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# Bioactive Compounds in Underutilized Tropical Fruits from Latin America

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von

André Gordon

aus Itzehoe

| | | |
|---|---|---|
| 1. | Gutachter: | Privatdozent Dr. Friedhelm Marx |
| 2. | Gutachter: | Professor Dr. Matthias Wüst |
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## Summary

A vast number of plant species originating from Latin America bear delicious fruits, which are hardly known beyond its regional borders. Mostly, these fruits represent an important source for an adequate nutrient supply to the local people and are established as a traditional good in their society. An increasing demand in Northern American and European countries for such exotic fruits is caused by different reasons. On the one hand, they satisfy not only the search for novel flavor experiences but provide also a valuable contribution to a well-balanced diet. Thus, epidemiological data have shown that high consumption of fruits and vegetables results in a diminished risk of suffering from civilization diseases. On the other hand, foodstuffs containing additives like colorants, preservatives, antioxidants and flavors from natural resources attract gaining popularity instead of those artificially produced. Therefore, many underutilized fruits offer an unknown potential for an intensified commercial use. In case their production is guided by aspects of sustainability, the cultivation can contribute to the preservation and diversification of the ecosystem. Crop growing of appropriate species would also make available additional sources of income to the people from prevalently rural regions.

The prerequisite for an enhanced perception of underutilized fruits by the consumer or the industry is a comprehensive knowledge on their value adding components. Hence, aim of this thesis is to shed light on physiologically important constituents of selected fruits and to disclose their bioactive potential by investigating their antioxidant capacity. Primarily, the identification of antioxidant phenolic compounds from these fruits is emphasized but macro- and micronutrients are studied as well. In cases of relevance, further bioactive substances like ascorbic acid or betalains are taken into account.

Studies on *Euterpe oleracea* (açaí), *Anacardium occidentale* (cashew apple), and *Myrtillocactus schenckii* (garambullo) revealed a species-specific antioxidant capacity at different maturity stages of the fruits. The antioxidant capacity of açaí and garambullo decreased in the course of ripening whereas cashew apple showed a reverse trend. Amounts of phenolic compounds of all fruits were the highest in unripe condition which may be reflected by the antioxidant behavior of açaí and garambullo. However, cashew apple was characterized additionally by a high ascorbic acid content that increased during maturity and influenced thereby the radical scavenging activity of this fruit.

A comprehensive chemical characterization was performed on ripe fruits of *Clidemia rubra*, which were scientifically described for the first time in a noteworthy extent. From a physiological point of view, a high content of dietary fiber as well as considerable amounts of minerals (Ca, Mn, and Zn) were found in comparison to other berry fruits. The identification and quantification of the

phenolic components showed high concentrations of anthocyanins and various flavonol glycosides.

As scientific data for *Byrsonima crassifolia* (muruci), *Syzygium cumini* (jambolão), *Psidium guineense* (araçá), and *Pouteria macrophylla* (cutite) could rarely be found, fruits of these species originating from the Amazon region were subjected to a qualitative investigation of polyphenolic ingredients. Different compounds were detected like gallotannins, ellagitannins, quinic acid gallates, flavanonols, flavonols, and proanthocyanidins. Thereof, 18 substances were identified in araçá, 37 in jambolão, 19 in muruci, and 22 in cutite. The determination of the antioxidant capacity resulted in following ranking: cutite > jambolão > araçá > muruci. In regard to its radical scavenging properties, cutite fruits could be put in part on a level with the extremely effective açaí.

From an industrial point of view, it can be interesting to develop methods in order to isolate phenolic compounds being used as dietary supplements, functional foods or natural additives. High speed countercurrent chromatography (HSCCC) provides the opportunity to separate phenolic substances in a semi-preparative scale from their complex plant matrix. Thus, cyanidin 3-O-rutinoside could exemplarily be isolated from *Clidemia rubra* berries in an appreciable purity (98%, measured by HPLC at 280 nm).

On the basis of an intervention study it was observed to what extend bioactive substances potentially exert effects on the antioxidative status and oxidative stress in human. Data about the influence of fruit or fruit product consumption *in vivo* are inconsistent to date and depend often on singly measured parameters. In this crossover study, twelve subjects ingested a fruit juice rich in vitamin C and polyphenols composed of açaí, Andean blackberries, and camu camu. A significant increase was only denoted for plasma levels of ascorbic acid. All other parameters (DNA single strand breaks, $F_2$-isoprostanes, TEAC, total phenolic content, triglycerides, vitamin E, β-carotene, uric acid, cholesterol) demonstrated no significant alterations. However, TOSC results of the intervention group did not show a significant decrease of the antioxidant capacity in plasma as it was observed for the control group. Thus, fruit juice consumption might have a stabilizing effect on the plasma antioxidant capacity. In addition, reducing substances were found in plasma after bolus ingestion of the juice by using HPLC-CEAD which suggests the formation of potent antioxidative metabolites. Further, gallic acid and a coumaric acid isomer were detected by HPLC-MS analysis after fruit juice consumption. Gallic acid seemed to be directly bioavailable from the juice blend whereas the coumaric acid isomer may moreover be derived from higher molecular polyphenols or coumaric acid conjugates.

## Zusammenfassung

In Lateinamerika sind zahlreiche Pflanzenarten beheimatet, die äußerst wohlschmeckende essbare Früchte tragen, deren Bekanntheitsgrad jedoch selten über regionale Grenzen hinausgeht. In ihren Verbreitungsgebieten sind diese Früchte eine wichtige Bezugsquelle für eine adäquate Nährstoffversorgung und zum Teil sogar als traditionelles Gut in der Gesellschaft fest verankert. Verschiedenste Gründe sorgen auch hierzulande für eine gesteigerte Nachfrage nach solchen exotischen Früchten. Sie bedienen nicht nur die Suche nach neuen Geschmackserlebnissen, sondern liefern auch einen wertvollen Beitrag zu einer ausgewogenen Ernährung. Schließlich ist aufgrund epidemiologischer Daten bekannt, dass ein hoher Verzehr von Obst und Gemüse das Risiko eindämmt, eine Zivilisationskrankheit zu erleiden. Des Weiteren erfahren Lebensmittel vermehrten Zuspruch, denen Zusatzstoffe wie Farbstoffe, Konservierungsstoffe, Antioxidantien und Aromen natürlicher Herkunft anstelle von künstlich erzeugten hinzugefügt werden. Viele wenig genutzte Früchte beherbergen dabei ein ungeahntes Potential für eine verstärkte kommerzielle Nutzung. Eine Kultivierung geeigneter Pflanzen würde den Menschen aus den häufig ärmeren ländlichen Regionen eine zusätzliche Einnahmequelle verschaffen und auch zum Erhalt und zur Diversifizierung unseres Ökosystems beitragen, sofern die Produktion unter Gesichtspunkten der Nachhaltigkeit verläuft.

Voraussetzung für eine gesteigerte Wahrnehmung wenig genutzter Früchte seitens des Verbrauchers oder der Industrie ist ein umfassendes Wissen u.a. über die wertgebenden Inhaltsstoffe. Diese Arbeit soll somit der Aufklärung ernährungsphysiologisch wichtiger Bestandteile dienen und über Untersuchungen der antioxidativen Kapazität der Früchte deren bioaktives Potential aufzeigen. In ausgewählten Früchten werden Makro- und Mikronährstoffe untersucht, wobei das Hauptaugenmerk auf der Identifizierung antioxidativ wirkender Komponenten aus dem Bereich der phenolischen Verbindungen liegt. Am Rande wurden auch weitere bioaktive Substanzen wie Ascorbinsäure oder Betalaine berücksichtigt.

Untersuchungen unterschiedlicher Reifegrade von *Euterpe oleracea* (Açaí), *Anacardium occidentale* (Cashewapfel) und *Myrtillocactus schenckii* (Garambullo) wiesen eine artspezifische antioxidative Kapazität auf. Während bei Açaí und Garambullo die antioxidative Kapazität im Verlauf der Reife abnahm, zeigte der Cashewapfel einen gegenläufigen Trend. Bei den drei Früchten lag im unreifen Zustand der höchste Gehalt phenolischer Verbindungen vor, was sich bei Açaí und Garambullo auch in der antioxidativen Kapazität widerspiegelte. Der Cashewapfel ist zusätzlich geprägt durch einen hohen Gehalt an Ascorbinsäure, deren Zunahme im Verlauf der Reife offensichtlich auch die Radikalfängereigenschaften dieser Frucht maßgeblich beeinflusst.

Erstmals wurde eine umfassende chemische Untersuchung an reifen Früchten der *Clidemia rubra* durchgeführt. Aus ernährungsphysiologischer Sicht konnten ein hoher Nahrungsfasergehalt sowie, im Vergleich zu anderen Beerenfrüchten, hohe Gehalte an einigen Mineralstoffen (Ca, Mn und Zn) festgestellt werden. Eine Identifizierung und Quantifizierung der phenolischen Komponenten zeigte eine hohe Konzentration an Anthocyanen sowie diversen Flavonolglykosiden.

Vier Früchte aus dem Amazonasraum wurden einer qualitativen Untersuchung der polyphenolischen Inhaltsstoffe unterzogen. Diesbezügliche Daten zu *Byrsonima crassifolia* (Muruci), *Syzygium cumini* (Jambolão), *Psidium guineense* (Araçá) und *Pouteria macrophylla* (Cutite) sind kaum vorhanden gewesen. Insgesamt wurden 18 phenolische Verbindungen in Araçá, 37 in Jambolão, 19 in Muruci und weitere 22 in Cutite identifiziert. Darunter waren unterschiedliche Verbindungen wie Gallotannine, Ellagtannine, Chinasäuregallate, Flavanonole, Flavonole, und Proanthocyanidine zu finden. Die Bestimmung der antioxidativen Kapazität zeigte folgendes Ranking: Cutite > Jambolão > Araçá > Muruci. Früchte der Cutite waren hinsichtlich ihrer Radikalfängereigenschaften z. T. der als antioxidativ äußerst wirksamen Açaí gleichzusetzen.

Da es aus lebensmittelindustrieller Sicht interessant sein kann, sich einzelne phenolische Verbindungen für den Einsatz als Nahrungsergänzungsmittel, funktionelles Lebensmittel oder natürlichen Zusatzstoff zu Nutze zu machen, müssen Methoden entwickelt werden, mit denen diese Stoffe isoliert werden können. Die Gegenstromextraktionschromatographie (HSCCC) bietet die Möglichkeit der Abtrennung phenolischer Substanzen aus der komplexen pflanzlichen Matrix in semipräparativem Umfang. So konnte das Anthocyan Cyanidin-3-O-Rutinosid in nennenswerter Reinheit (98%, gemessen mit HPLC bei 280 nm) aus *Clidemia rubra*-Beeren isoliert werden.

Inwiefern die in Früchten enthaltenen bioaktiven Substanzen eine Wirkung auf den antioxidativen Status bzw. oxidativen Stress beim Menschen ausüben, wurde anhand einer Interventionsstudie überprüft. Die Datenlage darüber, welchen Einfluss der Verzehr von Früchten oder Fruchtprodukten *in vivo* hat, ist bis heute widersprüchlich oder von den häufig nur einzeln gemessenen Parametern abhängig. In dieser Crossoverstudie bekamen 12 Probanden einen vitamin C- und polyphenolreichen Fruchtsaft bestehend aus Camu camu, Açaí und Andenbrombeeren zu trinken. Signifikante Veränderungen zeigten sich anhand eines transienten Anstieges des Ascorbinsäurespiegels im Verlauf des Messzeitraumes. Alle anderen Parameter (DNS-Strangbrüche, $F_2$-Isoprostane, TEAC, Gesamtphenolgehalt, Triglyceride, Vitamin E, β-Carotin, Harnsäure, Cholesterol) blieben unverändert. Allerdings kam es bei der TOSC-Untersuchung in der Interventionsgruppe nicht zu einem signifikanten Abfall der antioxidativen

Kapazität im Plasma, wie es parallel in der Kontrollgruppe beobachtet wurde. Der Konsum des Fruchtsaftes könnte somit einen stabilisierenden Einfluss auf die antioxidative Kapazität im Plasma haben. Mithilfe von HPLC-CEAD-Messungen wurden nach Bolusgabe des Saftes reduzierend wirkende Substanzen im Plasma gefunden, was auf die Entstehung von antioxidativ wirksamen Metaboliten schließen lässt.

Des Weiteren wurden anhand von LC-MS-Untersuchungen, durch den Fruchtsaft induziert, Gallussäure und ein Cumarsäureisomer im Plasma detektiert. Während die Gallussäure wohl direkt aus dem Saft bioverfügbar war, könnte das Cumarsäureisomer darüber hinaus aus höhermolekularen Polyphenolen oder Cumarsäurekonjugaten entstanden sein.

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## Abbreviations

| | |
|---|---|
| 8-iso $PGF_{2\alpha}$ | 8-Isoprostaglandine $F_{2\alpha}$ |
| AA | Ascorbic acid |
| AAS | Atomic absorption spectrometry |
| ABAP | 2,2'-Azobis-(2-methylpropionamidine) dichloride |
| ABTS | 2,2'-Azinobis-(3-ehtylbenzothiazoline-6-sulfonic acid)-diammonium salt |
| AE | Acetone-water extract |
| AUC | Area under the curve |
| BMI | Body mass index |
| cAMP | Cyclic adenosine monophosphate |
| CE | Cyanidin 3-O-glucoside equivalent |
| CEAD | Coulometric electrode array detector |
| CV | Coefficient of variation |
| DAD | Diode array detector |
| DM | Dry matter |
| DMSO | Dimethyl sulfoxide |
| DNA | Desoxyribonucleic acid |
| DTPA | Diethylenetriaminepentaacetic acid |
| EDTA | Ethylenediaminetetraacetic acid |
| ELISA | Enzyme-linked immunosorbent assay |
| ESI | Electrospray ionization |
| eNOS | Endothelial nitric oxide synthase |
| FAc | Formic acid |
| FCR | Folin-Ciocalteu reducing capacity |
| FRAP | Ferric reducing antioxidant power |
| FW | Fresh weight |
| GAE | Gallic acid equivalent |
| GC | Gas chromatography |
| GF | Glass fiber |
| HAc | Acetic acid |
| HAT | Hydrogen atom transfer |
| HHDP | Hexahydroxydiphenol |
| HPLC | High performance liquid chromatography |
| HSCCC | High speed countercurrent chromatography |

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|---|---|
| $IC_{50}$ | Half maximal inhibitory concentration |
| LC | Liquid chromatography |
| KMBA | α-Keto-γ-methiolbutyric acid |
| LDL | Low-density lipoprotein |
| MeOH | Methanol |
| MRM | Multiple reaction monitoring |
| MS | Mass spectrometry |
| MTBE | Methyl tertiary-buthyl ether |
| $m/z$ | Mass-to-charge ratio |
| n.d. | Not detected |
| ORAC | Oxygen radical absorbance capacity |
| PA | Polyamide |
| PDA | Photodiode array detector |
| pn | Peroxynitrite |
| px | Peroxyl radicals |
| PTFE | Polytetrafluoroethylene |
| SB | DNA single strand breaks |
| SD | Standard deviation |
| SET | Single electron transfer |
| SIN-1 | 3-Morpholinosydnonimine N-ethylcarbamide |
| SIR | Selected ion recording |
| SPE | Solid phase extraction |
| TE | Trolox equivalent |
| TBARS | Thiobarbituric acid reactive substances |
| TEAC | Trolox equivalent antioxidant capacity |
| TFA | Trifluoroacetic acid |
| TOSC | Total oxidant scavenging capacity |
| TRAP | Total radical-trapping antioxidant parameter |
| Trolox® | 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxyl acid |
| TTA | Total titratable acidity |
| UHQ | Ultra high quality |
| UPLC | Ultra performance liquid chromatography |
| UV | Ultraviolet |
| WE | Water extract |

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# 1 Introduction

## 1.1 What does 'underutilized' mean?

Tropical fruits are divided in different categories according to their economic importance. Firstly, the 'major tropical fruits' like banana, pineapple, and mango are cultivated in appropriate regional climates throughout the world. These fruits are well-known in both local and export markets. They have been subjected to comprehensive scientific data gathering including different aspects of agronomic practices, selection, breeding, plant protection and postharvest treatment (Galán-Saúco, 1996). Secondly, 'minor fruits' are regarded as those species having a small commercial value of production and trade compared to major agricultural commodities (Gruère *et al.*, 2006). These fruits require much effort to define general conditions on breeding, agronomic and postharvest constraints in order to obtain high yields (Galán-Saúco, 1996). According to Galán-Saúco (1996), there is a third group comprising the wild tropical fruits that need to undergo evaluation and collection of genetic material. However, wild fruits may also be categorized as minor fruits because both definitions flow smoothly into each other. Consequently, underutilized fruits cannot clearly be assigned to one of the two latter categories.

Even the term 'underutilized' has raised a discussion about a precise definition as it lacks any information on geographical, social and economic implications. For instance, species may be underutilized in some regions but not in others or they may be an important component of the daily diet for indigenous people but their poor marketing conditions make them underutilized in economic terms (Padulosi *et al.*, 2002). A definition for underutilized crops is provided by Gruère *et al.* (2006) who keep three characteristics ready. Underutilized plants are those which are rather locally than globally abundant. Secondly, the local growers have practical knowledge of the plant species but lack of scientific knowledge on plant physiology as well as plants agronomic and ecological properties. The third aspect is the limited use of a plant species in relation to its economic potential. The growing of these underutilized crops generates a significant local income but does not provide a significant share to the national or international trade. An official definition of the International Plant Genetic Resources Institute is given by Eyzaguirre *et al.* (1999). Here, underutilized crops are recognized as those that grew once in a broad scale but have fallen into disuse because of different agronomic, genetic, economic and cultural reasons. These crops are used less as they cannot compete with other species in the same agricultural environment. In differentiation to underutilized crops Eyzaguirre *et al.* (1999) make also use of the term 'neglected crop', which are those grown for the subsistence of local communities. Neglected crops may be globally distributed but occupy niches in the local ecology and in production and consumption systems. These crops remain inadequately characterized and

neglected by research despite their practical use. In accordance with Padulosi *et al.* (2002) the term 'underutilized' will be used in a broad sense in the following in reference to both underutilized and neglected species.

## 1.2 Socioeconomic importance of underutilized fruits and sustainable exploitation

The worldwide consumption of fresh fruits in 2007 is estimated at 69.9 kg/capita/year and rose by 1.7% from 2006 to 2007. Fruit consumption in Germany in 2007 is estimated at 88.0 kg/capita/year and grew by 2.8% towards 2006 (FAOSTAT, 2011a). Consumption saturation worldwide might be attained at 100-120 kg but is far from being reached (Janssens and Subramaniam, 2000). The minor tropical fruits, which include also underutilized fruits, were produced in an amount of 17.7 mio. t in 2009 representing a growth rate of 3.5 % in comparison to 2008 (FAOSTAT, 2011b). The developing countries produce 98% of the tropical fruits. The value of international trade for both fresh and processed fruits was estimated at about 4 billion US$ in 2004 with an indicated annual growth rate of 2.4% in export values. The bulk of the tropical fruits, which are imported into Germany, arrives from Latin America. The minor fruits come along with other sea freighted fruits, which are imported in particular with the banana shipments from that region. Although 90% of the tropical fruits are consumed in the producing countries their importance in trade cannot be over emphasized (FAO, 2011).

The cultivation of underutilized fruits provides both socioeconomic and environmental benefits to ensure the livelihood of farmers from marginal areas. Apart from being a valuable nutritional source to the people (Mitra *et al.*, 2010), many underutilized species are well adapted to harsh climate conditions (Bowe and Haq, 2010). Due to their natural occurrence, underutilized plants show a high degree of tolerance against biotic and abiotic stress (Mitra *et al.*, 2010) and enrich the biodiversity in their environment (Bowe and Haq, 2010). The contribution of fruit production may be significant to income generation and alleviation of poverty. Local markets offer the most realistic potential for an additional cash income for poor people as no large infrastructure, processing technology or capital is required to reach them. On the other hand, local markets generate only a limited demand, whereas national and global markets show a greater potential. However, promotion of underutilized plant species at this level requires a detailed understanding of the supply chain (Gündel *et al.*, 2003).

The production and commercial exploitation of minor tropical fruits should be attended by conventions of a sustainable use. Strategic elements need to be developed for the promotion of underutilized plant species (Gündel *et al.*, 2003; Vanhove and Van Damme, 2009). Such policy guidelines would prevent possible drawbacks like overexploitation and loss of genetic diversity as

a consequence of unsustainable monoculture plantations (Gündel *et al.*, 2003; Leakey *et al.*, 2005) or the takeover of exploitation and seizing of profits by a rich elite (Van Looy *et al.*, 2008).

## 1.3 Why is there demand for underutilized tropical fruits?

The growing demand for a diversified supply with tropical fruits is the consequence of technological advances, demographic changes and alterations in consumer's perception in the Western world. The enhancement in postharvest handling technologies results in ameliorated storage conditions, packaging and transportation of perishable products (Ahmad and Chwee, 2008). Immigration is attended by cultural and ethnic diversification and brings the world's cuisine and foreign cooking traditions to the autochthonous population (Hermann, 2009). All in all, changes in the perception of the consumer may be the main cause for an increasing trade volume of tropical fruits. Sabbe *et al.* (2009) emphasized that the introduction of innovative tropical fruit juices has substantial potential for a long-term market presence in West European countries. The number of implemented novel fruit beverages rose by 60% between 2003 and 2007 (Sabbe *et al.* 2009). Marketing aspects may play an important role for this development as fierce competition forces the companies to differentiate and add value to their products (Hermann, 2009). Furthermore, the consumer end desires not only for new tastes and flavors, but also the awareness of the consumer has arisen to attain beneficial effects for their health that is claimed to be derived from an increased consumption of fruits and fruit products (Ahmad and Chwee, 2008).

An important issue is also that consumer abandon the purchase of foods containing artificial ingredients used by the food industry. The demand tends to products containing additives from natural resources optionally attributed with functional or added value (Giusti and Wrolstad, 2003). Hence, a variety of colorants, antioxidants, preservatives, and flavors can be extracted from eligible underutilized fruits.

## 1.4 Bioactive compounds

There is large body of incidence that high consumption of fruits and vegetables can reduce the prevalence of degenerative illnesses like different types of cancer (Hertog *et al.*, 1994), cardiovascular diseases (Shivashankara *et al.*, 2010), and Alzheimer's disease (Singh *et al.*, 2008) or lifestyle diseases like obesity (Crujeiras *et al.*, 2010; Santos *et al.*, 2010) and its related disorders (McDougall *et al.*, 2005). These beneficial health effects may be exerted *inter alia* by biologically active compounds like certain essential dietary constituents such as vitamin C or non-nutritive phytochemicals like phenolic compounds (Crozier *et al.*, 2009; Szajdek and Borowska, 2008).

Vitamin C acts on the one hand as an important water-soluble antioxidant in biological fluids by scavenging physiologically relevant reactive oxygen species. In addition to this direct antiradical capacity, vitamin C is able to regenerate other antioxidants like α-tocopherol, glutathione, urate, and ß-carotene from their respective radical species (Carr *et al.* 1999). On the other hand, vitamin C exerts also regulatory functions on a cellular level by influencing gene expression and apoptosis (Balsano and Alisi, 2009).

Phenolic compounds occur ubiquitary in plants. They are products of the phenylpropanoid biosynthetic pathway (Williamson and Clifford, 2010). The vast number of approximately 8000 different phenolic compounds can be subdivided into flavonoids and non-flavonoids. Anthocyanins, chalcones and dihydrochalcones, flavanols, flavanones, flavones, flavonols, isoflavones, and proanthocyanins belong to the flavonoids (Williamson and Clifford, 2010). All these compounds show a diphenylpropane moiety ($C_6$-$C_3$-$C_6$-skeleton) as common structural feature (Balsano and Alisi, 2009). Benzoic acids, cinnamic acids and its conjugates, and hydrolyzable tannins are assigned to the non-flavonoids (Williamson and Clifford, 2010).

Because of the large number of phenolic compounds, it is difficult to elucidate all effects that result in different biological activities (Williamson and Clifford, 2010). Generally, polyphenols are attributed to have antimicrobial (Ferrazzano *et al.*, 2011; Martins *et al.*, 2011) and antioxidant properties (Holst and Williamson, 2008). They play a significant role in the prevention of oxidative stress, which is seen as the main cause in the development of degenerative diseases (Balsano and Alisi, 2009; Harfani and Souliman, 2007; Terao, 2009). Degenerative diseases can result from an imbalance between physiological free radical generation and insufficient free radical scavenging by enzymatic and non-enzymatic defense (Halliwell, 1997). Dietary phenolics and their metabolites perform also various physiological actions *in vivo* on different components of the intracellular signaling cascades, which are essential for cellular functions like growth, proliferation and apoptosis. They show regulatory effects on signaling pathways like nuclear factor-*κ*B, activator protein-1 or mitogen-activated protein kinases (Crozier *et al.*, 2009). By modulating these pathways, phenolic compounds may be seen as chemopreventive agents in cancerogenesis (Fresco *et al.*, 2006) and exert neuroprotective effects (Singh *et al.*, 2008). Further, numerous parameters involved in the pathogenesis of cardiovascular diseases are affected on a regulatory level by dietary phenolics as well. There is scientific evidence for effects on lipid metabolism disorders by decreasing total cholesterol, triglycerides and LDL-cholesterol, protection of platelet aggregation by increasing cAMP levels leading to lower intracellular $Ca^{2+}$ concentrations, and the avoidance of endothelial dysfunctions by preventing hypertension via eNOS stimulation (Harfani and Souliman, 2007).

## 1.5 How to measure bioactivity?

Many degenerative diseases are related to oxidative damages produced by radical species. The ability of a substance or a complex mixture to scavenge such radicals can be shown *in vitro* by determining the antioxidant capacity. Thus, the *in vitro* antioxidant capacity of a fruit may be used as a quality indicator demonstrating the content of bioactive compounds. However, it should be emphasized that results of these *in vitro* antioxidant capacity assays cannot replace *in vivo* studies which would be necessary for assessment of a potential physiological impact of a fruit. By initiating *in vivo* studies, for instance, the bioavailability of bioactive compounds or their transformation into metabolites can be taken into account (Espín *et al.*, 2007).

There is a great number of *in vitro* antioxidant capacity assays. Many of them underlie different principles in the radical scavenging process. For example, Trolox equivalent antioxidant capacity (TEAC), oxygen radical absorbance capacity (ORAC), ferric reducing antioxidant power (FRAP), total oxidant scavenging capacity (TOSC), total radical-trapping antioxidant parameter (TRAP) belong to the commonly used assays (Schaich, 2006). The TOSC assay was preferably chosen for analysis in this thesis as it enables the generation of different radicals that are physiologically relevant (Lichtenthäler *et al.*, 2005). The area under the curve technique allows further to take the kinetic of antioxidants like phenolic compounds or ascorbic acid into account. Mostly, other assays only measure the inhibition rate at a fixed time or the lag phase reaction like the widely used ORAC. By observing the time course of ethylene formation different types of antioxidants can be distinguished like fast acting antioxidants or retardants (Lichtenthäler *et al.*, 2003). As proposed by Schlesier *et al.* (2002) the use of at least two assays was preferred for most analysis due to the obvious differences between the test systems. The TOSC belongs to the hydrogen atom transfer (HAT) assays that measure the ability of antioxidants to donate hydrogen which, in turn, quenches radicals. In addition, the Folin-Ciocalteu total phenolic assay was chosen which generally performs a so called single electron transfer (SET). This assay measures the ability of antioxidants to transfer one or more electrons and to reduce the target compounds (Prior *et al.*, 2005). The Folin-Ciocalteu method is not only specific for phenolics and can be interfered by other reducing substances like sugars, amino acids, and ascorbic acid (Georgé *et al.*, 2007). A third assay used for analysis on the antioxidant capacity is the TEAC in which both HAT and SET systems are combined (Prior *et al.*, 2005).

## 1.6 Objective and outline of the study

The flora of Latin America bears a plethora of underutilized fruits containing compounds that have been shown to perform beneficial health effects and may additionally provide useful food technological properties. The knowledge on the composition of bioactive compounds and even

common nutritive ingredients like macronutrients and minerals in underutilized fruits is scarce. Thus, objective of this study was at first to contribute to the elucidation of biologically active and nutritive components in selected promising fruits as well as to evaluate their antioxidant capacity. Determinations will be performed on fruits of *Euterpe oleracea* (açaî), *Anacardium occidentale* (cashew apple), *Myrtillocactus schenckii* (garambullo) in the course of ripening in order to assess, which maturity stage provides the highest concentration of the target compounds (chapter 2.1.1). Further, ripe fruits of *Byrsonima crassifolia* (muruci), *Syzygium cumini* (jambolão), *Psidium guineense* (araçâ), and *Pouteria macrophylla* (cutite) from the Amazon region as well as scientifically little-noticed berries of *Clidemia rubra* from the Columbian highland will be subjected to a chemical characterization (chapter 2.1.2). Mass spectrometrical analysis was performed for identification of phenolic compounds in chapter 2.1.1 and 2.1.2. LC-UV-DAD-MS/MS experiments enable partial structure elucidation and unambiguous identification of phenolic compounds when used in combination with authentic standards. Fragmentation of the quasi-molecular ion leads to the aglycone enabling the mostly clear identification (Papagiannopoulos *et al.*, 2004).

The isolation of verifiable bioactive substances is the second goal of this thesis. Chapter 2.2 will exemplarily show a method how to separate anthocyanins from their plant matrix in a semi-preparative scale.

Chapter 2.3 will further contribute to the elucidation of *in vivo* effects after fruit juice consumption. There is striking epidemiological evidence for health benefits from a diet rich in fruits and vegetables, but shedding light on causes of these beneficial effects is still challenging. Possible changes of important plasma antioxidants and markers indicating oxidative stress as a consequence of the ingestion of a tropical fruit juice rich in phenolic compounds and vitamin C will be surveyed on the basis of a comprehensive intervention study with human participants. Eventually, the question of the bioavailability of individual phenolic compounds will also be explored.

## 1.7 References

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# 2 Results and discussion

## 2.1 Chemical characterization and antioxidant capacity of underutilized fruits

### 2.1.1 Changes of bioactive compounds during ripening

In general, underutilized fruits have been scientifically insufficiently studied although many of them bear a great potential for an intensified commercial use. This can be either as dietary supplement or functional food, for example, due to the food technological properties of their bioactive compound. Total amounts of the phytochemical constituents and the antioxidant capacity of a fruit depend on the degree of ripening. Hence, it might be more useful to harvest some species already during ripening since they provide better functional properties in immature condition. If a fruit is not only marketed for consumption, its phenolic compounds can be used as antioxidants (El-Hela and Abdullah, 2010) or inhibitors of undesirable microbial growth (Jalosinska and Wilczak, 2009). Vitamin C, which is known to be an important physiological antioxidant, has already been traditionally applied in foods (Bauernfeind and Pinkert, 1970).

In this study, evaluation of the bioactive potential in the course of ripening was performed on açaí (*Euterpe oleracea*), cashew apple (*Anarcadium occidentale*) and garambullo (*Myrtillocactus geometrizans*). Açaí and cashew apple have shown a big potential for intensified cultivation as they can be used in processed form or as functional food ingredient (Hoffmann-Ribani *et al.*, 2009; Messias, 2010; Espirito-Santo *et al.*, 2010). These fruits have already been studied to some extend in ripe condition e.g. by Kang *et al.* (2011) and Hoffmann-Ribani *et al.* (2009), respectively. However, nothing is known on the composition of bioactive substances during maturation. Scientific information on garambullos is scarce, in general.

#### 2.1.1.1 Açaí

##### 2.1.1.1.1 Bioactive compounds

The bioactive compounds evaluated in açaí fruits consist of a variety of different phenolic substances which were identified and quantified by HPLC-ESI-MS/MS.

Anthocyanins were found to be responsible for the deep purple color in açaí what is already known from previous reports (Lichtenthäler *et al.*, 2005; Schauss *et al.*, 2006). As expected, no anthocyanins were present in unripe açaí samples. Medium-ripe fruits contained pelargonidin 3-O-glucoside, peonidin 3-O-glucoside, peonidin 3-O-rutinoside, cyanidin 3-O-glucoside, and cyanidin 3-O-rutinoside. A total of six individual anthocyanins (cyanidin 3-O-sambubioside in addition to the four previously mentioned anthocyanins) was found in ripe fruits. Thereof, cyanidin 3-O-glucoside and cyanidin 3-O-rutinoside turned out to be the major anthocyanin

components, which is in accordance to a report of Schauss *et al.* (2006). An overview of the accumulation of anthocyanins in açaí is given in **table A.4.3** (p.42).

Beside the general increase of the anthocyanin concentration, it was also observed that the relation of the individual anthocyanin amounts was different in each ripening stage. The ratio of cyanidin 3-O-glucoside to cyanidin 3-O-rutinoside accounted for 1:1 in the intermediate ripening stage [cyanidin 3-O-glucoside: 0.29 ± 0.05 mg/100 g dry matter (DM), cyanidin 3-O-rutinoside: 0.31 ± 0.04 mg/100 g DM]. In ripe fruits, on the contrary, the ratio increased in favor of cyanidin 3-O-rutinoside to approximately 4:1 (cyanidin 3-O-rutinoside: 17.86 ± 1.59 mg/100 g DM, cyanidin 3-O-glucoside: 4.94 ± 0.55 mg/100 g DM). Siriwoharn *et al.* (2004) reported on changes in anthocyanin biosynthesis during ripening in two blackberry cultivars. Cyanidin 3-O-glucoside and cyanidin 3-O-rutinoside were determined as main anthocyanin compounds. But unlike in açaí, the content of cyanidin 3-O-glucoside increased steadily whereas amounts of cyanidin 3-O-rutinoside decreased from unripe to ripe fruits. Fernández-López *et al.* (1998) observed a decline of cyanidin 3-O-glucosid concentration in ripening grapes. They noted that in the biosynthesis of anthocyanidins in *Vitis* ssp. cyanidin typically occurs as a precursor molecule of peonidin derivatives. Conclusively, according to these results there are no general regularities deducible for the formation of individual anthocyanins during ripening in fruits. Only the increase of total anthocyanins in general is apparent.

Regarding the non-anthocyanin phenolic compounds in açaí, a total of eight phenolic acids and phenolic acid derivatives, eight flavones, and one flavanonol were identified (**table A.4.3**, p.42). With exception of p-coumaric acid all compounds were present in each of the three ripening stages. On the contrary to the anthocyanins, the highest concentrations of individual non-anthocyanin phenolics were generally found in unripe fruits (with exception of gallic acid). Amounts of the main flavones in each ripening stage could be ranked as follows: orientin >> homoorientin >> vitexin > isovitexin. Vanillic acid, p-hydroxybenzoic acid, and syringic acid were found in noteworthy amounts among the phenolic acids. Whereas amounts of all non-anthocyanin flavonoids and hydroxycinnamic acids consistently decreased during maturation, some exceptions could be observed in regard to the hydroxybenzoic acids. The concentration of gallic acid was the highest in intermediate ripe fruits. In case of syringic acid and protocatechuic acid, amounts decreased at first from unripe to intermediate ripe fruits and increased subsequently with further ripening.

In accordance with açaí, decreasing amounts of hydroxycinnamic acids in the course of fruit ripening were also reported by other authors (Dragovic-Uzelac *et al.*, 2007 and Gruz *et al.*, 2011). Interestingly, Xu *et al.* (2008) found that chlorogenic acid in *Citrus* cultivars vanished almost

completely during ripening which is in agreement with changes of this hydroxycinnamic acid derivative in açaí.

The biosynthesis of hydroxybenzoic acids in açaí during ripening was different to that reported by other authors. For instance, Gruz *et al.* (2011) observed in different ripening stages of medlar fruits that contents of free protocatechuic acid and syringic acid consistently decreased whereas an accumulation occurred in case of p-hydroxybenzoic acid.

Changes of individual flavones during ripening have not been reported previously. However, Dragovic-Uzelac *et al.* (2007) found the highest values of flavonols in the initial maturity stage in apricot cultivars.

The findings of phenolic acids and non-anthocyanin flavonoids in ripe açaí are in a great extend in common with other reports. On the contrary to findings of Lichtenthäler *et al.* (2005), Del Pozo-Insfran *et al.* (2004) and Pacheco-Palencia *et al.* (2009) the presence of ferulic acid and p-coumaric acid could not be constituted in our samples. On the other hand, chlorogenic acid and caffeic acid were mentioned for the first time. The identified flavones were previously constituted by Schauss *et al.* (2006) and Kang *et al.* (2010) with exception of luteolin 7-O-glucoside, chrysoeriol, and taxifolin.

#### 2.1.1.1.2 Antioxidant capacity

The antioxidant capacity in açaí was assessed by using Folin-Ciocalteu, TEAC, and TOSC. An overview of the results is given in **table A.4.4** (p.46). Unripe fruits showed the best antiradical behavior in all three assays. Further, a continuous decrease of the antioxidant capacity was observed in the course of ripening for TEAC and TOSC (TEAC: 16.99 ± 0.71 µmol Trolox/100 g DM in unripe fruits to 2.78 ± 0.10 µmol Trolox/100 g DM in ripe fruits; TOSC px: 12.1 mg DM/100 mL in unripe fruits to 24.0 mg DM/100 mL in ripe fruits; TOSC pn: 46.4 mg DM/100 mL in unripe fruits to 87.2 mg DM/100 mL in ripe fruits; note: TOSC values indicate the amount of lyophilized fruit sample that is needed to obtain a 50% inhibition of the radical activity). The Folin-Ciocalteu assay showed the lowest value in the intermediate ripening stage which was followed by a slight increase in ripe fruits (12316.5 ± 264.2 mg gallic acid equivalents/100 g DM in unripe fruits to 3437.0 ± 154.0 gallic acid equivalents/100 g DM in ripe fruits).

The TEAC antioxidant capacity of the ripe açaí fruits is in accordance with values found for different commercial açaí pulps. Depending on the water content of the pulps, the TEAC ranges approximately between 1.02 and 5.25 µmol Trolox equivalent/100 g DM (Dos Santos *et al.*, 2008). TOSC values of ripe açaí turned out being at least 1.5-fold higher regarding both radicals than those found in pulps of different harvest periods described in Lichtenthäler *et al.* (2005).

The trend of a decreasing antioxidant capacity during ripening was also observed in strawberries by Pineli *et al.* (2011). In opposite to these results, durian fruits showed the lowest antioxidant capacity in the immature ripening stage (Haruenkit *et al.*, 2010). Conclusively, there is no generalization or a consistent trend derivable for the antioxidant capacity in different ripening stages of fruits.

Furthermore, it was observed that the reduction of the antioxidant capacity in the course of ripening is less distinctive for TOSC than for TEAC. In order to elucidate which compounds are responsible for the antioxidant capacity, authentic standards of the main individual phenolic compounds in açaí, were evaluated by TOSC (**table A.4.5**, p.47). The results indicated that the radical scavenging system of the TOSC assay is more influenced by not identified compounds. Most likely is a contribution of condensed polyphenols (Lichtenthäler *et al.*, 2005).

Results of the antioxidant capacity and quantification of phenolic substances throughout ripening show that processing of açaí seems to be useful not only in ripe condition as health promoting product. Also extracts from unripe fruits may be interesting for the industry, e.g., as additive due to the considerably high content of polyphenols.

#### 2.1.1.2 Garambullo

##### 2.1.1.2.1 Bioactive compounds

In total ten phenolic constituents were identified by LC-ESI-MS/MS in three different ripening stages of garambullos. An overview of the phenolic constituents is given in **table B.3.1** (p. 55). Two phenolic acids were detected, but they were not present throughout the progress of ripening. Protocatechuic acid was only found in unripe and medium ripe fruits whereas caffeic acid was merely identified in ripe fruits. Furthermore, the occurrence of six different flavonols such as kaempferol and quercetin derivatives was constituted. All flavonols were present in each maturity stage as well as two different flavone glucosides of luteolin.

The presence of flavonols and flavones was constituted for the first time in garambullo fruits. One report is known on the detection of gallic acid and caffeic acid (Guzmán-Maldonaldo *et al.*, 2010). The finding of caffeic acid in ripe fruits is in accordance with our results. Gallic acid was not found in any of the three maturity stages. Quercetin 3-O-rutinoside, kaempferol 3-O-rutinoside, and quercetin 3-O-glucoside turned out to be the main compounds of the detected phenolic constituents throughout ripening.

Total amounts of the identified compounds in each maturity stage showed that the highest phenolic content occurred in unripe fruits (9.67 ± 0.83 mg/100 g DM). In the medium ripe fruits, the phenolic content decreased (6.98 ± 1.05 mg/100 g DM), but rose again in ripen fruits

(8.69 ± 0.80 mg/100 g DM). The decrease of the phenolic content from unripe to medium ripe fruits was significant for the most of the individually evaluated phenolic compounds. Kaempferol 3-O-rutinosed was the only compounds that significantly increased from medium ripe fruits to ripe fruits.

By comparing the ratio of the identified phenolic compounds in each ripening stage, it was found that percentages of kaempferol 3-O-rutinoside increased considerably (see **figure B.3.2**, p.57). As a consequence, a decline was observed for quercetin 3-O-glucoside and protocatechuic acid. Percentages of the quantitatively dominating quercetin 3-O-rutinoside alternated during ripening. From unripe to medium ripe fruits, an increase was observed from 38% to 44%. In ripe garambullo, quercetin 3-O-glucoside had a part of 40%of the identified phenolic compounds. All other phenolic compounds were present in more or less the same percentages in each ripening stage.

With regard to the biosynthesis of individual flavonols in fruits during ripening, the results of garambullo were different to that of bush butter fruits. On the contrary to garambullo, amounts of individual quercetin glycosides increased between two early ripening stages and showed subsequently a decline with progressing maturity (Missang *et al.*, 2003). Results were also different in common apples. High flavonol concentrations were found accordingly to garambullo at early maturity stage. But flavonols decreased gradually resulting in the lowest amounts in ripe fruits (Li *et al.*, 2002). In agreement with garambullo fruits, Awad *et al.* (2003) constituted some fluctuations in the ranking of individual quercetin glycosides. Conclusively, it can be stated that the rate of flavonol biosynthesis during ripening seems to depend on the fruit species.

#### 2.1.1.2.2 Antioxidant capacity

The antioxidant capacity was determined of two different extracts (aqueous and acetone) of garambullo by using TOSC assay (**table B.3.2**, p. 58). The acetone extract showed a higher radical scavenging activity in comparison to the water extract, probably in consequence of a better solubility of the phenolic compounds in the organic phase.

Concerning px, a steady decrease of the antioxidant capacity was observed for both extracts in the course of ripening. The same result could already be demonstrated in an aqueous açaí extract. In order to compare the radical scavenging properties of ripe garambullo to other betalain containing fruits, the antioxidant capacity of prickly pears was determined in this study. Results against px were similar for both fruits (2.50 g DM/L for garambullo and 2.41 g DM/L for prickly pear). Interestingly, the antioxidant capacity of these betalain containing fruits is rather moderate in comparison to those colored by anthocyanins such as *Clidemia rubra* berries (Gordon *et al.*, 2011a) or jambolão fruits (Gordon *et al.*, 2011b).

In regard to pn, a continuous decrease of the antioxidant capacity in the course of ripening was only observed for the acetone extract. The water extract of garambullo showed the highest radical scavenging activity in unripe fruits and the least in intermediate ripe fruits according to the total amount of the identified phenolic compounds.

