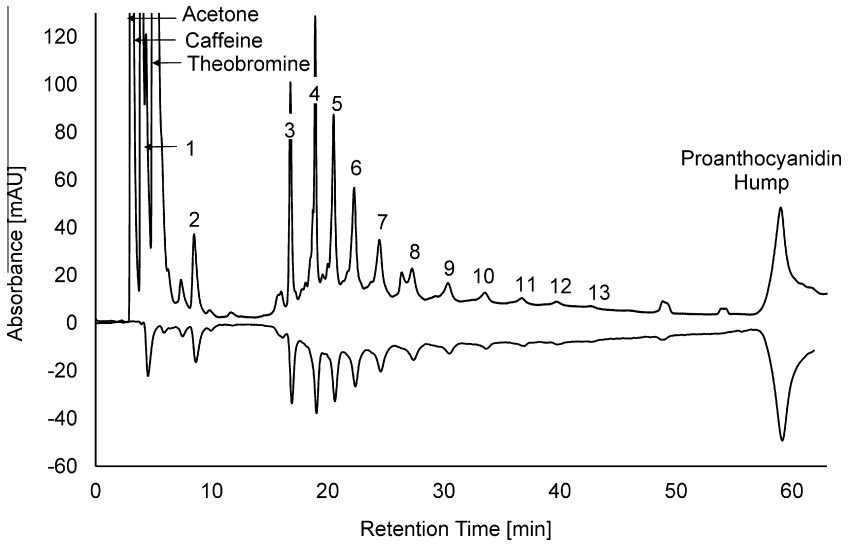

Fig. 2. Chromatogram of the oligomeric PAs from unroasted cocoa beans extract and their antioxidant activity using the NP-HPLC-online-DPPH (positive peaks at 280 nm, negative peaks at 515 nm). Identified PAs: epicatechin monomer (1), dimers (2), trimers (3), tetramers (4), pentamers (5), hexamers (6), heptamers (7), hexamers (8), nonamers (9), decamers (10), undecamers (11), dodecamers (12), tridecamers (13), and an unresolved 'PA hump'.

on the respective antioxidant activity demonstrate the changes of the different PAs and the corresponding changes of the antioxidant activity across the mentioned manufacturing stages. In general, PAs and their antioxidant capacity are expressed as monomeric (−)-epicatechin. Therefore, the results were expressed as (−)-epicatechin equivalents (ECE). The total PA content is reported as the total sum of the fractions ∑ DP1-DP13 for each processing step.

3.1.1. Fermentation process

In the present study, the initial acidity of the pulp (pH 6.6) resulting from the presence of citric acid, together with low oxygen levels, remained almost constant for three days, before it declined over the next six days to a final pH value of 5.5. First cocoa flavor precursors were noticed. Hereby, the initial total PA content (∑ DP1-DP13) of the raw cocoa bean seeds was 30.0 mg ECE/g non-fat dry matter which corresponds to an antioxidant capacity of 25.1 mg ECE/g. Furthermore, the amount of individual PAs detected in cocoa bean extract decreased from DP1 to DP13, whereby the monomeric fraction DP1 was more concentrated than polyphenols with DP > 1. However, according to Counet and Collin (2003), DP1 shows a higher extraction yield than higher molecular weight PAs, whereby the yield of higher oligomeric PAs can be increased due to optimized extraction solvents. In the present study, the unresolved HPLC signal identified as 'PA hump' in the chromatogram (Fig. 2) represents PAs with DP > 13, and/or other higher molecular PAs with different molecular structure. In this study, the so called 'PA hump' was responsible for up to 23.3 mg ECE/g. The sum of PAs from DP1-DP13 is approx. the same as that of the 'PA hump', illustrating the necessity to not neglect its overall quantitative and antioxidant contribution.

In the present study, the total PA content (∑ DP1-DP13) decreased only slightly to 26.7 mg ECE/g during the fermentation, which is about 11% less compared to the initial value (Table 1). The profile of PAs changed significantly and monomers appeared even more concentrated than the oligomers in the raw samples. In particularly, the amount of monomeric PAs increased to 8.8 mg ECE/g which corresponds to an increase of 60%. In parallel, the degree of polymerization decreased to a verifiable content of DP11. At the same time, the content of the compounds under the 'PA hump' doubled to 54.1 mg ECE/g.