Referring to the water extract, ripe garambullo can be attributed a higher antiradical activity against pn in comparison to prickly pears (3.33 g DM/L for garambullo and 4.33 g DM/L for prickly pear). However, the antioxidant capacity against pn is lower than that of *Clidemia rubra* berries (Gordon *et al.*, 2011a) or açaí fruits (Lichtenthäler *et al.*, 2005).

Interestingly, the TOSC values resulting from the water extract of ripe garambullo against px and pn were characterized by a relatively small difference. Usually, fruit extracts have been shown to be considerably more effective against px than against pn (Gordon *et al.*, 2011b).

HPLC analyses of garambullo fruits let assume an increase of the betalain content during ripening. Therefore, the influence of the betalains on the antioxidant capacity was evaluated by activity-guided fractionation. Those fractions, in which the betalains were suspected, showed an increasing antioxidant capacity from unripe to ripe fruits. Hence, an identifiable influence of betalains to the radical scavenging behavior could be constituted. However, the increasing antioxidant capacity of these fractions could not compensate the decline in the overall antioxidant capacity from unripe to ripe fruits. Decreasing amounts of unknown compounds (possibly phenolic acid conjugates and flavonoid glycosides) gave rise to a regressive antioxidant activity in the remaining fractions.

#### 2.1.1.3 Cashew apple

##### 2.1.1.3.1 Bioactive compounds

Ascorbic acid was found in all maturity stages of cashew apple. Results indicated a significant increase of ascorbic acid in the course of ripening (1038 ± 31 mg/100 g DM in unripe fruits to 1731 ± 45 mg/100 g DM in ripe fruits). Literature studies on ascorbic acid in different maturity stages of cashew apple are not known, but amounts of ascorbic acid in ripe fruits are in accordance to those found by Akinwale (2000). However, different cashew apple cultivars determined by Assunção and Mercadante (2003) showed approximately 50% lower contents of ascorbic acid. The ascorbic acid content in ripe cashew apple is remarkably high in general. Amounts are 4-5 times higher compared to those found in kiwi fruits or oranges and can be ranked at the same level with guavas (Souci *et al.*, 1989).

Accumulation of ascorbic acid during the ripening process was also observed in guavas (Mercado-Silva *et al.*, 1998). On the contrary, Celik *et al.* (2008) reported on decreasing

concentrations in ripening cranberries. Hence, the biosynthesis of ascorbic acid in the course ripening seems to depend on the fruit species.

In total, 14 phenolic constituents were mass spectrometrically identified during the ripening process of cashew apple (see **table C.4.1**, p. 69). Among these compounds, nine different flavonols were unambiguously assigned by using authentic standards and two quercetin pentosides were tentatively identified. Furthermore, epigallocatechin, epigallocatechin gallate and gallic acid were detected.

All detected phenolic compounds were present in unripe fruits. Their amounts decreased significantly from unripe to medium ripe fruits ($p < 0.05$). Kaempferol 3-O-glucoside, myricetin, and quercetin vanished completely. From medium ripe fruits to ripe fruits, a further decrease was observed for flavanols, quercetin pentosides, myricitrin, and quercetin hexosides of which quercetin 4'-O-glucoside was no longer detectable in ripe fruits. In contrast to these compounds, amounts of gallic acid and quercetin 3-O-rhamnoside increased. All changes from medium ripe fruits to ripe fruits were not found to be significant. The decline in the concentration of the phenolic compounds from the initial maturity stage to medium ripe fruits suggests that the biosynthesis becomes less intensive after stages of cell differentiation and during subsequent maturation as observed in bitter oranges (Castillo *et al.*, 1992). In accordance with the results of cashew apple, the highest values of flavonols were also found in the initial maturity stage of apricots (Dragovic-Uzelac *et al.*, 2007), common apples (Awad *et al.*, 2001), and camu camu (Chirinos *et al.*, 2010). Furthermore, the decrease of flavanols is in accordance with a report of Almeida *et al.* (2007) who found a higher activity for enzymes involved in the biosynthesis of these flavonoids rather in the early developmental stage of strawberries. In case of hydroxybenzoic acids, Gruz *et al.* (2011) observed in medlar fruits (*Mespilus germanica* L.) that concentrations of free protocatechuic acid and syringic acid decreased during maturation as observed for gallic acid in cashew apple from unripe to medium ripe fruits. The decrease of free phenolic esters in medlar fruits is explained by their integration into cell walls.

Interesting results were observed by comparing the ratios of the detected phenolic constituents in each ripening stage of cashew apple. Although concentrations of quercetin 3-O-galactoside, quercetin 3-O-rhamnoside, and myricitrin decreased during ripening, these flavonols were present in similar percentages in each ripening stage. E.g., quercetin 3-O-galactoside showed values of 17%, 19%, and 17% as observed from unripe to ripe fruits. The quercetin pentosides and gallic acid increased remarkably (e.g. quercetin 3-O-arabinoside from 4% to 8%; gallic acid from 11% to 19%) whereas a decrease was found for epigallocatechin (from 8% to 1%), epigallocatechin gallate, and quercetin 3-O-glucoside. In regard to the flavonols, Awad *et al.*

(2001) reported that the ratio of the principal quercetin glycosides in different cultivars of common apples (3-O-galactoside, 3-O-rhamnoside, 3-O-glucoside) undergo a permanent change during ripening which could only be constituted for quercetin 3-O-glucoside in cashew apple.

Two previously published reports provide information on individual phenolic compound in ripe cashew apple. Gallic acid, myricitrin, quercetin 3-O-galactoside, quercetin 3-O-glucoside, quercetin 3-O-rhamnosid, kaempferol 3-O-glucoside, myricetin, and quercetin were identified by Michodjehoun-Mestres *et al.* (2009). In accordance to our results, myricitrin, quercetin 3-O-galactoside, quercetin 3-O-glucoside, and quercetin 3-O-rhamnoside were quantified in similar amounts in cashew apple. De Brito *et al.* (2007) constituted the presence of the compounds myricitrin, quercetin 3-O-galactoside, quercetin 3-O-glucoside, quercetin 3-O-rhamnoside, and kaempferol 3-O-glucoside as well as three different quercetin pentosides. Epigallocatechin and epigallocatechin gallate are mentioned for the first time to be present in cashew apple.

#### 2.1.1.3.2 Antioxidant capacity

The antioxidant capacity increased during maturity in cashew apples measured by TOSC. The results are presented in **table C.4.2** (p. 71). Ripe cashew apples show approximately a twice as high radical scavenging activity against both px and pn in ripe condition (0.79 g DM/L and 1.00 g DM/L, respectively) when compared to unripe fruits (1.38 g DM/L and 1.88 g DM/L, respectively). Cashew apple shows high antioxidant properties against both radicals in comparison to other fruits from Latin America. The antioxidant capacity of ripe cashew pseudo fruits against px was higher than that of *Clidemia rubra* berries (Gordon *et al.*, 2011a) and was, in contrast, lower than that of cutite fruits (Gordon *et al.*, 2011b) and açaí (Lichtenthäler *et al.*, 2005). Further, cashew apple turned out to be a good scavenger of pn. Its antioxidant activity against pn is higher than that of açaí (Lichtenthäler *et al.*, 2005), *Clidemia rubra* berries (Gordon *et al.*, 2011a) or muruci, jambolão and araçá fruits from the Amazon region (Gordon *et al.*, 2011b).

An explanation for the antiradical behavior of cashew apple might be concluded from a report of Lichtenthäler *et al.* (2003). Studies on ascorbic acid showed a 4-5 times lower antioxidant activity against px in comparison to those of different phenolic standard compounds. Against pn, the difference between the polyphenols and ascorbic acid was less distinctive (only 1-2 times). Further, Lichtenthäler *et al.* (2003) found a nearly identical radical scavenging activity of ascorbic acid against px and pn. In consequence of these results, it becomes obvious that ascorbic acid has considerable influence on the antioxidant capacity of cashew apple. Firstly, the rising ascorbic acid concentration during ripening parallels the course of the antioxidant activity against both radicals. Amounts of ascorbic acid in each ripening stage are remarkably higher than those of the identified and quantified phenolic compounds (Total amounts - unripe: 20.40 mg/100 g DM,

medium ripe: 5.16 mg/100 g DM, ripe: 4.92 mg/100 g DM). Secondly, the high amounts of ascorbic acid in cashew apples are an explanation for the good antioxidant activity especially against pn.

### 2.1.2 Scientifically little-mentioned ripe fruits

#### 2.1.2.1 Berries of *Clidemia rubra* (Melastomatacea)

##### 2.1.2.1.1 Bioactive compounds

*Clidemia rubra* berries showed an ascorbic acid content of 8.44 ± 0.02 mg/100 g FW which is low compared to other berry fruits. Only grapes indicate lower ascorbic acid contents between 2.0-7.4 mg/100 g FW (Souci *et al.*, 2008).

Beside ascorbic acid, phenolic constituents were found to be another group of biologically active compounds in *Clidemia rubra* berries. Anthocyanins were found to be responsible for the bluish to black coloring of the berries. Delphinidin 3-O-glucoside, cyanidin 3-O-glucoside, and cyanidin 3-O-rutinoside could be identified by mass spectrometrical analysis (**table D.4.2**, p. 84). Delphinidin 3-O-rutinoside was at least tentatively assigned. After quantification, the 3-O-rutinosides of cyanidin and delphinidin (39.45 ± 1.66 mg/100 g fresh weight [FW] and 23.74 ± 1.18 mg/100 g FW, respectively) turned out to be the main anthocyanins followed by the respective 3-O-glucosides (11.68 ± 0.56 mg/100 g FW for cyanidin 3-O-glucoside and 6.08 ± 0.35 mg/100 g FW for delphinidin 3-O-glucoside).
Lowry (1975) showed the presence of malvidin glycosides in flowers and mainly delphinidin and pelargonidin glycosides in fruits of different Melastomataceae varieties. In this report, an acylated delphinidin 3,5-O-diglucoside was found in *Clidemia hirta* Don.
The total anthocyanin content of *Clidemia rubra* berries is about five-fold higher compared to that of red currants or different gooseberry cultivars (Wu *et al.*, 2004). However, amounts are approximately five times lower than those of black currant cultivars (Wu *et al.*, 2004) and 1.75-2.75 times lower than those found in different Andean blackberry cultivars (Mertz *et al.*, 2007).

Non-anthocyanin phenolic constituents are presented in **table D.4.3** (p. 86). In total, five phenolic acids were identified in *Clidemia rubra* berries. Quantitatively, vanillic and gallic acid (1.43 ± 0.02 and 0.56 ± 0.01 mg/100 g FW, respectively) were the most dominant phenolic acids. In addition, three flavan-3-ols were found of which epigallocatechin gallate was the dominating compound (2.99 ± 0.16 mg/100 g FW). Eleven different flavonols that are derived from myricetin and quercetin are present in berries of *Clidemia rubra*. Nine of these compounds could be identified by using authentic standards whereas two substances were only tentatively assigned as quercetin pentosides. Quercetin 3-O-arabinoside and quercetin 3-O-rhamnoside showed the

highest amounts among the non-anthocyanin flavonoids (5.26 ± 0.16 and 5.06 ± 0.08 mg/100g FW). Information on flavonoids in fruits of Melastomataceae varieties is not known. Only a few reports were found on flavonoids in Melastomataceae flowers or leaves. Mimura *et al.* (2004) identified 17 different flavone and flavonol glycosides in *Huberia* leaves. The same compound classes were found in leaves of *Miconia alypifolia* (Mancini *et al.*, 2008). One report describes the identification of kaempferol glycosides in *Melastoma malbathricum* L. flowers (Susanti *et al.*, 2007). The presence of kaempferol and kaempferol glycosides as well as the presence of flavones like apigenin and luteolin could not be approved in *Clidemia rubra* berries.

The content of the non-anthocyanin phenolic compounds in *Clidemia rubra* berries seems to be considerably high. The amount of identified flavonols is comparable to that found in Andean blackberries (Mertz *et al.*, 2007), cranberries, and bog whortleberries and is four times higher than that of bilberries, blueberries, and red gooseberries from Finland (Häkkinen *et al.*, 1999).

#### 2.1.2.1.2 Antioxidant capacity

The antioxidant activity measured by TOSC resulted in 0.9 g DM/L and 2.0 g DM/L. This amount of lyophilized *Clidemia rubra* sample was needed to inhibit the ethylene formation of 50% against px and pn, respectively (see also **figure D.4.3**, p.88). According to a report by Lichtenthäler and Marx (2005), high values for the antioxidant capacity against px and pn have generally been exhibited by anthocyanin-containing fruit juices. *Clidemia rubra* berries can be ranked between elderberry juice and sour cherry nectar. Compared to frequently consumed juices like that of oranges or apples, the antioxidant activity against pn is about three times higher.

The main part of the antioxidant capacity is ascribed to ascorbic acid and polyphenols, especially to anthocyanins as assessed by activity-guided fractionation. The fraction containing ascorbic acid and two fractions containing the anthocyanins showed the highest antioxidant capacity against px (**figure D.4.4**, p.89)

#### 2.1.2.1.3 Evaluation of nutritive compounds

In addition to the determination of bioactive compounds, a proper evaluation of nutritive compounds in *Clidemia rubra* berries is presented in this thesis. *Clidemia rubra* berries showed a fat content of 1.03 ± 0.01 g/100 g FW. This result is comparable to that of other berries like blackberries, blueberries or raspberries which generally contain 1% or less of fat (Souci *et al.*, 2008).

The nitrogen content was 0.19 ± 0.01 g/100 g FW and protein content accounted for 1.18 ± 0.03 g/100 g FW. The results of the protein content of berries of *Clidemia rubra* are in accordance with commonly cultivated berries. The highest amount of protein is found in black currants

whereas blueberries offer an averaged protein content of only 0.60 g/100 g FW (Souci *et al.*, 2008).

The dominant sugars found in *Clidemia rubra* berries were glucose and fructose. Amounts of glucose and fructose were 3.24 ± 0.06 g/100 g FW and 3.75 ± 0.10 g/100 g FW, respectively. The sugar content as well as the glucose-fructose ratio is comparable to that of other berries like black currant, blueberry or gooseberry (Souci *et al.*, 2008). Saccharose was found to be < 0.1 g/100 g FW.

*Clidemia rubra* berries are found to be a good source of dietary fibers. The determined content was 8.85 g±0.02 g/100 g FW which is about twice the amount contained in blueberries and raspberries (Souci *et al.*, 2008). An explanation for this high value might be the dry matter content that is likewise higher when compared to that of blueberries and raspberries. The dry matter of black currants (Souci *et al.*, 2008) and camarinha fruits (Bramorski *et al.*, 2010) is comparable to that of *Clidemia rubra* berries. Hence, the total dietary fiber of black currants (6.8 g/100 g FW) and camarinha (6.5 g/100 g FW) is higher than that of blueberries and raspberries but nonetheless lower compared to that of *Clidemia rubra* berries.

Mineral analysis by AAS showed high concentrations of calcium, zinc, and manganese when compared to other common berry fruits (Souci *et al.*, 2008). **Table D.4.1** (p. 82) shows in detail all determined minerals.

*Clidemia rubra* berries indicated a pH value of 3.0. The amount of TTA was determined as 138.8 mmol/L and 8.9 g/L calculated as citric acid. Compared to results of other berries from the literature, the acid concentration in berries of *Clidemia rubra* is low. For example, Rubinskiene *et al.* (2006) and Zatylny *et al.* (2005) found almost up to 5 times higher amounts for TTA in black currant. Even lower amounts of TTA (up to 0.35 g/100 g FW) were reported for other *Vaccinium* cultivars (Saftner *et al.*, 2008).

#### 2.1.2.2 Underutilized fruits from the Amazon region

##### 2.1.2.2.1 Bioactive compounds

The polyphenolic constituents in the edible part of four underutilized fruits from the Amazon region were investigated by HPLC/DAD-ESI-$MS^n$. Only a few studies exist on the phenolic composition of jambolão fruits (*Syzygium cumini*), and no studies have been published on individual phenolic substances in fruits of araçá (*Psidium guineense*), muruci (*Byrsonima crassifolia*), and cutite (*Pouteria macrophylla*).

Different compounds like gallotannins, ellagitannins, quinic acid gallates, flavanonols, flavonols, and proanthocyanidins were detected in the four fruits. Thereof, 18 substances were identified in araçá, 37 in jambolão, 19 in muruci, and 22 in cutite. Interestingly, no flavonoids could be found

in araçá but only gallic acid derivatives. Cutite and muruci present different galloylquinic acid derivatives, which have rarely been proven in fruits. All (tentatively) identified constituents in araçá, jambolão, muruci, and cutite are listed in **tables E.4.1** (p. 101), **E.4.2** (p. 104), **E.4.3** (p. 106), and **E.4.4** (p. 108), respectively.

Results of the ascorbic acid determination showed that noticeable amounts were only found in cutite (247.5 ± 23.5 mg/100 g DM). Jambolão and araçá contained less than half of the concentration present in cutite (93.5 ± 12.0 and 101.3 ± 9.8 mg/100 g DM, respectively). Ascorbic acid in muruci could not unambiguously be identified.

#### 2.1.2.2.2 Antioxidant capacity

In order to evaluate the bioactive potential of the fruits, aqueous extracts were determined on their radical scavenging activity against px and pn by TOSC. Additionally, the total phenolic content was measured by Folin-Ciocalteu. The results are summarized in **table E.4.5** (p. 110). The highest antioxidant capacity against both radicals was assessed for cutite followed by jambolão, araçá, and muruci. Against px, cutite bore a 9-fold higher antioxidant capacity than muruci. Against pn, even the 12-fold amount of muruci sample is needed to obtain an inhibition of 50% of the ethylene generation when compared to that of cutite. According to both radicals, antioxidant properties between jambolão and araçá were less distinctive. However, both fruits showed an approximately 3 times (px) and 4-5 times (pn) lower radical scavenging capacity than cutite.

Results of the TOSC assay were interrelated with those of the total phenolic content. The amounts of determined total phenols in the four fruits gave rise to the same ranking as described for px and pn. Hence, the antioxidant properties of each fruit can be ascribed to the total phenolic content in the meaning of the Folin-Ciocalteu test. The lowest phenolic content was found in muruci being roughly comparable to that of banana pulp (Faller and Fialho, 2010). The 12-fold amount of total phenols was constituted in cutite matching with that of tropical highland blackberries (Acosta-Montoya *et al.*, 2010).

Beside the phenolic content, ascorbic acid may significantly contribute to the antioxidant behavior of cutite fruits. As described by Lichtenthäler *et al.* (2003) a similar concentration of ascorbic acid standard is needed to obtain a radical inhibition of 50% against both px and pn. This could explain the less pronounced difference of the antioxidant capacity of cutite against the two radicals (0.57 g DM/L against px and 0.83 g/L against pn). Jambolão showed a higher antioxidant capacity than araçá, although the content of ascorbic acid was slightly lower. Thus, antioxidant compounds other than ascorbic acid seem to significantly influence the radical scavenging behavior of jambolão. Finally, the comparably weak antioxidant activity of muruci

may be explained by the probable absence of ascorbic acid in this fruit in addition to the low total phenolic content.

Up to now only a few studies are known about the antioxidant capacity of the four fruits from the Amazon region. Two different articles reported on the free radical scavenging behavior of jambolão and muruci. DPPH$^{\bullet}$ assay conditions for the determination of both fruits were identical. Jambolão showed a 3-fold higher antioxidant capacity than muruci (Rufino *et al.*, 2009; Rufino *et al.*, 2010) which is in accordance to our findings.

In comparison to other fruits originating from the Amazon basin, the antioxidant properties of cutite against pn determined by TOSC assay were higher than those of açaí pulp. Different harvest years of açaí (1998, 2000, and 2002) required concentrations between 1.17 and 1.72 g/L to obtain an inhibition of 50%. In contrast, the radical scavenging potential of cutite against px is less effective than that of açaí (0.39-0.48 g/L) (Lichtenthäler *et al.*, 2005).

## 2.2 Separation of bioactive compounds in a semi-preparative scale

The use of phenolic constituents for medicinal or technological applications demands for a possibility to isolate individual compounds from complex matrices. A preparation of preferably authentic standards is also indispensable in order to assess possible pharmacological effects of particular phenolic substances. High speed countercurrent chromatography (HSCCC) is a state-of-the-art method for the isolation of polyphenols from plant extracts as it works without any adsorption losses. High sample loads and the use of cheap solvents instead of expensive solid phase columns are further advantages over e.g. preparative HPLC (Schwarz *et al.*, 2003).

The following study intends to isolate phenolic compounds in a semi-preparative scale. HSCCC procedure is exemplified by berries of *Clidemia rubra*. These fruits have been shown to be rich in anthocyanins (Gordon *et al.*, 2011a) which have gained interest due to their health beneficial effects and their technological applicability as food colorant (Coisson *et al.*, 2005; Pazmino-Duran *et al.*, 2001).

The elution of the *Clidemia rubra* berry extract by HSCCC resulted in three main fractions detected at 280 nm (see **figure F.3.2**, p. 118). A subsequent determination of these fractions by HPLC/DAD primarily disclosed the presence of anthocyanins at 520 nm. Main fraction I (92-104 min) contained cyanidin 3-O-glucoside, cyanidin 3-O-rutinoside, delphinidin 3-O-glucoside, and delphinidin 3-O-rutinoside which were all previously identified in *Clidemia rubra* berries (Gordon *et al.*, 2011a). Cyanidin 3-O-rutinoside and delphinidin 3-O-rutinoside were predominantly present in this fraction in a relation of 1:2, whereas cyanidin 3-O-glucoside and delphinidin 3-O-glucoside were found in traces. Delphinidin 3-O-rutinoside was the

quantitatively dominating anthocyanin in main fraction II (173-188 min). Further, lower concentrations of cyanidin 3-O-glucoside and cyanidin 3-O-rutinoside were constituted. An enrichment of an anthocyanin in high purity occurred in main fraction III (209-224 min). Cyanidin 3-O-rutinoside was the only detectable compound at 520 nm as determined by HPLC analysis. The more unspecific wave length at 280 nm showed the presence of only one further non-anthocyanin constituent ($\lambda_{max}$ at 367 nm) in fraction III in a low concentration. The peak area of the unknown compound, recorded at 280 nm, accounted for less than 2% in comparison to that of cyanidin 3-O-rutinoside (see **figure F.3.2**, p. 118).

Conclusively, HSCCC experiments have demonstrated that this separation method is an implementable instrument for the separation of bioactive compounds. The isolation of cyanidin 3-O-rutinoside as the quantitatively dominating anthocyanin in *Clidemia rubra* berries was practicable in a high purity grade. It should also be possible to separate other major and minor compounds, respectively, by choosing alternative solvents or changes of the relation of individual solvents in the solvent system as well as modifying of the flow rate of the mobile phase. Schwarz *et al.* (2003) demonstrated that the purity of fractions containing several anthocyanins can be improved by repeated separation by HSCCC with lower flow rates.

## 2.3 Effects of tropical fruit juice consumption *in vivo*

### 2.3.1 Bolus consumption of a specifically designed fruit juice rich in anthocyanins and ascorbic acid did not influence markers of antioxidative defence in healthy humans

The primary goal of this study was to investigate if bolus consumption of a specifically designed fruit juice rich in anthocyanins and ascorbic acid increases the plasma antioxidant capacity and reduces markers of oxidative stress in healthy non-smokers. To answer these questions, a randomized controlled study with crossover design was performed to avoid between-subjects effects. The fruit juice consisted of camu camu, Andean blackberries, and açaí. A sugar solution with equimolar amounts of monosaccharides served as control drink to exclude antioxidant effects which may result from a fructose-mediated increase of uric acid (see **table G.3.1**, p. 124). This study design should allow a reliable evaluation of fruit juice effects on the pro-/antioxidative balance.

#### 2.3.1.1 Results of the study

##### 2.3.1.1.1 Antioxidant capacity in plasma

In total three parameters were chosen to assess the plasma antioxidant capacity of the study subjects (**table G.4.1**, p. 128). Thereof, TEAC and Folin-Ciocalteu total phenolics were not

affected by beverage, time and interactions between beverage and time. TOSC values against px only decreased significantly over time after ingestion of the sugar solution ($P = 0.02$), and reached lower values after 0.5, 1, 2, 3 and 6 h compared to baseline (all p-values < 0.05). The area under curve (AUC) of TOSC was higher after ingestion of the juice compared to control beverage (266 ± 16 vs. 250 ± 11 %*h; $p = 0.032$).

#### 2.3.1.1.2 Antioxidant status in plasma

The ascorbic acid concentration of the subjects increased up to 117% (3 h vs. 0 h) and was significantly higher anytime after juice intake ($p < 0.001$) compared to baseline (see also **figure G.4.1**, p. 128). Three hours after ingestion of the sugar solution, ascorbic acid concentration was slightly higher (6.5%) compared to the initial value ($p = 0.011$). As expected, the AUC of the ascorbic acid concentration in plasma was higher after consumption of the test juice compared to control beverage (607±115 vs. 351±68 µmol*h/L; $p < 0.001$).

The concentration of β-carotene and uric acid as well as the α-tocopherol-to-cholesterol-ratio were not affected by interactions of time and beverage.

In order to assess the occurrence of metabolites derived from the juice blend, a HPLC-CEAD analysis of the plasma samples was performed. In addition to the known compounds indicating the antioxidant status in human plasma, substances with reducing capacity, which were not present in the juice blend, were detected after consumption of fruit juice in the plasma of the subjects (**figure G.4.2**, p. 130). These metabolites could not be measured before ingestion of the fruit juice or before and after consumption of sugar solution. Interestingly, retention times of these substances observed after juice consumption were different from those of known metabolites like protocatechuic acid, gallic acid, vanillic acid, ferulic acid, and caffeic acid. As these unknown metabolites have reducing capacities, they can be, in turn, assigned to antioxidant substances. As these substances induced only very low HPLC-CEAD signals it is questionable whether they contribute to the stabilizing effect of the fruit juice on the antioxidant status in plasma measured by TOSC.

#### 2.3.1.1.3 Oxidative stress

Values regarding measurements on oxidative stress are shown in **table G.4.1** (8-iso $PGF_{2\alpha}$, p. 128) and **table G.4.2** (DNA strand breaks, p. 129). The beverage did neither affect $F_2$-isoprotane generation (8-iso $PGF_{2\alpha}$) nor DNA single strand breaks (SB) *in vivo* and *ex vivo*. Time had an impact on SB *in vivo*, but did not modulate 8-iso $PGF_{2\alpha}$ and SB *ex vivo*. Differences between the AUC of 8-iso $PGF_{2\alpha}$, SB in vivo and ex vivo obtained after consumption of juice and sugar solution did not occur (data not shown).

#### 2.3.1.2 Discussion

TEAC and FCR did not increase after juice ingestion despite an obvious increase of ascorbic acid and unknown substances with reducing capacity in plasma. This phenomenon can be explained by the measurement of electron transfer which is performed by TEAC and FCR (Prior *et al.*, 2005). Ascorbic acid and, probably, the unknown substances function as hydrogen donators; this effect cannot be detected by these assays. Interestingly, TOSC, an assay detecting hydrogen transfer, decreased after the consumption of the control beverage. This phenomenon may be due to the polyphenol-poor diet at the day before the study (Müller *et al.*, 2010). Maintenance of TOSC levels after juice intake may be explained by the enhanced extracellular ascorbic acid levels.

Before the intervention and after consumption of the control beverage, ascorbic acid levels were below the desirable steady-state concentrations in healthy adults (70-85 μmol/L) (Padayatty *et al.*, 2004), probably due to dietary restrictions. As expected, plasma concentrations of ascorbic acid temporarily increased after verum. The extent, however, was relatively low considering the supraphysiological dose (> 900 mg/d) ingested. This observation may be explained by a reduced bioavailability at supraphysiological doses compared to physiological doses that have been shown by Levine *et al.* (1998).

The concentration of further exogenous antioxidants in plasma (β-carotene and α-tocopherol), which also contribute to plasma antioxidant capacity (Cao and Prior, 1998), did not change significantly. β-Carotene is generally ingested with açaí (Schauss *et al.*, 2006), camu camu (Zanatta and Mercadante, 2007), and blackberries (Marinova and Ribarova, 2007), but the dose in our study was obviously too low to increase the β-carotene level in plasma.

It is known that anthocyanins consumed as food ingredient can only marginally be detected in plasma. One explanation may be the low stability of the flavylium cation under physiological pH conditions (McGhie and Walton, 2007). Moreover, anthocyanins are degraded to low-molecular phenolic acids by the micro flora of the gut as shown *in vitro* and *in vivo* (Williamson and Clifford, 2010). A study of Vitaglione *et al.* (2007) confirms that protocatechuic acid detected in human plasma accounts for 73% of ingested cyanidin 3 O-glucoside. To evaluate mucosal uptake of polyphenolic ingredients of the fruit juice, the plasma appearance of known low-molecular anthocyanin metabolites like protocatechuic acid, gallic acid, vanillic acid, caffeic acid, and ferulic acid was evaluated by highly sensitive HPLC-CEAD analysis. Most surprisingly, these metabolites could not be detected in plasma. Instead, several unknown metabolites with antioxidative properties occurred in plasma after juice consumption. As we were not able to isolate these metabolites from plasma samples in amounts which are sufficient for identification, the chemical

structures are still unknown. Probably, phenolic acids are further degraded already in the gut and/or after mucosal uptake.

Lipid peroxidation *in vivo* assessed by plasma 8-iso $PGF_{2\alpha}$ did not change in our study. This observation is in contrast to results of recent bolus studies: 2 h after ingestion of a cyanidin-rich juice blend with açaí as predominant ingredient, lipid peroxidation measured by thiobarbituric acid reactive substances (TBARS) decreased in healthy non-smokers (Jensen *et al.*, 2008). However, TBARS are less specific for lipid peroxidation than isoprostanes (del Rio *et al.*, 2002) analyzed in our study.

SB *in vivo* were only affected by time ($p < 0.001$) and not by beverage. This fits to the results of a previous study in which only effects by time occurred after bolus ingestion of white tea, green tea, and water (Müller *et al.*, 2010). Time-dependent effects may simply reflect circadian rhythms. Contrary to SB *in vivo*, SB *ex vivo* were not modulated by time or beverage. Comparable bolus studies with juices investigating SB *ex vivo* are not available, but white and green tea did not show any changes by time or beverage either (Müller *et al.*, 2010). In regard to our results it should be mentioned that the broad inter-individual variation of SB *in vivo* and against oxidative challenge *ex vivo* limits the power of the study considering these markers of DNA damage.

### 2.3.2 Occurence of phenolic acids in human blood plasma after fruit juice ingestion

The bioavailability of phenolic acids in human has been shown only in a few reports. The occurence of four phenolic acids *in vivo* after bolus ingestion of a tropical juice blend was investigated in this small scale intervention study by UPLC-ESI-MS/MS. Additionally, their generation as metabolites from higher molecular flavonoids or conjugates was supposed to be proven.

The juice blend used for bolus consumption by a voluntary male person contains gallic acid (8.19 mg/serving), p-coumaric acid (0.4 mg/serving) and protocatechuic acid (1.48 mg/serving) in quantifiable amounts. Caffeic acid could only be qualitatively identified as its concentration was below the limit of quantification. Analysis of blood plasma after a 12 h overnight fast showed that the presence of gallic acid and p-coumaric acid could not be constituted in the sample that was withdrawn immediately before ingestion of the fruit juice. Surprisingly, caffeic acid and protocatechuic acid were detected despite the fasting condition of the participant (see **figure H.3.3**, p. 142). Probably, a longer period than an one-day abstinence from a diet free of phenolic compounds is needed to assure a proper total clearance of these phenolics from the blood stream. Unfortunately, information on pharmacokinetic studies shedding light on the total clearance of phenolic acids is scarce. Caffeic acid was studied in rabbits by Uang *et al.* (1997). In

this study, most of the unchanged caffeic acid was excreted in the urine within 2 h. An investigation in human showed that the maximal urinal excretion of cinnamic acid occurred in the first 4 h of a 48 h survey (Clifford, 2000). Information on the pharmacokinetic of protocatechuic acid and gallic acid was not found.

Generally, phenolic compounds absorbed from the small intestine usually appear in the plasma in maximum concentration within less than 2.5 h (Williamson and Clifford, 2010). Hence, a further blood withdrawal was obtained 2 h after juice intake to proof the bioavailability of the determined phenolic acids.

The presence of caffeic acid in the plasma 2 h after ingestion could only be tentatively constituted as the retention time in the sample deviates from that of a standard compound (**figure H.3.3**, p. 142). MRM fragmentation experiments are in accordance to that of an authentic caffeic acid standard, so that the untimely retention time might be caused by matrix effects of the sample or the presence of a caffeic acid isomer. The concentration of caffeic acid before and after fruit juice ingestion could not be quantified, but the mass specific ion current of the MS analysis at $m/z$ 179 suggests a concentration decline after juice intake due to its lower intensity (from $3.00 * 10^5$ to $2.10 * 10^5$). This may be explained by an ongoing clearance of caffeic acid compared to the fasting condition. Simultaneously, a negligible supply with free caffeic acid and its conjugates from the study drink as well as caffeic acid as a metabolite derived from higher polyphenols is obvious. An appropriate supply would result in higher concentrations of free caffeic acid in the blood stream compared to the fasting condition.
In a report of Simonetti *et al.* (2001), caffeic acid was not present in plasma prior to the intervention with red wine after a diet low in polyphenols for 3 days. Plasma level rose to maximum concentration 60 min after wine consumption.

A quantification of protocatechuic acid from the plasma samples could not be performed as the concentration was below the limit of quantification. However, an unchanged concentration of protocatechuic acid in plasma before fruit juice ingestion compared to fasting condition can be assumed as the mass specific ion current of the MS analysis at $m/z$ 179 shows a similar intensity ($4.80 * 10^3$ before juice intake and $4.83 * 10^3$ after juice intake). A possible decrease of the concentration as it was observed for caffeic acid might be compensated by free protocatechuic acid provided by the juice itself. Furthermore, protocatechuic acid can also be derived as microbial degradation product of the gut flora from cyanidin-3-O-glucoside (Vitaglione *et al.*, 2007) which is provided as constituent from all fruits present in the juice (Lichtenthäler *et al.*, 2005; Mertz *et al.*, 2007; Rodrigues and Marx, 2006). Results from a bioavailability study, in which participants obtained a moderate amount of berries over 8 weeks, showed an increase of

protocatechuic acid in plasma. The increase accounted for 21% in the berry group in comparison to the control group (Koli *et al.*, 2010).

Gallic acid showed the highest concentration of the target compounds in our study drink (20.48 mg/L and 120.36 μmol/L, respectively) and gave rise to intestinal absorption. Gallic acid was also unambiguously identified in plasma after fruit juice consumption by LC-MS analysis (**figure H.3.3**, p. 142). The concentration after ingestion of the study drink in plasma was 5.88 μmol/L. Free gallic acid was previously reported to be well absorbed compared to other phenolic compounds which is in agreement with our findings (Manach *et al.*, 2005). As shown in a bioavailability study in healthy humans, peak plasma levels of gallic acid were reached after 1.3 h and 1.4 h subsequent to administration as tablets and as black tea drink, respectively. An oral dose consisting of 50 mg gallic acid gave rise to plasma concentrations of 1.83 μmol/L for the tablets and 2.09 μmol/L for the tea (Shahrzad *et al.*, 2001).

The most interesting aspect of this study is the occurence of a p-coumaric acid isomer in the plasma after fruit juice ingestion (**figure H.3.3**, p. 142). The presence of p-coumaric acid could not be constituted as the retention time of the compound found in the plasma was not in accordance with that of a p-coumaric acid standard. Thus, matrix effects may have affected the detection of p-coumaric acid. Furthermore, the presence of isomers is possible as ion currents from SIR and MRM measurements corresponded to that of a p-coumaric acid standard. The concentration of the p-coumaric acid isomer in the plasma after fruit juice intake was 0.13 μmol/L. As this compound is not derived in its free form by the fruit juice, it might be seen as a metabolite from other phenolic substances. MS data suggest that p-coumaric acid derivatives may be preferably present as conjugates in the fruit juice. As stated by Clifford *et al.* (2000) conjugates of phenolic acids are not absorbed as such, but they are cleaved by esterases of the gut flora prior to absorption. A study in rats also constituted the occurrence of p-coumaric acid as a metabolite from conjugated derivatives (Gonthier *et al.*, 2003). Furthermore, p-coumaric acid was found to be a metabolite of other polyphenolic compounds like rosmarinic acid (Baba *et al.*, 2005) and chlorogenic acid (Monteiro *et al.*, 2007) in human. As a consequence of the monohydroxylation, p-coumaric acid is reported to be less susceptible to glucuronidation than other polyphenols. Thus, the aglycone occurs in relatively high amounts in plasma after intestinal absorption (Spencer *et al.*, 1999).

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# 3 Outlook

Studies on cashew apple, açaí, and garambullos have shown that the development of both total and individual phenolics as well as their antioxidant potential during the course of ripening is not liable to general principles. Thus, all fruits that contain bioactive compounds of interest have to be particularly investigated in order to assess the highest concentration of potential target substances. The recently increasing number of scientific publications confirms the strong interest in this scope of research.

The phenolic composition of garambullo und *Clidemia rubra* berries was investigated for the first time. However, only those compounds were identified of which authentic standards were available. Further research with high resolution and accurate mass spectrometry has to be performed for detailed knowledge on the phenolic composition.

*Clidemia rubra* berries should be subjected to further investigations of essential ingredients like vitamins. Agronomic research in plant physiology, genetics like hybridization can increase yields. The commercial potential of these berries can be assessed by subsequent agri-food chain analysis in order to market not only fresh but also processed fruits as jams, juices, smoothies or as functional ingredient due to its anthocyanin content.

The isolation of individual phenolic substances succeeds verifiably by countercurrent chromatographic methods. HSCCC has been applied to separate a vast number of non-flavonoids and flavonoids like tea catechins, proanthocyanidins, anthocyanins, and flavonols from their plant matrices. Application methods for compounds rarely occurring in fruits like galloyl quinic acids (found in muruci and jambolão), HHDP derived ellagitannins (found in araçá), galloyl flavonols, and methylated flavonols (found in jambolão) should be developed in order to assess their contribution to the overall antioxidant capacity of the fruits and to survey their technological (e.g. antimicrobial) properties.

The health beneficial effects of fruit and vegetable consumption have been proven by epidemiological data. However, the question which part of these effects can be attributed to phenolic compounds is still challenging. Further research on a biochemical level *in vivo* should be conducted in order to assess the influence of the polyphenols. Also in this case, high resolution and accurate mass spectrometry is a useful instrument by what metabolites derived from polyphenols can be identified. Additional preferably long-term designed human studies will enlighten the contribution of polyphenols to the observed epidemiological phenomenon.

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# A Chemical characterization and evaluation of antioxidant properties of açaí fruits (*Euterpe oleraceae* Mart.) during ripening (accepted by *Food Chemistry*)

André Gordon[a], Ana Paula Gil Cruz[b], Lourdes Maria Corrêa Cabral[c], Sidinéa Cordeiro de Freitas[c], Cristina Maria Araujo Dib Taxi[d], Carmen Marino Donangelo[b], Rafaella de Andrade Mattietto[e], Mirko Friedrich[a], Virgínia Martins da Matta[c], & Friedhelm Marx[a]

[a]University of Bonn, Department of Nutrition and Food Sciences, Endenicher Allee 11-13, 53115 Bonn, Germany

[b]Federal University of Rio de Janeiro, Institute of Chemistry, Avenida Athos da Silveira Ramos 149, 21941-909 Rio de Janeiro, Brazil

[c]Embrapa Food Technology, Av. das Américas POB 29.501, 23020-470 Rio de Janeiro, Brazil

[d]Institute of Agricultural Science, Federal Rural University of Amazon, Avenida Presidente Tancredo Neves 2501, 66077-901 Belém, Brazil

[e]Embrapa Eastern Amazon, Trav. Dr. Enéas Pinheiro POB 48, 66095-100 Belém, Brazil

**Corresponding author:**

Friedhelm Marx

Address: University of Bonn, Department of Nutrition and Food Sciences, Endenicher Allee 11-13, 53115 Bonn, Germany

Telephone: +49-228-733713; *Fax*: +49-228-733757

E-Mail: f.marx@uni-bonn.de

Own contribution: preparation of the manuscript, identification and quantification of phenolic compounds, TOSC analysis

## A.1 Abstract

Growing interest in consumption of açaí has arisen as its fruits are linked to positive health effects due to the phenolic content and the nutritive value. The objective of this study was to characterize açaí fruits chemically and to determine the antioxidant capacity at three different maturity stages. With exception of fat, amounts of macronutrients, minerals and titratable acids decreased during the ripening process. The same trend was observed for the most of the phenolic constituents identified by HPLC-ESI-MS/MS. A consistent decline was shown for flavones and hydroxycinnamic acids. The concentration of the anthocyanins increased in the course of ripening. In accordance with the total amount of the identified phenolic compounds, the antioxidant capacity measured by TEAC and TOSC also decreased. However, the contribution of the identified main phenolic compounds to the overall antioxidant capacity evaluated by TOSC was estimated to be low.

## A.2 Introduction

Açaí (*Euterpe oleracea* Mart.) is a tropical palm tree that naturally occurs in the Amazon region. Its spherical grape-sized fruits are green when young and ripen usually to a dark purple (Strudwick and Sobel, 1988). An important reloading point for açaí is the city of Belém in Pará State, Brazil, where fruits can be collected all over the year. However, a main harvesting period exists during the "dry-months" from August to December. Usually, the fruits are used to prepare a liquid of creamy texture by macerating the pericarp and mixing it with different amounts of water yielding in commercially available açaí pulp (Lichtenthäler *et al.*, 2005). In the production region, açaí is integrated in the daily dietary habits of the native people and is normally used in main meals for lunch or dinner. In modern Brazilian society, it has gained interest as a nutritionally valuable wellness product (Strudwick & Sobel, 1988). Meanwhile, açaí is favored as an ingredient in fruit beverages beyond Brazilian borders and is exported mainly to the USA or to Europe (Sabbe *et al.*, 2009).

Attention on açaí has arisen especially due to its high *in vitro* antioxidant activity explained by the considerably high content of phenolic compounds like different anthocyanins, flavones, and phenolic acids (Lichtenthäler *et al.*, 2005; Pacheco-Palencia *et al.*, 2009). Phenolic constituents are generally associated with health promoting properties and the prevention of several degenerative diseases (Xia *et al.*, 2010). Additionally, phenolic compounds become more and more interesting for the food industry due to manifold properties. Because of legislators and consumers' growing concern over the use of artificial food additives there is a growing demand for additives from natural resources (Giusti and Wrolstad, 2003). For instance, anthocyanins can be used as a food

colorant (Pazmino-Duran *et al.*, 2001). Moreover, plant extracts containing a broad range of polyphenols may act as antioxidants or antimicrobial agents (El-Hela and Abdullah, 2010).

Generally, the ripening process of fruits is indicated by intensive metabolism of primary and secondary plant compounds. This study gives information on quantitative changes of macronutrients, individual phenolic compounds, and the antioxidant activity in açaí fruits during ripening. In addition, the influence of individual phenolic main compounds to the overall antioxidant capacity was estimated.

## A.3 Materials and methods

### 1. Chemicals

Ultrahigh quality (UHQ) water was prepared with a Direct-Q 3 system (Millipore, Billerica, USA). Protocatechuic acid (≥ 97%) and p-hydroxybenzoic acid (≥ 99%) were purchased from Merck (Darmstadt, Germany), caffeic acid (purum) from SERVA Feinbiochemica (Heidelberg, Germany). Gallic acid (≥ 97.5%), vanillic acid (≥ 97%), syringic acid (≥ 95%), chlorogenic acid (5-O-(3,4-dihydroxycinnamoyl)-L-quinic acid) (≥ 97%), taxifolin (≥ 85%), diethylene-triaminepentaacetic acid (DTPA) (≥ 99 %), α-keto-γ-methiolbutyric acid (KMBA) (≥ 97%), 2,2'-azobis(2-methylpropionamidine) dichloride (ABAP) (≥ 97%), 3-morpholino-sydnonimine N-ethylcarbamide (SIN-1), 2,2-azinobis-(3-ehtylbenzothiazoline-6-sulfonic acid)-diammonium salt (ABTS), Folin-Ciocalteu reagent, and 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox®) were obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Cyanidin 3-O-glucoside (≥ 96%), cyanidin 3-O-rutinoside (≥ 96%), peonidin 3-O-glucoside (≥ 95%), luteolin (≥ 99%), isovitexin (≥ 99%), chrysoeriol (≥ 99%), orientin (≥ 99%), homoorientin (≥ 99%) were purchased from Extrasynthèse (Genay, France). Luteolin 7-O-glucoside and chrysoeriol 7-O-glucoside standards were a gift of Professor Dr. Galensa (University of Bonn). They were self-isolated and had a purity grade of ≥ 84% and ≥ 70%, respectively.

### 2. Raw material, sampling, and preparation of the pulp

Açaí fruits of three different maturity stages (unripe – green fruits, intermediate – reddish-brown fruits, ripe – deep purple fruits) were collected at the experimental station of the Federal Rural University of Amazon in Castanhal, PA, Brazil. Fruits of each maturity stage were processed separately following the specifications that are usually applied by the industry. After reception, the fruits were selected, washed, weighted, and sanitized in chlorinated water. The subsequent maceration step consisted of steeping the fruits in hot water (50 °C) for 30 minutes to facilitate the separation of the exo- and mesocarp from the seeds. This separation was carried out in an

açaí specific extractor by adding water in proportion of 0.6 L/kg fruit. The obtained pulp was stored at -30 °C.

**3. Determination of ash, minerals, lipids, proteins, carbohydrates, and titratable acids**

Minerals were quantified by using flame atomic absorption spectrometry according to official AOAC methods (2005). Proximal composition of açaí pulp samples at three maturity stages was determined according to official AOAC methods (1998). Ash content was measured in a gravimetric assay. Lipids were determined by acid hydrolysis and gravimetric measure of the final ether solution. Protein content was measured following Kjeldahl method. Total protein was calculated by multiplication of the obtained nitrogen content with the fruit-specific conversion factor of 6.25. Carbohydrates were calculated as centesimal difference of the previous determinations. All results are referred to dry matter (DM) basis. Titratable acids were determined by a titration method using an automatic titrator with sodium hydroxide. Results are expressed in malic acid equivalent/100 g of DM.

**4. Identification and quantification of phenolic compounds by HPLC-ESI-MS/MS**

***Extraction of phenolic compounds***

Açaí pulp was lyophilized and defatted by Soxhlet extraction with petroleum ether. The extraction of phenolic compounds was performed using a modified sample preparation according to a method described in Pacheco-Palencia *et al.* (2009). For two times, sample of each maturity stage (500 mg) was dissolved in 10 ml of acetone-water-formic acid (70% + 29% + 1%; v/v/v), sonicated for 10 min, then centrifuged for 10 min with 10,000 rpm at 10 °C. The residue was extracted once more with 10 ml acetone-water-formic acid. Afterwards the supernatants were combined. To get rid of the organic solvent, the extract was vaporized using a rotary evaporator (Rotavapor R-210, Büchi, Essen, Germany) at 30 °C and the aqueous supernatant was shaken with 10 ml ethyl acetate. The received ethyl acetat fraction was vaporized to dryness. The residue was solubilized in 1 ml methanol-water-formic acid (50% + 49% + 1%; v/v/v) and filtered through a 1.0/0.45 µm syringe filter (Chromafil GF/PET-45/25, Macherey-Nagel, Düren, Germany) prior to application to HPLC-ESI-MS/MS.

Anthocyanins were extracted using a modified method explicitly described in Wu *et al.* (2004). Briefly, freeze-dried sample (250 mg) of each maturity stage was extracted in duplicate with 4 ml of methanol-UHQ water-acetic acid (MeOH-$H_2O$-HAc) (50% + 49.5% + 0.5%; v/v/v). After vortexing, sonication and centrifugation, the supernatant was removed and the sample once more extracted but with 2.5 ml of MeOH-$H_2O$-HAc. Both sample solutions were combined and

filtered through a 1.0/0.45 µm syringe filter (Chromafil GF/PET-45/25, Macherey-Nagel, Düren, Germany) prior to application to HPLC-ESI-MS/MS.

***Analysis of phenolic compounds***

Quantification of the phenolic compounds was performed following a method described in Gordon *et al.* (2011). HPLC instruments consisted of a pump system and a UV-detector of the HP 1050 series (Hewlett Packard, Waldbronn, Germany), a degasser Degasys Populair DP3010 (Uniflows, Tokyo, Japan) and an analytical column Aqua 3 µm C18, 150 mm, 2 mm i.d., with a guard column Security Guard, C18, 4 mm, 2 mm i.d. (both Phenomenex, Aschaffenburg, Germany). The solvents were UHQ water with 1% (v/v) formic acid (mobile phase A) and 1% (v/v) formic acid in acetonitrile (mobile phase B). The HPLC gradient using a flow rate of 0.2 ml/min started at 5% B and rose to 35% B after 55 min, 100% B after 65 min and re-equilibrated for 15 min at 5% B. Another gradient was used to obtain a better separation for quantification of the luteolin derivatives orientin and homoorientin. Starting at 5% B, the gradient rose to 17.5% B after 50 min with subsequent washing and re-equilibration procedure. 20 µl of each sample extract were injected for analysis. The coupled API 2000 HPLC-ESI-MS/MS system was controlled with Analyst 1.5 Software (both Applied Biosystems, Darmstadt, Germany). Mass spectra for the determination of anthocyanins were obtained by using positive ionization whereas all other phenolic compounds were detected in negative ionization mode.

Identification of phenolic compounds was performed by comparing fragmentation patterns in multiple reaction mode and retention times with those of authentic standard substances. Standards were also used to create calibration curves for quantification. Results were recalculated to the non-defatted material and expressed in mg/100 g DM.

**5. Antioxidant capacity**

***Total oxidant scavenging capacity (TOSC) assay***

Analysis of the TOSC assay was performed as described in Lichtenthäler *et al.* (2003). Briefly, the measurement of the antioxidant capacity is based on an ethylene yielding reaction of KMBA with either generated peroxyl radicals or peroxynitrite. Antioxidative compounds present in the sample inhibit the ethylene formation. Ethylene is measured gas chromatographically (GC-17A, Shimadzu, Tokyo, Japan) over a time course of one hour. Quantification of generated ethylene results in a kinetic curve of which the area under the curve (AUC) is calculated. Only those data with a variance (standard deviation/arithmetic mean) of the AUC after repeat determination below 5% are further processed. Mean data of a sample are compared to those of an uninhibited reaction with water, which gives rise to the TOSC values.

For TOSC analysis, authentic standard compounds were solubilized in DMSO and subsequently diluted with UHD water. Lyophilized sample (1 g) of each maturity stage was suspended in UHQ water to obtain a total weight of 10 g (w/w). The suspension was sonicated for 10 min and centrifuged for 7 min with 10000 rpm at 10 °C. The supernatant of the water extract was stored until further application at -30 °C. Results indicate the sample concentration in mg/100mL which is needed to obtain a radical inhibition of 50%.

***Trolox equivalent antioxidant capacity (TEAC)***

The antioxidant activity with $ABTS^{\bullet+}$ radicals was determined after an extraction of the pulp in a methanol/acetone solution described in Rufino *et al.* (2007). Trolox was used as reference compound. Results were expressed in μmol Trolox equivalent/100 g DM.

***Folin-Ciocalteu total phenols***

Total phenolic compounds were determined by the Folin-Ciocalteu assay based on Georgé *et al.* (2005). Results are expressed in mg gallic acid equivalent/100 g DM.

**6. Data analysis**

To prove significant differences between maturity stages, statistical analysis of data was accomplished by one-way analysis of variance followed by Tukey test at 95% of probability using XLSTAT 7.5 software. Results were significantly different between maturity stages unless otherwise indicated.

## A.4 Results and discussion

**1. Nutrients and titratable acidity**

Variations among the samples of different ripeness are as expected because maturation can

| Parameter (g/100 g DM) | Unripe | Intermediate | Ripe |
|---|---|---|---|
| Ash | 14 ± 0 | 8 ± 0 | 4 ± 0 |
| Lipids | 7 ± 0 | 31 ± 0 | 48 ± 4 |
| Total protein | 21 ± 0 | 13 ± 0 | 12 ± 0 |
| Carbohydrates | 58 ± 14 | 48.0 ± 2.6 | 36 ± 4 |
| Titratable acidity[a] | 2.8 ± 0.0 | 1.5 ± 0.0 | 1.2 ± 0.0 |

**Table A.4.1.** Centesimal specification of nutrients and titratable acids in three maturity stages of açaí. Data: mean ± standard deviation referred to the dry matter (DM) content of the fruit. [a]Expressed in malic acid equivalents.

generally be defined as a sequence of changes in color, flavor, and texture of fruits and vegetables (Chitarra and Chitarra, 2005). As shown in **table A.4.1**, the ash content decreases during the

ripening process. This development is concordant with the results of the mineral composition (**table A.4.2**). With exception of sodium and zinc, all other values of the determined minerals decline with increasing maturity. Amounts of total proteins, carbohydrates as well as titratable acids indicate the same regressive trend.

| Parameter (mg/100 g DM) | Unripe | Intermediate | Ripe |
|---|---|---|---|
| Sodium | n.d. | 51.3 ± 7.2 | 6.8 ± 0.7 |
| Magnesium | 397.0 ± 0.4 | 287.1 ± 0.8 | 172.1 ± 0.3 |
| Phosphorus | 262.1 ± 0.5 | 232.3 ± 1.8 | 185.5 ± 1.5 |
| Potassium | 4271.3 ± 21.7 | 2314.4 ± 11.4 | 929.9 ± 9.9 |
| Calcium | 962.3 ± 2.3 | 846.4 ± 1.7 | 423.2 ± 1.2 |
| Manganese | 30.9 ± 0.1 | 17.7 ± 0.1 | 13.3 ± 0.1 |
| Iron | 23.9 ± 0.9 | 12.8 ± 0.0 | 7.8 ± 0.2 |
| Zinc | n.d. | 1.2 ± 0.2 | 2.1 ± 0.0 |

**Table A.4.2.** Minerals in three different maturity stages of açaí. Data: mean ± standard deviation referred to the dry matter (DM) content. N.d.: not detected.

Values of total carbohydrates are approximately in the same range as reported previously for açaí pulp obtained from fruits harvested in February (31.6 g/100 g DM). However, values are lower than those of pulp from fruits harvested in July (48.0 g/100 g DM) (Sanabria and Sangronis, 2007). In opposite to the aforementioned parameters, the lipid content increased during ripening generating partly the expected alterations in flavor and texture. Our findings represent an average content of total lipids of 48% in the DM of ripe fruits similar to the value of 42% found by Sanabria and Sangronis (2007).

## 2. Identification and quantification of phenolic compounds

### *Anthocyanins*

Monomeric anthocyanins were mass spectrometrically identified by comparing fragmentation patterns and retention times with those of authentic standards. According to **figure A.4.1**, cyanidin 3-O-glucoside (peak 18) and cyanidin 3-O-rutinoside (peak 19) were assigned to the main anthocyanins in ripe açaí. Cyanidin 3-O-sambubioside (peak 17), pelargonidin 3-O-glucoside (peak 20), and peonidin 3-O-glucoside (peak 21) were found in traces. In addition to these anthocyanins, the presence of peonidin 3-O-rutinoside (peak 22) was tentatively constituted. Peak 22 showed a molecular ion $[M+H]^+$ at $m/z$ 609 and a product ion at $m/z$ 301. The mass difference of $m/z$ 308 suggests the loss of a hexosyl-deoxyhexoside. This assignment also agrees with regularities in the elution order of anthocyanins. Generally, the 3-O-rutinoside of an anthocyanidin is retained after the 3-O-glucoside moiety (Wu and Prior 2005a). Additionally, a sugar moiety bound to a certain anthocyanidin will likely be linked to another anthocyanidin

distributed in the same fruit (Wu and Prior 2005b). As the presence of cyanidin 3-O-rutinoside was already constituted, the linkage of a rutinoside to peonidin is certain.

The overall findings concerning anthocyanins in açaí are in accordance with other published papers. Lichtenthäler *et al.* (2005) assessed cyanidin 3-O-glucoside and cyanidin 3-O-rutinoside as the major anthocyanin components. Peonidin 3-O-rutinoside was found as a minor component in their açaí sample. Schauss *et al.* (2006) also found mainly cyanidin 3-O-glucoside and cyanidin 3-O-rutinoside. Additionally, cyanidin 3-O-sambubioside, peonidin 3-O-glucoside, and peonidin 3-O-rutinoside were found as minor anthocyanins. Del Pozo-Insfran *et al.* (2004) found pelargonidin 3-O-glucoside as main anthocyanin component in açaí beside cyanidin 3-O-glucoside. Further, pelargonidin 3-O-glucoside was present in fruits of *Euterpe precatoria* (Pancheco-Palencia *et al.*, 2009).

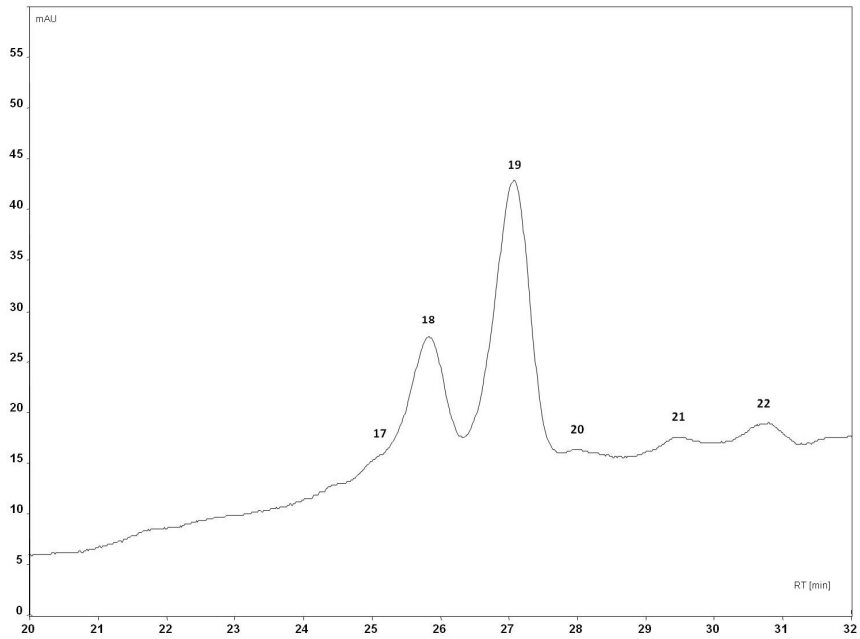


**Figure A.4.1.** HPLC chromatogram of ripe açaí recorded at 520 nm.

Quantification in the intermediate maturity stage of açaí could only be performed for cyanidin 3-O-glucoside and 3-O-rutinoside (**table A.4.3**). Peonidin 3-O-glucoside, peonidin 3-O-rutinoside, and pelargonidin 3-O-glucoside were also identified but just achieved the limit of detection defined by a peak/noise-ratio of 3:1. Cyanidin 3-O-sambubioside was not detected in the intermediate maturity stage. No monomeric anthocyanins were present in unripe açaí. Thus, the anthocyanin concentration rises in açaí during maturity as well as in many other plant species probably since the anthocyanin biosynthesis proceeds faster than fruit expansion (Bureau *et al.,* 2009).

| Peak | Compound | $[M-H]^-$/ product ion *m/z* | Maturity stage | | |
|---|---|---|---|---|---|
| | | | **Unripe** | **Intermediate** | **Ripe** |
| 1 | Gallic acid | 169/125 | 0.01 ± 0.00 | 0.04 ± 0.00 | 0.02 ± 0.00 |
| 2 | Protocatechuic acid | 153/109 | 0.75 ± 0.00 | 0.63 ± 0.06 | 0.65 ± 0.02 |
| 3 | p-Hydroxybenzoic acid | 137/93 | 6.48 ± 0.27 | 2.56 ± 0.18 | 1.90 ± 0.07 |
| 4 | Vanillic acid | 167/152 | 25.90 ± 0.54 | 12.30 ± 0.73 | 6.97 ± 0.30 |
| 5 | Chlorogenic acid | 353/191 | 1.64 ± 0.20 | 0.06 ± 0.02 | 0.02 ± 0.00 |
| 6 | Caffeic acid | 179/135 | 0.56 ± 0.03 | 0.06 ± 0.01 | 0.02 ± 0.00 |
| 7 | Syringic acid | 197/182 | 4.95 ± 0.38 | 0.46 ± 0.08 | 1.10 ± 0.12 |
| 8 | Orientin | 447/327 | 108.86 ± 5.01 | 18.99 ± 0.7 | 11.16 ± 0.84 |
| 9 | Homoorientin | 447/327 | 67.12 ± 1.85 | 13.96 ± 0.32 | 3.06 ± 0.26 |
| - | p-Coumaric acid | 163/119 | Traces | n.d. | n.d. |
| 10 | Luteolin 7-O-glucoside[a] | 447/285 | ≤0.04[a,b] | ≤0.02[a,b] | ≤0.01[a,b] |
| 11 | Vitexin | 431/311 | 24.65 ± 0.99 | 11.30 ± 0.18 | 3.41 ± 0.04 |
| 12 | Isovitexin | 431/311 | 28.95 ± 0.69 | 10.80 ± 0.94 | 2.66 ± 0.14 |
| 13 | Chrysoeriol 7-O-glucoside | 461/255 | 0.44 ± 0.03 | 0.08 ± 0.01 | 0.03 ± 0.00 |
| 14 | Taxifolin | 303/285 | 0.98 ± 0.05 | 0.46 ± 0.01 | 0.20 ± 0.01 |
| 15 | Luteolin | 285/133 | 4.98 ± 0.15 | 1.32 ± 0.09 | 0.24 ± 0.07 |
| 16 | Chrysoeriol | 299/285 | 5.27 ± 0.19 | 2.53 ± 0.11 | 0.68 ± 0.04 |
| | | $[M+H]^+$/ product ion *m/z* | | | |
| 17 | Cyanidin 3-O-sambubioside | 581/287 | n.d. | n.d. | 0.02 ± 0.00 |
| 18 | Cyanidin 3-O-glucoside | 449/287 | n.d. | 0.29 ± 0.05 | 4.94 ± 0.55 |
| 19 | Cyanidin 3-O-rutinoside | 595/287 | n.d. | 0.31 ± 0.04 | 17.86 ± 1.59 |
| 20 | Pelargonidin 3-O-glucoside | 433/271 | n.d. | Traces[b] | 0.06 ± 0.01[b] |
| 21 | Peonidin 3-O-glucoside | 463/301 | n.d. | Traces[b] | 0.08 ± 0.01[b] |
| 22 | Peonidin 3-O-rutinoside[c] | 609/301 | n.d. | Traces[b] | 0.29 ± 0.00[b] |
| Sum of the identified phenolic compounds | | | 281.54 ± 10.35[b] | 77.15 ± 3.53[b] | 55.37 ± 4.07[b] |

**Table A.4.3.** Content of phenolic compounds in three maturity stages of açaí. Data: mean ± standard deviation referred to mg/100 g dry matter. [a]estimated, n.d.: not detected. [b]Significance not evaluated between mean values in each row. [c]Values expressed as peonidin 3-O-glucoside equivalents.

Beside the general increase of the anthocyanin concentration, it was also observed that the relation of the individual anthocyanin amounts was different in each ripening stage. The ratio of cyanidin 3-O-glucoside to cyanidin 3-O-rutinoside accounted for 1:1 in the intermediate ripening stage. In ripe fruits, on the contrary, the ratio increased in favor of cyanidin 3-O-rutinoside to approximately 4:1. Siriwoharn *et al.* (2004) reported on changes in anthocyanin accumulation in two blackberry cultivars. Cyanidin 3-O-glucoside and cyanidin 3-O-rutinoside were determined as main anthocyanin compounds. But unlike in açaí, the content of cyanidin 3-O-glucoside increased steadily whereas amounts of cyanidin 3-O-rutinoside decreased from unripe to ripe

fruits. Fernández-López *et al.* (1998) observed a decline of cyanidin 3-O-glucosid concentration in ripening grapes. They noted that in the biosynthesis of anthocyanidins in *Vitis* ssp. cyanidin typically occurs as a precursor molecule of peonidin derivatives. Conclusively, according to these results there are no general regularities deducible for the formation of individual anthocyanins during ripening in fruits. Only the increase of total anthocyanins in general is apparent.

***Phenolic acids***

According to **table A.4.3** and **figure A.4.2**, in total eight phenolic acids and phenolic acid derivatives were identified in açaí: gallic acid (peak 1), protocatechuic acid (peak 2), p-hydroxybenzoic acid (peak 3), vanillic acid (peak 4), p-coumaric acid (peak not present in figure 2), caffeic acid (peak 6), syringic acid (peak 7), and chlorogenic acid (peak 5). With exception of p-coumaric acid, all compounds were present in each of the three different ripening stages. During the ripening process, the individual phenolic acids were found in different amounts. The highest concentrations of individual phenolic acids were generally found in unripe açaí. Vanillic acid, p-hydroxybenzoic acid, syringic acid were quantitatively the most dominating phenolic acids. Gallic acid, protocatechuic acid, caffeic acid and chlorogenic acid were found in lower amounts. P-coumaric acid was only present in traces. Amounts of hydroxycinnamic acids decreased during ripening. Chlorogenic acid and caffeic acid vanished nearly completely. Similar findings were reported by Dragovic-Uzelac *et al.* (2007) and Gruz *et al.* (2011). Interestingly, Xu *et al.* (2008) found the biggest quantitative loss during ripening in two *Citrus* varieties for chlorogenic acid, which is in agreement with results found in açaí. However, amounts of caffeic acid and syringic acid increased during ripening in *Citrus* fruits.

In opposite to the hydroxycinnamic acids, there was no clear trend observable for the hydroxybenzoic acids in açaí (**table A.4.3**). Amounts of vanillic acid and p-hydroxybenzoic acid decreased from immature fruits to ripe fruits. A decline of 90% and 16% was observed for syringic acid and protocatechuic acid, respectively, from unripe fruits to the intermediate ripening stage. Subsequently, the concentrations increased with further ripeness. In regard to gallic acid, the highest amount was noted in the intermediate ripening stage. Beside gallic acid, the biggest losses of the phenolic acids were observed between unripe and intermediate ripe fruits. With exception of syringic acid, a further quantitative decrease between the intermediate and ripe maturity stage was generally less distinctive. Different amounts of hydroxybenzoic acids during the ripening process were also observed in medlar fruits (*Mespilus germanica* L). Contents of free protocatechuic acid and syringic acid became less whereas amounts of p-hydroxybenzoic acid increased (Gruz *et al.*, 2011).

Concerning the findings of phenolic acids in ripe açaí, our results are in a great extend in common with previously published reports. Protocatechuic acid was identified by Lichtenthäler *et al.* (2005). Del Pozo-Insfran *et al.* (2004) identified and quantified ferulic acid, p-hydroxybenzoic acid, gallic acid, protocatechuic acid, vanillic acid, and p-coumaric acid in fresh açaí pulp. Furthermore Pacheco-Palencia *et al.* (2009) found protocatechuic acid, p-hydroxybenzoic acid, vanillic acid, syringic acid, and ferulic acid in *Euterpe oleracea* and *Euterpe precatoria* fruits. Contrary to these reports, the presence of ferulic acid and p-coumaric acid could not be constituted in our samples. On the other hand, chlorogenic acid and caffeic acid were detected for the first time in açaí fruits.

***Flavones and flavanonols***

In total eight flavones and one flavanonol were detected in açaí. All compounds were present throughout the three different maturity stages. According to **figure A.4.2** and **table A.4.3**, peaks 11 and 12 were assigned to vitexin and isovitexin, respectively, which showed a deprotonated molecular ion $[M–H]^-$ at $m/z$ 431 and a fragment ion at $m/z$ 311.

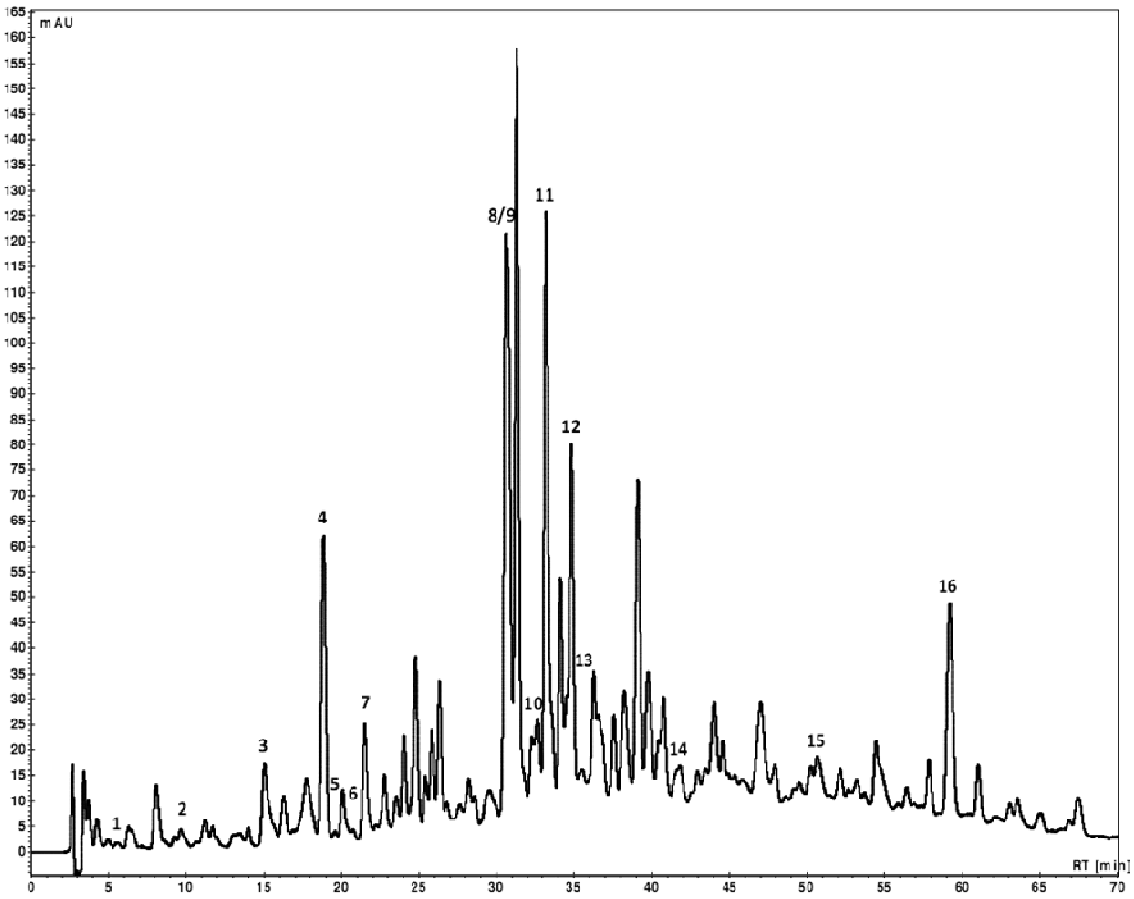


**Figure A.4.2.** HPLC chromatogram of açaí at intermediate maturity stage recorded at 280 nm.

Four luteolin derivatives were constituted in peaks 8, 9, 10, and 15. Peaks 8 and 9 showed a base peak $[M-H]^-$ at $m/z$ 447 and a product ion at $m/z$ 327 which is typically found for orientin and

homoorientin due to the likely fragmentation of the attached C-glycoside (Pacheco-Palencia *et al.*, 2009). Peak 10 had a base peak $[M–H]^-$ at $m/z$ 447 as well but produced a daughter ion at $m/z$ 285 indicating the presence of luteolin 7-O-glucoside as consequence of a corresponding retention time and fragmentation pattern to that of a standard solution. Luteolin was found in peak 15 providing a deprotonated molecular ion $[M–H]^-$ at $m/z$ 285 and a product ion at $m/z$ 133. Chrysoeriol and a chrysoeriol glycoside were found in peaks 16 and 13, respectively. Peak 16 exhibited a base peak $[M–H]^-$ at $m/z$ 299 and a fragment ion at $m/z$ 285 after loss of the methyl group indicating the presence of the chrysoeriol. A deprotonated molecular ion $[M–H]^-$ at $m/z$ 461 and a fragment ion at $m/z$ 255 found in peak 13 showed the presence of chrysoeriol 7-O-glucoside. The flavanonol taxifolin was detected in peak 14 providing a deprotonated molecular ion $[M–H]^-$ at $m/z$ 303 and a corresponding daughter ion at $m/z$ 285.

The findings of flavones in açaí were partially consistent with those of previous reports. The presence of homoorientin, orientin, and isovitexin were already constituted by Schauss *et al.* (2006). Additionally, Kang *et al.* (2010) mentioned vitexin, luteolin, and chrysoeriol as polyphenolic constituents in açaí pulp. No reports are known about the 7-O-glucosides of luteolin and chrysoeriol as constituents of açaí. In addition, the presence of the flavanonol taxifolin could be constituted for the first time. Foregone reports were only based on the occurrence of taxifolin derivatives (Pacheco-Palencia *et al.*, 2009; Schauss *et al.*, 2006).

According to **table A.4.3**, amounts of all flavones and taxifolin constantly decreased throughout ripening. C-glycosidic derivatives of luteolin (homoorientin, orientin) and apigenin (isovitexin, vitexin) were found to be quantitatively dominating in all three maturity stages followed luteolin and chrysoeriol, taxifolin, and the 7-O-glycosides of luteolin and chrysoeriol. Especially in unripe açaí, orientin and homoorientin occurred in outstandingly high concentrations of 108.86 and 67.12 mg/100 g DM showing three- to four-fold amounts when compared to other polyphenolic ingredients. Also in another report, homoorientin, orientin, and isovitexin were calculated as the highest concentrated non-anthocyanin polyphenols in ripe açaí (Pacheco-Palencia *et al.*, 2009).

To our knowledge, literature on changing amounts of non-anthocyanin flavonoid compounds in the course of fruit ripening is scarce. No reports are available on flavones or flavanonols. Flavanols and flavonol glycosides were determined in different maturity stages of apricots. The quantities of flavonoids varied between the ripening stages but it was generally not possible to establish a correlation between flavonoid content and ripening stages. However, regarding flavonols the highest values were mostly found in the initial maturity stage of apricot cultivars (Dragovic-Uzelac *et al.*, 2007). Amounts of ellagic acids and total flavonols decreased during

ripening in camu camu fruits whereas values of total flavanols, and total flavanones did not remarkably change (Chirinos *et al.*, 2010).

### 3. Antioxidant capacity (Folin-Ciocalteu, TEAC, TOSC)

As shown in **table A.4.4**, a change during ripening was observed for the Folin-Ciocalteu assay. The highest value of Folin-Ciocalteu total phenols was found in unripe açaí. Only a fourth of the concentration was present in the intermediate ripening stage followed by a slight increase in the ripe fruits.

| Parameter | | Maturity stage | | |
|---|---|---|---|---|
| | | Unripe | Intermediate | Ripe |
| Folin-Ciocalteu total phenols (in mg GAE[a]/100 g DM) | | 12316.5 ± 264.2 | 3038.7 ± 148.5 | 3437.0 ± 154.0 |
| TEAC (in µmol Trolox/100 g DM) | | 16.99 ± 0.71 | 4.04 ± 0.05 | 2.78 ± 0.10 |
| TOSC[b] (mg DM/100 mL) | px | 12.1 | 15.0 | 24.0 |
| | pn | 46.4 | 60.3 | 87.2 |

**Table A.4.4.** Antioxidant capacity of açaí fruits at three different maturity stages. Results are referred to the dry matter (DM) content. [a]Gallic acid equivalent, [b]TOSC values represent the concentration of the sample containing the antioxidants which is needed to obtain an inhibition rate of 50% against peroxyl radical (px) and peroxynitrite (pn).

Comparing the results of the Folin-Ciocalteu assay to those of other fruits during ripening is difficult. Results are often presented in wet matter by other authors not taking into account likely alterations of the water content during the ripening process. A determination of the total phenolic content in blackberries at three different maturity stages showed e.g. a continuous decrease from unripe to ripe fruits (Tosun *et al.*, 2008). On the contrary, some raspberry cultivars behaved like açaí or showed even a reversed trend by offering highest amounts in ripe condition (Wang and Lin, 2000).

The antioxidant capacity for both TEAC and TOSC was the highest in unripe açaí fruits and decreased continuously with increasing ripening as shown in **table A.4.4**. The TEAC antioxidant capacity showed four times higher values in unripe fruits than in the intermediate ripening stage. A further decline of the TEAC value of around 30% was observed from intermediate to ripe fruits. TOSC results indicate the sample concentration that is needed to obtain a radical inhibition of 50%. Regarding peroxyl radicals (px) and peroxynitrite (pn), the antioxidant capacity bisected from the unripe to the ripe maturity stage. For both radicals, the decline was the most distinctive between the intermediate and ripe fruits.

The TEAC antioxidant capacity of the ripe açaí fruits is in accordance with values found for different commercial açaí pulps. Depending on the water content of the pulps, the TEAC ranges approximately between 1.02 and 5.25 µmol Trolox equivalent/100 g dry matter (Dos Santos *et al.*,

2008). Regarding both radicals, TOSC values of ripe açaí turned out being at least 1.5-fold higher than those found in pulps of different harvest periods described in Lichtenthäler *et al.* (2005).

The trend of a decreasing antioxidant capacity during ripening was also observed in strawberries by referring the data of Pineli *et al.* (2011) on dry matter basis. In opposite to these results, durian fruits showed the lowest antioxidant capacity in the immature ripening stage (Haruenkit *et al.*, 2010). Corresponding to the discussion of the total phenolic content there is also no generalization or a consistent trend derivable for the antioxidant capacity in different ripening stages of fruits.

**4. Contribution of the phenolic content to the antioxidant capacity**

The totaled amount of the individual phenolic compounds in unripe fruits is four times higher when compared to the intermediate ripening degree (**table A.4.3**). Amounts decreased further during the ripening process of approximately 30%. Interestingly, TEAC values reduced in the same relation.

| Compound | TOSC (in mg/L) |
|---|---|
| Luteolin | 4.8 |
| Cyanidin 3-O-glucoside | 5.65 |
| Orientin | 6.52 |
| Cyanidin 3-O-rutinoside | 6.72 |
| Homoorientin | 7.11 |
| Chrysoeriol | 7.49 |
| Isovitexin | 7.87 |
| Vitexin | 9.12 |
| Vanillic acid | 10.8 |

**Table A.4.5.** Antioxidant capacity of standard compounds. TOSC values indicate the concentration that is needed to obtain an inhibition rate of 50% against peroxyl radicals.

It was observed that the reduction of the antioxidant capacity in the course of maturity especially between unripe and medium ripe fruits is less distinctive for TOSC than for TEAC. In order to elucidate which compounds are responsible for the antioxidant capacity, authentic standard compounds that occur in relevant amounts in açaí fruits were determined by TOSC against px. Results of the determination are given in **table A.4.5**. It turned out that the antioxidant capacity of these standard compounds depended on the extent of hydroxylation, position of glycosylation, and the kind of glycosylation: Orientin and homoorientin differ by the presence of one additional hydroxyl group in 3'-position (B-ring) from vitexin and isovitexin showing consequently a higher antioxidant capacity. Vanillic acid had the weakest antioxidant capacity presenting the fewest hydroxyl groups in comparison to all measured standard compounds. Due to the varying position of the glucose residue between homoorientin (6-C-glucoside) and orientin (8-C-glucoside) both

compounds showed a different radical scavenging behavior. Orientin turned out to be a more efficient radical scavenger than homoorientin. *Vice versa,* vitexin (8-C-glucoside of apigenin) exhibited weaker antioxidant properties than isovitexin (6-C-glucoside). In comparison to the flavones, anthocyanins tend to be more potent radical scavengers. In agreement with Lichtenthäler *et al.* (2005), cyanidin 3-O-glucoside showed higher TOSC values than cyanidin 3-O-rutinoside. Our results are in accordance with those of Kang *et al.* (2010) to a certain extent. In this report, flavonoids isolated from açaí were measured with ORAC. Luteolin also showed e.g. higher ORAC values than its corresponding C-glycosides. In opposite to our findings, vitexin exhibited a 2-10 times higher antioxidant capacity than luteolin, orientin, and homoorientin.

Despite the determination of the antioxidant capacity of individual phenolic compounds, it is difficult to estimate their contribution to the overall antioxidant capacity. The decrease of these quantitatively dominating compounds from unripe and to medium ripe fruits is not in relation with the decline of the TOSC antioxidant capacity, which is less distinctive. Regarding the standard compounds, a bigger reduction of the TOSC values should be expected. Further, the TOSC antioxidant capacity decreased of approximately 75% from intermediate ripe fruits to ripe fruits whereas the total amount of identified phenolic compounds was reduced only by 30%. The reduction of TOSC values was also not compensated by increasing amounts of anthocyanins, which are potent radical scavengers. Thus, it is striking that the antioxidant capacity of açaí fruits against px during ripening is strongly influenced by unknown compounds. Most likely is a contribution of proanthocyanidins (Lichtenthäler *et al.*, 2005).

## A.5 Conclusion

The chemical characterization of açaí fruits at three different maturity stages passed significant alterations in all determined parameters. Concentrations of nutritional relevant compounds like minerals, proteins, and carbohydrates as well as acidity decreased in the course of ripening. An increase was observed for the fat content. Manifold alterations also occurred regarding the phenolic composition. The concentration of anthocyanins increased during ripening whereas amounts for hydroxycinnamic acids and other flavonoids steadily decreased. Hydroxybenzoic acids did not show a consistent trend. A tremendous high phenolic content was found in unripe açaí whereof the flavones orientin and homoorientin were quantitatively dominating. Values of the antioxidant capacity determined by TOSC and TEAC decreased consistently with increasing maturity. All in all, changes of the determined parameters generate expected alterations in flavor and texture of açaí fruits during ripening. Processing of açaí fruits seems to be useful not only in ripe condition as health promoting product. Extracts of unripe fruits may also be interesting for

the industry as e.g. food additive, dietary supplement or ingredient in cosmetics due to the high content of bioactive compounds.

## A.6 Acknowledgment

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# B Phenolic constituents and antioxidant capacity of *Myrtillocactus geometrizans* (garambullo) in three different stages of maturity (manuscript not intended for publication)

## B.1 Introduction

Mexico shows a high level of species endemicity as a result of the wide variation of physiographic, geological and climatic conditions (Palomino, 2000). *Myrtillocactus geometrizans* is one of in total four identified *Myrtillocactus* species belonging to the family of Cactaceae (Hernández-López *et al.*, 2008). The plant is endemic to Mexico and grows on the slopes of cliffy mountain ranges in arid regions of the Mexican states Guanajuato, Jalisco, México, Michoacán, Puebla, Oaxaca, Tlaxcala, Hidalgo, Querétaro and San Luis Potosí (Céspedes *et al.*, 2005). *Myrtillocactus* species are also known as blueberry cacti according to size and color of their fruits. *Myrtillocactus geometrizans* yields berry fruits of approximately up to 1.5 cm in diameter which are locally known as garambullo. Garambullos show a thin peel and a gel-like endocarp of reddish to bluish color due to the presence of betalains. The fruits contain numerous small black seeds (< 1 mm) scattered throughout the flesh (Hernández-López *et al.*, 2008).

Garambullos still grow with poor or even without agronomic management. Fruits are under-utilized and consumed only locally. Nevertheless, they are offered in all public markets around their production site. Garambullos are either eaten fresh, sun-dried or they are processed as ice-cream and jam. Interest by the industry has been arisen due to the potential as food colorant (Hernández-López *et al.*, 2008).

Coloration of garambullos has been ascribed to betalains (Guzmán-Maldonaldo *et al.*, 2010), which are secondary plant compounds belonging to the alkaloids (Brossi, 1990). Betalains have been shown to be biologically active with considerable antioxidant activity (Cai *et al.*, 2003) albeit it has not been investigated whether they act as antioxidants in plant tissues (Stintzing and Carle, 2004). Interestingly, the occurrence of betalains is limited to only ten families of the plant order Caryophyllales and to the genus *Amanita* of the Basidiomycetes. E.g. prickly pears (*Opuntia ficus-indica*) were found to be a source of both betalain subgroups betacyanins and betaxanthins. Research on betalains has also been restricted due to the lack of commercially available standards (Stintzing, *et al.*, 2002).

Scientific information on *Myrtillocactus* species is scarce. *Myrtillocactus geometrizans* has shown biological activity as some terpenoids from the roots and aerial parts were evaluated as insect growth inhibitors (Céspedes *et al.*, 2005). Only one report is known on the nutritional value, betalain content, and the phenolic acid composition (Guzmán-Maldonaldo *et al.*, 2010). No information is available on further phenolic constituents of garambullos.

Aim of this study was to evaluate the antioxidant capacity and phenolic composition at three stages of maturity in order to assess the bioactivity of *Myrtillocactus geometrizans* fruits for potential dietary or food technological application. The contribution of betalains to the antioxidant capacity was approximated by activity-guided HPLC-fractionation of garambullos.

## B.2 Materials and methods

### 1. Chemicals

Ultrahigh quality (UHQ) water was prepared with a Direct-Q 3 system (Millipore, Billerica, USA). Protocatechuic acid (≥ 97%) was purchased from Merck (Darmstadt, Germany). Caffeic acid (purum) was purchased from SERVA Feinbiochemica (Heidelberg, Germany). Diethylenetriaminepentaacetic acid (DTPA) (≥ 99%), α-keto-γ-methiolbutyric acid (KMBA) (≥ 97%), 2,2'-azobis(2-methylpropionamidine) dichloride (ABAP) (≥ 97%), 3-morpholinosydnonimine N-ethylcarbamide (SIN-1), and quercetin were obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Flavonol glycoside standard compounds used for identification were a gift of Prof. Dr. Galensa, University of Bonn. They were self-isolated and of different purity grade.

### 2. Sampling

*Myrtillocactus geometrizans* fruits were harvested in 2007 at different sites in Guanajuato and Querétaro. Fruits were lyophilized and subsequent thoroughly ground. Freeze-dried samples were air-shipped to Germany and stored at -30 °C prior to analysis.

*Opuntia ficus-indica* (prickly pears) fruits were bought in a local supermarket. The fruits were peeled, de-seeded, lyophilized, and thoroughly ground. The freeze-dried samples were stored at -30 °C until analysis.