Generally, during the fermentation, microorganisms and endogenous enzymes lead to a degradation of the pulp that surrounds the fresh beans and limit the development of pathogenic microorganisms. Flavor precursors are formed and polyphenols undergo an enzymatic transformation by polyphenol oxidase(s). In detail, polyphenols diffuse from the vacuoles through the cotyledons and interact covalently as wells non-covalently with proteins or polysaccharides (De Brito et al., 2002; Forsyth, Quesnel, & Roberts, 1958). Following the enzymatic oxidation, high molecular weight polymers get formed (Albertini et al., 2015). Afoakwa et al. (2013) observed through Folin-Ciocalteu assay a decrease of the total polyphenol content ranging from 169.1 mg/g to 148.8 mg/g for a 3 day fermentation and a further decrease to 119.4 mg/g in 7 days and to 83.5 mg/g after 10 days. Forsyth (1952) stated a loss of the total polyphenols by 24% after 60 h of fermentation, and 58% after 8 days. A similar percentage was found by De Brito et al. (2000) during their investigation whereby the total phenolic content slightly decreased from 231 ± 5 mg/g non-fat dry matter at time 0 h to 213 ± 5 mg/g after 72 h. Aikpokpodion and Dongo (2010) observed a more pronounced decrease of the polyphenol content from day 0 with 161.1 mg/g to 60.1 mg/g at day 6. A predominance of (−)-epicatechin was observed for some cocoa cultivars during fermentation with an initial value of 3.88 ± 0.20 mg/g at timepoint 0 h towards a doubling of the value to 7.25 ± 1.07 mg/g after 72 h (Cruz, Leite, Soares, & Bispo, 2015). By extending the fermentation time, the increase of epimerization products was more pronounced. In the present study, the strong increase of the monomers could be explained as result of the decrease of DP11, DP12, and DP13. Cruz et al. (2015) reported also a sharp decrease of polyphenolic compounds of about 70% and especially, a loss of (−)-epicatechin of up to 90%. Also Camu et al. (2008) described a linear decrease of (−)-epicatechin due to diffusion, polyphenol oxidation, and condensation started from 11 mg/g at the beginning of fermentation to a >70% loss after 144 h of fermentation. In general, the decrease in the amount of polyphenols is accompanied by enzymatic and non-enzymatic browning resulting in quite complex polymers. The increase of the 'PA hump' can be an indicator for this polymerization. PAs with a DP > 13 and further UV-active polymers (e.g., complex browning products) may co-elute as a big hump at the end of the chromatographic run. Afoakwa et al. (2013) suggested that during the fermentation,

Table 1
Oligomeric proanthocyanidins and their antioxidant activity (AA) obtained by NP-HPLC-online-DPPH from different chocolate manufacturing stages. The values are expressed as mg (−)-epicatechin equivalents per gram of the non-fat dry matter. PA content and its antioxidant activity are expressed as (−)-epicatechin equivalents (using (−)-epicatechin as standard, ECE). Values represent value mean, with n = 3 (±standard deviation). The bold entries refer to further explanations in the text. The asterisks indicate a single determination.