### 3. Extraction and analysis of phenolic compounds

Extraction of phenolic compounds was performed as described in Gordon *et al.* (2011a). Briefly, freeze-dried sample (250 mg) of each ripening stage was extracted in three treatments with acetone-water-formic acid (70 + 29 + 1, v/v/v) by using an Accelerated Solvent Extractor (ASE 200, Dionex, Idstein, Germany). The following solid-phase extraction (SPE) was performed by help of a Gilson ASPEC XL system (Automated Sample Preparation with Extraction Cartridges, Abimed, Langenfeld, Germany) on polyamide solid phase extraction cartridges (500 mg PA, 3 mL cartridge, Macherey Nagel, Düren, Germany). The extract was eluted with dimethyl sulfoxide-formic acid-trifluoroacetic acid (98.7 + 1 + 0.3; v/v/v).

Polyphenol identification and quantification was performed by HPLC-ESI-MS/MS as described in Gordon *et al.* (2011a). Extracts were measured in duplicate. Identification was performed by comparing fragmentation patterns and/or retention time of standard substances which were also used for creation of calibration curves for quantification of the phenolic compounds. Results are presented in mg/100 g dry matter (DM).

**4. Antioxidant capacity by TOSC**

Lyophilized sample of *Myrtillocactus geometrizans* fruits were reconstituted to the original weight with UHQ water (WE) and acetone-water (70 + 30, v/v) (AE) according to the dry matter content of 15% (w/w) for each maturity stage. Freeze dried material of prickly pears was taken to prepare a WE. Sample was reconstituted to the original weight under consideration of the dry matter content of 18.4% (w/w). The suspensions were sonicated for 5 min and centrifuged for 10 min at 12,000 rpm with a Heraeus Biofuge Stratos (Kendro, Langenselbold, Germany). The supernatants of the WE were stored for further analysis at -30 °C. As the organic solvents interfere the gas chromatographic procedure of the TOSC assay, supernatants of AE were taken, weight and vaporized to dryness using a rotary evaporator (Rotavapor R-210, Büchi, Essen, Germany) at 40 °C. Subsequent, the vaporized solvents were replaced by UHQ water (w/w). Analysis of the extracts by TOSC was performed as described in Gordon *et al.* (2011b).

**5. Activity-guided fraction by HPLC**

Fractionation was modified according to a method previously described by Rodrigues *et al.* (2006). The HPLC-DAD system of Pro Star series (Varian, Walnut Creek, USA) was equipped with an analytical column Max-RP 4 μm C18, 150 mm, 4.6 mm i.d., combined with a guard column Security Guard, C18, 4 mm, 2 mm i.d. (both Phenomenex, Aschaffenburg, Germany). The solvents were UHQ water with 2% (v/v) formic acid (mobile phase A) and 2% (v/v) formic acid in acetonitrile (mobile phase B). The HPLC gradient using a flow rate of 1.0 mL/min started at 0% B and rose to 5% B after 10 min, 25% after 35 min, and 32.5% B after 40 min. The column was flushed with 100% B and re-equilibrated for 15 min at 0% B. 20 μL were injected for analysis.

Fractions of the WE were collected in periods of 5 min starting directly after injection for a total time of 40 min. All collected samples were freeze-dried, dissolved in 500 μL UHQ water, and sonicated prior to analysis with the TOSC assay. The antioxidant capacity of the fractions was determined against peroxyl radicals.

### 6. Statistical Analysis

To prove significant differences between maturity stages, statistical analysis of data from measurements on phenolic composition was performed by one-way analysis of variance. Means were compared by Bonferroni test at 95% of probability using PASW Statistics 18.

## B.3 Results and discussion

### 1. Identification and quantification of phenolic constituents during ripening

In total, ten phenolic constituents were mass spectrometrically identified in three different maturity stages of *Myrtillocactus geometrizans* (**table B.3.1**). All compounds were assigned by comparison of fragmentation pattern and retention time with those of authentic standards.

| Peak | Compound | $[M-H]^-$/ product ion *m/z* | Maturity stage | | |
|---|---|---|---|---|---|
| | | | Unripe | Intermediate | Ripe |
| 1 | Protocatechuic acid | 154/109 | $0.70 \pm 0.19^a$ | $0.14 \pm 0.03^b$ | n.d. |
| - | Caffeic acid | 179/135 | n.d. | n.d. | $0.02 \pm 0.00$ |
| 2 | Quercetin 3-O-rutinoside | 609/300 | $3.69 \pm 0.21^a$ | $3.08 \pm 0.42^a$ | $3.47 \pm 0.36^a$ |
| 3 | Quercetin 3-O-galactoside | 463/300 | $0.73 \pm 0.05^a$ | $0.48 \pm 0.05^b$ | $0.49 \pm 0.07^b$ |
| 4 | Luteolin 7-O-glucoside | 447/285 | $0.44 \pm 0.02^a$ | $0.39 \pm 0.03^a$ | $0.44 \pm 0.04^a$ |
| 5 | Quercetin 3-O-glucoside | 463/300 | $1.83 \pm 0.08^a$ | $0.99 \pm 0.16^b$ | $1.01 \pm 0.05^b$ |
| 6 | Kaempferol 3-O-rutinoside | 593/285 | $1.93 \pm 0.23^a$ | $1.65 \pm 0.31^a$ | $3.02 \pm 0.26^b$ |
| 7 | Kaempferol 3-O-glucoside | 447/285 | $0.11 \pm 0.02^a$ | $0.08 \pm 0.03^a$ | $0.08 \pm 0.00^a$ |
| 8 | Luteolin 4-O-glucoside | 447/285 | $0.11 \pm 0.02^a$ | $0.07 \pm 0.01^b$ | $0.08 \pm 0.01^b$ |
| 9 | Quercetin | 301/151 | $0.13 \pm 0.01^a$ | $0.10 \pm 0.01^b$ | $0.08 \pm 0.01^b$ |
| Total | | | $9.67 \pm 0.86$ | $6.98 \pm 1.05$ | $8.69 \pm 0.80$ |

**Table B.3.1.** Content of phenolic compounds in three maturity stages of garambullo. Values are presented as mean ± standard deviation in mg/100 g dry matter**.** N.d.: not detected. $^{a,b}$Comparison of the means was performed by analysis of variance and Bonferroni test. Values with the same letters are not significantly different at level of $p < 0.05$. Means were compared within each row.

According to **table B.3.1**, two phenolic acids were detected in garambullos, but they were not present throughout ripening. Protocatechuic acid (peak 1) was only found in unripe and medium ripe fruits. Caffeic acid (no peak in **figure B.3.1**) was merely identified in ripe fruits. In addition to the phenolic acids, the occurrence of six different flavonols such as kaempferol (peaks 6 and 7) and quercetin (peaks 2, 3 5, and 9) derivatives was constituted (**figure B.3.1**). All flavonols were present in each of the three determined maturity stages as well as two different flavone glucosides derived from luteolin (peaks 4 and 8). By comparing the amounts of unripe and medium ripe fruits, significant changes were only evaluated for quercetin, quercetin 3-O-galactoside, quercetin

3-O-glucoside, and luteolin 4-O-glucoside. In addition, amounts of kaempferol 3-O-rutinoside increased significantly from medium ripe fruits to ripe fruits.

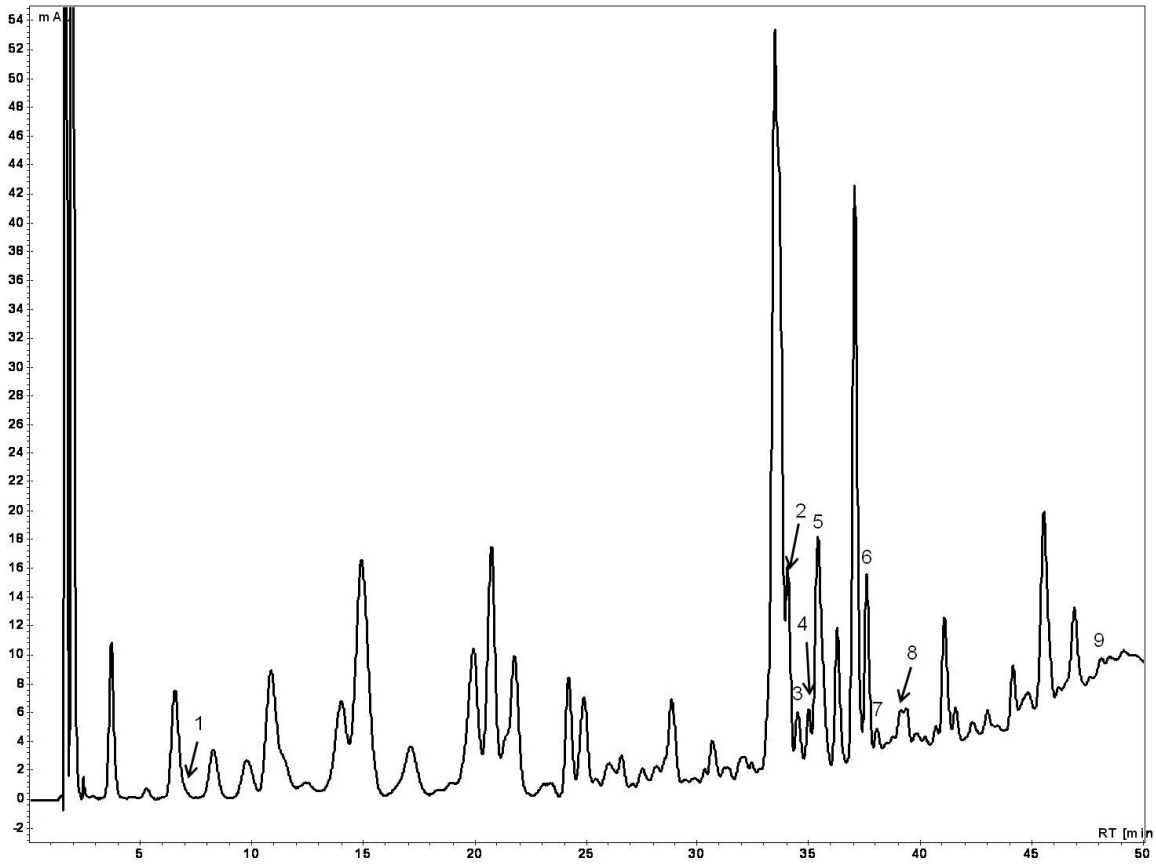


**Figure B.3.1.** Chromatogram of an aqueous acetone extract of unripe garambullo fruits at 320 nm.

According to **table B.3.1**, quercetin 3-O-rutinoside, kaempferol 3-O-rutinoside, and quercetin 3-O-glucoside turned out to be quantitatively the most important compounds of the detected phenolic constituents throughout ripening. Total amounts of the identified compounds in each maturity stage showed that the highest phenolic content occurred in unripe (9.67 mg/100 g DM) fruits (see **table B.3.1**). In the next maturity stage, the phenolic content decreased (6.98 mg/ 100 g DM), but rose again in ripen fruits (8.69 mg/100 g DM).

With regard to the biosynthesis of individual flavonols in fruits during ripening, the results of garambullo were different to that of bush butter fruits. On the contrary to garambullo, amounts of individual quercetin glycosides increased between two early ripening stages and showed subsequently a decline with progressing maturity (Missang *et al.*, 2003). Results were also different in common apples. High flavonol concentrations were found accordingly to garambullo at early maturity stage. But flavonols decreased gradually resulting in the lowest amounts in ripe fruits (Li *et al.*, 2002). In agreement with garambullo fruits, Awad *et al.* (2003) constituted some fluctuations in the ranking of individual quercetin glycosides. Conclusively, it can be stated that the rate of flavonol biosynthesis during ripening seem to depend on the fruit species.

The presence of flavonols and flavones was constituted for the first time in garambullo fruits. One report is known on the detection of gallic acid and caffeic acid garambullos (Guzmán-Maldonaldo *et al.*, 2010). The finding of caffeic acid in ripe fruits is in accordance with our results. Gallic acid was not found in any of the three maturity stages.

By comparing the ratio of the identified phenolic compounds in each ripening stages (**Figure B.3.2**) it was found that percentages of kaempferol 3-O-rutinoside increased considerably. As a consequence, a decline was observed for quercetin 3-O-glucoside and protocatechuic acid. Percentages of the quantitatively dominating quercetin 3-O-rutinoside alternated during ripening. From unripe to medium ripe fruits, an increase was observed from 38% to 44%. In ripe garambullo, quercetin 3-O-glucoside had a part of 40% in comparison to other identified phenolic compounds. All other phenolic compounds were present in more or less the same percentages in each ripening stage.

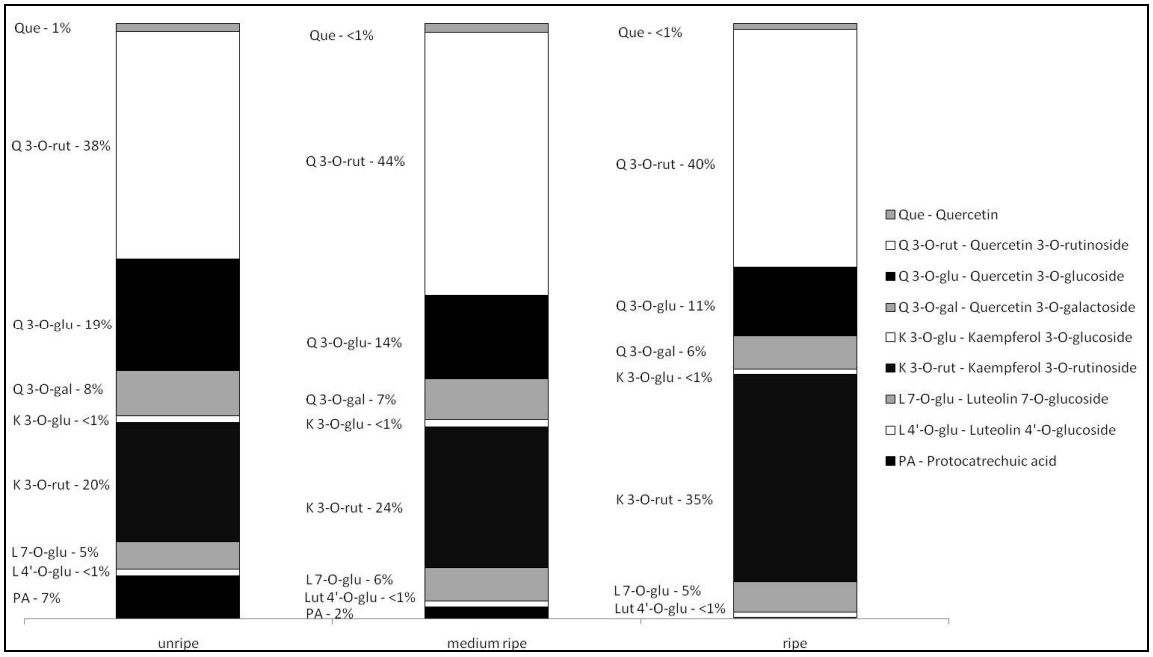


**Figure B.3.2.** Relative amounts of detected phenolic compounds in three maturity stages of garambullo fruits.

## 2. Antioxidant capacity and activity-guided fractionation

Results of the antioxidant capacity measurement of both aqueous and acetone extracts from garambullo is shown in **table B.3.2**. Generally, the AE showed a higher radical scavenging activity than the WE probably in consequence of a better solubility of the phenolic compounds in the organic phase. Concerning px, a steady decrease was observed in course of ripening with regard to both extracts.

The same result could already be demonstrated in water extracts prepared from açaí pulp as discussed in manuscript of appendix A.4. On the contrary to garambullo and açaí, results of ripening cashew apples have shown a rising antioxidant capacity in the course of ripening

(compare Annex C.4). Ripe garambullo had only moderate radical scavenging properties against px when compared to fruits from the Amazon region. Only muruci fruits showed lower TOSC values (Gordon *et al.*, 2011b).

| | Px | | Pn | |
|---|---|---|---|---|
| **Maturity stage** | **WE** | **AE** | **WE** | **AE** |
| Unripe | 1.56 | 0.94 | 2.88 | 1.22 |
| Intermediate | 2.03 | 1.35 | 3.49 | 2.08 |
| Ripe | 2.50 | 1.38 | 3.33 | 2.94 |

**Table B.3.2.** Antioxidant capacity of garambullo fruits at three stages of maturity. TOSC values are expressed in g/L indicating the concentration of antioxidants that is needed to obtain a radical inhibition of 50%. WE: water extract, AE: acetone extract, px: peroxyl radicals, pn: peroxynitrite.

Due to lack of information on betalaine containing fruits assessed by TOSC, prickly pears were evaluated in this study. Prickly pears have been shown to contain considerable amounts of betalains (Stintzing *et al.*, 2002). The antioxidant capacity of prickly pears against px (2.41 g dry matter/L) was similar to that of ripe garambullo. Interestingly, results showed only a moderate antioxidant capacity for both betalain containing fruits in comparison to those colored by anthocyanins like açaí (Lichtenthäler *et al.*, 2005), *Clidemia rubra* berries (Gordon *et al.*, 2011a) or jambolão (Gordon *et al.*, 2011b).

With regard to pn, a gradual decrease of the antioxidant capacity was only observed for the acetone extract. The water extract of garambullo showed the highest radical scavenging activity in unripe fruits and the least in the intermediate ripening stage which is in accordance to the total amount of the identified phenolic compounds (see **table B.3.1**)

Referring to the water extract, ripe garambullo can be attributed a good antiradical behavior against pn in comparison to prickly pears (3.33 g DM/L for garambullo and 4.33 g DM/L for prickly pear). However, the antioxidant capacity against pn is lower than that of *Clidemia rubra* berries (Gordon *et al.*, 2011a) or açaí fruits (Lichtenthäler *et al.*, 2005). Interestingly, the TOSC values resulting from the water extract of ripe garambullo against px and pn were characterized by a relatively small gap. Usually, fruit extracts have been shown to be considerably more effective against px than against pn (Gordon *et al.*, 2011b).

The contribution of betalains to the overall antioxidant capacity of garambullo fruits might be derived from an activity-guided fractionation, whose result is shown in **figure B.3.3**. Betalains were tentatively assigned by additional HPLC detection at a wavelength of 520 nm. The highest antioxidant activity was found in the first fraction (min 0-5) likely influenced by ascorbic acid that

was found to be present in garambullos in variety depending concentrations of 22.6 to 49.8 mg/100 g fresh weight (Guzmán-Maldonaldo *et al.*, 2010).

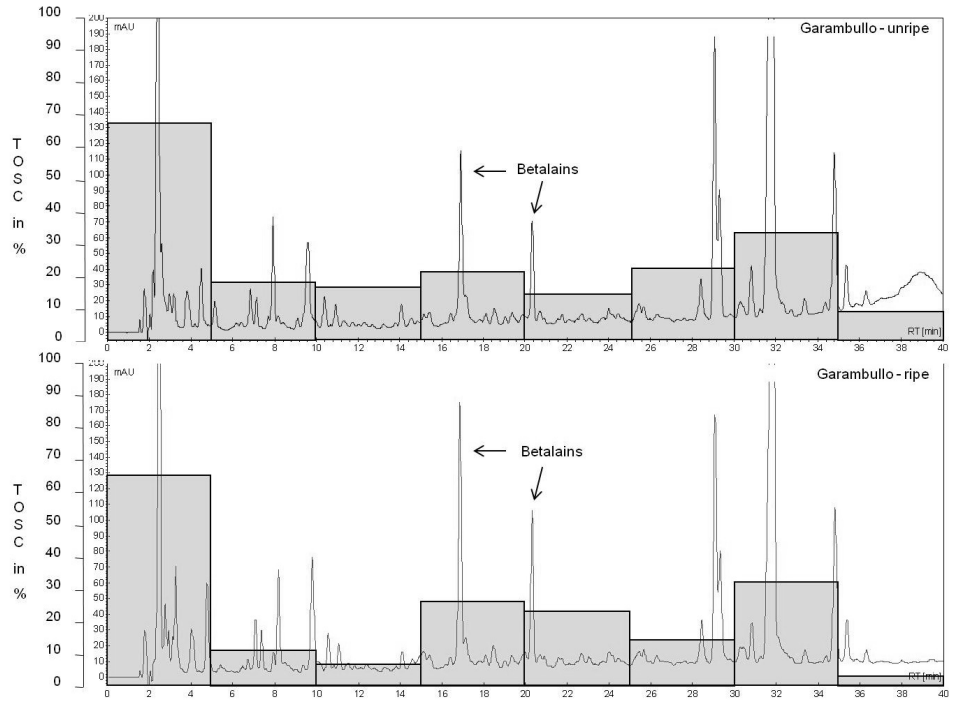


**Figure B.3.3.** Activity-guided fractionation of garambullo fruits at two different ripening stages. The HPLC chromatogram of a water extract is recorded at 260 nm. TOSC values (bars) of the fractions are given in %. The antioxidant capacity was measured against peroxyl radicals.

By comparing the peak intensity of the betalains in fractions 4 (min 15-20) and 5 (min 20-25), it can be assumed that their concentration increases in the course of ripening. This would be in accordance with the rising antioxidant capacity in these fractions and shows consequently an identifiable influence of betalains to the radical scavenging behavior of garambullos. However, the increasing antioxidant capacity of fractions 4 and 5 cannot compensate the decline in the overall antioxidant capacity from unripe to ripe fruits. The concentration decrease of unknown compounds gives rise to a regressive antioxidant activity in the remaining fractions. This becomes obvious e.g. in fraction 2 (min 5-10) and 3 (min 10-15) comprising the retention range of possible phenolic acid conjugates or fraction 6 (min 25-30) and 7 (min 30-35) being the retention range of the flavonoid glycosides.

## B.4 Conclusion

In conclusion, two phenolic acids and eight different flavonoids were identified throughout three different ripening stages of garambullo fruits. The rutinosides of quercetin and kaempferol were the quantitatively most dominant of the detected compounds. Unripe fruits showed a slightly higher concentration of phenolic compounds in comparison to the other determined ripening

stages. The highest antioxidant capacity was also assessed in unripe fruits. Betalains show an identifiable contribution to the antioxidant capacity. However, a concentration increase of betalains could not compensate the decrease of other antioxidants giving rise to regressive TOSC values in the course of ripening. The antioxidant capacity of garambullos in ripe condition was similar to that of prickly pears when measured against peroxyl radicals and distinctively higher against peroxynitrite. In consequence of these results, garambullo fruits seem to be a reasonable alternative to other betalain containing fruits for possible technological and functional applications.

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# C Changes of phenolic composition, ascorbic acid and antioxidant capacity in cashew apple (*Anacardium occidentale* L.) during ripening (accepted by *Fruits*)

André Gordon[a], Mirko Friedrich[a], Virgínia Martins da Matta[b], Carlos Farley Herbster Moura[c] & Friedhelm Marx[a]

[a]University of Bonn, Department of Nutrition and Food Sciences, Endenicher Allee 11-13, 53115 Bonn, Germany

[b]Embrapa Food Technology, Av. das Américas POB 29.501, 23020-470 Rio de Janeiro, Brazil

[c]Embrapa Tropical Agroindustry, Rua Dra. Sara Mesquita, 2270, Pici, 60511-110 Fortaleza, Brazil

**Corresponding author:**

Friedhelm Marx

Address: University of Bonn, Department of Nutrition and Food Sciences, Endenicher Allee 11-13, 53115 Bonn, Germany

Telephone: +49-228-733713; *Fax*: +49-228-733757

E-Mail: f.marx@uni-bonn.de

Own contribution: Preparation of the manuscript, identification and quantification of phenolic compounds, guidance of TOSC and ascorbic acid analysis

## C.1 Abstract

**Introduction.** Cashew apple is a rich source of sugars, vitamin C, and polyphenols. In spite of its nutritional value the pseudo fruits have been left unexploited in a large extend on the crop growing areas. Some reports on chemical characteristics of cashew apple have been published. However, nothing is known on changes of the composition of its bioactive compounds in the course of ripening. **Material and methods.** Cashew apples at three different maturity stages were examined with respect to their ascorbic acid content, phenolic compounds and antioxidant capacity. Ascorbic acid was quantified by HPLC. Phenolic compounds were identified and quantified by using HPLC-ESI-MS/MS by comparison with authentic standard compounds. The antioxidant capacity was measured by TOSC assay against peroxyl radicals and peroxynitrite. **Results.** Amounts of identified phenolic compounds were the highest in unripe cashew apple and decreased in the course of ripening. Myricetin 3-O-rhamnoside, quercetin 3-O-glactoside and quercetin 3-O-rhamnoside turned out to be the main flavonoids in all maturity stages. The antioxidant capacity and the concentration of ascorbic acid increased in the course of ripening. The antioxidant activity was considerably influenced by ascorbic acid more than by the content of phenolic compounds. **Conclusion.** This study provides for the first time information on changes of bioactive compounds and the antioxidant capacity in cashew apple during ripening. A dietary or technological exploitation of ascorbic acid is useful in ripe condition. The unripe pseudo fruits are a good source for the extraction of polyphenols with regard to possible food technological purposes or the preparation of food supplements.

## C.2 Introduction

*Anacardium occidentale* L. (Cashew) is an evergreen shrub or tree up to 15 m in height that originates from the coastal strip of northern and north-eastern Brazil (FAO, 1986). Recently, cashew is distributed across tropical America, the West Indies, India and Africa (Michodjehoun-Mestres *et al.*, 2009). The cashew tree bears two food products, the 'cashew nut' and the 'cashew apple'. The cashew nut is demanded on international markets due to its sweet flavor. Botanically, the cashew nut is the embryo of the kidney-shaped drupe which has a length of 3-5 cm. The cashew apple is attached as an enlarged peduncle to the drupe. This false fruit shows a yellow to red skin and a juicy flesh. It is 6-8 cm long and approximately 4.5 cm in diameter (FAO, 1986).

Despite its promising economical potential, cashew apples are still underutilized. Only 10% of the production is used in either fresh or processed form as ice cream and jellies (Akinwale, 2000). The largest part rots on the crop growing areas although cashew apple juice is palatable because of its strong exotic flavor. In addition, cashew apples are nutritive due to the high content of

vitamin C and sugars (Garruti *et al.*, 2010). A reason for the low attention to cashew apple is the astringent taste. Cashew apple juice has to be prepared technologically prior to consumption due to the content of tannins (Campos *et al.*, 2002). Both, the clarified product 'cajuina' and fresh cashew apple juice have been reported to be antimicrobially active because of the bioactive constituents such as flavonols, tannins, carotenoids, and ascorbic acid (Melo Cavalcante *et al.*, 2003).

Dietary phenolics have been ascribed to contribute to the prevention of some degenerative diseases due to their health promoting properties (Gollucke, 2010; Xia *et al.*, 2010). On the other hand, phenolic compounds have also become interesting for food technological purposes as they can be applied as antioxidants or antimicrobial agents (El-Hela and Abdullah, 2010; Jalosinska and Wilczak, 2009).

Vitamin C is regarded to be the most important vitamin in human nutrition. Approximately 90% of vitamin C in the human diet is supplied by fruits and vegetables (Lee and Kader, 2000). Beside a number of important physiological functions it acts *inter alia* as a dietary antioxidant (Lee and Kader, 2000) and is used in a large scale as antioxidant agent in foods and drinks (Beitollahi *et al.*, 2009).

Aim of this study was to evaluate the ascorbic acid content, the phenolic composition, and the antioxidant capacity in the course of ripening of *Anacardium occidentale* pseudo fruits in order to assess the bioactive potential for either dietary or food technological applications.

## C.3 Material and methods

### 1. Chemicals

Ultrahigh quality (UHQ) water was prepared with a Direct-Q 3 system (Millipore, Billerica, USA). Gallic acid (≥ 97.5%), quercetin (≥ 98%), myricetin (≥ 96%), diethylenetriamine-pentaacetic acid (DTPA) (≥ 99%), α-keto-γ-methiolbutyric acid (KMBA) (≥ 97%), 2,2'-azobis(2-methylpropionamidine) dichloride (ABAP) (≥ 97%), 3-morpholino-sydnonimine N-ethylcarbamide (SIN-1), (–)-epigallocatechin (≥ 95%), (–)-epigallocatechin gallate (≥ 95%), were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Myricitrin (≥ 95%) was obtained by Extrasynthése (Genay, France). Ascorbic acid (≥ 95%) was purchased from Kraemer & Martin GmbH (Sankt Augustin, Germany). Standards of quercetin 3-O-galactoside, quercetin 3-O-glucoside, quercetin 3-O-arabinoside, quercetin 3-O-rhamnoside, quercetin 4'-O-glucoside, and kaempferol 3-O-glucoside were a gift of Professor Dr. Galensa, University of Bonn. These standards were self-isolated and of different purity grades (≥ 42% in case of quercetin 3-O-

arabinoside, ≥ 90% for the remaining flavonols). Purity grades were taken into account for the quantification.

**2. Sampling**

Cashew apple pseudo fruits of the commercial variety CCP 76 were harvested at three maturity stages by visual analysis according to the classification described in Figueiredo (2000). Unripe fruits grew 33-36 days, medium ripe fruits 45-50 days, and ripe fruits 52 days. Fruits were collected at Embrapa's experimental station in Pacajus, Ceará, Brazil. The harvested fruits of each ripening stage were randomly divided in three sub-sets (each representing an independent replicate) of at least five fruits. Fruits were lyophilized immediately after harvest. Freeze-dried samples were air-shipped to Germany and ground by ball milling (MM2000, Retsch, Haan, Germany) prior to storage at -30 °C.

**3. Polyphenol analysis**

***Extraction of phenolic compounds***

Extraction of phenolic compounds was carried out by using a modified pressurized liquid extraction (PLE) method previously described in (Papagiannopoulos *et al.*, 2004). Freeze-dried sample (250 mg) of each ripening stage was extracted in triplicate with acetone-water-formic acid (70 + 29 + 1; v/v/v) in an Accelerated Solvent Extractor (ASE 200, Dionex, Idstein, Germany) at room temperature, for 20 min, in two cycles. The subsequent solid-phase extraction (SPE) was performed by using a Gilson ASPEC XL system (Automated Sample Preparation with Extraction Cartridges, Abimed, Langenfeld, Germany) following a modified method described in (Papagiannopoulos *et al.*, 2004). Polyamide (PA) SPE cartridges (500 mg PA, 3 ml cartridge, Macherey-Nagel, Düren, Germany) were conditioned with 3 ml of dimethyl sulfoxide-formic acid-trifluoroacetic acid (DMSO-FAc-TFA) (98.7 + 1 + 0.3; v/v/v) and washed with 5 ml of UHQ water. Prior to loading the cartridge, the sample extract was diluted to contain less than 15% (v/v) of organic solvent. The cartridge was loaded with sample extract in volumetric steps of 20 ml until exhaustion and washed with 10 ml of water after each load. While eluting with DMSO-FAc-TFA solvent, the first 0.5 ml were discarded and the next 1.25 ml collected. Prior to application to HPLC-MS/MS, the samples were filtered through a 1.0/0.45 µm syringe filter (Chromafil GF/PET-45/25, Macherey-Nagel, Düren, Germany).

***Identification and quantification of phenolic compounds***

Quantification of the phenolic compounds was performed following a previously described method (Gordon *et al.*, 2011). HPLC instruments consisted of a pump system and a UV-detector of the HP 1050 series (Hewlett Packard, Waldbronn, Germany), a degasser Degasys Populair

DP3010 (Uniflows, Tokyo, Japan) and an analytical column Aqua 3 µm C18, 150 mm, 2 mm i.d., combined with a guard column Security Guard, C18, 4 mm, 2 mm i.d. (both Phenomenex, Aschaffenburg, Germany). The solvents were UHQ water with 1% (v/v) formic acid (mobile phase A) and 1% (v/v) formic acid in acetonitrile (mobile phase B). The HPLC gradient using a flow rate of 0.3 ml/min started at 5% B, was hold isocratic for 10 min, and rose to 40% B after 60 min. Subsequently, the column was flushed for 10 min at 100% B and re-equilibrated for 25 min at 5% B. 20 µl were injected for analysis. Each sample extract was analyzed in duplicate (n = 6). The coupled API 2000 HPLC-ESI-MS/MS system was controlled with Analyst 1.5 Software (both Applied Biosystems, Darmstadt, Germany). Mass spectra for the determination of phenolic compounds were generated in negative ionization mode.

Identification was performed by comparing retention times and fragmentation patterns of phenolic compounds in multiple reaction mode with those of authentic standard substances. Standards were also used to create calibration curves for quantification. Results are presented in mg/100 g dry matter (DM).

**4. Antioxidant capacity by TOSC**

For TOSC analysis, freeze-dried sample of each ripening stage was reconstituted with UHQ water under consideration of the DM content of 12.9% (w/w). The suspension was sonicated for 5 min and centrifuged for 10 min at 12,000 rpm with a Heraeus Biofuge Stratos (Kendro, Langenselbold, Germany). The aqueous supernatant (WE) was stored for further analysis at -30 °C. Extraction procedure was performed in duplicate for each ripening stage.

The TOSC assay was performed as described in Lichtenthäler *et al.* (2003). Briefly, the TOSC assay is based on an ethylene-yielding reaction of KMBA with either peroxyl radicals (px) or peroxynitrite (pn). Antioxidant compounds present in the sample can inhibit the ethylene formation that is recorded in a time course of 1 h using automatically repeated headspace GC analysis (GC-17A, Shimadzu, Tokyo, Japan). Each ripening stage was analyzed in duplicate (n = 4). Quantification of generated ethylene results in a kinetic curve of which the area under the curve (AUC) is calculated. Mean data of a sample are compared to those of an uninhibited reaction with water which gives rise to the TOSC values. Results of this study indicate the concentration of the sample in gram per liter that is needed to obtain a radical inhibition of 50%.

**5. Ascorbic Acid**

The ascorbic acid content in the WE of each maturity stage was determined chromatographically. The HPLC-DAD system of PRO Star series (Varian, Walnut Creek, USA) was equipped with an analytical column Synergi 4 µ Hydro RP, 150 mm, 2 mm i.d., and with a guard column Security

Guard, C 18, 4 mm, 2 mm i.d. (both Phenomenex, Aschaffenburg, Germany). Separation was performed with acidified UHQ water (2% formic acid, v/v) at isocratic condition using a flow rate of 0.8 mL/min. The injection volume was 20 µL. Confirmation of ascorbic acid in the WE was arranged by standard, retention time, and doping of standard to the sample. A five-point calibration curve (5-100 mg/100 mL, $r^2 = 0.9996$) was created for quantification with authentic standard. Ascorbic acid was quantified at a wavelength of 260 nm. Sample runs of each WE were performed in duplicate (n = 4).

**6. Statistical Analysis**

To prove significant differences between maturity stages, statistical analysis of the data was performed by one-way analysis of variance. Means were compared by Bonferroni test at 95% of probability using PASW Statistics 18.

## C.4 Results and discussion

**1. Ascorbic acid**

Ascorbic acid was found in all maturity stages of cashew apple. Unripe fruits contained 1038 ± 31 mg/100 g DM, medium ripe fruits 1392 ± 52 mg/100 g DM, and ripe fruits 1731 ± 45 mg/100 g DM. Hence, results indicate an increase of the ascorbic acid content during ripening. A comparison of the mean values of each ripening stage demonstrated that the maturation process had a significant effect on the ascorbic acid content ($p < 0.05$). Literature studies on ascorbic acid levels at different ripening stages of cashew apple are not known but amounts of ascorbic acid in ripe fruits are in accordance with those found by Akinwale (2000). Different cashew apple cultivars determined by Assunção and Mercadante (2003) showed approximately 50% lower contents of ascorbic acid. The ascorbic acid content in ripe cashew apples is remarkably high in general. Amounts are 4-5 times higher in comparison to those of kiwi fruits or oranges (Souci *et al.*, 1989) and can be ranked at the same level with guavas (Mercado-Silva *et al.*, 1998).

Ascorbic acid is generally present in plant tissues that undergo active growth and development (Lee and Kader, 2000). Increasing amounts of ascorbic acid were also observed in ripening guavas (Mercado-Silva *et al.*, 1998). On the contrary, a decrease in the ascorbic acid content was reported by Celik *et al.* (2008) during ripening of cranberries. Hence, the ascorbic acid formation during fruit ripening seems to depend in particular on the species.

**2. Phenolic constituents**

A total of 14 phenolic constituents were detected in cashew apples (see **table C.4.1** and **figure C.4.1**) by HPLC-MS/MS analysis. With the exception of peaks 7 and 8 all of them could be

identified by comparison of their retention times and mass spectrometric data with those of the authentic standard compounds. Accordingly, peak 1 consists of a phenolic acid, namely gallic acid. Peaks 2 and 3 correspond to flavanols: epigallocatechin and epigallocatechin gallate, respectively. Nine different flavonols (peaks 4-6, 9-14) were identified – their identity is described in table **C.4.1**). Peaks 7 and 8 could only be tentatively assigned to quercetin pentosides. These compounds showed $[M–H]^-$ ions at $m/z$ 433 and product ions at $m/z$ 301 which is in agreement with the fragmentation pattern of quercetin 3-O-arabinoside. However, the retention times are differing.

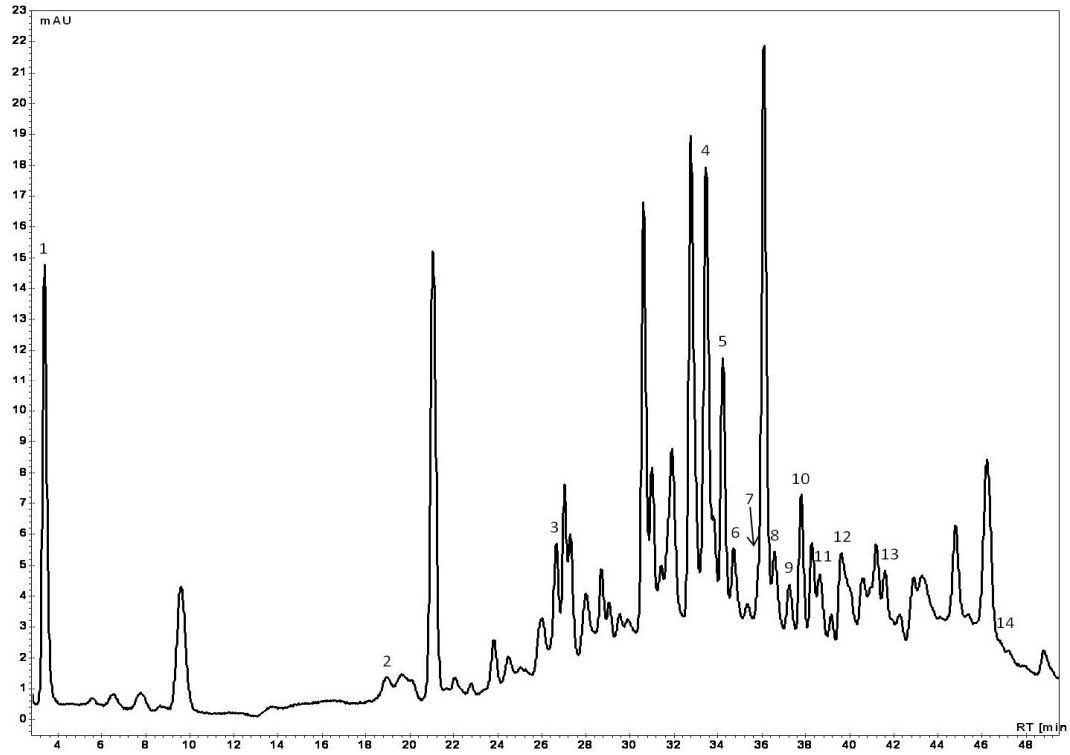


**Figure C.4.1.** Chromatogram of an aqueous acetone extract of unripe cashew apple recorded at 280 nm.

All detected phenolic compounds were present in unripe fruits. Their amounts decreased significantly from unripe to medium ripe fruits ($p < 0.05$). Kaempferol 3-O-glucoside (peak 12), myricetin (peak 13), and quercetin (peak 14) vanished completely. From medium ripe fruits to ripe fruits, a further decrease was observed for flavanols, quercetin pentosides, myricitrin, and quercetin hexosides of which quercetin 4'-O-glucoside (Peak 11) was no longer detectable in ripe fruits. In contrast to these compounds, amounts of gallic acid and quercetin 3-O-rhamnoside increased. All changes from medium ripe fruits to ripe fruits were not found to be significant. The decline in the concentration of the phenolic compounds from the unripe stage to medium ripe fruits suggests that the biosynthesis becomes less during growth and subsequent maturation as observed in bitter oranges (Castillo *et al.*, 1992). Changes on flavonols were also determined in different maturity stages of apricots (Gruz *et al.*, 2011) and common apples (Awad *et al.*, 2001; Li

*et al.*, 2002). In accordance with the results of cashew apple, the highest values of flavonols were mostly found in the initial maturity stage. Decreasing amounts of total flavonols during ripening were also found in camu camu fruits. On the contrary to cashew apple, values of total flavanols did not remarkably change (Chirinos *et al.*, 2010). The decrease of flavanol amounts in cashew apple is in accordance with a report of Almeida *et al.* who found a higher activity for enzymes involved in the biosynthesis of these flavonoids in the early developmental stage of strawberries (Almeida *et al.*, 2007). In case of hydroxybenzoic acids, Gruz *et al.* (2011) observed in medlar fruits (*Mespilus germanica* L.) that concentrations of free protocatechuic acid and syringic acid decreased during maturation as observed for gallic acid in cashew apple from unripe to medium ripe fruits. The decrease of free phenolic esters in medlar fruits is explained by their integration into cell walls.

| Peak | Compound | $[M-H]^-$/ product ion $m/z$ | Maturity stage | | |
|---|---|---|---|---|---|
| | | | Unripe[b] | Intermediate[b] | Ripe |
| 1 | Gallic acid | 169/125 | 2.22 ± 0.46 | 0.64 ± 0.06 | 0.94 ± 0.15 |
| 2 | Epigallocatechin | 305/125 | 0.61 ± 0.13 | 0.11 ± 0.03 | 0.02 ± 0.01 |
| 3 | Epigallocatechin gallate | 457/125 | 1.59 ± 0.31 | 0.07 ± 0.01 | 0.04 ± 0.00 |
| 4 | Myricitrin | 463/316 | 4.48 ± 0.68 | 0.91 ± 0.36 | 0.86 ± 0.14 |
| 5 | Quercetin 3-O-galactoside | 463/300 | 3.38 ± 0.53 | 0.99 ± 0.12 | 0.83 ± 0.23 |
| 6 | Quercetin 3-O-glucoside | 463/300 | 1.95 ± 0.57 | 0.44 ± 0.07 | 0.31 ± 0.10 |
| 7 | Quercetin pentoside 1 | 433/301 | 1.12 ± 0.28[a] | 0.45 ± 0.02[a] | 0.42 ± 0.05[a] |
| 8 | Quercetin pentoside 2 | 433/301 | 0.80 ± 0.12[a] | 0.40 ± 0.03[a] | 0.36 ± 0.08[a] |
| 9 | Quercetin 3-O-arabinoside | 433/301 | 0.73 ± 0.11 | 0.37 ± 0.05 | 0.38 ± 0.06 |
| 10 | Quercetin 3-O-rhamnoside | 447/301 | 2.15 ± 0.28 | 0.65 ± 0.10 | 0.69 ± 0.18 |
| 11 | Quercetin 4-O-glucoside | 463/300 | 0.13 ± 0.04 | 0.04 ± 0.01 | n.d. |
| 12 | Kaempferol 3-O-glucoside | 447/285 | 0.05 ± 0.02 | n.d. | n.d. |
| 13 | Myricetin | 317/151 | 0.64 ± 0.01 | n.d. | n.d. |
| 14 | Quercetin | 301/151 | 0.17 ± 0.02 | n.d. | n.d. |
| Total | | | 20.40 ± 3.56 | 5.16 ± 0.86 | 4.92 ± 1.00 |

**Table C.4.1.** Phenolic compounds in three maturity stages of cashew apple detected by HPLC-ESI-MS/MS. Data: mean ± standard deviation in mg/100 g dry matter. [a]expressed as quercetin 3-O-arabinoside equivalents. [b]Means of the compounds 1-11 differed significantly ($p < 0.05$) as assessed by analysis of variance and Bonferroni test.