Degree of polymerization	Raw cocoa beans		Fermented cocoa beans		Roasted cocoa mass		1 h conching		4 h conching		Chocolate	
	PA [mg/g]	AA [mg/g]	PA [mg/g]	AA [mg/g]	PA [mg/g]	AA [mg/g]	PA [mg/g]	AA [mg/g]	PA [mg/g]	AA [mg/g]	PA [mg/g]	AA [mg/g]
DP 1	**5.26 ± 0.55**	3.81 ± 0.75	**8.78 ± 0.28**	6.84 ± 0.71	**4.21 ± 0.15**	2.84 ± 0.47	**2.59 ± 0.75**	3.20 ± 1.07	**2.70 ± 0.02**	1.82 ± 0.39	**1.77 ± 0.46**	1.49 ± 0.52
DP 2	2.65 ± 0.33	2.59 ± 0.70	4.05 ± 0.14	4.22 ± 0.36	1.68 ± 0.03	1.34 ± 0.39	1.06 ± 0.22	1.50 ± 0.59	1.02 ± 0.03	0.82 ± 0.13	0.84 ± 0.21	0.80 ± 0.05
DP 3	**4.39 ± 0.56**	4.20 ± 0.92	4.15 ± 0.09	4.66 ± 0.20	1.78 ± 0.02	1.74 ± 0.42	1.18 ± 0.28	1.72 ± 0.57	1.11 ± 0.03	0.93 ± 0.04	0.95 ± 0.27	0.90 ± 0.06
DP 4	**4.17 ± 0.50**	3.53 ± 0.95	2.99 ± 0.31	3.09 ± 0.06	1.40 ± 0.03	1.02 ± 0.16	0.85 ± 0.20	1.06 ± 0.53	0.72 ± 0.03	0.51 ± 0.15	0.60 ± 0.14	0.50 ± 0.16
DP 5	**4.31 ± 0.48**	3.54 ± 0.97	2.64 ± 0.12	2.27 ± 0.18	1.06 ± 0.02	0.69 ± 0.20	0.66 ± 0.17	0.86 ± 0.42	0.63 ± 0.05	0.27 ± 0.22	0.56 ± 0.17	0.37 ± 0.13
DP 6	3.20 ± 0.57	2.71 ± 0.72	1.22 ± 0.04	1.68 ± 0.13	0.62 ± 0.02	0.35 ± 0.29	0.50 ± 0.23	0.52 ± 0.32	0.45 ± 0.18	0.14 ± 0.10	0.34 ± 0.12	0.19 ± 0.10
DP 7	2.14 ± 0.37	1.98 ± 0.54	1.21 ± 0.14	0.87 ± 0.11	0.47 ± 0.06	0.12 ± 0.05	0.28 ± 0.05	0.21 ± 0.18	0.23 ± 0.00	0.03 ± 0.03	0.22 ± 0.03	0.07 ± 0.02
DP 8	1.51 ± 0.31	1.27 ± 0.31	0.85 ± 0.14	0.49 ± 0.01	0.40 ± 0.04	0.07 ± 0.11	0.20 ± 0.02	0.12 ± 0.15	0.20 ± 0.01	0.07*	0.16 ± 0.06	0.02*
DP 9	0.64 ± 0.19	0.99 ± 0.95	0.47 ± 0.06	0.31 ± 0.13	0.25 ± 0.01	–	0.13 ± 0.03	0.11*	0.14 ± 0.02	–	0.13 ± 0.03	–
DP 10	0.65 ± 0.14	0.47 ± 0.16	0.23*	–	0.14 ± 0.02	–	0.08 ± 0.02	–	0.10 ± 0.02	–	0.08 ± 0.01	–
DP 11	0.52 ± 0.10	–	0.36*	–	0.07 ± 0.06	–	0.03 ± 0.05	–	0.07 ± 0.06	–	0.09*	–
DP 12	0.26 ± 0.11	–	0.49*	–	–	–	–	–	–	–	–	–
DP 13	0.27 ± 0.09	–	–	–	–	–	–	–	–	–	–	–
PA Hump	**23.33 ± 3.92**	24.96 ± 3.13	**54.12 ± 3.47**	77.27 ± 3.92	29.49 ± 5.79	68.31 ± 4.17	20.31 ± 3.91	44.93 ± 2.61	16.99 ± 4.47	27.57 ± 9.94	19.68 ± 4.57	24.95 ± 4.56
∑ (1–13)	**29.98 ± 3.98**	25.08 ± 6.56	**26.72 ± 0.28**	24.43 ± 4.71	**12.08 ± 0.23**	8.16 ± 2.08	7.57 ± 1.84	9.30 ± 3.82	7.36 ± 0.09	4.60 ± 1.02	5.74 ± 1.56	4.34 ± 1.06

Table 2
Oligomeric PA and their AA obtained by NP-HPLC-online-DPPH from different chocolate manufacturing stages. The values are expressed as mg proanthocyanidin equivalents per gram of the non-fat dry matter. PA content and PA equivalents expressed as mean values with n = 3 (±standard deviation). The bold entries refer to further explanations in the text.

Degree of polymerization	Raw cocoa beans		Fermented cocoa beans		Roasted cocoa mass		1 h conching		4 h conching		Chocolate	
	PA [mg/g]	AA [mg/g]	PA [mg/g]	AA [mg/g]	PA [mg/g]	AA [mg/g]	PA [mg/g]	AA [mg/g]	PA [mg/g]	AA [mg/g]	PA [mg/g]	AA [mg/g]
DP 1	**5.02 ± 0.53**	1.86 ± 0.48	**8.43 ± 0.27**	3.81 ± 1.36	4.01 ± 0.14	1.24 ± 0.30	**2.46 ± 1.03**	1.64 ± 0.69	**2.57 ± 0.02**	0.75 ± 0.25	**1.66 ± 0.45**	0.53 ± 0.34
DP 2	3.58 ± 0.45	1.41 ± 0.43	5.47 ± 0.20	2.40 ± 0.60	2.25 ± 0.04	0.65 ± 0.24	1.42 ± 0.43	0.79 ± 0.36	1.36 ± 0.04	0.38 ± 0.08	1.13 ± 0.29	0.37 ± 0.03
DP 3	5.55 ± 0.69	2.09 ± 0.49	5.24 ± 0.12	2.34 ± 0.27	2.31 ± 0.02	0.80 ± 0.22	1.53 ± 0.49	0.82 ± 0.30	1.45 ± 0.04	0.41 ± 0.02	1.25 ± 0.33	0.39 ± 0.03
DP 4	6.81 ± 0.81	2.15 ± 0.59	4.91 ± 0.50	1.88 ± 0.10	2.36 ± 0.05	0.58 ± 0.10	1.44 ± 0.46	0.63 ± 0.33	1.23 ± 0.04	0.28 ± 0.09	1.03 ± 0.23	0.27 ± 0.10
DP 5	**7.04 ± 0.77**	2.16 ± 0.60	**4.35 ± 0.19**	1.37 ± 0.29	1.81 ± 0.03	0.37 ± 0.12	1.14 ± 0.40	0.49 ± 0.26	1.08 ± 0.08	0.13 ± 0.14	0.97 ± 0.27	0.19 ± 0.08
DP 6	5.26 ± 0.92	1.64 ± 0.45	2.07 ± 0.06	0.99 ± 0.22	1.10 ± 0.03	0.16 ± 0.18	0.87 ± 0.54	0.29 ± 0.20	0.79 ± 0.29	0.05 ± 0.06	0.63 ± 0.20	0.08 ± 0.06
DP 7	3.54 ± 0.59	1.18 ± 0.34	2.05 ± 0.23	0.49 ± 0.18	0.86 ± 0.10	0.02 ± 0.03	0.52 ± 0.11	0.15 ± 0.06	0.45 ± 0.00	–	0.43 ± 0.05	–
DP 8	2.53 ± 0.50	2.02 ± 0.19	1.47 ± 0.23	0.25 ± 0.01	0.74 ± 0.07	0.27 ± 0.07	0.39 ± 0.05	0.10*	0.39 ± 0.02	–	0.33 ± 0.09	–
DP 9	1.13 ± 0.31	0.56 ± 0.60	0.85 ± 0.09	0.14 ± 0.21	0.50 ± 0.02	0.11 ± 0.05	0.28 ± 0.07	–	0.30 ± 0.04	–	0.28 ± 0.06	–
DP 10	1.15 ± 0.22	0.24 ± 0.10	–	–	0.33 ± 0.04	–	0.21 ± 0.05	–	0.23 ± 0.04	–	0.20 ± 0.02	–
DP 11	0.94 ± 0.17	–	–	–	0.22 ± 0.10	–	0.22 ± 0.12	–	0.24*	–	0.21*	–
DP 12	1.02 ± 0.17	–	–	–	–	–	–	–	–	–	–	–
DP 13	0.54 ± 0.14	–	–	–	–	–	–	–	–	–	–	–
PA Hump	**37.65 ± 6.31**	15.56 ± 1.96	87.19 ± 5.59	48.29 ± 2.46	47.55 ± 9.31	42.69 ± 2.61	32.75 ± 6.30	28.07 ± 1.63	27.41 ± 7.19	17.21 ± 6.22	31.75 ± 7.35	15.57 ± 2.85
$\sum$ (1–13)	**43.59 ± 5.79**	14.03 ± 4.02	35.52 ± 0.72	13.67 ± 2.91	16.45 ± 0.20	3.84 ± 1.23	10.50 ± 2.37	4.92 ± 2.29	10.08 ± 0.16	2.00 ± 0.60	8.12 ± 2.10	1.84 ± 0.64

polyphenols are released from their storage cell organelles, and become substrates for enzymes leading to oxidation and polymerization reactions. Further condensation with amino acids, proteins and flavonoids leads to high molecular weight fractions.

3.1.2. Drying process

During the drying process beans were placed in shallow trays. The oxidation reaction that began with the fermentation continues any further during drying. As a result, the amount of polyphenols further decreased, accompanied by a reduction in astringency. During the drying process, the moisture content dropped significantly saving the cocoa bean from mold infestations. According to Albertini et al. (2015), the (−)-epicatechin content is reduced to approximately 75% at the end of the fermentation and drying process. Results obtained in the present study confirmed the high impact of fermentation and drying on the PA content. Being the first processing steps which contribute directly to the typical cocoa flavor, attention should be paid to these two steps to obtain high polyphenol content chocolate.

3.1.3. Roasting process

Additional loss of the polyphenol content is described to occur during roasting (Kealey et al., 2001). Preferably, oligomeric PAs were lost during roasting process whereby in the present study the total PA content (∑ DP1-DP13) continued falling to 12.1 mg ECE/g. Despite the high level of monomeric PAs found in the fermented cocoa beans, only 4.2 mg ECE/g were detected in the roasted cocoa beans but the remaining amount can be considered high. However, degradation also occurred for the 'PA hump' with 45.5% to 29.5 mg ECE/g. No DP12/13 polymers were found in the roasted cocoa beans (Fig. 3a and b).

Roasting has a high impact on cocoa flavor resulting from the Maillard reaction and Strecker degradation. High temperatures and low moistures are pre-requisites for the Maillard reaction. Convectional roasting temperature varies from 130 to 150 °C and last from 15 to 45 min, whereby in the present study roasting conditions were kept to a minimum on physical treatment. Traditional manufacturers either use whole-bean roasting or nib roasting, although cocoa-mass roasting is much more precise concerning heat transfer. As the cocoa-mass is more homogenous and degradation of valuable substances through longer heat impact can be avoided. Kothe, Zimmermann, and Galensa (2013) analyzed the stability of phenolic compounds during roasting and observed a 40% loss of flavanols during roasting temperatures beyond 120 °C and duration of 30 min. In detail, the sum of flavanols ranged from 7.75 mg/g in unroasted cocoa beans to 4.77 mg/g in roasted cocoa beans. Furthermore, they observed a loss of the primary monomers (−)-epicatechin ranged from 4.77 ± 0.08 mg/g to 3.35 ± 0.2 mg/g, as well as the PA dimers B2 ranged from 2.03 ± 0.02 mg/g to 1.33 ± 0.04 mg/g. A diminution was also confirmed for higher PAs. Furthermore, they found evidence for an epimerization of flavanol monomers and dimers as a function of temperature, whereby (−)-epicatechin decreased substantially with growing temperature. On the other hand, as already mentioned above, (+)-catechin can increase due to heat-induced epimerization.