Interesting results were observed by comparing the ratios of the detected phenolic constituents in each ripening stage of cashew apple (**figure C.4.2**). Although concentrations of quercetin 3-O-galactoside, quercetin 3-O-rhamnoside, and myricitrin decreased during ripening, these flavonols were present in similar percentages in each ripening stage. The quercetin pentosides and gallic acid increased remarkably whereas a decrease was found for epigallocatechin, epigallocatechin gallate, and quercetin 3-O-glucoside. In regard to flavonols, Awad *et al.* (2001) reported that the

ratio of the individually identified main quercetin glycosides in different cultivars of common apples (3-O-galactoside, 3-O-rhamnoside, 3-O-glucoside) undergo a permanent change during ripening which could in cashew apple only be constituted for quercetin 3-O-glucoside.

A determination of phenolic compounds during the ripening process of cashew apple is performed for the first time. Two previously published reports are known on individual phenolic compounds in ripe cashew apple. Compounds 1, 4, 5, 6, 10, 12, 13, 14 and two quercetin pentosides were identified by Michodjehoun-Mestres *et al.* (2009). In accordance to our results, myricitrin, quercetin 3-O-galactoside, quercetin 3-O-glucoside, and quercetin 3-O-rhamnoside were quantified in similar amounts in the flesh of cashew apple cultivar CCP 76 (De Brito *et al.*, 2007).

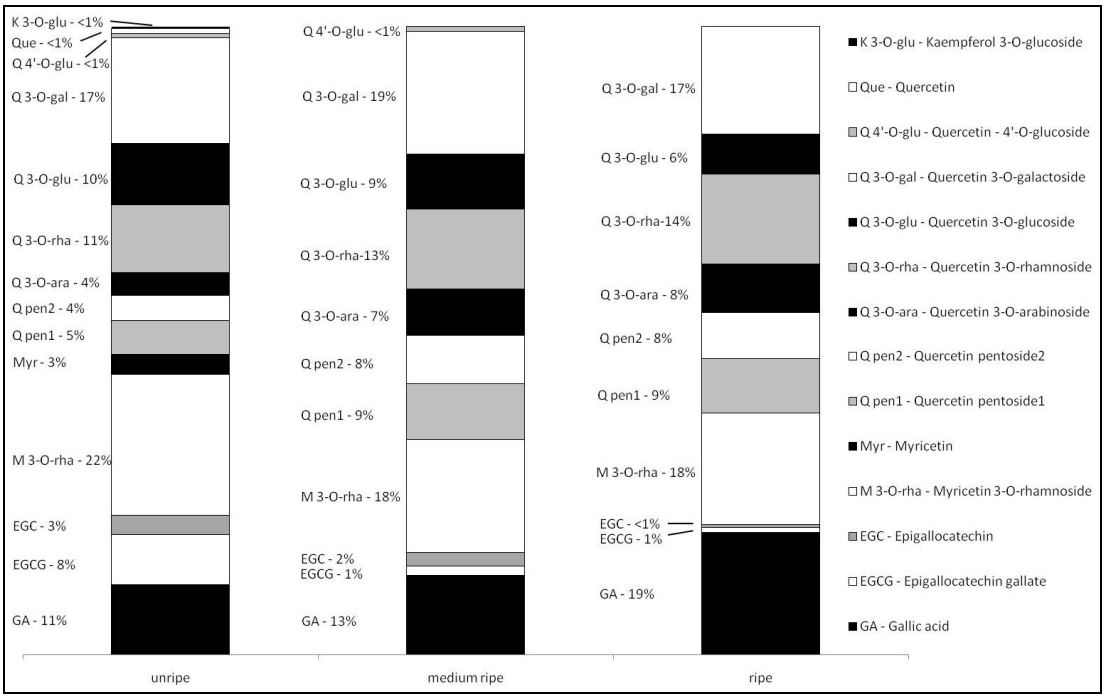


**Figure C.4.2.** Ratio of detected phenolic compounds at each stage of maturity in cashew apple.

De Brito *et al.* (2007) constituted also the presence of compound 4, 5, 6, 10, and 12 as well as three different quercetin pentosides. Epigallocatechin and epigallocatechin gallate are reported for the first time to occur in cashew apple total, 14 phenolic constituents were detected in unripe cashew apple.

### 3. Antioxidant capacity

The antioxidant capacity of cashew apple increased during maturity as shown in **table C.4.2**. Means were significantly different ($p < 0.05$) with exception of values between unripe and medium ripe fruits regarding px. Ripe cashew apples show approximately a twice as high radical scavenging activity against both radicals in ripe condition when compared to unripe fruits. Cashew apples show high antioxidant properties against both radicals in comparison to other

fruits from Latin America. The radical scavenging activity of ripe cashew pseudo fruits against px is higher than that of *Clidemia rubra* berries (0.9 g/L) (Gordon *et al.*, 2011). However, the antioxidant activity was lower when compared to that of açaí fruits from different harvest years (0.39-0.48 g/L) (Lichtenthäler *et al.*, 2005). Further, cashew apple turned out to be a good radical scavenger against pn as lower concentrations are needed to obtain a radical inhibition of 50% in comparison to açaí (1.17-1.72 g/L) (Lichtenthäler *et al.*, 2005) and *Clidemia rubra* berries (2.0 g/L) (Gordon *et al.*, 2011).

| | Maturity stage | | |
|---|---|---|---|
| | **Unripe** | **Intermediate** | **Ripe** |
| Peroxyl | $1.38 \pm 0.19^{a}$ | $1.22 \pm 0.16^{a}$ | $0.79 \pm 0.08^{b}$ |
| Peroxynitrite | $1.88 \pm 0.23^{a}$ | $1.37 \pm 0.10^{b}$ | $1.00 \pm 0.13^{c}$ |

**Table C.4.2.** TOSC values of cashew apple at three maturity stages in g dry matter/L. TOSC values indicate the concentration of cashew apple (in g dry matter/L) that is needed to obtain a radical inhibition of 50%. $^{a\text{-}c}$Comparison of the means was performed by analysis of variance and Bonferroni test. Values with the same letters are not significantly different at level of $p < 0.05$. Means were compared within each row.

A report of Lichtenthäler *et al.* (2003) might provide an explanation for the antiradical behavior of cashew apple in the course of ripening because of the antioxidant capacity which was determined of different flavonoid standards and ascorbic acid. Briefly, ascorbic acid showed a 4-5 times lower antioxidant activity against px in comparison to the phenolic standard compounds. Against pn, the difference between the polyphenols and ascorbic acid was less distinctive (only 1-2 times). Additionally, the radical scavenging activity of ascorbic acid towards px and pn was nearly identical. In consequence of these results, it becomes obvious that ascorbic acid has considerable influence on the antioxidant capacity of cashew apple. Firstly, the rising ascorbic acid concentration during ripening parallels the increase of the antioxidant activity against both radicals. Amounts of ascorbic acid in each ripening stage are remarkably higher than those of the identified and quantified phenolic compounds (the sum is given in **table C.4.1**). Secondly, the high ascorbic acid content is an explanation for the good antioxidant activity of cashew apple especially against pn.

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# D Chemical characterization and antioxidant capacity of berries from *Clidemia rubra* (Aubl.) Mart. (Melastomataceae) (published in Food Research International, 2011, 44, 2120-2127)

André Gordon[a], Benjamin Schadow[a], Clara E. Quijano[b], Friedhelm Marx[a]

[a]University of Bonn, Department of Nutrition and Food Sciences, Endenicher Allee 11-13, 53115 Bonn, Germany

[b]Universidad de los Andes, Facultad de Ciencias, Dpto. de Química, Cra. 1 a Este No. 18-A-10 Edif. (Q-826), Bogotá, Colombia

**Corresponding author:**

Friedhelm Marx

Address: University of Bonn, Department of Nutrition and Food Sciences, Endenicher Allee 11-13, 53115 Bonn, Germany

Telephone: +49-228-733713; Fax: +49-228-733757

E-Mail: f.marx@uni-bonn.de

Own contribution: preparation of the manuscript, identification and quantification phenolic compounds, guidance of TOSC and centesimal analysis

## D.1 Abstract

The flora of Latin America attracts gaining interest as it provides a plethora of still unexplored or underutilized fruits that can contribute to human well-being due to their nutritional value and their content of bioactive compounds. *Clidemia rubra* (Aubl.) Mart. is a shrub belonging to the family of the Melastomataceae that grows preferably in a tropical climate. This paper comprises a nutritional characterization of the berries from *Clidemia rubra* and provides data on the phenolic compounds as well as the antioxidant capacity of the fruit. Findings in macronutrients like protein, carbohydrates, and fat were comparable to that of common berry fruits. *Clidemia rubra* berries seemed to be a good source for dietary fibers and some minerals (Ca, Mn, and Zn). In contrast, contents of titratable acids and ascorbic acid in were low. The polyphenolic profile was determined by using HPLC-MS/MS in comparison to standard compounds. Noteworthy amounts of cyanidin 3-O-rutinoside (39.43 ± 1.66 mg/100 g fresh weight (FW)), delphinidin 3-O-rutinoside 23.74 ± 1.18 mg/100 g FW), cyanidin 3-O-glucoside (11.68 ± 0.56 mg/100 g FW), and delphinidin 3-O-glucoside (6.08 ± 0.35 mg/100 g FW) were found. Non-anthocyanin phenolic constituents were phenolic acids (gallic, protocatechuic, p-hydroxybenzoic, vanillic, and caffeic acid), flavan-3-ols (epigallocatechin, epigallocatechin gallate, and epicatechin gallate), and 11 different myricetin- and quercetin derivatives of which quercetin 3-O-arabinoside (5.26 ± 0.16 mg/100 g FW) and quercetin 3-O-rhamnoside (5.06 ± 0.08 mg/100 g FW) were dominating. Anthocyanins and ascorbic acid were mainly responsible for the antioxidant capacity of *Clidemia rubra* berries assessed with the total oxidant scavenging capacity (TOSC) assay.

## D.2 Introduction

There is considerable epidemiological evidence that a diet rich in fruits and vegetables reduces the risk of developing degenerative diseases like cardiovascular disorders (Wang *et al.*, 2010), Alzheimer's disease (Singh *et al.*, 2008), and cancer (Hertog *et al.*, 1994). Especially berry fruits are a rich source of antioxidant compounds like vitamin C and polyphenols that have been implicated in promoting these protective effects (Szajdek and Borowska, 2008). Due to the significant health benefits of bioactive compounds in berry fruits, there has been gaining interest in recent years in exploring new and exotic types of berries (Schreckinger *et al.*, 2010). A large number of underutilized exotic fruit species are of special interest to the agroindustry and may potentially supplement the income of the local population. However, only limited information is available on the nutritional value and polyphenolic content of many exotic species (Contreras-Calderón *et al.*, 2010).

*Clidemia rubra* is a shrub belonging to the family of Melastomataceae (Krasser, 1893). The bluish or black colored berries are of oval shape and 4-5 mm in diameter (Standley and Williams, 1963). Like all species of Melastomataceae, *Clidemia rubra* prefers a warm, tropical climate (Raffauf, 1996). The growth area extends in Central America from Oaxaca in Mexico to Panama. In South America it is mainly scattered in the northern part of the continent like Columbia, Ecuador, Venezuela, French-Guiana whereas different varieties can also be found in Bolivia and in the south of Brazil (Gleason, 1939; Naudin, 1849). The edible and juicy fruits (Hanelt, 2001; Standley and Williams, 1963) of *Clidemia rubra* are either collected from wild growing plants or cultivated in greenhouses (Krasser, 1893) before they are offered at local markets (Ternes *et al.*, 2005). Usually, the berries are eaten fresh (Krasser, 1893).

Literature about the family of Melastomataceae is generally scarce. To our knowledge, there is no information available on the distribution of nutrients and phenolic compounds in *Clidemia rubra*. This work will give a first appraisal of the characterization of primary and secondary plant compounds as well as the antioxidant activity of its berry fruits.

## D.3 Material and methods

### 1. Chemicals

Ultrahigh quality (UHQ) water was prepared with a Direct-Q 3 system (Millipore, Billerica, USA). All mineral Titrisol® standards, D-(–)-fructose (≥ 99%), protocatechuic acid (≥ 97%) and p-hydroxybenzoic acid (≥ 99%) were purchased from Merck (Darmstadt, Germany). Bile extract, pancreatin, Na2HPO4·H2O (≥ 99%), KH2PO4 (≥ 99%), D-(+)-glucose (puriss.), gallic acid (≥ 97.5%), vanillic acid (≥ 97%), (–)-epicatechin gallate (≥ 98%), (–)-epigallocatechin (≥ 95%), quercetin (≥ 98%), myricetin (≥ 96%), diethylenetriaminepentaacetic acid (DTPA) (≥ 99%), α-keto-γ-methiolbutyric acid (KMBA) (≥ 97%), 2,2'-Azobis(2-methylpropionamidine) dichloride (ABAP) (≥ 97%), and 3-morpholinosydnonimine N-ethylcarbamide (SIN-1), K3O4P·H2O (≥ 95%) were obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Ascorbic acid (≥ 95%) was purchased from Kraemer & Martin GmbH (Sankt Augustin, Germany). Cyanidin 3-O-glucoside (≥ 96%), cyanidin 3-O-rutinoside (≥ 96%), delphinidin 3-O-glucoside (≥ 95%), (–)-epigallocatechin gallate (≥ 95%) and myricitrin (≥ 95%) were purchased from Extrasynthèse (Genay, France). Caffeic acid (purum) was obtained from SERVA Feinbiochemica (Heidelberg, Germany). HPLC and HPLC-MS solvents in the necessary purity grade were obtained from J.T. Baker (Griesheim, Germany). All flavonol glycoside standards were a gift of Professor Dr. Galensa (University of Bonn). They were self-isolated and of different purity grades.

**2. Sampling**

Berries of *Clidemia rubra* were collected in Restrepo (Meta) near Bogotá, Columbia. After harvest, the fruits were directly homogenized and lyophilized. Until analysis, the freeze-dried samples were stored at -30 °C.

**3. Determination of fat, protein, carbohydrates, and dietary fibers**

Fat content was determined using a Soxhlet method described in Carpenter *et al.* (1993). For analysis 1 g of freeze-dried sample was extracted with petroleum ether for 2 h.

Total nitrogen was measured with an automated combustion method based on that of Dumas being in accordance with the official AOAC method 990.03 (AOAC, 1998). 100 mg of the lyophilized sample of *Clidemia rubra* were transferred into a Nitrogen/Protein Analyser FP-328 (Leco, Mönchengladbach, Germany). Protein concentration was calculated by multiplication of obtained nitrogen concentration with the conversion factor of 6.25 (Simonne *et al.*, 1997).

For determination of carbohydrates, lyophilized fruit sample of *Clidemia rubra* was dissolved in UHQ water according to their original water content of 81.4%, sonicated for 10 min and centrifuged for the same time at 15,000 rpm with a Heraeus Biofuge Stratos (Kendro, Langenselbold, Germany). The supernatant of the water extract (WE) was filtered through a 0.45 µm syringe filter (Chromafil PET-45/25, Macherey-Nagel, Düren, Germany). The filtrate was stored until further application at -30 °C.

Glucose, fructose and saccharose were quantified by an enzymatic-photometric method. The enzyme kit was purchased from R-Biopharm AG (Darmstadt, Germany). Analysis was performed following the instruction manual. The spectroscopical measurement was carried out by a Cary 50 UV-Vis Spectrophotometer (Varian, Walnut Creek, USA).

Dietary fiber was determined using a modified method of Al-Hasani *et al.* (1993). The dietary fiber content is considered as the residue of the dry crucible less ashed crucible and crude protein. Therefore, freeze-dried sample (1 g) was accurately weighed in a 250 mL PE-flask and suspended in 50 mL phosphate buffer, pH 7.4. After adding of 2.5 mL of bile solution and 5 mL of pancreatic enzyme solution, pH was adjusted to 7.8 with 1 N sodium hydroxide. The flask was placed in an incubator shaker at 40 °C for 6 h. Following the incubation 100 mL reagent grade alcohol was added, the suspension was than centrifuged for 10 min at 4000 rpm and the supernatant was rejected. The residue was elutriated with 30 mL alcohol, centrifuged and the supernatant rejected again. This procedure was repeated once more with 30 mL alcohol and following with 20 mL acetone. The residue was dried in an oven set at 130 °C for 1 h and weighed accurately. Ash content of the residue was determined in a muffle furnace set at 525 °C

for 6 h. The crude protein content of the residue was determined as described in AOAC method 981.10 for protein determination (AOAC, 1998).

Determination of macronutrients and dietary fibers were carried out in a duplicate.

**4. Determination of total titratable acid (TTA*)***

Determination of TTA was performed as described in AOAC standard method 942.15 with some modifications (AOAC, 1998). 4 mL of WE was filled up to a volume of 10 mL with UHQ water. The titration was accomplished with 0.5 M KOH. After addition of several droplets of KOH the pH-value was recorded by using an inoLab Level 2 pH-meter (WTW, Weilheim, Germany). This procedure was repeated twice. By help of data evaluation software SPSS TableCurve 2D v5.1 (SYSTAT, Erkrath, Germany) the stoichiometric point was calculated and the results were expressed as citric acid equivalent.

**5. Determination of minerals by atomic absorption spectrometry (AAS)**

Determination of minerals was performed following official European standard methods. For the pressure digestion, an acid hydrolysis was conducted in order to separate the anorganic minerals from their organic ligands. Five times, lyophilized fruit sample (350 mg) was mixed with 2 mL hydrogen peroxide (30%) and 3 mL nitric acid (70%), given in cartridges and transferred into a microwave digestion system MEGA 1200 (MLS, Leutkirch, Germany) (DIN EN 13805, 2002).

As described in DIN EN 1134 (1994) eight elements (Ca, Mg, K, Na, Zn, Mn, Cu, Fe) were determined by using flame AAS (acetylene and air). Analyses were operated with an AA240FS Atomic Absorption Spectrometer (Varian, Walnut Creek, USA) equipped with mineral specific hollow cathode lamps. Quantification was done with aid of specific calibration curves.

**6. Determination of ascorbic acid by HPLC**

Ascorbic acid content of the WE was determined basing on a modified method of Romero-Rodriguez *et al.* (1992). The HPLC-DAD system of Pro Star series (Varian, Walnut Creek, USA) was equipped with an analytical column Aqua 3 µm C18, 150 mm, 2 mm i.d., and with a guard column Security Guard, C18, 4 mm, 2 mm i.d. (both Phenomenex, Aschaffenburg, Germany). The solvent for isocratic separation was UHQ water with 1% formic acid (v/v). Measurements were accomplished with a flow rate of 0.4 mL/min and a detection wavelength of 260 nm. Confirmation of ascorbic acid in the WE was arranged by authentic standard, retention time, and doping of standard to the sample. For quantification a calibration curve was created. All sample runs were carried out in duplicate.

## 7. Identification and quantification of phenolic compounds by HPLC-MS/MS

### *Polyphenol extraction*

Anthocyanins of *Clidemia rubra* were extracted using a modified method explicitly described in Wu *et al.* (2004). Briefly, freeze-dried sample (250 mg) was extracted with 4 mL of methanol-UHQ water-acetic acid (MeOH-$H_2O$-HAc) (50 + 49.5 + 0.5; v/v/v). After vortexing, sonication and centrifugation the supernatant was removed and the sample once more extracted but with 2.5 mL of MeOH-$H_2O$-HAc. Both sample solutions were combined and filtered through a 1.0/0.45 µm syringe filter (Chromafil GF/PET-45/25, Macherey-Nagel, Düren, Germany) prior to application into HPLC.

For determination of phenolic acids and flavanols a modified sample preparation was used as described in Pacheco-Palencia *et al.* (2009). For two times, lyophilized sample (500 mg) was dissolved in 10 mL of acetone-water-formic acid (70 + 29 + 1; v/v/v), sonicated for 10 min, then centrifuged for 10 min with 10,000 rpm at 10 °C. The sample was extracted once more with 10 mL acetone-water-formic acid. Afterwards, the supernatants were combined. To get rid of the organic solvent, the extract was vaporized using a rotary evaporator (Rotavapor R-210, Büchi, Essen, Germany) at 30 °C and the aqueous supernatant was shaken with 10 mL ethyl acetate. The received ethyl acetate fraction was vaporized to dryness. The residue was solubilized in 1 mL methanol-water-formic acid (50 + 49 + 1; v/v/v) and filtered through a 1.0/0.45 µm syringe filter (Chromafil GF/PET-45/25, Macherey-Nagel, Düren, Germany) before application to HPLC-MS/MS.

Due to a low recovery of glycosylated compounds in the ethyl acetate fraction, the preparation for flavonol determination was carried out by using a modified pressurized liquid extraction (PLE) method previously described in Papagiannopoulos *et al.* (2004). Freeze-dried sample (250 mg) were extracted with acetone-water-formic acid (70 + 29 + 1; v/v/v) in an Accelerated Solvent Extractor (ASE 200, Dionex, Idstein, Germany) at room temperature, for 20 min, in two cycles. The following solid-phase extraction (SPE) was performed using a modified method described in Papagiannopoulos *et al.* (2004) by help of a Gilson ASPEC XL system (Automated Sample Preparation with Extraction Cartridges, Abimed, Langenfeld, Germany). Polyamide (PA) SPE cartridges (500 mg PA, 3 mL cartridge, Macherey-Nagel, Düren, Germany) were conditioned with 3 mL of dimethyl sulfoxide-formic acid-trifluoroacetic acid (DMSO-FAc-TFA) (98.7 + 1 + 0.3; v/v/v) and washed with 5 mL of UHQ water. Prior to loading the cartridge the sample extract was diluted to contain less than 15% (v/v) of organic solvent. The cartridge was loaded with sample extract in volumetric steps of 20 mL until exhaustion and washed with 10 mL of water after each load. While eluting with DMSO-FAc-TFA solvent, the first 0.5 mL were

discarded and the next 1.25 mL collected. Before application to HPLC-MS/MS the samples were filtered through a 1.0/0.45 µm syringe filter (Chromafil GF/PET-45/25, Macherey-Nagel, Düren, Germany).

***Polyphenol analysis***

The determination of the phenolic compounds was performed using a modified method of Papagiannopoulos *et al.* (2004). HPLC instruments consisted of a pump system and an UV-detector of the HP 1050 series (Hewlett Packard, Waldbronn, Germany), a degasser Degasys Populair DP3010 (Uniflows, Tokyo, Japan) and an analytical column Aqua 3 µm C18, 150 mm, 2 mm i.d., with a guard column Security Guard, C18, 4 mm, 2 mm i.d. (both Phenomenex, Aschaffenburg, Germany). The solvents were UHQ water with 1% (v/v) formic acid (mobile phase A) and 1% (v/v) formic acid in acetonitrile (mobile phase B). The HPLC gradient using a flow rate of 0.2 mL/min started at 5% B and rose up to 35% B after 55 min, 100% B after 65 min and re-equilibrated for 15 min at 5% B. 20 µL of each sample extract were injected for analysis. The coupled API 2000 HPLC-MS/MS system was controlled with Analyst 1.5 Software (both Applied Biosystems, Darmstadt, Germany). Mass spectra for the determination of anthocyanins were obtained by using positive ionization whereas all other polyphenols were detected in negative ionization mode.

Identification was performed by comparing fragmentation patterns and/or retention time of standard substances which also were used for creation of calibration curves for quantification of the non-anthocyanin phenolic compounds.

For quantification of the anthocyanins, a HPLC-DAD system of Pro Star series (Varian, Walnut Creek, USA) equipped with an analytical column Aqua 3 µm C18, 150 mm, 2 mm i.d., combined with a guard column Security Guard, C18, 4 mm, 2 mm i.d. (both Phenomenex, Aschaffenburg, Germany) was used. Calibration curves were created with authentic standards of the individual anthocyanins.

**8. Antioxidant activity**

***Total oxidant scavenging capacity (TOSC) assay***

Analyses of the TOSC assay were performed as described in Lichtenthäler *et al.* (2003). Briefly, the measurement of the antioxidant capacity is based on an ethylene yielding reaction of KMBA with either generated peroxyl radicals or peroxynitrite. Antioxidative compounds present in the sample can inhibit the ethylene formation. Samples without antioxidant properties have a TOSC value of 0%. A complete suppression of ethylene formation corresponds to a TOSC value of

100%. Ethylene is measured gas chromatographically (GC-17A, Shimadzu, Tokyo, Japan) over a time course of 1 h.

***Fractionation by HPLC***

Fractionation was performed as described in Rodrigues *et al.* (2006) with some modifications. The HPLC-DAD system of Pro Star series (Varian, Walnut Creek, USA) was equipped with an analytical column Max-RP 4 µm C18, 150 mm, 4.6 mm i.d., combined with a guard column Security Guard, C18, 4 mm, 2 mm i.d. (both Phenomenex, Aschaffenburg, Germany). The solvents were UHQ water with 2% (v/v) formic acid (mobile phase A) and 2% (v/v) formic acid in acetonitrile (mobile phase B). The HPLC gradient using a flow rate of 0.8 mL/min started at 0% B and was hold isocratic for 10 min. After 10 min the gradient rose up to 40% B after 60 min. The column was flushed with 100% B and re-equilibrated for 15 min at 0% B. 20 µL were injected for analysis.

Fractions of the WE were collected in periods of 4 min starting directly after injection for a total time of 44 min. All collected samples were freeze-dried, dissolved in 500 µL UHQ-water, and sonicated prior analysis with the TOSC-assay. The antioxidant capacity of the fractions was determined against peroxyl radicals.

## D.4 Results and discussion

### 1. Macronutrients

*Clidemia rubra* berries showed a fat content of 1.03 ± 0.01 g/100 g FW. This result is comparable to that of other berries like blackberries, blueberries or raspberries which generally contain 1% or less of fat (Souci *et al.*, 2008).

The nitrogen and protein content denoted as arithmetic mean were 0.19 ± 0.01 g/100 g fresh weight (FW) for nitrogen and 1.18 ± 0.03 g/100 g FW for protein, respectively. The results of the protein content of berries of *Clidemia rubra* are in accordance with commonly cultivated berries. The highest amount of protein is found in black currants whereas blueberries offer an averaged protein content of only 0.60 g/100 g FW (Souci *et al.*, 2008).

The dominant sugars in berries of *Clidemia rubra* were glucose and fructose. Glucose was found in a concentration of 3.24 ± 0.06 g/100 g FW. The amount of fructose added up to 3.75 ± 0.10 g/100 g FW. The sugar content as well as the glucose-fructose ratio is comparable to that of other berries like black currant, blueberry or gooseberry (Souci *et al.*, 2008). Saccharose was found to be < 0.1 g/100 g. Generally, saccharose occurs in berries only in low concentrations which can be explained by its transport function inside the plant and the immediate inversion to fructose

and glucose at place of use (Talcott, 2007). The amount of saccharose in *Clidemia rubra* is even lower than in black currant, blueberry or gooseberry (Souci *et al.*, 2008).

**2. Dietary fibers**

Clidemia rubra berries are found to be a good source for dietary fibers. The determined content was 8.85 ± 0.02 g/100 g FW which is about twice the amount contained in blueberries and raspberries (Souci *et al.*, 2008). An explanation for this high value might be the dry matter content that is likewise higher when compared to blueberries and raspberries. The dry matter of black currants (Souci *et al.*, 2008) and camarinha fruits (Bramorski *et al.*, 2010) is comparable to that of Clidemia rubra berries. Hence, the total dietary fiber of black currants (6.8 g/100 g FW) and camarinha (6.5 g/100 g FW) is higher than in blueberries and raspberries but nonetheless lower than in Clidemia rubra berries.

**3. Minerals**

The results of mineral analysis by AAS are illustrated in **table D.4.1**. For calcium, zinc and manganese the concentrations are high compared to those of other common berry fruits (Souci *et al.*, 2008).

| **Mineral content in mg/100 g fresh weight** | |
|---|---|
| Na | 0.85 ± 0.02 |
| K | 163.42 ± 2.55 |
| Ca | 43.62 ± 2.50 |
| Mg | 9.21 ± 0.21 |
| Fe | 1.73 ± 0.07 |
| Zn | 0.63 ± 0.02 |
| Cu | 0.01 ± 0.00 |
| Mn | 9.61 ± 0.15 |

**Table D.4.1.** Minerals in *Clidemia rubra* berries.

**4. pH value and TTA**

*Clidemia rubra* berries indicated a pH value of 3.0. The amount of TTA was determined as 138.8 mmol/L and 8.9 g/L calculated as citric acid.

Compared with results of other berries from literature, the acid concentration in berries of *Clidemia rubra* is low. For example, Rubinskiene *et al.* (2006) and Zatylny *et al.* (2005) found almost up to 5 times higher amounts for TTA in black currant calculated as citric acid. Depending on stage of maturity Famiani *et al.* (2005) found 2.5 fold amounts in red currants. In case of ripe blueberries, Giovanelli and Buratti (2009) obtained results comparable to that of *Clidemia rubra* berries. Even lower amounts of TTA up to 0.35 g/100 g FW were reported for other Vaccinium

cultivars (Saftner *et al.*, 2008). Beside differences in fruit ripeness and cultivars, the large variation in TTA depends on hydrothermal conditions like sun exposure and temperature (Bergqvist *et al.*, 2001; Famiani *et al.*, 2005).

**5. Ascorbid acid**

*Clidemia rubra* berries showed an ascorbic acid content of 8.44 ± 0.02 mg/100 g FW which is low compared to other berry fruits. Blueberries, gooseberries as well as red currants show more than two-fold higher concentrations in ascorbic acid (Pantelidis *et al.*, 2007; Souci *et al.*, 2008). Only grapes indicate lower ascorbic acid contents between 2.0-7.4 mg/100 g FW (Souci *et al.*, 2008).

**6. Identification and quantification of phenolic compounds**

***Anthocyanins***

A typical chromatogram of the determined anthocyanins at 520 nm is given in **figure D.4.1**. A list of the identified anthocyanins is given in **table D.4.2**. In total four anthocyanins were detected in *Clidemia rubra* berries.

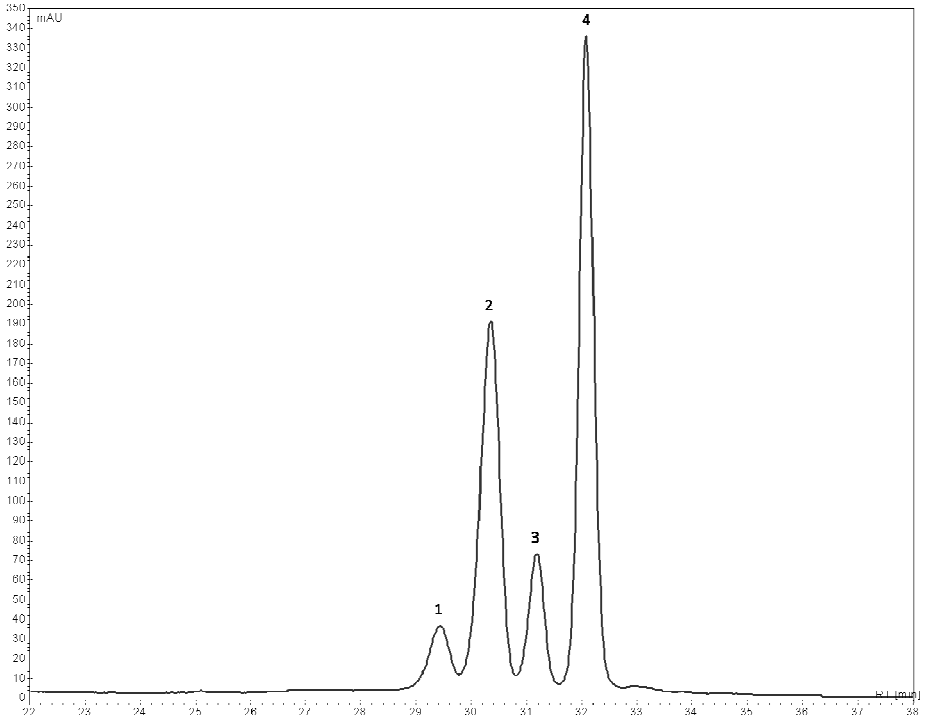


**Figure D.4.1**. Anthocyanins in *Clidemia rubra* berries. HPLC chromatogram at 520 nm of an aqueous methanol extract.

Peaks 1, 3, and 4 were mass spectrometrically identified by comparison of fragmentation pattern and retention time with authentic standards of delphinidin 3-O-glucoside, cyanidin 3-O-glucoside, and cyanidin 3-O-rutinoside. Peak 2 was tentatively assigned to delphinidin 3-O-rutinoside showing a molecular ion $[M+H]^+$ at $m/z$ 611. The positive product ion was at $m/z$ 303 indicating an agylcone fragment of delphinidin and the loss of $m/z$ 308 is in accordance with the

mass of a hexosyl-deoxyhexoside. Further, the presence of rutinoside in *Clidemia rubra* berries was already proven as a sugar moiety of cyanidin. This suggestion agrees with regularities in the determination of anthocyanins. At first, the sequence of the elution order depends on the sugar moiety. Generally, an anthocyanidin 3-O-rutinoside is retained after an anthocyanidin 3-O-glucoside (Wu and Prior, 2005a). Secondly, if a certain sugar moiety is linked to an anthocyanidin it will also be distributed to other anthocyanidins present in the fruit. Beside this, larger sugar moieties tend to link to 3-position of the flavylium ring. For instance, diglucose and rutinose were found to be exclusively bond at that position (Wu and Prior, 2005b). After quantification, the 3-O-rutinosides of cyanidin and delphinidin (calculated as delphinidin 3-O-glucoside equivalents) turned out to be the main anthocyanins in *Clidemia rubra* berries followed by the 3-O-glucosides. A report of anthocyanins in different Melastomataceae varieties showed the presence of malvidin glycosides in the flowers and mainly delphinidin and pelargonidin glycosides in the fruits. Here, an acylated delphinidin 3,5-O-diglucoside was found in Clidemia hirta Don (Lowry, 1975).

| Peak | Retention time | $[M+H]^+$/ product ion (m/z) | Compound | Content in mg/100 g fresh weight |
|---|---|---|---|---|
| 1 | 29.4 | 465/303 | Delphinidin 3-O-glucoside | 6.08 ± 0.35 |
| 2 | 30.3 | 611/303 | Delphinidin 3-O-rutinoside | 23.74 ± 1.18[a] |
| 3 | 31.2 | 449/287 | Cyanidin 3-O-glucoside | 11.68 ± 0.56 |
| 4 | 32.0 | 595/287 | Cyanidin 3-O-rutinoside | 39.43 ± 1.66 |

**Table D.4.2.** Anthocyanins in an aqueous methanol extract of *Clidemia rubra* berries. Data: mean ± standard deviation. [a]expressed as delphinidin 3-O-glucoside equivalents.

The total anthocyanin content of *Clidemia rubra* berries is about five-fold higher compared to that of red currants or different gooseberry cultivars (Wu *et al.*, 2004). However, amounts are approximately five times lower than those of black currant cultivars (Wu *et al.*, 2004) and 1.75-2.75 times lower than those found in different Andean blackberry cultivars (Mertz *et al.*, 2007).

### *Non-anthocyanin phenolic compounds*

**Figure D.4.2** shows the HPLC-UV-Vis chromatogram of an extract of *Clidemia rubra* berries at 280 nm. Results are presented in **table D.4.3**. In total five phenolic acids were identified. Quantitatively, vanillic (peak 9) and gallic acid (peak 5) were the most dominant phenolic acids, followed by lower quantities of protocatechuic, p-hydroxy-benzoic, and caffeic acid (peaks 6, 7, and 10). Three different flavan-3-ols were found in *Clidemia rubra* berries, namely epigallocatechin (peak 8), epigallocatechin gallate (peak 11) as dominating flavan-3-ols, and epicatechin gallate (peak 13). Berries of *Clidemia rubra* contain different flavonols that are derived from myricetin and quercetin. Peak 22 showed a base peak $[M-H]^-$ at $m/z$ 317 and a product ion at $m/z$ 151 which

was in agreement with an authentic standard of myricetin. By comparing the fragmentation pattern and the conforming retention time this peak can positively be recognized as myricetin.

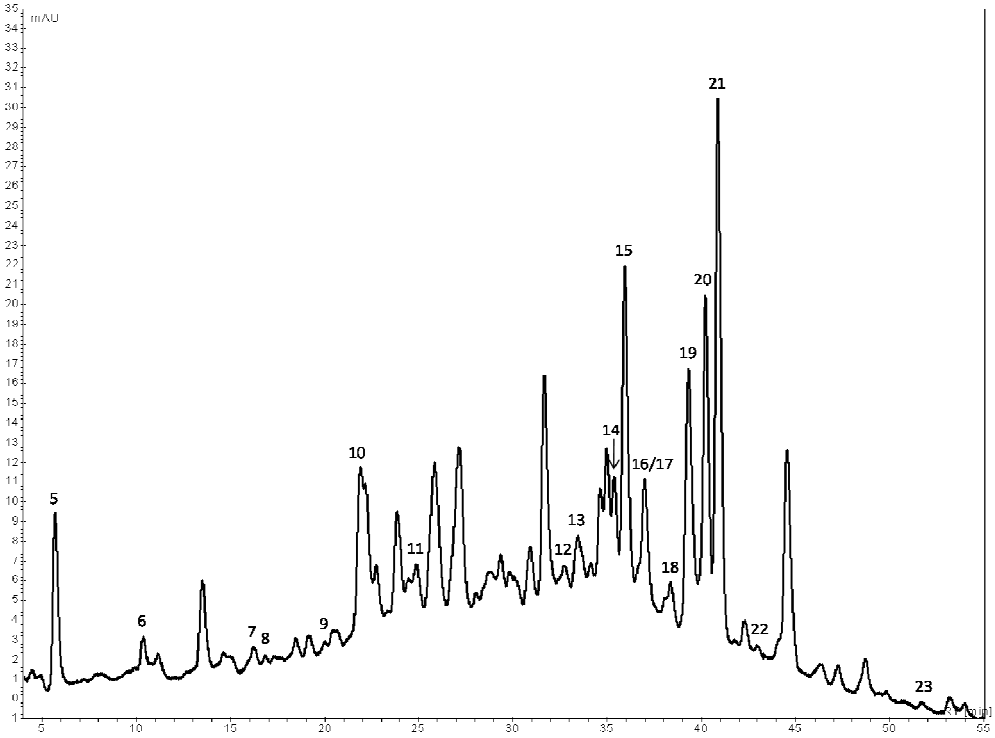


**Figure D.4.2.** HPLC chromatogram at 280 nm of an aqueous acetone extract prepared with ASE-ASPEC.

The presence of a myricetin aglycone confirmed the identification of peaks 12 and 14 as myricetin 3-O-glucoside and myricetin 3-O-rhamnoside, respectively. Deprotonated molecular ions were found $[M–H]^-$ at $m/z$ 479 and 463, respectively. Their corresponding deprotonated product ion showed a peak at $m/z$ 316. As stated in Hvattum and Ekeberg (2003) both an aglycone product ion $[M–H]^-$ after heterolytic cleavage of the O-glycosidic bond and a radical aglycone fragment ($[M–H]^{-\bullet}$) are obtained after homolytic cleavage of non-methoxylated flavonoids in consequence of negative ion collision-induced dissociation. In this case, fragmentation pattern and retention time matched with that of myricetin 3-O-glucoside and myricetin 3-O-rhamnoside standards. Quantitatively, myricetin 3-O-rhamnoside turned out to be the dominating myricetin compound. 1.04 mg/100 g fresh weight (FW) were found for myricetin 3-O-rhamnoside and only 0.05 mg/100 g FW for the aglycone. The amount of myricetin 3-O-glucoside could not be quantified due to an impurity of the standard. Peak 23 was ascribed to quercetin indicated by a deprotonated molecular ion $[M–H]^-$ at $m/z$ 301 matching with the fragmentation pattern and the retention time of an authentic standard.

| Peak | Retention time | $[M–H]^-$/ product ion *(m/z)* | Compound | Content in mg/100 g fresh weight |
|---|---|---|---|---|
| 5 | 5.7 | 169/125 | Gallic acid | 0.56 ± 0.01 |
| 6 | 10.4 | 153/109 | Protocatechuic acid | 0.11 ± 0.01 |
| 7 | 16.2 | 137/93 | p-Hydroxy-benzoic acid | 0.06 ± 0.00 |
| 8 | 16.9 | 305/125 | Epigallocatechin | 0.45 ± 0.03 |
| 9 | 20.0 | 167/152 | Vanillic acid | 1.43 ± 0.02 |
| 10 | 21.9 | 179/135 | Caffeic acid | 0.004 ± 0.000 |
| 11 | 24.9 | 457/125 | Epigallocatechin gallate | 2.99 ± 0.16 |
| 12 | 32.5 | 479/316 | Myricetin 3-O-glucoside | Not quantified |
| 13 | 33.4 | 441/169 | Epicatechin gallate | 0.09 ± 0.01 |
| 14 | 35.4 | 463/316 | Myricetin 3-O-rhamnoside | 1.04 ± 0.04 |
| 15 | 35.9 | 609/300 | Quercetin 3-O-rutinoside | 0.59 ± 0.18 |
| 16 | 37.0 | 463/300 | Quercetin 3-O-glucoside | 1.23 ± 0.14 |
| 17 | 37.0 | 477/301 | Quercetin 3-O-glucuronide | 0.14 ± 0.01 |
| 18 | 38.4 | 433/301 | Quercetin pentoside | 0.47 ± 0.05[a] |
| 19 | 39.3 | 433/301 | Quercetin pentoside | 2.42 ± 0.03[a] |
| 20 | 40.2 | 433/301 | Quercetin 3-O-arabinoside | 5.26 ± 0.16 |
| 21 | 40.9 | 447/300 | Quercetin 3-O-rhamnoside | 5.06 ± 0.08 |
| 22 | 42.9 | 317/151 | Myricetin | 0.05 ± 0.00 |
| 23 | 52.7 | 301/151 | Quercetin | Trace amounts |

**Table D.4.3.** Retention times, HPLC/MS/MS data, and amounts of non-anthocyanin polyphenols in *Clidemia rubra* berries identified from an aqueous acetone extract. Data: mean ± standard deviation. [a]expressed as quercetin 3-O-arabinoside equivalents.

Several quercetin 3-O-glycosides were found as well as the quercetin aglycone. In regard to **table D.4.3**, peaks 15, 16, and 21 showed a deprotonated molecular ion $[M–H]^-$, a radical aglycone fragment and a retention time which was in accordance with standard solutions of quercetin 3-O-rutinoside, quercetin 3-O-glucoside, and quercetin 3-O-rhamnoside, respectively. Peak 17 co-eluted with peak 16 but indicated a deprotonated molecular ion $[M–H]^-$ at $m/z$ 477 and an aglycone fragment at $m/z$ 301. Fragmentation pattern and elution time of peak 17 coincided with that of a quercetin 3-O-glucuronide standard solution. Peak 20 showed a deprotonated molecular ion $[M–H]^-$ at $m/z$ 433 and a product ion at $m/z$ 301, which corresponded positively also in regard to the retention time to a quercetin 3-O-arabinoside standard. The same fragmentation behavior accounted for peak 18 and 19. Hence, the loss of $m/z$ 132 indicated the cleavage of a pentoside of the molecular ion $[M–H]^-$ at $m/z$ 433 and the product ion at $m/z$ 301 so that these compounds could be assigned to a quercetin pentoside. Quantitatively, the quercetin pentosides and quercetin 3-O-rhamnoside showed the highest amounts of flavonol glycosides in *Clidemia rubra* berries.