However, studies done by Ioannone et al. (2015) focused on the determination of monomeric and oligomeric PAs and the devolution of the total antioxidant activity when using different roasting conditions. In that study, a total PA content (∑ DP1-DP10) of 13.1 mg/g non-fat dry matter after six minutes at 125 °C has been determined, whereby a decrease from DP1 with 4.22 mg/g to DP10 with 0.12 mg/g was observed. Furthermore, the group observed a general reduction with a more cascade shape declination from DP1 to DP10.

In a further recent study, Gültekin-Özgüven, Berktaş, and Özçelik (2016) demonstrated under similar roasting temperatures a lower content of monomeric (−)-epicatechin compared to the present study with 1.97 ± 0.16 mg/g, a dimeric B2 content with 1.11 ± 0.12 mg/g and the trimeric PA C1 content with 0.26 ± 0.02 mg/g. However, no information about roasting periods was given. The same study also described a total phenolic content of 8.79 ± 0.30 mg catechin equivalents per gram when using the Folin-Ciocalteu assay. When using similar roasting conditions, the group of Payne, Hurst, Miller, Rank, and Stuart (2010) found a total content of monomers in fermented cocoa beans ranging from 0.78 to 1.02 mg/g and an (−)-epicatechin content of 0.46–0.50 mg/g at 120 °C. Also here, no further information about roasting duration was provided.

3.1.4. Conching process

In the present study, cocoa nibs were firstly thoroughly separated from the outer shell before packed into the grinder. The longer the nibs are ground, the smoother the chocolate appears during consumption. Generally, conching periods can vary from a few hours to a few days. An adequate conching time is for the most Western European chocolatiers at least 72 h while American chocolatiers conch their chocolate for only 18–20 h (Alberts & Cidell, 2006). One of the main aims of the conching process is to remove unwanted acids such as acetic, propionic, and butyric acid. In the present study, samples were taken after 1 h and 4 h of conching, whereby the total PA content (∑ DP1-DP13) remained stable with from 7.6 to 7.4 mg ECE/g. A monomeric fraction of 2.6 mg ECE/g after 1 h conching and a monomeric fraction of 2.7 mg ECE/g after 4 h conching were obtained. The 'PA hump' with 20.3 mg ECE/g declined after 1 h and further decreased slightly to 16.9 mg ECE/g after 4 h. The results were calculated on the basis of sugar and fat-free cocoa mass.

Contrary to Di Mattia et al. (2014), who did not find compounds with DP10 during a short time conching process, the present study observed more complex PAs with a DP10/11. With regard to conching parameters, they furthermore observed a slight increase of the total PA content from the initial raw chocolate mass to the conched product ranged from 8.03 ± 0.13 mg ECE/g before conching to 8.42 ± 0.08 mg ECE/g after the conching process. In accordance to the study done by Mazor Jolić, Radojčić Redovniković, Marković, Ivanec Šipušić, and Delonga (2011), the total PA content of cocoa samples changes from 9.6 mg/g for cocoa beans to 7.7 mg/g for roasted cocoa beans to 5.6 mg/g for cocoa liquor when determined by using Bate-Smith assay.

3.1.5. Chocolate

In the present study, after a 12 h conching time and a short tempering process, the chocolate bars have a total PA content (∑ DP1-DP13) of 5.7 mg ECE /g calculated on the basis of sugar and fat-free cocoa mass which is approx. one fifth of the initial total PA content. The monomeric PA fraction decreased just slightly to 1.8 mg ECE/g per gram non-fat dry matter. The additional conching process seems to affect in a significant way the content of monomeric PAs. Nevertheless, the 'PA hump' remained stable with 19.7 mg ECE/g. As obvious from Table 1, the PA content correlates with the antioxidant activity. The contribution of ∑ DP1-DP6 to the total antioxidant activity is about 80% for the raw cocoa beans and increased to a contribution of 98% for the final chocolate. The contribution of these PAs slowly increased during chocolate manufacturing process. However, this result is quite contrary to the reports of Counet and Collin (2003) claiming for ∑ DP1-DP6 a contribution of only 40% to the total antioxidant activity.

A similar distribution of PAs and antioxidant activity was found in commercial chocolate bars. Studies done by Gu, House, Wu, Ou, and Prior (2006) compared different chocolate products from major brands and determined a total PA content ranging from 8.5 to 19.8 mg/g. When using non-specific colorimetric assays

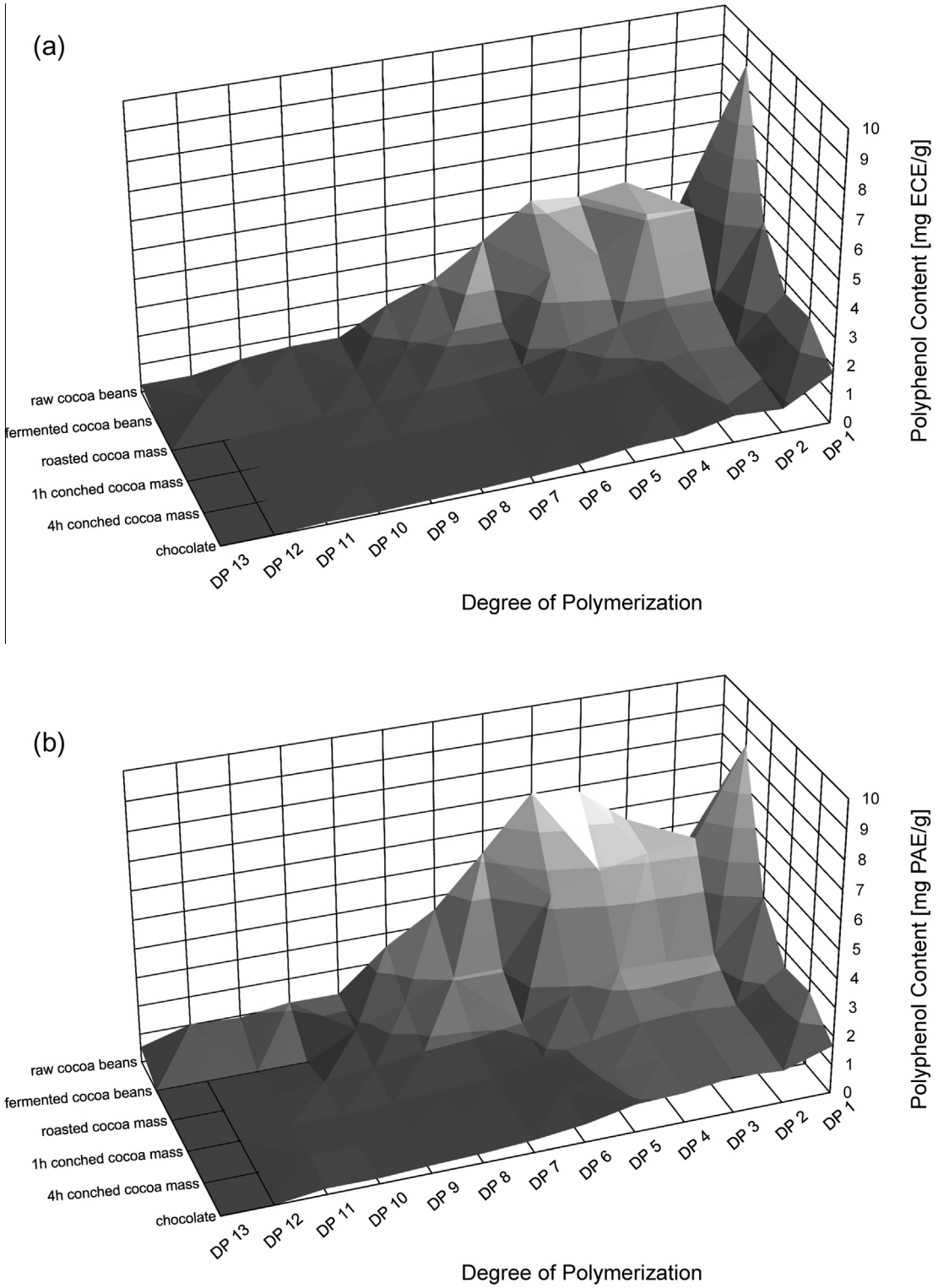

Fig. 3. (a) Oligomeric proanthocyanidin profile of the model chocolate manufacturing depending on the different stages of the chocolate manufacturing process and the degree of polymerization expressed as (−)-epicatechin equivalent. (b) Oligomeric proanthocyanidin profile of the model chocolate manufacturing and the degree of polymerization expressed as proanthocyanidin equivalent.