With exception of anthocyanins, no reports have been found on flavonoids in fruits of *Clidemia* and accordingly Melastomataceae varieties, in general. Only few reports are known about flavonoids in Melastomataceae flowers or leaves. Mimura *et al.* (2004) identified 17 different flavone and flavonol glycosides in *Huberia* leaves. The same compound classes were found in leaves of *Miconia alypifolia* (Mancini *et al.*, 2008). One report describes the identification of kaempferol glycosides in *Melastoma malbathricum* L. flowers (Susanti *et al.*, 2007). The presence of kaempferol and kaempferol glycosides as well as the presence of flavones like apigenin and luteolin could not be approved in *Clidemia rubra* berries.

The content of non-anthocyanin phenolic compounds in *Clidemia rubra* berries seems to be considerably high. The amount of the identified flavonols is comparable to that found in Andean blackberries (Mertz *et al.*, 2007), cranberries, and bog whortleberries and is four times higher than that of bilberries, blueberries and red gooseberries from Finland (Häkkinen *et al.*, 1999).

**7. Antioxidant activity**

The results of the determination of the antioxidant activity of *Clidemia rubra* berries are demonstrated in **figure D.4.3**. The antioxidant activity was measured with TOSC assay against peroxyl radicals (px) and peroxynitrite (pn). To suppress the ethylene formation for 50%, a concentration of 0.9 g/L and 2.0 g/L of freeze-dried sample was needed against px and pn, respectively. Consequently, the WE of *Clidemia rubra* showed an about 2-fold higher radical scavenging potential towards px than against pn. The varying results between the two reactive oxygen species (ROS) can partially be explained by the difference in the reactivity of the two radicals (Halliwell *et al.*, 1995; Lichtenthäler and Marx, 2005). Generally, px have shown a half-life of several seconds. In regard to this relatively high stability, the radicals can easily be inactivated by potent antioxidant compounds even in low concentrations (Lichtenthäler *et al.*, 2003). In opposite to px, pn has exhibited a half-life of $10^{-2}$ sec. This lower lifetime and the following shorter period for a possible reduction require, on the one hand, a higher concentration of antioxidants or, on the other hand, antioxidants of a higher radical scavenging potential in order to obtain the inhibition rate like against px (Rodrigues *et al.*, 2006).

Lichtenthäler and Marx (2005) analyzed the antioxidant capacity of various commercially available fruit and vegetable juices with the TOSC assay. High values for the antioxidant capacity have been exhibited by anthocyanin-containing fruit juices. To achieve a TOSC value of 50% against px, lingonberry, blueberry, elderberry and sour cherry nectar correspond to Trolox equivalents (TE) in this order between 40.0 and 13.3 mmol/L. Juices of other fruits like oranges, grapes, lemons and apples indicate far lower antioxidant activity as they do not exceed a TE of 2.9 mmol/L. According to these results, the WE of *Clidemia rubra* can be ranked between the

elderberry juice and the sour cherry nectar. Unpublished data of a WE of Ecuadorian blackberries showed similar results regarding the antioxidant capacity of *Clidemia rubra* against px. The inhibitory effect of the WE of blackberries was equivalent to that of a Trolox solution of 15.5 mmol/L.

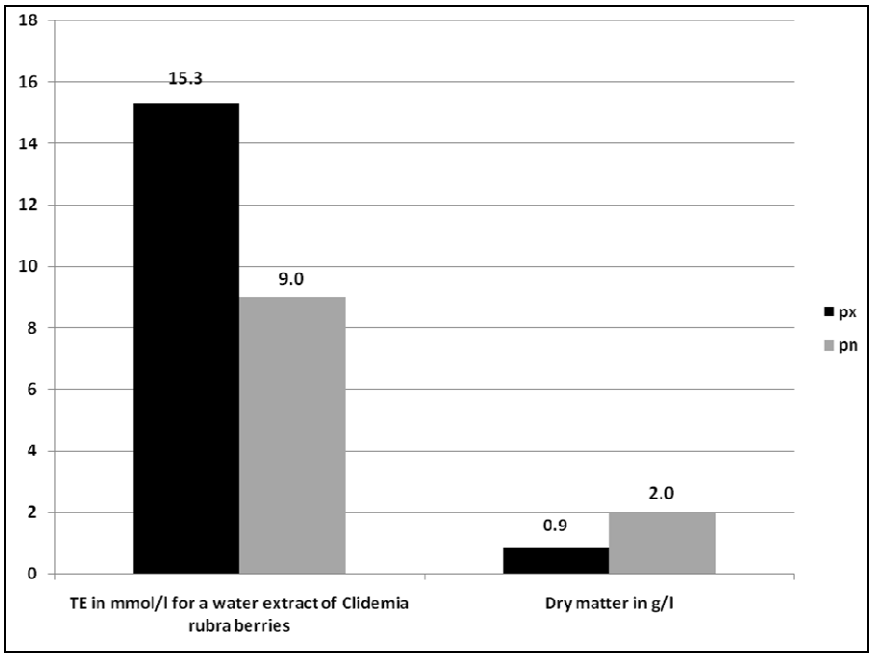


**Figure D.4.3.** Antioxidant capacity of *Clidemia rubra* berries. Values are expressed in Trolox equivalents (TE) and corresponding concentration of freeze-dried sample that is needed to obtain an inhibition rate of 50% with TOSC assay against peroxyl radicals (px) and peroxynitrite (pn).

Towards pn, a classification of *Clidemia rubra* berries is similar to px. In this order, blueberry juice, elderberry juice, lingonberry juice, sour cherry nectar, orange juice, and apple juice resemble a TE between 14.5 and 2.3 mmol/L. *Clidemia rubra* berries account for 9.0 mmol/L TE by what they can be ranked between lingonberry juice and sour cherry nectar. Compared to frequently consumed juices like that of oranges or apples, the antioxidant activity against pn is about three times higher.

The main part of the antioxidant capacity is ascribed to ascorbic acid and the polyphenols, especially to the anthocyanins. In order to estimate the contribution of the anthocyanins and ascorbic acid to the overall antioxidant capacity the WE was fractionated with HPLC. The first fraction (0-4 min) containing ascorbic acid and two fractions containing the anthocyanins (28-32 min and 32-36 min) exhibit the highest antioxidant capacity against px with 41%, 56%, and 42% TOSC, respectively (see **figure D.4.4**).

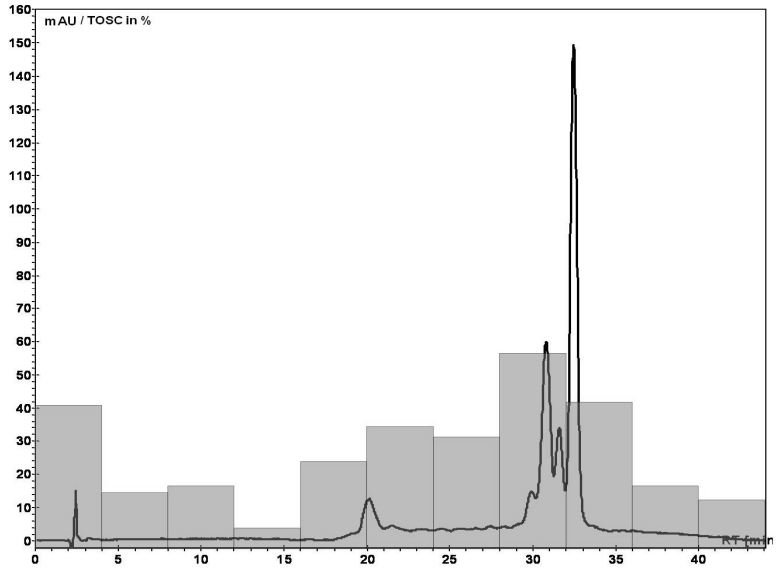


**Figure D.4.4.** Activity-guided fractionation of *Clidemia rubra* berries. HPLC chromatogram of a water extract recorded at 520 nm and TOSC values (bars) of the fractions against peroxyl radicals in %.

## D.5 Conclusion

This is the first report on a chemical characterization of the berries from *Clidemia rubra* (Aubl.) Mart. (Melastomataceae). From a nutritional point of view *Clidemia rubra* berries turned out be a good source for dietary fibers and some minerals (Ca, Mn, Zn) compared to other common berries. Ascorbic acid as well as different polyphenols were determined as secondary plant compounds with known antioxidant properties. Five phenolic acids, three flavan-3-ols and 11 different myricetin- and quercetin compounds were identified and quantified. Four different anthocyanins turned out to be mainly responsible for the bluish color as well as for the antioxidant capacity of the fruit. Due to the considerably high content in anthocyanins and other phenolic compounds *Clidemia rubra* berries are promising fruits that could contribute to the prevention of degenerative diseases.

## D.6 Acknowledgement

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# E Phenolic constituents and antioxidant capacity of four underutilized fruits from the Amazon region (published in Journal of Agricultural and Food Chemistry, 2011, 59, 7688–7699)

André Gordon[a], Elvira Jungfer[a], Bruno Alexandre da Silva[b], José Guilherme S. Maia[b], & Friedhelm Marx[a]

[a]University of Bonn, Department of Nutrition and Food Sciences, Endenicher Allee 11-13, 53115 Bonn, Germany

[b]Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos, Universidade Federal do Pará, 66075-900 Belém, PA, Brazil

Corresponding author:

Friedhelm Marx

Department of Nutrition and Food Sciences, University of Bonn, Endenicher Allee 11-13, 53115 Bonn, Germany.

Tel.: +49 228 733713; fax: +49 228 733757.

E-mail address: f.marx@uni-bonn.de

Own contribution: preparation of the manuscript, LC-MS data evaluation, antioxidant capacity analysis

## E.1 Abstract

The Amazon region comprises a plethora of fruit-bearing species of which a large number are still agriculturally unimportant. Because fruit consumption has been attributed to an enhanced physical well-being, interest in the knowledge of the chemical composition of underexplored exotic fruits has increased during recent years. This paper provides a comprehensive identification of the polyphenolic constituents of four underutilized fruits from the Amazon region by HPLC/DAD-ESI-$MS^n$. Araçá (*Psidium guineense*), jambolão (*Syzygium cumini*), muruci (*Byrsonima crassifolia*), and cutite (*Pouteria macrophylla*) turned out to be primarily good sources of hydrolyzable tannins and/or flavonols. Additionally, different flavanonols and proanthocyanidins were identified in some fruits. The antioxidant capacity was determined by using the total oxidant scavenging capacity (TOSC) assay. Cutite showed the highest antioxidant capacity followed by jambolão, araçá, and muruci.

## E.2 Introduction

The Amazon region is the largest tropical forest area in the world, and its flora bears a plenty of still unexplored or underutilized fruit species. Due to the postulated contribution to an enhanced human well-being and promotion of beneficial health effects against degenerative diseases, interest has arisen in exploiting new and exotic types of fruits during recent years (Schreckinger *et al.*, 2010). Promising species may also represent an opportunity for local growers to reach niche markets to increase their revenues (Alves *et al.*, 2008). However, many edible fruits have not attained economic importance as they are insufficiently studied with regard to their possibilities of commercialization, crop growing conditions, and chemical composition (Rodrigues *et al.*, 2006). As well, scientific information is scarce about the bioactive compounds of the locally popular Brazilian fruits araçá, jambolão, muruci, and cutite.

*Psidium guineense* Sw. (Myrtaceae), known as araçá, is a shrub or small tree between 4 and 6 m in height. The berry fruit is of spherical to egg-like shape, usually 1-3 cm in diameter with numerous 2-3 mm stony seeds. The pulp is sweet acetous in taste and is particularly used for preparing jellies, juices, and ice-cream (Lederman *et al.*, 1997). The fruit pericarp of araçá showed antimicrobial activity against *Staphylococcus aureus* and *Escherichia coli* (Anesini *et al.*, 1993).

*Syzygium cumini* (L.) Skeels (Myrtaceae), known as jambolão, is a tree that originates from India and Southeast Asia but is also widespread in some states across Brazil (Migliato *et al.*, 2010). The edible fruits are of oval shape and 2-3 cm long. The color of the peel is deep purple to black. Jambolão pulp has a grayish white color and embeds a big purple seed. Ripe fruits possess an aromatic sour astringent taste and are either eaten fresh (Kratochvil, 1995) or processed to

preserves, jellies and vine (Zhang and Lin, 2009). Fruits as well as bark, seeds, and leaves are traditionally used for diabetes treatment and different gastrointestinal disorders. Additionally, a fruit extract showed antimicrobial and cytotoxic activities and may potentially be used in topical antimicrobial products (Migliato *et al.*, 2010). In comparison to other nontraditional fruits from Brazil, jambolão showed considerably high antioxidant activity (Rufino *et al.*, 2010), which can be at least partly ascribed to the phenolic constituents such as anthocyanins (De Brito *et al.*, 2007), tannins (Zhang and Lin, 2009) and flavonols (Faria *et al.*, 2011).

*Byrsonima crassifolia* (L.) Kunth (Malpighiaceae), known as muruci, as well as a number of related species occur in the Amazon basin, suggesting that this may be its center of origin. It is a large shrub to a small tree of 2-6 m in height. Drupes are yellow with a diameter of 1.5-2 cm containing one or, rarely, two to three seeds (FAO, 1986). The soft pulp develops an exotic, very distinctive cheese-like aroma and is preferably consumed as a juice, jelly, confectionary, or liquor (Alves and Franco, 2003). Compared to six other exotic fruits including the well-known açaí, cashew apple, and acerola, muruci showed a high content of extractable polyphenols, although its radical scavenging capacity was reported to be low (Rufino *et al.*, 2009).

*Pouteria macrophylla* (Lam.) Eyma (Sapotaceae), known as cutite, is a small to medium forest tree up to 20-25 m in height. It develops egg-shaped berry fruits up to 6 cm in diameter with a starchy, yellow, soft pulp embedding a long ovoid seed. Cutite is always eaten as a fresh fruit characterized by an agreeable and generally sweet taste that is not always immediately appreciated by those who do not know it. Because of the starch content cutite supplies a reasonable amount of calories (FAO, 1986).

Only a few studies exist on the phenolic composition of jambolão fruits, and no studies have been published about individual phenolic substances in fruits of araçá, muruci, and cutite. Therefore, the aim of the study was to provide a comprehensive characterization of the phenolic constituents in the edible part of the four Amazonian fruits by HPLC/DAD-ESI-$MS^n$. In addition, the antioxidant capacity was assessed to evaluate their biological activity.

## E.3 Materials and methods

### 1. Chemicals

Ultrahigh quality (UHQ) water was prepared with a Direct-Q 3 system (Millipore, Billerica, USA). HPLC and extraction solvents were obtained from J.T. Baker (Griesheim, Germany). Diethylenetriaminepentaacetic acid (≥ 99%), α-keto-γ-methiolbutyric acid (KMBA) (≥ 97%), 2,2'-Azobis(2-methylpropionamidine) dichloride (≥ 97%), 3-morpholinosydnonimine N-ethylcarbamide, gallic acid (≥ 99%), and Folin-Ciocalteu's phenol reagent were obtained from

Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Ascorbic acid (≥ 95%) was purchased from Kraemer & Martin GmbH (Sankt Augustin, Germany).

**2. Fruit material and sample preparation**

Fruits were harvested at a particular farm, located in the municipality of São João de Pirabas, northeastern Pará, Brazil, in the months of November and December 2009. The fruits were identified with authentic samples deposited in the Herbarium of Museu Emilio Goeldi, city of Belém, state of Pará, Brazil.

After deseeding, the edible parts of the fruits (peel and flesh) were deep-frozen and freeze-dried immediately subsequent to harvest. Samples were air-shipped to Germany and stored at -30 °C prior to analysis.

**3. Identification of phenolic compounds by HPLC/DAD-ESI-MS$^n$**

Polyphenol extraction was carried out by using a modified pressurized liquid extraction method according to Papagiannopoulos *et al.* (2004). Freeze-dried sample (500 mg) was thoroughly ground and extracted with acetone-water-formic acid (70 + 29 + 1; v/v/v) in an accelerated solvent extractor (ASE 200, Dionex, Idstein, Germany) at room temperature, for 20 min in two cycles. The following solid-phase extraction (SPE) was performed by using a Gilson ASPEC XL system (Automated Sample Preparation with Extraction Cartridges, Abimed, Langenfeld, Germany). Polyamid (PA) SPE cartridges (500 mg PA, 3 mL cartridge, Macherey Nagel, Düren, Germany) were conditioned with 3 mL of dimethyl sulfoxide-formic acid-trifluoroacetic acid (DMSO-FAc-TFA) (98.7 + 1 + 0.3; v/v/v) and washed with 5 mL of UHQ water. Prior to cartridge loading, the sample extract was diluted to contain < 15% (v/v) of organic solvent. The cartridge was loaded with sample extract in volumetric steps of 20 mL until exhaustion and washed with 10 mL of water after each load. During elution with DMSO-FAc-TFA solvent, the first 0.5 mL was discarded and the next 1.25 mL collected. Before application to HPLC/DAD-ESI-MS$^n$, the samples were filtered through a 1.0/0.45 µm syringe filter (Chromafil GF/PET-45/25, Macherey-Nagel).

Analysis of polyphenolic compounds was performed following a HPLC/DAD-ESI-MS$^n$ method according to Papagiannopoulos *et al.* (2004). The liquid chromatograph was a Summit system (Dionex, Germering, Germany) consisting of a P-580 A HPG pump, an ASI-100 T automated sample injector, a STH-585 column oven, and an UVD-340S detector equipped with a capillary cell. Chromeleon software package v6.7 SP2 (Dionex) was used for system control and data evaluation. Separation was carried out with the help of an analytical column Aqua RP 18, 150 mm, 2 mm i.d., 3 µm with a guard column Security Guard, C18, 4mm, 2mm i.d. (both Phenomenex, Aschaffenburg, Germany) kept at 25 °C. Solvents were UHQ water with 1% acetic

acid (v/v) (mobile phase A) and acetonitrile with 1% acetic acid (v/v) (mobile phase B). The gradient elution program using a flow rate of 0.2 mL/min started with 0.5% B, rose to 40% B after 32 min and to 100% B after 34 min, and was kept at 100% B for 9 min. The column was re-equilibrated for 15 min with initial conditions. For analysis, 20 µL of each sample was injected. An LCQ classic ion trap mass spectrometer (MS) equipped with an electrospray interface was coupled to the HPLC and controlled with Xcalibur software v1.2 (all Thermo Fisher Scientific, Dreieich, Germany). Settings for the negative ionization with MS were as follows: source voltage -4.0 kV, sheath gas flow 90, auxillary gas flow 60, capillary voltage -10 V, capillary temperature 300 °C, tube lens offset +20 V, first octapole offset +4 V, interoctapole lens +30 V, second octapole offset +10 V, and trap DC offset +10 V.

The identification of phenolic compounds was performed with authentic standards in cases of gallic acid, quercetin, and myricetin. All other compounds were tentatively identified by combining characteristic data of HPLC elution order of compounds and UV spectra with those of mass spectrometrical fragmentation analysis. Additionally, compound assignment was supported by comparison with data from the literature when available.

**4. Total oxidant scavenging capacity (TOSC) assay**

The antioxidant capacity of the fruits was determined with the TOSC assay performed as described by Lichtenthäler *et al.* (2003). Briefly, the TOSC assay is based on an ethylene-yielding reaction of KMBA with either peroxyl radicals or peroxynitrite. Antioxidant compounds present in the sample can inhibit the ethylene formation that is recorded in a time course of 1 h using automatically repeated headspace GC analysis (GC-17A, Shimadzu, Tokyo, Japan). Each fruit was analyzed in duplicate. Quantification of generated ethylene results in a kinetic curve of which the area under the curve (AUC) is calculated. Only those data with a variance (standard deviation/arithmetic mean) of the AUC after repeat determination below 5% are further processed. Mean data of a sample are compared to those of an uninhibited reaction with water, which gives rise to the TOSC values. Results of this study indicate the concentration of antioxidants present in the sample in grams per liter that is needed to obtain a radical inhibition of 50%.

For TOSC analysis, freeze-dried sample (1 g) of each fruit was suspended in UHQ water to obtain a total weight of 10 g (w/w). The suspension was sonicated for 10 min and centrifuged for 7 min at 10000 rpm with a Heraeus Biofuge Stratos (Kendro, Langenselbold, Germany). The supernatant of the water extract (WE) was stored until further application at -30 °C.

**5. Total phenolic content**

Total phenolic content was determined by using the Folin-Ciocalteu assay described by Georgé *et al.* (2005). Briefly, 500 µL of water-diluted Folin-Ciocalteu reagent (9 + 1, v/v) and 100 µL of the WE were mixed. After incubation for 2 min at room temperature, 400 µL of sodium carbonate (7.5 g/100 mL) was added. The mixture was incubated at 50 °C for 15 min and subsequently photometrically measured (Cary 50, Varian, Walnut Creek, USA) at 760 nm. In total, two water extracts were prepared per fruit and analyzed in duplicate. Seven dilutions (10-100 mg/L) of a gallic acid standard were used to create a calibration curve ($r^2 = 0.9980$). Results are expressed as gallic acid equivalents in milligrams per 100 gram dry matter.

**6. Determination of ascorbic acid**

Ascorbic acid was determined by HPLC after modification of a method previously described by Gordon *et al.* (2011). The HPLC-DAD system of PRO Star series (Varian) was equipped with an analytical column Synergi 4 µ Hydro RP, 150 mm, 2 mm i.d. and with a guard column Security Guard, C 18, 4 mm, 2 mm i.d. (both Phenomenex, Aschaffenburg, Germany). The separation was performed with acidified UHQ water (1% FAc, v/v) at isocratic condition using a flow rate of 0.3 mL/min. The injection volume was 20 µL. Confirmation of ascorbic acid in the fruits was arranged by standard, retention time, and doping of standard to the sample. A five-point calibration curve (5-100 mg/100 mL, $r^2 = 0.9995$) was created for quantification with authentic standard. Ascorbic acid was quantified at a wavelength 260 nm. Two sample extracts were prepared and measured in duplicate.

## E.4 Results and discussion

**1. HPLC/DAD-ESI-MS$^n$ analysis of phenolic compounds**

Most of the detected compounds shown in **tables E.4.1-E.4.4** can be classified into hydrolyzable tannins (gallotannins, galloylquinic acids, and ellagitannins), condensed tannins (proanthocyanidins), flavonols, and flavanonols. At first, spectral data were used for a distinction of these different compound groups. According to Cantos *et al.* (2003) and Boulekbache-Makhlouf *et al.* (2010), the obtained UV spectra of the hydrolyzable tannins can generally be arranged into two groups. The first group comprises compounds derived from ellagic acid with two absorption maxima at $\lambda_{max}$ ~250 nm and ~365 nm. The second group has only one maximum available at $\lambda_{max}$ ~275 nm, typically found for galloyl and hexahydroxydiphenoyl (HHDP) derivatives. A condensed HHDP molecule gives rise to ellagic acid, for which reason they are also considered to be ellagitannins (Cantos *et al.*, 2003). Exemplary structures of these compounds are shown in **figure E.4.1**. UV spectra of proanthocyanidins are identical with those

of catechins showing two maxima at ~230 nm and ~280 nm. Flavonol and Flavanonol glycosides come with two absorption maxima derived from the conjugated system of the aglycones. The first maximum of ~260 nm is attributed to the benzoyl system (ring A), the second maximum of ~350 nm to the cinnamoyl system (ring B) (Engelhardt and Galensa, 1997).

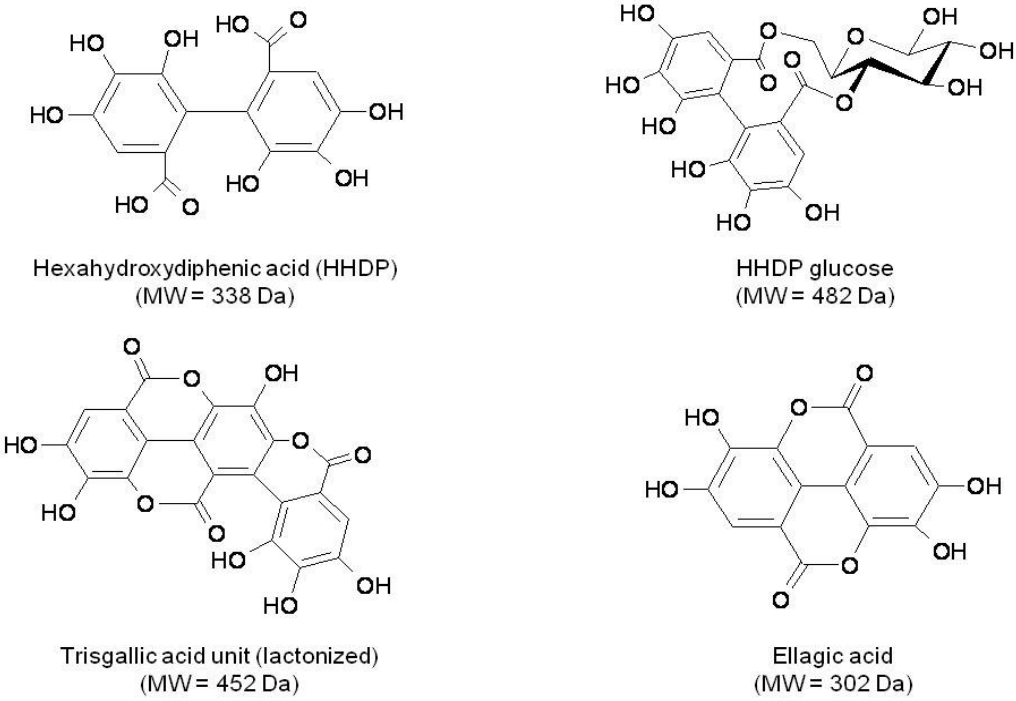


**Figure E.4.1**. Gallic acid derivatives [according to Hager *et al.* (2008)] occurring in araçá, cutite, or jambolão with corresponding molecular weight (MW).

In addition to the UV spectra, mass spectrometrical fragmentation experiments enable at least a tentative identification of the phenolic compounds. $MS^n$ analysis allows the distinction between individual flavonols or flavanonols, the elucidation of proanthocyanidins and the composition of hydrolyzable tannins (Papagiannopoulos *et al.*, 2004).

In the following, all mass spectrometrically identified sugar moieties of gallotannins and HHDP hexosides of will be tentatively characterized as glucose due to its predominant abundance within these compound groups (Hagerman, 2002).

### *Araçá* (*Psidium guineense*)

The HPLC chromatogram of the araçá extract is shown in **figure E.4.2**. According to **table E.4.1**, a total of 18 polyphenolic compounds could be at least tentatively identified. All of them were classified as ellagitannins with exception of peaks 8, 9, and 12, which belong to the gallotannins. Identification of gallic acid in peak 1 was assured by using an authentic standard. Peaks 8 and 12 were presumably assigned to derivatives of galloyl glucose. Peak 8 provides parental $[M\text{-}H]^-$ ions at $m/z$ 483 and $MS^2$ fragments typically found for digalloylglucose (Sandhu and Gu, 2010). As peak 12 shows a $[M\text{-}H]^-$ ion at $m/z$ 635 and a fragment at $m/z$ 483, the neutral

loss of 152 Da gives rise to the presence of an additional esterified galloyl residue conform to a trigalloylglucose. Peak 9 provides a $[M-H]^-$ ion at $m/z$ 453 and gives two $MS^2$ fragments at $m/z$ 313 and 169. Due to the mass difference of 30 Da, conforming UV data and the similarity of some fragments in comparison to peak 8, this compound is tentatively assigned to digalloylpentoside.

Mass spectrometric data of peaks 4, 7, and 10 correspond to those of galloyl-HHDP glucose derivatives in grapes (Sandhu and Gu, 2010) and fruits of *Eucalyptus* (Boulekbache-Makhlouf *et al.*, 2010). Peak 4 shows $[M-H]^-$ ions at $m/z$ 633 and produces daughter ions at $m/z$ 421, 275, and 301 matching with those of HHDP galloylglucose (Boulekbache-Makhlouf *et al.*, 2010; Sandhu and Gu, 2010). Peaks 7 and 10 correspond to HHDP digalloylglucose isomers having a parental $[M-H]^-$ ion at $m/z$ 785 and characteristic product ions at $m/z$ 633, 483, and 301 (*23*). Peaks 13 and 14 show $[M-H]^-$ ions at $m/z$ 933 and give among others daughter fragments at $m/z$ 451 and 301. These compounds were tentatively assigned to castalagin/vescalagin isomers as proposed by Hager et al. (2008) due to the according fragmentation pattern. On the basis of UV data, the product ion at $m/z$ 301 indicates the presence of a HHDP derivative rather than that of ellagic acid. The neutral loss of 482 Da from the parent ion suggests the existence of a HHDP glucose unit. The resulting fragment ion after this neutral loss at $m/z$ 451 is consistent with that of a trisgalloyl unit (see **figure E.4.1**) after undergoing lactonization (Hager *et al.*, 2008). In return, the neutral loss of 452 Da accounting for a lactonized trisgalloyl unit is indicated by the daughter ion at $m/z$ 481 in peak 14. The fragment ion at $m/z$ 631 in peak 13 may result from the loss of two galloyl units from the quasi-molecular ion (Hager *et al.*, 2008).

The occurrence of di-HHDP glucose derivatives was presumably assessed in peaks 2, 3, 5, 6, and 11. All compounds have a shift in the UV spectrum to $\lambda_{max}$ ~260 nm in common. In peaks 2 and 5, a parental $[M-H]^-$ ion at $m/z$ 783 is present producing fragment ions at $m/z$ 301, 481, and 275 in $MS^2$ and additionally two fragment ions at $m/z$ 257 and 229 in $MS^3$. These fragments are characteristic for di-HHDP glucose found also in cork of *Quercus suber* (Fernandes *et al.*, 2011), in blackberries (Hager *et al.*, 2008), and strawberries (Seeram *et al.*, 2006). Peaks 3, 6, and 11 show $[M-H]^-$ ions at $m/z$ 951 that yield fragment ions at $m/z$ 907 and 783. Compounds with the same fragmentation pattern were suggested to be trisgalloyl HHDP glucose isomers (Boulekbache-Makhlouf *et al.*, 2010; Barry *et al.*, 2001). UV data and the fragment ion at $m/z$ 783 indicate the occurrence of di-HHDP glucose. The fragment $[M-H-168]^-$ accounts for the presence of an additional galloyl residue but only with a C-C linkage to one of the HHDP molecules. The loss of 44 Da ($CO_2$) agrees with the presence of a free, unesterified carboxyl group (Barry *et al.*, 2001).

UV spectra of peaks 15-18 match with those of ellagic acid (Cantos *et al.*, 2003). Peak 16 shows $[M-H]^-$ ions at $m/z$ 447 and yields fragment ions at $m/z$ 301 ($MS^2$) and 257 ($MS^3$) that were also found for ellagic acid (Sandhu and Gu, 2010). Due to the neutral loss of 146 Da, peaks 15 and 16 are tentatively assigned to ellagic acid deoxyhexoside isomers. The late retention time is an argument for the occurrence of dimethylated ellagic acid hexoside in peak 17. Parental $[M-H]^-$ ions at $m/z$ 491 and fragmentation pattern ($m/z$ 329, 313) coincide to some extent with that of a dimethylated ellagic acid glucoside described by Boulekbache-Makhlouf et al. (2010). Peak 18 shows $[M-H]^-$ ions at $m/z$ 461 and produces $MS^n$ fragment ions at $m/z$ 315, 301, and 300 corresponding to a methylated ellagic acid (Boulekbache-Makhlouf *et al.*, 2010). Due to the mass difference of 30 Da from peak 17, this compound is tentatively assigned to dimethylellagic acid pentoside.

| Peak | Retention time | Compound[a, b] | HPLC-DAD $\lambda_{max}$ [nm] | $[M-H]^-$ $m/z$ | Fragments ($m/z$) |
|---|---|---|---|---|---|
| 1 | 9.84 | Gallic acid | 273 | 169 | $MS^2$ [169]: - |
| 2 | 13.83 | Di-HHDP glucose[3, 4, 5] | 228, 260 | 783 | $MS^2$ [783]: 301, 481, 275<br>$MS^3$ [783 → 301]: 257, 229 |
| 3 | 15.34 | Trisgalloyl HHDP glucose isomer[1, 6] | 227, 262 | 951 | $MS^2$ [951]: 907, 783<br>$MS^3$ [951 → 907]: 783 |
| 4 | 15.86 | HHDP galloylglucose[1, 2] | 226, 275 | 633 | $MS^2$ [633]: 301, 275, 421 |
| 5 | 17.54 | Di-HHDP glucose[3, 4, 5] | 228, 260 | 783 | $MS^2$ [783]: 301, 481, 275<br>$MS^3$ [783 → 301]: 257 |
| 6 | 18.63 | Trisgalloyl HHDP glucose isomer[1, 6] | 232, 263 | 951 | $MS^2$ [951]: 907, 783<br>$MS^3$ [951 → 907 ]: 783 |
| 7 | 19.47 | HHDP digalloylglucose isomer[1, 2] | 225, 280 | 785 | $MS^2$ [785]: 301, 633, 275, 483, 615, 419 |
| 8 | 20.83 | Digalloyl glucose[2] | 224, 273 | 483 | $MS^2$ [483]: 439, 313, 271, 331, 169<br>$MS^3$ [483 → 439]: 287, 313 |
| 9 | 21.59 | Digalloyl pentose | 224, 280 | 453 | $MS^2$ [453]: 391, 313, 169 |
| 10 | 22.58 | HHDP digalloylglucose isomer[1, 2] | 225, 276 | 785 | $MS^2$ [785]: 301, 483, 633, 275<br>$MS^3$ [785 → 301]: 257 |
| 11 | 22.87 | Trisgalloyl HHDP glucose isomer[1, 6] | 235, 258 | 951 | $MS^2$ [951]: 907, 783<br>$MS^3$ [951 → 907]: 783 |
| 12 | 24.83 | Trigalloyl glucose[2] | 224, 281 | 635 | $MS^2$ [635]: 423, 483, 271, 465, 193<br>$MS^3$ [635 → 423]: 271 |
| 13 | 25.26 | Castalagin/vescalagin isomer[3] | 226, 282 | 933 | $MS^2$ [933]: 451, 631, 301<br>$MS^3$ [933 → 451]: 351, 433, 285, 407, 311 |
| 14 | 27.38 | Castalagin/vescalagin isomer[3] | 225, 289 | 933 | $MS^2$ [933]: 451, 351, 301, 481<br>$MS^3$ [933 → 451]: 351, 285, 433, 407, 335, 379 |
| 15 | 28.73 | Ellagic acid deoxyhexoside | 252, 371 | 447 | $MS^2$ [447]: 301 |
| 16 | 29.13 | Ellagic acid deoxyhexoside | 256, 362 | 447 | $MS^2$ [447]: 301<br>$MS^3$ [ 447 → 301]: 257 |
| 17 | 30.63 | Dimethylellagic acid hexoside | 249, 368 | 491 | $MS^2$ [491]: 328, 313, 329, 454, 476<br>$MS^3$ [491 → 328]: 313, 285 |
| 18 | 34.18 | Dimethylellagic acid pentoside | 252, 363 | 461 | $MS^2$ [461]: 315, 300<br>$MS^3$ [ 461 → 315]: 300, 301 |

**Table E.4.1.** UV and mass spectrometric data of phenolic constituents extracted from araçá (*Psidium guineense*) fruits. [a]Italic superscript numbers indicate the literature in which the compounds were previously described: [1]Boulekbache-Maklouf *et al.*, 2010; [2]Sandhu and Gu, 2010; [3]Hager *et al*, 2008; [4]Fernandes *et al.*, 2011, [5]Seeram *et al.*, 2006; [6]Barry *et al.,* 2001. [b]Gallic acid was identified with authentic standard; all other compounds were tentatively identified.

### *Jambolão (Syzygium cumini)*

The HPLC chromatogram of the jambolão extract is shown in **figure E.4.2**. According to **table E.4.2**, a total of 37 non-anthocyanin polyphenolic compounds could be identified or at

least tentatively assigned. They were classified as gallotannins, ellagitannins, flavonols, and flavanonols. Identification of gallic acid in peak 1 was assured by using an authentic standard.

As $MS^n$ data of peaks 8-10, 12, 13, 15, 16, 18, 20, 24a, 26a, and 29 agree with those of different gallotannins described by Sandhu and Gu (2010). Hence, these compounds were tentatively assigned to a series of galloylglucose esters starting from isomers of digalloylglucose ($[M-H]^-$ at $m/z$ 483) to hexagalloylglucose ($[M-H]^-$ at $m/z$ 1091). Peak 14 shows to some extent mass spectrometric attributes of HHDP galloylglucose previously found in grape seeds (Sandhu and Gu, 2010). However, elucidation of this compound could only conditionally be ascertained due to unutilizable UV data. Peak 17 gives parental $[M-H]^-$ ions at $m/z$ 775 and produces dominating $MS^2$ fragment ions at $m/z$ 613 and 451. Both fragments indicate the sequential loss of hexosyl units with $[M-H-162]^-$ and $[M-H-162-162]^-$. The product ion at $m/z$ 451 suggests the existence of a trisgalloyl residue (Hager *et al.*, 2008) which was already discussed for ellagitannins occurring in araçá (**figure E.4.1**). Hence, this compound is tentatively identified as trisgalloyldiglucoside.

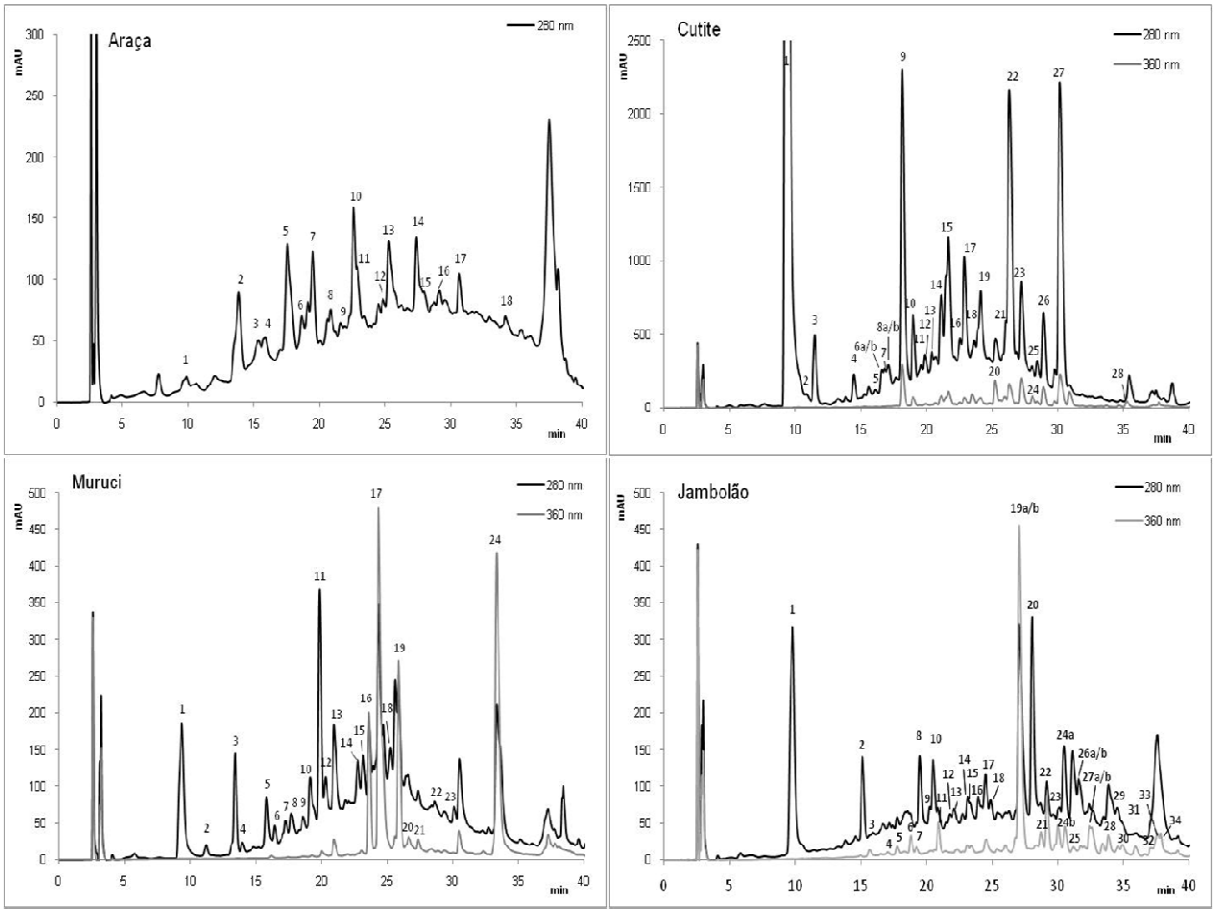


**Figure E.4.2.** HPLC Chromatograms of four different fruits from the Amazon region. The chromatograms of araçá, jambolão, muruci, and cutite correspond to **tables E.4.1-E.4.4**. The numbered peaks are denoted in the accordant table.