(e.g., p-dimethylaminocinnamaldehyde) for the determination of the total PA content, Payne et al. (2010) observed an amount of 7 mg/g for chocolate sample. Cooper et al. (2007) reported for 68 different chocolates an (−)-epicatechin content ranged from 0.071 to 1.942 mg/g, a PA dimer B2 content in the range of 0.041–1.174 mg/g, a PA trimer C1 in the range of not detectable – 0.905 mg/g, and PA tetramer A2 in the range of not detectable – 0.387 mg/g. Furthermore they observed that (−)-epicatechin accounted for a mean percentage of 39.0% of the total PA content. Counet et al. (2006) found a total PA content ($\sum$ DP1-DP6) of 85.4 mg/g in a dark chocolate extract, a DP1 of 42.9 mg/g and a DP2 of 14.6 mg/g.

Data from the present study cannot be directly compared with data found in other publications. Differences in the methodology of the phenolic compound extraction generate a wide range of data. Study done by Patras, Milev, Vrancken, and Kuhnert (2014) performed extraction with 70% aqueous methanol at room temperature overnight. Hammerstone et al. (1999) determined that 70% acetone in water, followed by two additional extractions with 70% methanol in water are most effective in extracting higher oligomeric cocoa PAs. Camu et al. (2008) used boiled water for phenolic compound extraction. Besides the different extraction methods, varying origins or processing parameters make a direct comparison even more difficult.

Furthermore, suggestions from other studies (Cooper et al., 2007) to use the (−)-epicatechin content for quantification of oligomeric procyanidins cannot be maintained from our point of view. A linear relationship among (−)-epicatechin and condensed polyphenols, as described there, would lead to assume that these molecules were affected the same way during cocoa processing. According to our results (Table 1) the relationship among PA monomers and PA dimer B2, trimers or tetramers is not a linear correlation. For an accurate quantification still NP-HPLC analysis must be done on the basis of higher molecular PAs as standard substances.

3.1.6. Correlation of concentration and antioxidant capacity

Attention must be paid when comparing the content of individual PAs with their corresponding antioxidant capacity (Table 1, Table 2). The values for the 'PA hump' of roasted cocoa mass and for four hours conching (Table 1) can be exemplarily explained: It is evident that the concentrations do not correspond linearly with the measured antioxidant capacities. A probable reason could be that the individual peaks contain more than one component with different antioxidant capacities, and that changes in this profile take place during processing. The insufficient separation into groups of similar molecular weight rather than individual components is due to the specific separation mode of the normal phase chromatography (Pedan et al., 2015).

3.2. Oligomeric proanthocyanidins expressed as proanthocyanidin equivalents

Traditionally, (−)-epicatechin or gallic acid are used as standards for the estimation of the total phenolic content of cocoa and related products (Hii, Law, Suzannah, Misnawi, & Cloke, 2009). At equal concentrations, (−)-epicatechin has a higher response to UV radiation than (+)-catechin (McMurrough & McDowell, 1978). Among PAs, the monomeric (−)-epicatechin has the highest UV/Vis response followed by proanthocyanidin dimer B2, trimer C1 while the more complex tetramer A2 is the least UV-active compound (Pedan et al., 2015). Consequently, using (−)-epicatechin as standard underestimates the amount of oligomeric PAs in a sample. To minimize the problem of using less suitable standards, it is proposed to calculate the content of PAs expressed at hand of closely related standard compounds, e.g., for monomers as mg (−)-epicatechin, for dimers as proanthocyanidin B2 equivalents, for trimers as proanthocyanidin C1 equivalents and for fractions more complex or equal than tetramers as mg cinnamtannin A2 equivalents, as long as the corresponding standards are not available.

Most of the studies reported in the literature showed cascading, diminished levels of the polyphenol content from monomers to decamers. In this study, the calculation at hand of related standards provided evidence that raw cocoa beans possess a high amount of pentameric PAs with 7.1 mg cinnamtannin A2 equivalents/g compared to pentameric PAs with 4.3 mg ECE/g when calculated as (−)-epicatechin (Table 2). During the chocolate manufacturing process, the levels additionally decrease, whereby already after fermentation the pentameric PAs decreased significantly to 4.3 mg cinnamtannin A2 equivalents/g and more monomeric PAs are formed. There is a >1.5-fold difference in the response between the PAs expressed as (−)-epicatechin equivalent or expressed as related standard compounds. Furthermore, this kind of evaluation indicates that pentameric PAs are the most abundant compounds in raw cocoa beans with the highest antioxidant capacity.

As presented in Fig. 3a, the oligomeric PA profile of the model chocolate manufacturing expressed as (−)-epicatechin equivalent is more shaped as a cascade with a decrease from monomeric to oligomeric PAs. In contrast, Fig. 3b shows the oligomeric PA profile of the model chocolate manufacturing and the degree of polymerization expressed as proanthocyanidin equivalents, whereby the profile occurs more accented to tetrameric up to heptameric PAs.