A large number of myricetin-derived compounds were presumptively identified on the basis of UV and mass spectrometric fragmentation data in peaks 19a, 19b, 22, 28, and 33. Myricetin was

identified in peak 30 by comparing fragments with those of an authentic standard. Mass spectra obtained from the other myricetin derived constituents indicate at least the presence of the aglycone at $m/z$ 317. The occurrence of two coeluting myricetin compounds is supposably revealed in peak 19a/b. Peak 19a yields fragments of $[M-H-162]^-$ and peak 19b fragments of $[M-H-176]^-$ corresponding to a myricetin hexoside and myricetin glucuronide, respectively. In previous studies, glucose was the only identified hexoside in jambolão (Faria *et al.*, 2011). Hence, peak 19a may be assigned to myricetin glucoside, which was already described by Faria *et al.* (2011). Peak 22 is ascribed to myricetin deoxyhexoside due to the loss of 146 Da from $[M-H]^-$ ions at $m/z$ 463. According to Faria *et al.* (2011), myricetin rhamnoside (myricitrin) likely occurs in this peak as rhamnose makes up the only deoxyhexoside commonly found in fruits. The loss of 42 Da from $[M-H]^-$ ions at $m/z$ 505 in peak 28 indicates the presence of acylated myricetin deoxyhexoside that was already constituted in jambolão fruits (Faria *et al.*, 2011) and jambolão leaves (Mahmoud *et al.*, 2001). Peak 33 shows parent $[M-H]^-$ ions at $m/z$ 657 and produces dominating fragment ions at $m/z$ 505. These mass data are in agreement with that of acylated galloylmyricetin deoxyhexoside previously found in leaves of jambolão (Mahmoud *et al.*, 2001). Peaks 21, 23, 24b, 25, 26b, 32, and 34 were presumably identified as methylmyricetin derivatives. Peak 23 indicates the presence of methylmyricetin hexoside as the parent ion at $m/z$ 493 results in fragment ions at $m/z$ 331 after neutral loss of 162 Da. The dominating daughter ion at $m/z$ 331 would account for the aglycone methylmyricetin (Mahmoud *et al.*, 2001). Because the flavonol mearnsetin (myricetin 4'-methyl ether) was found in jambolão leaves (Mahmoud *et al.*, 2001), it likely occurs also in the fruits. As reported by Faria *et al.* (2011) glucose is the verisimilar occurring hexoside in jambolão. Peak 21 was tentatively identified as galloylmethylmyricetin hexoside showing $[M-H]^-$ ions at $m/z$ 645 and dominating daughter ions at $m/z$ 493. The neutral loss of 132 Da in peaks 24b and 26b as well as the loss of 176 Da in peak 25 would be in agreement with the presence of methylmyricetin pentoside isomers and methylmyricetin glucuronide, respectively. The sequential neutral loss of 42 Da and 146 Da in peak 32 may account for the occurrence of acylated methylmyricetin deoxyhexoside. This compound could be more precisely ascribed to acylated mearnsetin rhamnoside, which was identified in jambolão leaves (Mahmoud *et al.*, 2001). Finally, peak 34 shows $[M-H]^-$ ions at $m/z$ 645 and produces fragments at $m/z$ 331 and 505. This corresponds to the loss of a galloyl residue (152 Da) in addition to an acylated pentose unit (42 Da + 132 Da) tentatively resulting in acylated galloyl ester of methylmyricetin pentoside.

| Peak | Retention time | Compound[a, b] | HPLC-DAD $\lambda_{max}$ [nm] | [M-H]$^-$ *m/z* | Fragments (*m/z*) |
|---|---|---|---|---|---|
| 1 | 9.78 | Gallic acid | 225, 273 | 169 | MS² [169]: 125, 151 |
| 2 | 15.10 | Gallic acid derivative | 226, 277 | 285 | MS² [285]: 133, 169, |
| 3 | 15.65 | Dihydromyricetin dihexoside[1] | 237, 338 | 643 | MS² [643]: 463, 481, 283, 355 |
| 4 | 16.68 | Methyldihydromyricetin dihexoside[1] | 224, 277 | 657 | MS² [657]: 495, 477, 315, 355<br>MS³ [657 → 495]: 315, 333, 369 |
| 5 | 17.75 | Unknown compound | 225, 277 | 625 | MS² [625]: 419, 257, 463, 581<br>MS³ [625 → 419]: 257, 404, 242 |
| 6 | 18.79 | Methyldihydromyricetin dihexoside[1] | 253, 342 | 657 | MS² [657]: 495, 315, 477, 333, 297, 355<br>MS³ [657 → 495]: 315, 333, 369 |
| 7 | 19.23 | Dimethyldihydromyricetin dihexoside[1] | 243, 345 | 671 | MS² [671]: 509<br>MS³ [671 → 509]: 347, 329 |
| 8 | 19.48 | Digalloylglucose[2] | 224, 278 | 483 | MS² [483]: 271, 331, 211, 169<br>MS³ [483 → 271]: 211, 169 |
| 9 | 20.20 | Trigalloylglucose[2] | 227, 291 | 635 | MS² [635]: 465, 483, 313, 271<br>MS³ [635 → 483]: 271 |
| 10 | 20.47 | Digalloylglucose[2] | 224, 271 | 483 | MS² [483]: 439, 313, 287, 465<br>MS³ [483 → 313]: 169 |
| 11 | 20.87 | Dimethyldihydromyricetin dihexoside[1] | 223, 335 | 671 | MS² [671]: 509<br>MS³ [671 → 509]: 347, 371, 329 |
| 12 | 21.76 | Trigalloylglucose[2] | 225, 285 | 635 | MS² [635]: 465, 483, 313<br>MS³ [635 → 483]: 271 |
| 13 | 22.04 | Trigalloylglucose[2] | 224, 284 | 635 | MS² [635]: 483, 465, 271<br>MS³ [635 → 483]: 423 |
| 14 | 23.13 | HHDP galloylglucose[2] | - | 633 | MS² [633]: 615, 463, 505, 283, 571, 301<br>MS³ [633 → 615]: 463, 505, 571 |
| 15 | 23.40 | Trigalloylglucose[2] | - | 635 | MS² [635]: 465, 483, 313, 617<br>MS³ [635 → 465]: 131, 169 |
| 16 | 23.10 | Trigalloylglucose[2] | 222, 278 | 635 | MS² [635]: 465, 483, 313, 617<br>MS³ [635 → 465]: 313, 169 |
| 17 | 24.49 | Trisgalloyl diglucose | 242, 267, 359 | 775 | MS² [775]: 613, 451, 285<br>MS³ [775 → 613]: 451, 407, 285 |
| 18 | 24.88 | Tetragalloyl glucose[2] | 225, 283 | 787 | MS² [787]: 635, 617, 465, 447 |
| 19a | 27.05 | Myricetin hexoside[1] | - | 479 | MS² [479]: 317 |
| 19b | 27.05 | Myricetin glucuronide | - | 493 | MS² [493]: 317<br>MS³ [493 → 317]: 179, 151, 194 |
| 20 | 28.03 | Tetragalloylglucose[2] | 224, 280 | 787 | MS² [787]: 617, 635, 465, 313<br>MS³ [787 → 617]: 465, 573, 447, 403, 313 |
| 21 | 28.69 | Galloylmethylmyricetin hexoside | 224, 262, 360 | 645 | MS² [645]: 493, 331, 479, 316<br>MS³ [645 → 493]: 331 |
| 22 | 29.14 | Myricetin deoxyhexoside[1] | 224, 265, 352 | 463 | MS² [463]: 317<br>MS³ [463 → 317]: 179, 272, 151 |
| 23 | 29.99 | Methylmyricetin hexoside | 225, 264, 360 | 493 | MS² [493]: 331, 301, 315<br>MS³ [493 → 331]: 315, 301, 179 |
| 24a | 30.47 | Pentagalloylglucose[2] | 224, 264 | 939 | MS² [939]: 769, 787, 617, 599<br>MS³ [939 → 769]: 617, 599 |
| 24b | 30.47 | Methylmyricetin pentoside | 224, 264, 360 | 463 | MS² [463]: 331, 301<br>MS³ [463 → 331]: 301 |
| 25 | 31.10 | Methylmyricetin glucuronide | 224, 287, 352 | 507 | MS² [507]: 331, 317<br>MS³ [507 → 331]: 301 |
| 26a | 31.57 | Pentagalloylglucose[2] | 224, 287 | 939 | MS² [939]: 787, 769, 617<br>MS³ [939 → 787]: 617, 635, 465 |
| 26b | 31.57 | Methylmyricetin pentoside | 224, 287, 352 | 463 | MS² [463]: 331, 301<br>MS³ [463 → 331]: 315 |
| 27a | 32.41 | Dimethylmyricetin hexoside | 225, 260, 355 | 507 | MS² [507]: 345<br>MS³ [507 → 345]: 330, 301, 315, 271 |
| 27b | 32.41 | Dimethylmyricetin pentoside | 225, 260, 355 | 477 | MS² [477]: 331, 315<br>MS³ [477 → 331]: 316 |
| 28 | 33.85 | Acylated myricetin deoxyhexoside[1, 3] | 223, 267, 354 | 505 | MS² [505]: → 316, 463<br>MS³ [505 → 316]: 271, 287, 179 |
| 29 | 34.1 | Hexagalloylglucose[2] | - | 1091 | MS² [1091]: 939, 787<br>MS³ [1091 → 787]: 617, 635, 465 |
| 30 | 34.89 | Myricetin | 260, 376 | 317 | MS² [317]: 179, 151 |
| 31 | 35.89 | Dimethylmyricetin pentoside | 228, 362 | 477 | MS² [477]: 344, 329 |
| 32 | 37.07 | Acylated methylmyricetin deoxyhexoside[3] | 263, 350 | 519 | MS² [519]: 315, 331, 477 |
| 33 | 37.56 | Acylated galloylmyricetin deoxyhexoside[3] | 223, 283 | 657 | MS² [657]: 505, 317, 597<br>MS³ [657 → 317]: 179, 271 |
| 34 | 37.83 | Acylated galloyl ester of methylmyricetin pentoside | 257, 363 | 657 | MS² [657]: 517, 331, 505<br>MS³ [657 → 331]: 316 |

**Table E.4.2.** UV and mass spectrometric data of phenolic constituents extracted from jambolão (*Syzygium cumini*) fruits. [a]Italic superscript numbers indicate the literature in which the compounds were previously described: [1]Faria *et al.*, 2011; [2]Sandhu and Gu, 2010; [3]Mahmoud *et al.*, 2001. [b]Gallic acid and myricetin were identified with authentic standard; all other compounds were tentatively identified.

Due to $MS^2$ ($m/z$ 345) and $MS^3$ ($m/z$ 330 and 315) data, a dimethylmyricetin is presumptively identified in peak 27a. The loss of 162 Da from $[M-H]^-$ ions at $m/z$ 507 indicates the presence of dimethylmyricetin hexoside. $MS^n$ data of peaks 27b and 31 as well as the mass difference of 30 Da in comparison to peak 27a give rise to the likely occurrence of two dimethylmyricetin pentoside isomers.

All flavanonols in jambolão fruits occur as dihexosides. Mass spectrometric data of the flavanonols are in agreement with those described by Faria *et al.* (2011). Aglycones of methyldihydromyricetin ($[M-H-162-162]^-$ at $m/z$ 333) were tentatively identified in peaks 4 and 6 after neutral loss of two hexose units. Peaks 7 and 11 are presumably assigned to be isomers of dimethyldihydromyricetin dihexoside ($[M-H-162-162]^-$ at $m/z$ 347). Elution time and parental $[M-H]^-$ ions at $m/z$ 643 indicate the presence of dihydromyricetin dihexoside in peak 3. However, peak 3 could only tentatively be denoted as dihydromyricetin dihexoside. Our $MS^2$ data are significantly in accordance with those previously found by Faria *et al.* (2011) but lack the presence of the aglycone fragment.

***Muruci (Byrsonima crassifolia)***

The HPLC chromatogram of the muruci extract is shown in **figure E.4.2**. According to **table E.4.3**, a total of 19 polyphenolic compounds could be at least tentatively identified as gallotannins, quinic acid gallates, proanthocyanidins, and quercetin derivatives. Five compounds could only be specified as gallic acid derivatives. Identification of gallic acid in peak 1 was assured by comparison of the fragments with those of an authentic standard.

As already discussed in the section on araçá peaks 7 and 8 were tentatively assigned to digalloyl glucose and digalloyl pentose, respectively. Both peaks basically coincide in terms of their fragmentation pattern with these already described compounds. Peaks 2, 4-6, 10, and 11 were presumably found to be a series of quinic acid gallates showing a typical UV spectrum of gallic acid. Peak 2 produces $[M-H]^-$ ions at $m/z$ 343 that yield fragment ions at $m/z$ 169 and 125. The neutral loss of 174 Da corresponds to quinic acid (192 Da – $H_2O$) accounting for galloylquinic acid. Peak 11 is supposedly assigned to tetragalloylquinic acid producing $[M-H]^-$ ions at $m/z$ 799. Yielded $[M-H-152]^-$ ions of this peak at $m/z$ 647 are also shown in peaks 5 and 10 as parental $[M-H]^-$ ions suggesting the presence of trigalloylquinic acid. Peaks 4 and 6 suffer from the loss of a galloyl residue resulting in $[M-H-152]^-$ ions at $m/z$ 343. Hence, these compounds are tentatively assigned to digalloylqunic acid. The presence of galloylquinic acid esters in muruci fruits is supported by Maldini *et al.* (2011). In this paper, 5-O-galloylquinic acid, 3-O-galloylquinic acid, 3,4-di-O-galloylquinic acid, 3,5-O-galloylquinic acid, and 3,4,5-tri-O-galloylquinic acid were identified in *B. crassifolia* bark by NMR and MS, which let assume the occurrence of these

compounds also in the fruit. Mass spectrometric data of galloylquinic acids found in this study are in accordance with those determined in green tea (Clifford *et al.*, 2007).

| Peak | Retention time | Compound[a, b] | HPLC-DAD $\lambda_{max}$ [nm] | [M-H]$^-$ *m/z* | Fragments (*m/z*) |
|---|---|---|---|---|---|
| 1 | 9.35 | Gallic acid | 225, 273 | 169 | MS$^2$ [169]: 151, 125 |
| 2 | 11.21 | Galloylquinic acid[2] | 226, 276 | 343 | MS$^2$ [343]: 169, 125 |
| 3 | 13.41 | Gallic acid derivative | 225, 277 | 285 | MS$^2$ [285]: 169, 133 |
| 4 | 13.96 | Digalloylquinic acid[2] | 225, 276 | 495 | MS$^2$ [495]: 343, 325, 169<br>MS$^3$ [495 → 343]: 169 |
| 5 | 15.80 | Trigalloylquinic acid[2] | 225, 275 | 647 | MS$^2$ [647]: 477, 325<br>MS$^3$ [647 → 477]: 325, 169, 307 |
| 6 | 16.40 | Digalloylquinic acid[2] | 226, 279 | 495 | MS$^2$ [495]: 343, 325, 169<br>MS$^3$ [495 → 343]: 169 |
| 7 | 17.27 | Digalloyl glucose[1] | 225, 272 | 483 | MS$^2$ [483]: 439, 313, 271<br>MS$^3$ [483 → 439]: 313, 287 |
| 8 | 17.67 | Digalloyl pentose | 226, 280 | 453 | MS$^2$ [453]: 313, 327, 285, 169<br>MS$^3$ [453 → 313]: 169 |
| 9 | 18.58 | Proanthocyanidin dimer[3] | 228, 282 | 577 | MS$^2$ [577]: 425, 407, 451, 289<br>MS$^3$ [577 → 425]: 407 |
| 10 | 19.12 | Trigalloylquinic acid[2] | 226, 277 | 647 | MS$^2$ [647]: 495, 477, 343<br>MS$^3$ [647 → 495]: 343, 325, 169 |
| 11 | 19.83 | Tetragalloylquinic acid[2] | 227, 277 | 799 | MS$^2$ [799]: 601, 629, 477, 647,<br>MS$^3$ [799 → 601]: 431, 449, 261 |
| 12 | 20.28 | Unknown gallic acid derivative | 226, 291 | 617 | MS$^2$ [617]: 285, 313, 599, 447<br>MS$^3$ [617 → 285]: 241 |
| 13 | 20.94 | Galloylproanthocyanidin dimer[1] | 228, 295 | 729 | MS$^2$ [729]: 407, 559, 577, 451, 603, 289<br>MS$^3$ [729 → 407]: 285 |
| 14 | 22.75 | Unknown gallic acid derivative | 226, 287 | 601 | MS$^2$ [601]: 583, 269, 313, 439<br>MS$^3$ [601 → 583]: 313, 269, 431 |
| 15 | 23.13 | Digalloylproanthocyanidin dimer | 227, 295 | 881 | MS$^2$ [881]: 729, 559, 711, 577<br>MS$^3$ [881 →729]: 407, 577, 559 |
| 16 | 23.59 | Quercetin deoxyhexosylhexoside | 258, 357 | 609 | MS$^2$ [609]: 300, 271, 343<br>MS$^3$ [609 → 300]: 271, 255, 179, 151 |
| 17 | 24.32 | Quercetin hexoside | 258, 358 | 463 | MS$^2$ [463]: 301<br>MS$^3$ [463 → 301]: 271, 255, 179, 151 |
| 18 | 24.87 | Galloylquercetin hexoside | 227, 271, 366 | 615 | MS$^2$ [615]: 301, 463, 313<br>MS$^3$ [615 → 313]: 169 |
| 19 | 25.83 | Quercetin pentoside | 259, 356 | 433 | MS$^2$ [433]: 301<br>MS$^3$ [433 → 301]: 271, 255 |
| 20 | 26.58 | Quercetin pentoside | 274, 361 | 433 | MS$^2$ [433]: 301<br>MS$^3$ [433 → 301]: 271, 255 |
| 21 | 27.33 | Galloylquercetin pentoside | 226, 268, 356 | 585 | MS$^2$ [585]: 301<br>MS$^3$ [585 → 301]: 179, 151 |
| 22 | 28.61 | Unknown | 246, 316 | 677 | MS$^2$ [677]: 645, 617, 585<br>MS$^3$ [677 → 645]: 489 |
| 23 | 30.07 | Unknown | 248, 316 | 675 | MS$^2$ [675]: 643, 599<br>MS$^3$ [677 → 643]: 599, 625 |
| 24 | 33.33 | Quercetin | 257, 370 | 301 | MS$^2$ [301]: 179, 151 |

**Table E.4.3.** UV and mass spectrometric data of phenolic constituents extracted from muruci (*Byrsonima crassifolia*) fruits. [a]Italic superscript numbers indicate the literature in which the compounds were previously described: [1]Sandhu and Gu, 2010; [2]Clifford *et al.*, 2007; [3]Friedrich *et al.*, 2000. [b]Gallic acid and quercetin were identified with authentic standard; all other compounds were tentatively identified.

Peak 9 was identified as a proanthocyanidin dimer. The typical UV spectrum and fragmentation pattern match with those previously described by Friedrich *et al.* (2000). Data were produced with the same MS instrument. The parent [M-H]$^-$ ion at *m/z* 729 of peak 13 produces fragment ions at *m/z* 577, 451, and 407 corresponding to those of peak 9. Due to the neutral loss of 152 Da, this compound was presumably assessed as a galloylproanthocyanidin dimer. Fragments of compounds found in peaks 9 and 13 are also in accordance with those reported by Sandhu and Gu (2010). Peak 15 suffers from the loss of 152 Da as well. On the basis of peak 13, yielded fragment ions at *m/z* 729 and 577 give rise to the presence of a digalloylproanthocyanidin dimer.

Geiss *et al.* (1995) reported about different proanthocyanidins with (+)-epicatechin units occurring in the bark of *B. crassifolia,* which argues for the presence of (+)-epicatechin units also in the fruits.

Peaks 16-21 belong to a series of quercetin derivatives. All of these peaks show in part characteristic fragment ions of a quercetin aglycone (e.g., $MS^n$ data at $m/z$ 301, 300, 271, 255, 179, and/or 151) which were generated from fragmentation of an authentic quercetin standard. Hence, simply quercetin was identified in peak 24. Data of quercetin are consistent with those found by Hvattum and Ekeberg (2003). Peak 16 was tentatively identified as quercetin deoxyhexosylhexoside ($m/z$ 609). The yielded product ion at $m/z$ 300 resulted probably from the homolytic cleavage of the O-glycosidic bond, which gave rise to the formation of a radical aglycone anion (Hvattum and Ekeberg, 2003). Quercetin hexoside was presumably present in peak 17 ($m/z$ 463) indicated by the neutral loss of 162 Da. Peaks 19 and 20 were assigned to be isomers of quercetin pentoside ($m/z$ 433), which is designated by the neutral loss of 132 Da. Two quercetin gallates were found in peaks 18 and 21, resulting in an additional absorption maximum (~270 nm) to the distinctive flavonol spectrum. Consequently, peak 18 was tentatively identified as galloylquercetin hexoside after sequential loss of 152 Da and 162 Da accounting for a galloyl and a hexosyl unit, respectively. The presence of galloylquercetin pentoside is likely in peak 21. The loss of 284 Da may be derived from a galloyl and a pentoside unit (152 Da + 132 Da) resulting into the aglycone ion of quercetin at $m/z$ 301.

Peaks 3, 12, and 14, could not clearly be identified. Nevertheless, these peaks embed characteristics typically found for gallic acid. Peak 3 shows an UV spectrum similar to that of gallic acid. The parent $[M-H]^-$ ions at $m/z$ 285 result in $MS^2$ data among other at $m/z$ 169 accounting for the presence of gallic acid. Peaks 12 and 14 show interesting parallels. Peak 14 produces $[M-H]^-$ ions at $m/z$ 601 and yields fragment ions ($m/z$ 313, 439) that were previously found in $MS^2$ data of digalloyl glucoside. $MS^3$ data of peak 14 indicate a neutral loss of a galloyl residue ($m/z$ 583 to $m/z$ 431) and a neutral loss of a hexoside ($m/z$ 431 to $m/z$ 269). Peak 12 might be a derivative of peak 14. Its parent ions $[M-H]^-$ at $m/z$ 617 suggest the presence of an additional hydroxyl group. $MS^2$ data also account for an additional hydroxyl group as the same neutral losses occur like in peak 14 but with an increase of 16 Da. The difference between $m/z$ 599 and $m/z$ 447 suggests the loss of a galloyl residue. Eventually, the difference between $m/z$ 447 and $m/z$ 285 assumes the loss of a hexoside. The same substances occur obviously also in cutite in the same elution order (compare peaks 21 and 25 of cutite in **table E.4.4**).

### *Cutite (Pouteria macrophylla)*

The HPLC chromatogram of the cutite extract is shown in **figure E.4.2**. Regarding **table E.4.4**,

| Peak | Retention time | Compound[a, b] | HPLC-DAD $\lambda_{max}$ [nm] | [M-H]$^-$ *m/z* | Fragments (*m/z*) |
|---|---|---|---|---|---|
| 1 | 9.35 | Gallic acid | 225, 273 | 169 | MS$^2$ [169]: 151, 125 |
| 2 | 9.64 | Gallic acid derivative | 229, 274 | 483 | MS$^2$ [483]: 465, 368, 174, 303, 350, 393, 229<br>MS$^3$ [483 →368]: 350 |
| 3 | 11.48 | Galloylquinic acid[2] | 226, 276 | 343 | MS$^2$ [343]: 169, 173 |
| 4 | 14.45 | Digalloylglucose[1] | 226, 278 | 483 | MS$^2$ [483]: 313, 331, 169, 271<br>MS$^3$ [483 →313]: 169 |
| 5 | 15.60 | Digalloylglucose[1] | 226, 273 | 483 | MS$^2$ [483]: 331, 271, 169, 241, 423, 313<br>MS$^3$ [483 →331]: 169, 271 |
| 6a | 16.58 | Digalloylglucose[1] | 229, 279 | 483 | MS$^2$ [483]: 331, 169, 271, 313<br>MS$^3$ [483 →331]: 169, 271, 241 |
| 6b | 16.58 | Digalloylquinic acid[2] | 229, 279 | 495 | MS$^2$ [495]: 343, 191<br>MS$^3$ [495 →343]: 191, 169 |
| 7 | 16.81 | Galloyl(epi)gallocatechin dimer | 228, 284 | 761 | MS$^2$ [761]: 423, 609, 575, 305, 405, 287<br>MS$^3$ [761 →423]: 283, 297 |
| 8a | 17.10 | Digalloylglucose[1] | 226, 277 | 483 | MS$^2$ [483]: 331, 169, 241, 271, 313<br>MS$^3$ [483 →331]: 241, 169, 271 |
| 8b | 17.10 | Digalloylquinic acid[2] | 226, 277 | 495 | MS$^2$ [495]: 343, 325, 169<br>MS$^3$ [495 →343]: 169, 191, 125 |
| 9 | 18.13 | HHDP glucose[1] | 233, 296 | 481 | MS$^2$ [481]:301, 355, 463, 151<br>MS$^3$ [481 →301]: 257, 215, 283 |
| 10 | 18.96 | HHDP glucose[1] | 231, 295 | 481 | MS$^2$ [481]:301, 355, 463, 151<br>MS$^3$ [481 →301]: 257, 215, 283 |
| 11 | 19.58 | Digalloyl(epi)gallocatechin dimer | 229, 276 | 913 | MS$^2$ [913]:761, 423, 743, 591, 573, 609<br>MS$^3$ [913 →761]: 609, 591 |
| 12 | 19.86 | HHDP galloylglucose[1] | 228, 290 | 633 | MS$^2$ [633]: 507, 301, 481, 271, 331, 615<br>MS$^3$ [633 →507]: 271, 175, 355 |
| 13 | 20.39 | Trigalloyl glucose[1] | 228, 277 | 635 | MS$^2$ [635]: 465, 483, 617, 313<br>MS$^3$ [635 →465]: 313, 169, 211 |
| 14 | 21.12 | HHDP galloylglucose[1] | 231, 296 | 633 | MS$^2$ [633]: 301, 507, 481, 271, 331<br>MS$^3$ [635 →507]: 175, 355, 271, 331 |
| 15 | 21.65 | Unknown compound | 233, 295 | 467 | MS$^2$ [467]: 286, 285, 340, 151 |
| 16 | 22.55 | HHDP galloylglucose[1] | 229, 293 | 633 | MS$^2$ [633]: 481, 301, 471, 355, 507, 463<br>MS$^3$ [633 →481]: 301, 355, 151, 463 |
| 17 | 22.88 | HHDP galloylglucose[1] | 226, 293 | 633 | MS$^2$ [633]: 331, 301, 481, 507, 271<br>MS$^3$ [633 →331]: 271, 169, 211, 193 |
| 18 | 23.60 | HHDP galloylglucose[1] | 231, 296 | 633 | MS$^2$ [633]: 331, 301, 481, 507, 271, 215, 355<br>MS$^3$ [633 →331]: 271, 169, 211, 193 |
| 19 | 24.11 | Unknown compound | - | 449 | MS$^2$ [449]: 269, 316<br>MS$^3$ [449 → 269]: 225, 151, 197, 183 |
| 20 | 25.24 | Myricetin deoxyhexoside | 268, 355 | 463 | MS$^2$ [463]: 316<br>MS$^3$ [463 → 316]: 271, 287, 179, 151 |
| 21 | 26.03 | Unknown gallic acid derivative | 235, 295 | 617 | MS$^2$ [617]: 331, 285, 465, 491, 507<br>MS$^3$ [617 → 285]: 241, 199, 217, 175, 257 |
| 22 | 26.30 | Dihydroquercetin | 295, 337 | 303 | MS$^2$ [303]: 285, 177, 125, 179, 241, 276 |
| 23 | 27.20 | Unknown compound | 231, 308 | 263 | MS$^2$ [263]: 219, 191<br>MS$^3$ [ 263→ 219]: 191 |
| 24 | 28.05 | Quercetin deoxyhexoside | 266, 297, 352 | 447 | MS$^2$ [447]: 301<br>MS$^3$ [ 447→ 301]: 179, 271, 255, 151 |
| 25 | 28.41 | Unknown gallic acid derivative | 228, 294 | 601 | MS$^2$ [601]: 287, 259, 331, 475, 313, 269<br>MS$^3$ [ 601→ 287]: 259, 243 |
| 26 | 28.89 | Unknown compound | 229, 301 | 575 | MS$^2$ [575]: 395, 449<br>MS$^3$ [ 575→ 395]: 367, 243, 449, 269 |
| 27 | 30.14 | Unknown compound | 234, 297 | 287 | MS$^2$ [287]: 259, 243, 269, 201<br>MS$^3$ [ 287→ 259]: 215, 173, 125, 241, 151 |
| 28 | 35.42 | Quercetin | 268, 370 | 301 | MS$^2$ [301]: 179, 151, 273 |

**Table E.4.4.** UV and mass spectrometric data of phenolic constituents extracted from cutite (*Pouteria macrophylla*) fruits. [a]Italic superscript numbers indicate the literature in which the compounds were previously described: [1]Sandhu and Gu, 2010; [2]Clifford *et al.*, 2007. [b]Gallic acid and quercetin were identified with authentic standard; all other compounds were tentatively identified.

a total of 22 polyphenolic compounds could be at least tentatively identified as gallotannins, quinic acid gallates, ellagitannins, proanthocyanidins, flavonols, and a flavanonol. Identification of gallic acid in peak 1 was assured by using an authentic standard.

A galloylquinic acid (peak 3) and two digalloylquinic acid isomers (peaks 6b and 8b) were tentatively identified in cutite due to mass spectrometric data that were already discussed for galloylquinic acids in muruci. Findings agree with MS data of quinic acid gallates reported by Clifford *et al.* (2007). The same accounts for different digalloyl glucoside isomers (peaks 4-6a, 8a) and a trigalloyl glucoside (peak 13). $[M-H]^-$ ions at $m/z$ 483 and $m/z$ 635, respectively, produce characteristic fragment ions that are present in muruci, araçá, and jambolão, too.

Peaks 9, 10, 12, 14, and 16-18 show fragmentation patterns distinctive for ellagitannins (Sandhu and Gu, 2010). The presence of two HHDP glucose isomers (**figure E.4.1**) is indicated in peaks 9 and 10 by the production of $[M-H]^-$ ions at $m/z$ 481 and accordant dominating daughter ions at $m/z$ 301. Peaks 12, 14, and 16-18 yielded fragment ions at $m/z$ 481 accounting for HHDP glucose after loss of a galloyl residue of 152 Da. Hence, these compounds are tentatively ascribed to be isomers HHDP galloylglucose. The occurrence of the dominating fragment ion at $m/z$ 301 for these compounds is in agreement with the report by Sandhu and Gu (2010)

Different groups of flavonoids are detectable in cutite. Peaks 7 and 11 were recognized as proanthocyanidins. Both peaks show typical $MS^n$ ions for a (epi)gallocatechin dimer at $m/z$ 609, 423, and 305. Congruent data obtained under the same instrumental conditions were previously published (Friedrich *et al.*, 2000). The parent $[M-H]^-$ ions at $m/z$ 761 of peak 7 and $[M-H]^-$ ions at $m/z$ 913 of peak 11 indicate the likely presence of a galloyl(epi)gallocatechin dimer and a digalloyl(epi)gallocatechin dimer, respectively. This assignment is derived from the neutral loss of a galloyl unit (152 Da) in peak 7 and the sequential loss of two galloyl units in peak 11. Three flavonols were found in peaks 20, 24, and 28. Peak 20 was tentatively identified as myricetin deoxyhexoside and peak 24 as quercetin deoxyhexoside. The neutral loss of 146 Da yielded in the particular (radical) aglycone ion ($m/z$ 316 and $m/z$ 301, respectively). Simply quercetin was specified in peak 28 on the basis of an authentic standard. Due to $MS^2$ data, peak 22 was tentatively identified as the flavanonol dihydroquercetin ($[M-H]^-$ at $m/z$ 303)..

**2. Antioxidant capacity**

**Table E.4.5** shows the results of the antioxidant capacity measurement of the four Amazonian fruits. Aqueous extracts were determined on their radical scavenging activity against peroxyl radicals (px) and peroxynitrite (pn) by the TOSC assay. TOSC results indicate the concentration of antioxidants present in the sample that is needed to attain a radical inhibition of 50% ($IC_{50}$). The total phenolic content was measured by Folin-Ciocalteu. The concentration of ascorbic acid was determined in these fruits as ascorbic acid affects results of the total phenolic content (Georgé *et al.*, 2005) and shows a perceivable impact on the antioxidant capacity measured by TOSC (Lichtenthäler *et al.*, 2003). The results are also given in **table E.4.5**.

The highest antioxidant capacity against both radicals was assessed for cutite followed by jambolão, araçá, and muruci. Against px, cutite bore a 9-fold higher antioxidant capacity in comparison to muruci. Against pn, even the 12-fold amount of muruci sample is needed to attain the $IC_{50}$ when compared to cutite. According to both radicals, antioxidant properties between jambolão and araçá were less distinctive. However, both fruits showed an approximately 3 times (px) and 4-5 times (pn) lower radical scavenging capacity than cutite.

| | Peroxyl radicals[a] | Peroxynitrite[a] | Total phenolic content[b] | Ascorbic acid[c] |
|---|---|---|---|---|
| Cutite | 0.57 | 0.83 | 2915.1 ± 0.0 | 247.5 ± 23.5 |
| Jambolão | 1.49 | 3.13 | 786.8 ± 6.9 | 93.5 ± 12.0 |
| Araçá | 1.58 | 4.00 | 754.4 ± 12.5 | 101.3 ± 9.8 |
| Muruci | 5.26 | 10.00 | 254.7 ± 15.2 | n.q. |

**Table E.4.5**. Antioxidant capacity (TOSC) against two different radicals, total phenolic content, and ascorbic acid content of four fruits from the Amazon region. [a]Concentration of freeze-dried sample (g/L) that is needed to obtain an inhibition rate of 50%. TOSC values imply a variance < 5%. [b]Data expressed as mean ± standard deviation (n = 4) in mg gallic acid equivalent/100 g dry matter. [c]Data expressed as mean ± standard deviation (n = 4) in mg/100 g dry matter; n.q., not quantifiable.

It becomes obvious that the fruit extracts generally performed higher against px than against pn. By comparing the two radicals, the results indicate differences in the effectiveness of the antioxidants contained in the fruit extracts. The difference between the antioxidant potential of px and pn is less distinctive for cutite in comparison to the other fruits. The 1.5-fold amount of cutite sample is required for the $IC_{50}$ of pn in comparison to px. Double the amount of sample is necessary for jambolão and muruci and even a 2.5-fold amount of araçá sample is needed. Consequently, the antioxidants present in cutite show the most effective impact against pn, whereas the antioxidants in araçá are the least effective against pn when compared to px.

Results of the TOSC assay are interrelated with the total phenolic content. The amounts of determined total phenols of the four fruits give rise to the same ranking as described for px and pn. Hence, the antioxidant properties of each fruit can be ascribed to the total phenolic content in the meaning of the Folin-Ciocalteu test. The lowest phenolic content was found in muruci being roughly comparable to that of banana pulp (Faller and Fialho, 2010). The 12-fold amount of total phenols was constituted in cutite matching that of tropical highland blackberries (Acosta-Montoya *et al.*, 2010).

Results of the ascorbic acid determination showed that noticeable amounts were found only in cutite. Jambolão and araçá contained less than half of the concentration present in cutite. Ascorbic acid in muruci could not unambiguously be identified. Besides the phenolic content,

ascorbic acid may significantly contribute to the antioxidant behavior of cutite fruits. As described by Lichtenthäler *et al.* (2003) a similar concentration of ascorbic acid standard is needed to obtain a radical inhibition of 50% against both radicals. This could explain the less pronounced difference of the antioxidant capacity of cutite against the two radicals. Jambolão shows a higher antioxidant capacity than araçá, although the content of ascorbic acid is slightly lower. Thus, antioxidant compounds other than ascorbic acid seem to significantly influence the radical scavenging behavior of jambolão. Finally, the comparably weak antioxidant activity of muruci may be explained by the probable absence of ascorbic acid in this fruit in addition to the low total phenolic content.

Up to now only a few studies are known about the antioxidant capacity of the four fruits from the Amazon region. Two different papers reported the free radical scavenging behavior of jambolão and muruci. $DPPH^{\cdot}$ assay conditions for the determination of both fruits were identical. Results showed a 3-fold higher antioxidant capacity for jambolão in comparison to muruci, which is accordance with our findings (Rufino *et al.*, 2009; Rufino *et al.*, 2010).
In comparison to other fruits originating from the Amazon basin, the antioxidant properties determined by the TOSC assay of cutite against pn were better than those of açaí pulp. Different harvest years of açaí (1998, 2000, and 2002) require concentrations between 1.17 and 1.72 g/L to attain an inhibition of 50%. In contrast, the radical scavenging potential of cutite against px is less effective than that of açaí (0.39-0.48 g/L) (Lichtenthäler *et al.*, 2005). Cutite also shows a lower antioxidant capacity with regard to both radicals than the outstanding fruits of camu camu (Rodrigues *et al.*, 2006) but its antioxidant capacity was higher when compared to berries of *Clidemia rubra* from Columbia (Gordon *et al.*, 2011).

In conclusion, a large number of phenolic constituents were detected in the Amazonian fruits. Thereof, 18 compounds were found in araçá, 37 in jambolão, 19 in muruci, and 22 in cutite. The compounds can be ascribed to hydrolyzable tannins, proanthocyanidins, flavonols, and flavanonols. Interestingly, no flavonoids could be found in araçá but only gallic acid derivatives. Cutite and muruci present different galloylquinic acid derivatives, which have rarely been proven in fruits. Studies on the antioxidant capacity revealed the best bioactive potential for cutite followed by jambolão, araçá, and muruci.

## E.5 Acknowledgment

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# F Separation of bioactive compounds in a semi-preparative scale (manuscript not intended for publication)

## F.1 Introduction

On the basis of epidemiological data, an increased consumption of fruits and vegetables has been associated with a reduced risk of contracting degenerative and chronic illnesses such as cardiovascular diseases (Hertog *et al.*, 1993; Harnafi and Amrani, 2007), Alzheimer's disease (Singh *et al.*, 2008), inflammatory diseases (Holt *et al.*, 2009) or cancer (Hertog *et al.*, 1994). The health promoting biological activities have been ascribed to dietary phenolics which show antioxidant properties and exert regulatory influence on diverse cellular mechanisms. Attention to phenolic compounds has also arisen in regard to existing antimicrobial activities. Phenolic compounds may be helpful in the control of the wild spectra of pathogens, in view of recent problems associated with antibiotic resistance (Paredes-Lopéz *et al.*, 2010). Apart from the medicinal aspects, the bioactive properties render phenolic compounds interesting for food technological purposes. There is, for instance, an increasing demand for food additives from natural resources because of consumers growing concern over the use of artificial additives (Giusti and Wrolstad, 2003). In this context, plant extracts containing a broad range of polyphenols can be applied as antioxidants or antimicrobial agents as well (Anastasiadi *et al.*, 2009; El-Hela and Abdullah, 2010; Jalosinska and Wilczak, 2009).

The use of phenolic constituents for medicinal or technological applications demands for a possibility to isolate individual compounds from complex matrices. A preparation of preferably authentic standards is also indispensable in order to assess possible pharmacological effects of particular phenolic substances. A state-of-the-art method for the isolation of phenolic compounds from plant extracts is high speed countercurrent chromatography (HSCCC) as it works without any adsorption losses. High sample loads and the use of cheap solvents instead of expensive solid phase columns are further advantages over e.g. preparative HPLC (Schwarz *et al.*, 2003).

The following study intends to isolate phenolic compounds in a semi-preparative scale. HSCCC procedure is exemplified by berries of *Clidemia rubra* as they have been shown to be rich in anthocyanins (Gordon *et al.*, 2011). Dietary anthocyanins have gained interest due to their health beneficial effects against coronary heart diseases (Wallace, 2011), obesity and hyperglycemia (Tsuda *et al.*, 2003; Sasaki *et al.*, 2007). Moreover anthocyanins can be technologically applied as food colorant (Coisson *et al.*, 2005; Pazmino-Duran *et al.*, 2001).

## F.2 Materials and methods

### 1. Chemicals

Following solvents were used to perform the chromatography: 1-Butanol and methyl tert-butyl ether (MTBE) were purchased from (Merck, Darmstadt, Germany). Acetonitrile was delivered by Th. Geyer GmbH & Co KG (Renningen, Germany). Trifluoroacetic acid (≥ 99%) was purchased by Acros Organics (Geel, Belgium).

### 2. Sample

Lyophilized sample of *Clidemia rubra* berries was taken for analysis. Samples were prepared as described in Gordon *et al.* (2011).

### 3. High speed countercurrent chromatography (HSCCC)

#### *Theory of HSCCC*

Countercurrent chromatography is a liquid-liquid separation method suitable for complex sample matrices. Elution occurs due to repeated partitioning of the analyte between two immiscible phases (stationary and mobile phase) by vigorous mixing in separatory funnel. The chromatography takes place in a multilayer coil that consists of wrapped PTFE tubing around a holder. The tubing can reach a length of 160 m. Multiple coils can be connected in series to increase the total volume of the instrument and sample capacity. During separation, the coil is rotated in a planetary fashion. It rotates between 800-1000 rpm around its own central axis and simultaneously around a parallel coil axis as shown in the instrument setup in **figure F.2.1**.

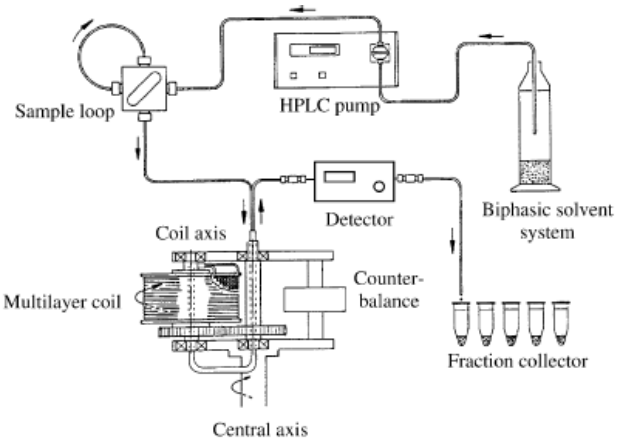


**Figure F.2.1.** Instrumental HSCCC setup (Schwarz *et al.*, 2003).

The planetary rotation results in two effects. Firstly, the rotation creates a fluctuating acceleration field which enables intense mixing of the two phases followed by settling within the coil. The force field is weak in areas of the coil which are close to the centre of rotation. Consequently, the phases are mixed. At a further point of the orbit, in the opposite to the centre of rotation the force field becomes stronger by what the phases are separated. Secondly, the retention of the

stationary phase is enabled due to the rotation of the coil. During rotation, it can be observed that the two immiscible phases move towards opposite ends of the coil also known as 'head' and 'tail'. Normally, the less dense layer displaces the heavier phase towards the tail until a hydrodynamic equilibrium is reached. When the heavier layer is selected as the mobile phase, the proper elution mode is 'head-to-tail' and the mobile phase is introduced from the head of the system. By choosing the lighter phase as the mobile phase, the elution order of the compounds is reversed, the correct elution mode is 'tail-to-head'. Hence, the mobile phase is pumped into the tail of the system (Schwarz *et al.*, 2003). Choosing the correct elution mode depends on the partition coefficient of the sample in the mobile and the stationary phase, respectively. Analytes with higher affinity to the mobile phase will be moved faster through the coil system as those interacting preferably with the stationary phase (McAlpine and Morris, 2006).

***Procedure***

A P.C. Inc. Model HSCCC (Zinsser Analytics, Frankfurt am Main, Germany) was equipped with a triple coil with total volume of 325 mL. According to Schwarz *et al.* (2003), the solvent system consisted of 1-butanol-MTBE-acetonitrile-water (2:2:1:5, v/v/v/v) acidified with 0.1% trifluoroacetic acid. The less dense layer of this solvent system was used as stationary phase. Conditioning of the coil with the heavy dense layer used as mobile phase was performed. Solvents were delivered by a Milipore Waters Model 510 (Milipore, Billerica, USA) using a flow rate of 1.0 mL/min. Lyophilized *Clidemia rubra* berries (1 g) were solubilized in 10 mL of mobile phase. After filtration through a 1.0/0.45 µm syringe filter (Chromafil GF/PET-45/25, Macherey-Nagel, Düren, Germany), the sample was injected into 5 mL sample loop. Elution mode was head-to-tail with revolution speed of the central axis set to 850 rpm in reversed direction. Separation was monitored by a UV-detector (Knauer, Berlin, Germany) at 280 nm. Fractions were collected with a Frac-100 (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) in steps of 3 min. Determination of the purity of the fractions was performed using HPLC/DAD instruments and conditions described previously in Gordon *et al* (2011).