The complexity of oligomeric PAs and an appropriate evaluation of analytical methods are also discussed by Schofield, Mbugua, and Pell (2001). They analyzed the relationship between the tannin concentration and the absorbance of three main standard compounds delphinidin, cyanidin, and quebracho at 550 nm using the butanol/HCl assay. They showed a 30-fold difference in response between the UV-reactive delphinidin and the least UV-reactive quebracho. As a result, tannin concentration is underestimated when quebracho is used as standard for tannin-containing plants.

4. Conclusion

This study contributes to understand the influence of the different chocolate manufacturing stages on the content and composition of oligomeric PAs and the antioxidant activity as well as provides information about stages which are important for loses of PAs.

The highest content of phenolic compounds can be obtained for raw unfermented cocoa beans. During the chocolate manufacturing process, the total PA content decreased continuously, whereby the final chocolate contained only about 20% of the initial PA content. In the present study, the degree of polymerization also changed from a maximum of DP13 to DP11. The present study could confirm the main impact on the PA profile caused by fermentation resulted in a loss of the total PA content ($\sum$ DP1-DP13) of approx. 11% and a declination of the total PA content ($\sum$ DP1-DP13) during roasting resulted of approx. 60%. In comparison with other literature, this study helps to understand the influence of time/temperature combination, which is often used in order to modulate and increase the functional properties of some foods. For a general understanding, more emphasis should be paid to the 'PA hump' and along with it oligomeric PAs with DP > 13. This study clearly showed that the 'PA hump' increased during the fermentation process and dropped during roasting, whereby higher oligomeric PAs of higher molecular weight are formed during fermentation and decreased during roasting.

For the quantitative determination of the PA amounts, it should be considered that a calculation as epicatechin equivalents (ECE) leads to other results than the calculation as PA equivalents (PAE) up to DP4. Through calculation as ECE, the PA profile of raw cocoa beans appears to cascade down with DP1 > DP2 > DP3 etc. In contrast, when calculating the PA as PAE, the PA profile appears to have a peak at DP5 > DP4 > DP3 etc.

Finally, the major extractable PAs in cocoa could be determined using NP-HPLC-online-DPPH. A positive relationship was shown between DPPH scavenging activity and oligomeric PAs of higher molecular weight. Despite the complexity of cocoa polyphenols, a large number of novel cocoa flavonoids have already been

identified recently (e.g., Patras et al., 2014). But further efforts need to be directed towards the analysis of polymeric PAs and the investigation of the 'PA hump'.

Acknowledgments

We thank Esther Kokabi for providing the manufactured chocolate samples.

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Danksagung

Ganz besonders möchte ich mich bei Herrn Dr. Norbert Fischer (des Instituts für Lebensmittel- und Getränkeinnovation der Zürcher Hochschule für Angewandte Wissenschaften) für die Ermöglichung der Durchführung der vorliegenden Arbeit sowie die wertvollen Denkanstöße und moralische Unterstützung bedanken.

Ebenfalls gilt mein herzlicher Dank meinem Doktorvater Herrn Prof. Dr. Sascha Rohn (Institut für Lebensmittelchemie der Universität Hamburg) für die wissenschaftlichen Ratschläge und das arbeitsame Korrekturlesen der Manuskripte, die letzten Endes alle Publikationen zur Welt brachten.

Großer Dank gilt auch allen Mitarbeitern des Instituts für Lebensmittel- und Getränkeinnovation für die Zusammenarbeit, die Hilfestellungen und das angenehme Arbeitsklima.

An dieser Stelle möchte ich auch meiner Familie für ihre fortwährende Unterstützung über die ganzen Jahre hinweg danken. Im Besonderen danke ich Frau Mirjam Holinger für ihre unermüdliche Unterstützung die ganze Zeit über.

Insgesamt möchte ich allen danken, die zum Gelingen dieser Arbeit beigetragen haben.

Eidesstattliche Versicherung

Hiermit erkläre ich an Eides statt, die vorliegende Dissertation selbst verfasst und keine anderen als die angegebenen Hilfsmittel benutzt zu haben.

Zürich, den
30.03.2017

Unterschrift

Curriculum Vitae

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EDUCATION AND TRAINING

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• Title of qualification awarded	PhD
PUBLICATIONS	Pedan, V., Fischer, N., Bernath, K., Hühn, T., & Rohn, S. (2017). Determination of oligomeric proanthocyanidins and their antioxidant activity from different chocolate manufacturing stages using the NP-HPLC-online-DPPH methodology. Food Chem. DOI: 10.1016/j.foodchem.2016.07.094. Pedan, V., Fischer, N., & Rohn, S. (2016). Extraction of cocoa proanthocyanidins and their fractionation by sequential centrifugal partition chromatography and gel permeation chromatography. Anal. Bioanal. Chem. DOI: 10.1007/s00216-016-9705-7 Pedan, V., Fischer, N., & Rohn, S. (2015). An online NP-HPLC-DPPH method for the determination of the antioxidant activity of condensed polyphenols in cocoa. Food Res. Int. DOI:10.1016/j.foodres.2015.10.030

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