## F.3 Results and discussion

The elution of the *Clidemia rubra* berry extract by HSCCC resulted in three main fractions detected at 280 nm as demonstrated in **figure F.3.1**. A subsequent determination of these fractions by HPLC/DAD primarily disclosed the presence of anthocyanins. Main fraction I (92-104 min) contained cyanidin 3-O-glucoside, cyanidin 3-O-rutinoside, delphinidin 3-O-glucoside, and delphinidin 3-O-rutinoside which were all previously identified in *Clidemia rubra* berries (Gordon *et al.*, 2011). Cyanidin 3-O-rutinoside and delphinidin 3-O-rutinoside were predominantly present in this fraction in a relation of 1:2 whereas cyanidin 3-O-glucoside and

delphinidin 3-O-glucoside were found in traces. Delphinidin 3-O-rutinoside was the quantitatively dominating anthocyanin in main fraction II (173-188 min). Further, lower concentrations of cyanidin 3-O-glucoside and cyanidin 3-O-rutinoside were constituted. An enrichment of high purity of an anthocyanin occurred in main fraction III (209-224 min). Cyanidin 3-O-rutinoside was the only detectable compound at 520 nm. The more unspecific wave length at 280 nm showed the presence of only one further non-anthocyanin constituent ($\lambda_{max}$ at 367 nm) in fraction III in a low concentration as shown in **figure F.3.2**. The peak area of the unknown compound, recorded at 280 nm, accounted for less than 2% in comparison to that of cyanidin 3-O-rutinoside.

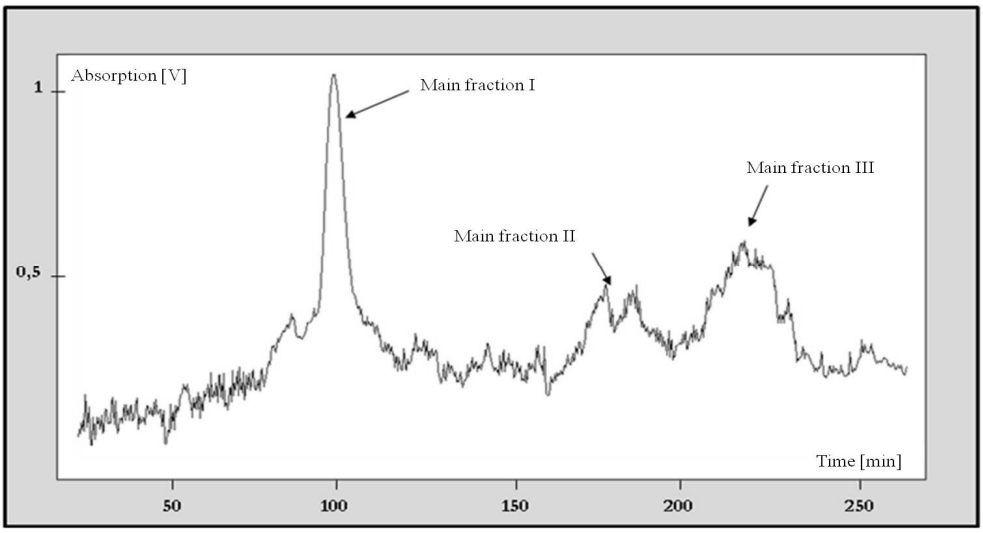


**Figure F.3.1.** HSCCC chromatogram of a *Clidemia rubra* berry extract recorded at 280 nm.

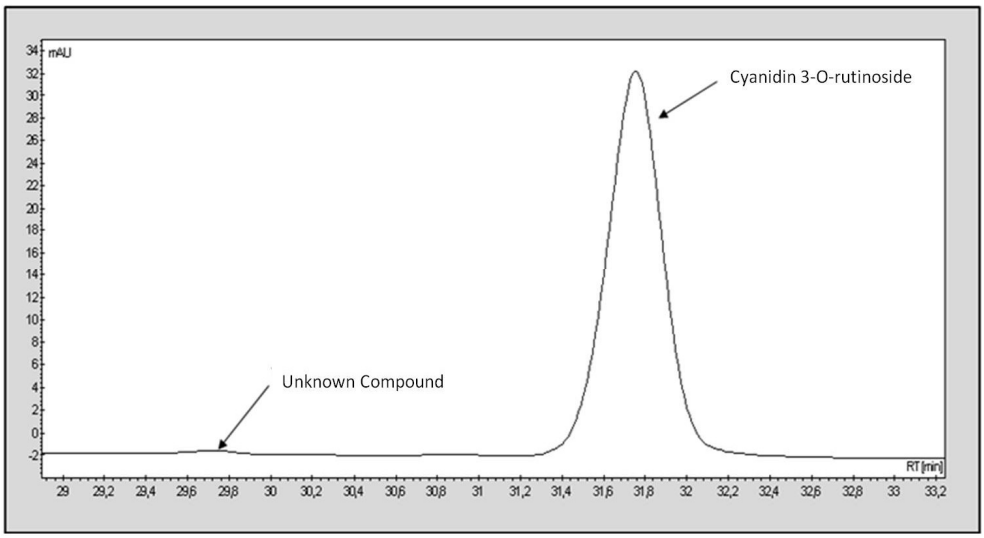


**Figure F.3.2.** Detail of a HPLC chromatogram of main fraction III at 280 nm.

Conclusively, HSCCC experiments have demonstrated that this separation method is an implementable instrument for the separation of bioactive compounds. The isolation of cyanidin 3-O-rutinoside as the quantitatively dominating anthocyanin in *Clidemia rubra* berries was

practicable in a high purity grade. By choosing alternative solvents or changes of the relation of individual solvents in the solvent system as well as modifying of the flow rate of the mobile phase, it would also be possible to separate other major and minor compounds, respectively. Schwarz *et al.* (2003) demonstrated that the purity of fractions containing several anthocyanins can be improved by repeated separation by HSCCC with lower flow rates.

## F.4 References

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# G Bolus consumption of a specifically designed fruit juice rich in anthocyanins and ascorbic acid did not influence markers of antioxidative defence in healthy humans (submitted to Agricultural and Food Chemistry)

Sabine Ellinger[a], André Gordon[b], Mira Kürten[a,b], Berndt Zur[c], Jörg Ellinger[d], Friedhelm Marx[b], Peter Stehle[a]

[a]Department of Nutrition and Food Sciences, Nutritional Physiology, University of Bonn, Endenicher Allee 11-13, 53115 Bonn, Germany

[b]Department of Nutrition and Food Sciences, Food Chemistry, University of Bonn, Endenicher Allee 11-13, 53115 Bonn, Germany

[c]Department of Urology and Children Urology, University of Bonn, Sigmund-Freud-Str. 25, 53105 Bonn, Germany

[d]Department of Clinical Chemistry and Clinical Pharmacology, University of Bonn, Sigmund-Freud-Str. 25, 53105 Bonn, Germany

Corresponding author:

PD Dr. Sabine Ellinger

Department of Nutrition and Food Sciences, Nutritional Physiology, Endenicher Allee 11-13, 53115 Bonn, Germany

Phone: ++49 228 73 3814, Fax: ++49 228 73 3217

E-mail: ellinger@uni-bonn.de

Own contribution: study design, study implementation, revision of the manuscript, designing of the figures, TOSC analysis, preparation of plasma samples for HPLC-CEAD analysis

## G.1 Abstract

Exotic fruits like açaí, camu camu, and blackberries rich in natural antioxidants (ascorbic acid, anthocyanins) are marketed as "functional" food supporting a pro-/antioxidant balance. Confirming data from human studies are lacking. Within a randomized controlled crossover trial, 12 healthy non-smokers ingested 400 mL of a blended juice of these fruits or a sugar solution (control). Blood was drawn before and afterwards to determine antioxidants in plasma, markers of antioxidant capacity [Trolox equivalent antioxidant capacity, Folin-Ciocalteu reducing capacity, total oxidant scavenging capacity (TOSC)] and oxidative stress [isoprostane, DNA strand breaks (SB) in leukocytes *in vivo*], and their resistance *vs.* $H_2O_2$-induced SB. Compared with sugar beverage, juice consumption increased plasma ascorbic acid and maintained TOSC levels ($p < 0.05$). SB *in vivo* increased after ingestion of both beverages ($p < 0.001$), probably due to postprandial and/or circadian effects. Exotic fruit juices cannot further improve a stable pro-/antioxidant balance in healthy non-smokers.

## G.2 Introduction

There is convincing epidemiologic evidence that regular fruit and vegetable consumption contributes to decrease the risk of several chronic diseases like coronary heart disease (Nikolic *et al.*) and probably certain kinds of cancer (AICR, 2007). It is hypothesized that antioxidative ingredients like polyphenols and water-soluble vitamins are the decisive factors explaining the health promoting properties of fruits and vegetables (Liu, 2003).

This scientific evidence is worldwide translated into public health initiatives like "5 a day" and school fruit programs with the goal to increase daily consumption of fruits and vegetables. Encouraged by these policy-driven actions, food industry is strongly engaged to launch novel fruit (and vegetable) based products on the market. Blended juices, juice concentrates and smoothies rich in polyphenols and water-soluble vitamins like ascorbic acid are marketed as health supporting food specifically preventing from radical-driven chronic diseases.

In this respect, economic and scientific interests are focused on fruits frequently consumed in South America. Camu camu (*Myrciaria dubia*) grows in the Amazon region and contains anthocyanins [30 – 50 mg/100 g, with cyanidin 3-O-glucoside as major (89% of total) anthocyanin], and an extraordinary high content of ascorbic acid (up to 3.0 g/100 g pulp) (Rodrigues and Marx). Another popular fruit in Central and South America is açaí (*Euterpe oleraceae* Mart.). It is also rich in anthocyanins, especially in cyanidin 3-O-glucoside (up to 456 mg/L pulp) and exhibit high antioxidant capacity in vitro (World Cancer Research Fund, 2007;

Lichtenthäler *et al.*, 2005). Andean blackberries similarly present a high antioxidant capacity because of their high content in ellagtannins (Acosta-Montoya *et al.*, 2010).

Controlled clinical trials to evaluate the protective effects of these fruits are scarce. Daily intake of 70 mL camu camu juice for one week reduced urinary 8-hydroxydesoxyguanosine, a biomarker of DNA damage, which did not occur after ingestion of equal amounts of isolated ascorbic acid (1050 mg/d) (Inoue *et al.*, 2008). Intervention studies in healthy non-smokers have shown that bolus consumption of açaí (pulp or juice) (Mertens-Talcott *et al.*, 2008) or a juice blend with açaí as predominant ingredient (Jensen *et al.*, 2008) increased antioxidant capacity in plasma (Mertens-Talcott *et al.*, 2008) and erythrocytes (Jensen *et al.*, 2008), respectively, and decreased lipid peroxidation (Jensen *et al.*, 2008). Even if individual markers on antioxidant capacity and oxidative stress improved in these studies, a comprehensive picture concerning antioxidant defense is lacking.

The aim of our study was, thus, to investigate the effects of a bolus consumption of a blended juice made of açaí, Andean blackberries and camu camu on the concentrations of plasma antioxidants, plasma antioxidant capacity, and markers of oxidative stress in healthy non-smokers. Secondary goal was to detect and characterize metabolites of anthocyanins in plasma using a newly developed HPLC technology.

## G.3 Materials and methods

### 1. Subjects and study design

To reach these study goals, we performed a randomized, controlled trial with crossover design. The primary endpoint was the assessment of total antioxidative capacity in blood. Sample size calculation was based on data from a preliminary trial with three healthy non-smokers ingesting a bolus of 400 mL of the juice blend under standardized conditions. Trolox equivalent antioxidant capacity (TEAC) increased from 1.25 mmol/l Trolox equivalents (TE; baseline) to 1.36 mmol/l TE (0.5 h; maximal TEAC). Considering a standard deviation of 0.075 mmol/l TE, a difference of 0.11 mmol/l TE could be detected with $\alpha = 0.05$ and a power of 90% if 11 participants were recruited. To account for dropouts, 12 participants were included in our study.

Participants (18 - 50 years, body-mass-index between 18.5 - 24.9 kg/m$^2$, non-smokers for at least 6 months) were recruited within our staff. Exclusion criteria (questionnaire) were: known hepatic/gastrointestinal disorders, pregnancy or breastfeeding, regular use of vitamin or flavonoid-rich supplements. Participants were asked on their usual intake of fruit and vegetables (portions per day).

The randomization into two equal groups was stratified by sex and was done by lots. Group A first ingested 400 mL of a fruit juice blend after 12 h overnight fast; after a wash-out period of 2 - 3 weeks, they received 400 mL of a solution with equal amounts of monosaccharides (13.0 g glucose, 7.2 g fructose) as control. Group B consumed the test beverages in reversed order.

The fruit juice blend consisted of 44% açaí, 12% camu camu and 44% blackberry juice *(Rubens ssp.)*. The fruit juice was produced according to the technological standards for the production of customary in trade from a commercially available frozen açaí puree (açaí juice pads; Açaí GmbH, Berlin, Germany), camu camu pulp [Brazilian Agricultural Research Corporation (Embrapa)] and Costa Rican blackberry juice [Centro National de Investigacion en Tecnologia de Alimentos (CITA), Costa Rica]. Ingredients of the juice blend are presented in **table G.3.1**.

| | Juice | Control |
|---|---|---|
| Anthocyanins (mg CE) | 276 | n.d. |
| Ascorbic acid (mg) | 936 | n.d. |
| Total phenolic content (mg GAE) | 1612 | n.d. |
| Glucose (g) | 13.0 | 13.0 |
| Fructose (g) | 7.2 | 7.2 |

**Table G.3.1.** Ingredients of a single portion (400 mL) of the study drinks. CE: cyanidin 3-O-glucoside equivalents, GAE: gallic acid equivalents, n.d.: not determined.

Fructose is degraded endogenously to uric acid and can, thus, exhibit antioxidative effects (Hallfrisch, 1990, Livesey, 2009). The control beverage, thus, contained amounts of fructose equal to the test juice. To avoid artefacts by other foods, subjects were instructed to abstain from foods rich in polyphenols (hand-out) starting 24 h before the first study day until completion. On the study day, participants received a standardized meal (two bread rolls with butter and cheese) 3 and 5 hours after consumption of the study beverages. Water was allowed to drink *ad libitum*.

Written informed consent was obtained by all participants. The study was conducted according to the Declaration of Helsinki and approved by the ethical Committee of the University of Bonn (No. 207/08).

**2. Blood sampling**

Blood samples (7.2 mL each) were collected before and 30, 60, 90, 120, 180 and 360 min after consumption of the study drink using tubes (S-Monovette®, Sarstedt, Nümbrecht, Germany) coated with EDTA [analyses of ascorbic acid, fat soluble pro-/vitamins, antioxidant capacity, 8-isoprostaglandine $F_{2\alpha}$ (8-iso $PGF_{2\alpha}$) and phenolic acids in plasma], heparin (determination of

DNA strand breaks in leukocytes), and tubes without anticoagulant (analysis of cholesterol, triglycerides, and uric acid).

**3. Preparation of plasma samples**

After blood withdrawal, EDTA tubes were placed on ice immediately. Then, blood was centrifuged at 3,000 × g for 20 min at 4 °C. For the determination of ascorbic acid, 500 µL of a cold 6% perchloric acid/2% metaphosphoric acid solution (v/v) was added to 500 µL fresh EDTA plasma in order to precipitate proteins and to stabilize ascorbic acid. After centrifugation (3,000 × g, 10 min, 4 °C), the supernatant was aliquoted and stored at -80 °C until analysis. To avoid oxidation, 10 µL butylhydroxytoluol (0.5% w/v in ethanol) was added to 1000 µL fresh EDTA plasma for later analysis of 8-iso $PGF_{2\alpha}$ and fat soluble pro-/vitamins. Ten µL of a solution of 0.4 M $NaH_2PO_4$ with 20% ascorbic acid and 0.1% EDTA (pH 3.6) were added to 500 µL plasma in which phenolic acids should be detected. Heparinized blood was used immediately for the determination of DNA strand breaks in leukocytes.

**4. Dietary intake of energy and nutrients**

The intake of energy, macronutrients, dietary fiber and antioxidant pro-/vitamins on the day before the study was calculated using Ebis Pro 4.0 software (University of Hohenheim, Stuttgart, Germany) based on German Nutrient database, version II.3. The flavonoid intake was calculated by using the USDA database (USDA, 2007).

**5. Analytical methods**

***Plasma antioxidant capacity***

Since no single assay truly reflects overall antioxidant capacity, multiple assays with different radicals and mechanisms (hydrogen or electron transfer which reflect radical quenching and radical reduction, respectively) should be used (Prior *et al.*, 2005). Thus, TEAC (Miller *et al.*, 1993) (CV 1.2%) was measured and expressed as Trolox equivalents. Furthermore, the Folin-Ciocalteu reducing capacity (FCR) (Prior *et al.*, 2005), was assessed with the modifications of Arendt *et al.* (2005) to avoid interferences with plasma proteins (CV 2.0%). The FCR of plasma was expressed as catechin equivalents. Additionally, total oxidant scavenging capacity (TOSC) in plasma (diluted 1:20 (v/v) with *aqua dest.*) was determined against peroxyl radicals (CV 3.6%) according to Lichtenthäler *et al.* (2003).

***Concentrations of antioxidants in plasma/serum***

Ascorbic acid in plasma was measured by HPLC with UV/VIS detection at 243 nm (CV 1.8%) according to Steffan (1999).

α-Tocopherol and β-carotene were also determined by HPLC. The protocol of Erhardt *et al.* (1999) was modified by using apocarotenal as internal standard, Nucleosil® 100-5 CN (Macherey-Nagel, Düren, Germany) as column and a solution of 98% hexane and 2% isopropanol as mobile phase. α-Tocopherol was detected at 292 nm (CV 4.1%) and β-carotene at 450 nm (CV 3.5%).

Uric acid in serum was determined photometrically within routine analysis (Urea Flex® reagents cassette, Siemens Healthcare Diagnostics, Eschborn, Germany) (CV 1% according to manufacturer).

***Phenolic acids in plasma***

A solid phase extraction using Supel® – Select HLB SPE tubes (bed wt., 60 mg, volume 3 mL) (Supelco, Steinheim, Germany) was performed to eliminate plasma proteins. After equilibration with 0.1% formic acid, the cartridge was loaded with plasma (450 µL). After washing with *aqua dest.*, a solution of methanol, acetonitrile and formic acid (50 + 49.9 + 0.1, v/v/v) was used for elution. The eluate was evaporated under nitrogen to dryness and reconstituted with a solution (50 µL) of methanol, water and trifluoroacetic acid (20 + 79.9 + 0.1, v/v/v). Thereafter, single compounds with reducing capacity were determined in the samples by HPLC-CEAD detection at 100, 200, 300 and 400 mV using the conditions (instrument settings, elution) previously described by Ritter *et al.* (2010). The analytical column was an Aqua 3 µm C18, 150 mm, 4.6 mm i.d. with a guard column (Security Guard, Aqua RP-18, 4 mm, 3 mm i.d.) (both from Phenomenex, Aschaffenburg, Germany). For analysis, 20 µL of the sample (plasma, standard and juice) were injected. Protocatechuic acid (≥ 97%) was obtained from Merck (Darmstadt, Germany), gallic acid (≥ 97.5%), vanillic acid (≥ 97%) and ferulic acid (≥ 99%) from Sigma-Aldrich (Steinheim, Germany) and caffeic acid (purum) from Serva (Heidelberg, Germany).

***Lipid peroxidation***

Total 8-iso $PGF_{2\alpha}$ concentration was determined as sum of free plus esterified 8-iso-$PGF_{2\alpha}$ in EDTA-plasma by an ELISA kit (Cayman Chemical, Ann Arbor, MI, USA, CV 10%) as described previously (Roggenbuck *et al.*, 2008).

***DNA strand breaks***

DNA single strand breaks (SB) were measured in leukocytes *in vivo* and after 20 min incubation at 4 °C with 300 µM $H_2O_2$ *ex vivo* using the single cell gel electrophoresis assay (also called Comet Assay). The protocol of Arendt *et al.* (2005) was used, however, electrophoresis period was extended to 20 min. Fifty nuclei per slide were evaluated for DNA damage by calculating tail moment with the Comet Assay III software (Perceptive Instruments, Suffolk, UK). The

difference in tail moment between untreated cells (SB *in vivo*) and cells challenged with $H_2O_2$ was calculated to determine the resistance of DNA *vs.* $H_2O_2$ *ex vivo* (CV 22%).

***Triglycerides***

Triglycerides in serum were measured within routine procedures by using Flex® reagent cartridges and the Dimension Vista® System (Siemens Healthcare Diagnostics; CV 3% according to manufacturer).

***Statistics***

Since data were normally distributed according to Kolmogorov-Smirnow-test, parametrical tests were used.

The effects of beverage, time, interactions of beverage and time (beverage × time) and the order of beverage intake on laboratory parameters were investigated with repeated measures ANOVA. In case of significant effects, a paired or unpaired *t*-test was performed subsequently.

Area under the curve (AUC) was calculated for all parameters by the trapezoidal rule for non-uniform intervals. AUC obtained after consumption of juice and sugar solution was compared with each other by an unpaired *t*-test.

Results are shown as mean and standard deviation. Statistical evaluation was performed with PASW Statistics, version 17.0 (SPSS Inc., Chicago, IL, USA).

## G.4 Results

Six males and six females with a mean age of 33 ± 7 years and a body-mass-index of 23.0 ± 3.1 kg/m2 participated in our study. Their usual intake of fruit plus vegetables was 3.3 ± 0.8 portions per day. The intake of energy (8.54 ± 2.27 MJ), protein (86 ± 28 g), fat (95 ± 38 g), carbohydrates (205 ± 65 g), dietary fiber (12 ± 5 g), ascorbic acid (18 ± 16 mg), β-carotene (0.4 ± 0.2 mg), vitamin E (5 ± 3 mg α-tocopherol equivalents), and flavonoids (0.5 ± 0.7 mg) was not significantly different between the days before each study day. This indicates an excellent compliance to dietary restrictions.

TEAC and FCR as parameters of plasma antioxidative capacity were not affected by beverage, time and interactions between beverage and time (**table G.4.1**) and AUC were not different (data not shown). TOSC values only decreased significantly over time after ingestion of sugar solution ($p = 0.02$), and reached lower values after 0.5, 1, 2, 3 and 6 h compared to baseline (all p-values $< 0.05$) (**table G.4.1**). The AUC of TOSC was higher after ingestion of juice compared to control beverage (266 ± 16 vs. 250 ± 11 %*h; $p = 0.032$).

| | TEAC (mM TE) | | FCR (mg CE/L) | | TOSC[a] | | 8-Iso-PGF2α (pg/mL) | |
|---|---|---|---|---|---|---|---|---|
| | Juice | Control | Juice | Control | Juice | Control | Juice | Control |
| 0 h | 1.26 ± 0.13 | 1.21 ± 0.14 | 21.4 ± 4.0 | 22.3 ± 5.3 | 43.7 ± 3.2 | 44.2 ± 2.4 | 17.1 ± 8.2 | 18.7 ± 8.5 |
| 0.5 h | 1.25 ± 0.11 | 1.21 ± 0.14 | 21.0 ± 3.6 | 21.3 ± 4.4 | 43.6 ± 3.0 | 41.3 ± 3.5** | 17.1 ± 5.3 | 15.4 ± 7.7 |
| 1.0 h | 1.29 ± 0.14 | 1.23 ± 0.14 | 20.8 ± 2.6 | 20.3 ± 3.5 | 44.9 ± 3.7 | 41.7 ± 3.1* | 16.1 ± 10.0 | 18.3 ± 10.5 |
| 1.5 h | 1.25 ± 0.13 | 1.23 ± 0.14 | 21.5 ± 3.9 | 19.5 ± 2.8 | 46.0 ± 4.1 | 41.6 ± 2.6 | 19.0 ± 14.3 | 22.2 ± 12.8 |
| 2.0 h | 1.25 ± 0.15 | 1.23 ± 0.16 | 21.1 ± 2.9 | 21.3 ± 3.2 | 44.1 ± 4.7 | 41.6 ± 2.3* | 15.2 ± 6.5 | 22.5 ± 15.5 |
| 3.0 h | 1.24 ± 0.12 | 1.22 ± 0.15 | 21.4 ± 3.1 | 21.8 ± 3.2 | 43.4 ± 3.0 | 41.6 ± 2.2** | 17.7 ± 8.1 | 16.8 ± 10.3 |
| 6.0 h | 1.24 ± 0.13 | 1.24 ± 0.14 | 21.7 ± 3.1 | 22.1 ± 2.9 | 46.6 ± 4.2 | 41.6 ± 3.0** | 17.7 ± 7.9 | 19.0 ± 12.1 |

**Table G.4.1.** Plasma antioxidant capacity and 8-isoprostaglandine $F_{2\alpha}$ before and after consumption of juice or control beverage. Data: Means ± SD, based on n = 12, except for TOSC (n = 9). CE, catechin equivalents; FCR, Folin-Ciocalteu reducing capacity; 8-Iso-$PGF_{2\alpha}$ , 8-iso-prostaglandine $F_{2\alpha}$; TE, Trolox equivalents; TEAC, Trolox equivalent antioxidant capacity; TOSC: total oxidant scavenging capacity. [a]Repeated measures ANOVA showed significant interactions by time × beverage ($p = 0.049$) for TOSC. Separate analysis of the changes for juice and sugar solution indicated that changes were only significant after ingestion of sugar solution. Significant differences *vs.* baseline were analyzed with a paired *t*-test with * $p < 0.05$ and ** $p < 0.01$.

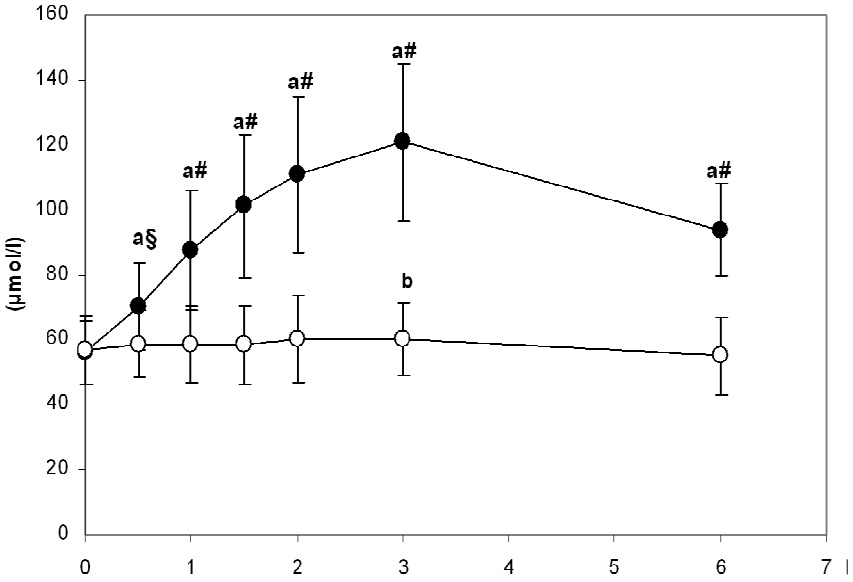


**Figure G.4.1.** Concentration of ascorbic acid in plasma before and after ingestion of fruit juice and control beverage. Data: Means ± SD, based on n = 12. Filled circles: after ingestion of juice, open circles: after ingestion of control beverage. Letters indicate significant differences compared to baseline ([a]$p < 0.001$, [b]$p < 0.011$, paired t-test). Symbols indicate significant differences between the ingestion of juice and sugar solution ([§]$p < 0.05$; [#]$p < 0.001$, unpaired t-test).

As shown in **figure G.4.1**, ascorbic acid concentration in plasma was dependent on interactions between beverage and time. Subsequent statistical analysis showed an impact of time on ascorbic acid for the ingestion of juice ($p < 0.001$) and sugar solution ($p = 0.008$): After juice intake, ascorbic acid concentration increased up to 117% (3 h vs. 0 h) and was significantly higher anytime ($p < 0.001$) compared to baseline. Three hours after ingestion of the sugar solution, ascorbic acid concentration was slightly (6.5%) higher compared with the initial value ($p = 0.011$) (**figure G.4.1**). As expected, the AUC of the ascorbic acid concentration in plasma was higher

after consumption of the test juice compared to control beverage (607 ± 115 vs. 351 ± 68 µmol*h/L; $p < 0.001$).

| | β-Carotene (µmol/L) | | α-Tocopherol: cholesterol - ratio (µmol/mmol) | | Uric acid (µmol/L) | |
|---|---|---|---|---|---|---|
| | Juice | Control | Juice | Control | Juice | Control |
| 0 h | 0.85 ± 0.52 | 0.84 ± 0.43 | 5.8 ± 1.7 | 5.3 ± 0.8 | 267 ± 51 | 265 ± 52 |
| 0.5 h | 0.80 ± 0.45 | 0.82 ± 0.46 | 5.6 ± 1.3 | 5.2 ± 0.7 | 272 ± 56 | 265 ± 53 |
| 1.0 h | 0.81 ± 0.48 | 0.83 ± 0.47 | 5.6 ± 0.8 | 5.3 ± 0.7 | 271 ± 53 | 266 ± 52 |
| 1.5 h | 0.81 ± 0.48 | 0.84 ± 0.49 | 5.4 ± 0.7 | 5.3 ± 0.8 | 268 ± 56 | 267 ± 53 |
| 2.0 h | 0.83 ± 0.46 | 0.82 ± 0.44 | 5.4 ± 0.9 | 5.3 ± 0.7 | 264 ± 57 | 262 ± 50 |
| 3.0 h | 0.82 ± 0.46 | 0.88 ± 0.48 | 5.5 ± 0.8 | 5.5 ± 0.7 | 265 ± 56 | 266 ± 56 |
| 6.0 h | 0.78 ± 0.46 | 0.83 ± 0.42 | 5.7 ± 0.9 | 5.3 ± 0.5 | 270 ± 55 | 258 ± 56 |

**Table G.4.2.** Status of antioxidants in plasma before and after ingestion of juice or control beverage. Data: Means ± SD, based on n = 12. Repeated measures ANOVA did not show any effects by time and beverage alone, and not by interactions of time and beverage.

The concentrations of β-carotene and of uric acid as well as the α-tocopherol-to-cholesterol-ratio were neither influenced by time and beverage alone nor by interactions with each other (**table G.4.2**).

In plasma, additional substances with reducing capacity not present in the juice blend were detected after consumption of fruit juice (**figure G.4.2/b**) compared to baseline (**figure G.4.2/a**). These metabolites could not be measured before (**figure G.4.2/c**) or after consumption of sugar solution (**figure G.4.2/d**). Interestingly, retention times of these substances observed after juice consumption were different from those of known metabolites like protocatechuic acid, gallic acid, vanillic acid, ferulic acid, and caffeic acid (**figure G.4.3**).

The beverage did neither affect 8-iso PGF2α (**table G.4.1**) nor SB in vivo and ex vivo (**table G.4.3**). Time had an impact on SB in vivo (**table G.4.3**), but did not modulate 8-iso PGF2α (**table G.4.1**) and SB ex vivo (**table G.4.3**). Differences between the AUC of 8-iso PGF2α, SB in vivo and ex vivo obtained after consumption of juice and sugar solution did not occur (data not shown).

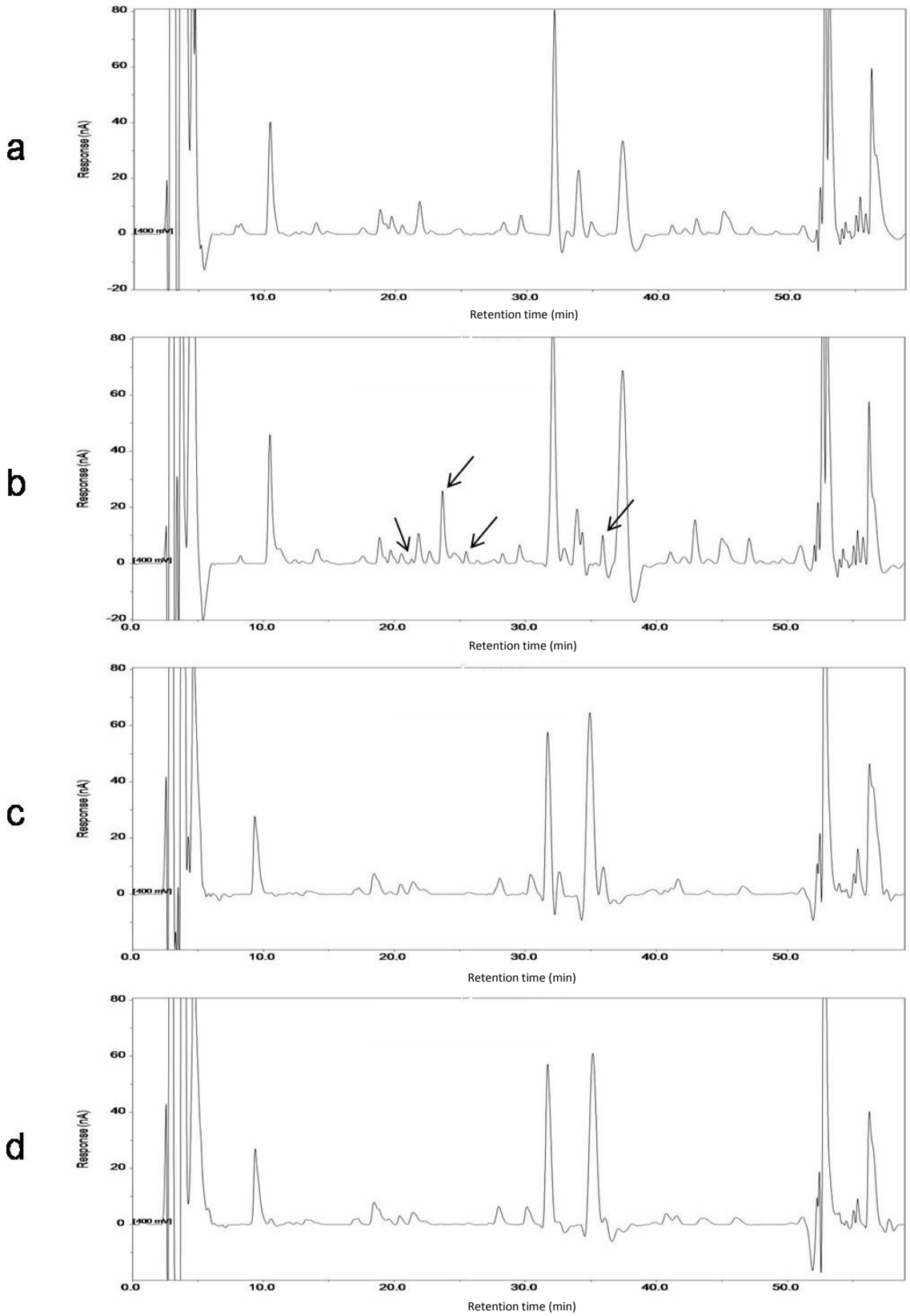


**Figure G.4.2.** Representative chromatograms of plasma samples obtained from one participant analyzed by HPLC-CEAD. The plasma samples were obtained after an overnight fast, just before juice consumption (a), 1 h after juice consumption (b), after an overnight fast, just before ingestion of sugar solution (c), and 1 h after consumption of sugar solution (d).

Triglycerides concentration in serum increased after both interventions ($p < 0.001$) with a significant increase after 6 h after ingestion of juice (1.30 ± 0.60 vs. 0.86 ± 0.38 mmol/L; $p = 0.002$) and sugar solution (1.56 ± 0.67 vs. 0.94 ± 0.21 mmol/L; $p = 0.004$) compared to baseline.

The order of the consumed study beverages did not affect any of the parameters investigated.

| | DNA strand breaks in vivo (TM)[a] | | DNA strand breaks ex vivo (TM) | |
|---|---|---|---|---|
| | Juice | Control | Juice | Control |
| 0 h | 14.4 ± 5.0 | 15.4 ± 5.7 | -1.7 ± 4.7 | -0.6 ± 3.8 |
| 0.5 h | 15.2 ± 5.5 | 14.4 ± 5.8 | -0.3 ± 3.9 | -0.6 ± 5.7 |
| 6.0 h | 22.4 ± 6.4 | 18.2 ± 7.3 | -0.8 ± 3.5 | 1.4 ± 5.8 |

**Table G.4.3.** DNA single strand breaks *in vivo* and *ex vivo* in peripheral leukocytes before and after ingestion of juice or control beverage. Data: Means ± SD, based on n = 12. TM: Tail moment. [a]Effects by time were only observed for DNA strand breaks *in vivo* ($p < 0.001$, repeated measures ANOVA). Interactions by time and beverage did not occur.

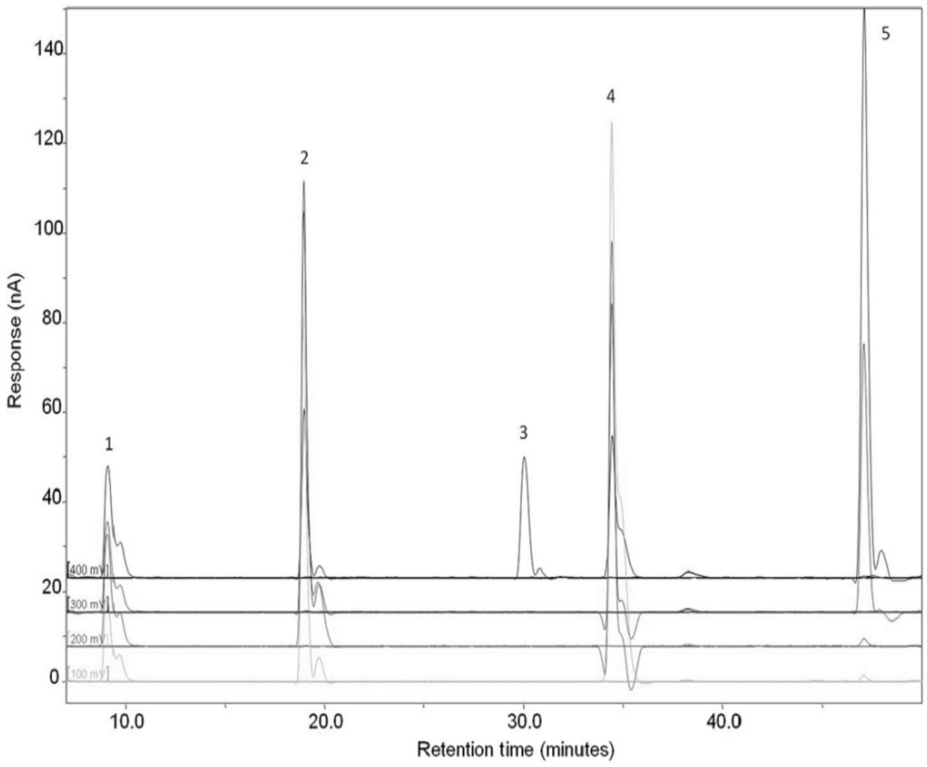


**Figure G.4.3.** Chromatogram of phenolic acid standards analyzed by HPLC-CEAD. Peaks represent (1) gallic acid, (2) protocatechuic acid, (3) vanillic acid, (4) caffeic acid, and (5) ferulic acid.

## G.5 Discussion

The primary goal of this study was to investigate if bolus consumption of a specifically designed fruit juice rich in anthocyanins and ascorbic acid increases plasma antioxidant capacity and

reduces markers of oxidative stress in healthy non-smokers. To answer these questions, a randomized controlled study with crossover design was performed to avoid between-subjects effects. A sugar solution with equimolar amounts of monosaccharides served as control drink to exclude antioxidant effects which may result from a fructose-mediated increase of uric acid. This study design should allow a reliable evaluation of fruit juice effects on the pro-/antioxidative balance.

Despite an obvious increase of ascorbic acid (**figure G.4.1**) and other substances with reducing capacity in plasma (**figure G.4.2/b**), TEAC and FCR did not increase after juice ingestion (**table G.4.1**). TEAC and FCR are based on measurements of electron transfer (Prior *et al.*, 2005). Ascorbic acid and, probably, the unknown substances function as hydrogen donators; this effect cannot be detected by these assays. Interestingly, TOSC, an assay detecting hydrogen transfer, decreased after the consumption of the control beverage (**table G.4.1**). This phenomenon may be due to the polyphenol-poor diet at the day before the study (Müller *et al.*, 2010). Maintenance of TOSC levels after juice intake (**table G.4.1**) may be explained by the enhanced extracellular ascorbic acid levels.

Before the intervention and after consumption of the control beverage, ascorbic acid levels were below the desirable steady-state concentrations in healthy adults (70-85 μmol/L) (Padayatty *et al.*, 2004), probably due to dietary restrictions. As expected, plasma concentrations of ascorbic acid temporarily increased after verum (**figure G.4.1**); the extent, however, was, relatively low considering the supraphysiological dose (> 900 mg/d) ingested. Reduced bioavailability at supraphysiological compared to physiological doses, but also exceeding the threshold plasma concentration for urinary excretion (55-60 μmol/L) (Levine *et al.* 1998) may explain this observation.

The concentration of further exogenous antioxidants in plasma (β-carotene and α-tocopherol), which also contribute to plasma antioxidant capacity (Cao and Prior, 1998), did not change significantly (**table G.4.3**). β-Carotene is generally ingested with açaí (Schauss *et al.*, 2006), camu camu (Zanatta and Mercadante, 2007) and blackberries (Marinova and Ribarova, 2007), but the dose in our study was obviously too low to increase the β-carotene level in plasma.

It is known that anthocyanins consumed as food ingredients cannot or only in marginal concentrations be detected in plasma. One explanation may be the low stability of the flavylium cation under physiological pH conditions (McGhie and Walton, 2007). Moreover, anthocyanins are degraded to low-molecular phenolic acids by the micro flora of the gut as shown *in vitro* and *in vivo* (Williamson and Clifford, 2010). The recent study of Vitaglione *et al.* (2007) confirms that

protocatechuic acid detected in human plasma accounts for 73% of ingested cyanidin 3-O-glucoside. To evaluate mucosal uptake of fruit juice polyphenol ingredients we, thus, evaluated plasma appearance of known low-molecular anthocyanin metabolites like protocatechuic acid, gallic acid, vanillic acid, caffeic acid, and ferulic acid with a newly developed highly sensitive HPLC technology (**figure G.4.3**). Most surprisingly, these metabolites could not be detected in plasma. Instead, several unknown metabolites with antioxidative properties occurred in plasma after juice consumption (**figure G.4.2/b**). Since we were not able to isolate these metabolites in quantitative amounts from plasma samples, the chemical structures are still unknown. Probably, phenolic acids are further degraded already in the gut and/or after mucosal uptake.

Lipid peroxidation *in vivo* assessed by plasma 8-iso $PGF_{2a}$ did not change in our study. This observation is in contrast to results of recent bolus study: 2 h after ingestion of a cyanidin-rich juice blend with açaí as predominant ingredient, lipid peroxidation measured by thiobarbituric acid reactive substances (TBARS) decreased in healthy non-smokers (Jensen *et al.*, 2008). However, TBARS are less specific for lipid peroxidation than isoprostanes (del Rio *et al.*, 2002) analysed in our study.

SB *in vivo* were only affected by time ($p < 0.001$) and not by beverage (**table G.4.3**). This fits to the results of an own previous study where only effects by time occurred after bolus ingestion of white tea, green tea and water (Müller *et al.*, 2010). Time-dependent effects may simply reflect circadian rhythms. Contrary to SB *in vivo*, SB *ex vivo* were not modulated by time or beverage (**table G.4.3**). Comparable bolus studies with juices investigating SB *ex vivo* are not available, but white and green tea did not show any changes by time or beverage either (Müller *et al.*, 2010). Cell based antioxidant capacity, determined in erythrocytes, increased after bolus consumption of an açaí-rich juice, probably due to an intracellular accumulation of antioxidants (Jensen *et al.*, 2008), but did not change leukocytes' resistance against challenge with reactive oxygen species *ex vivo* after single intake of açaí pulp or juice (Mertens-Talcott *et al.*, 2008). Primarily, endogenous glutathione determines the protection against oxidative cell injury (Shan *et al.*, 1990). It is to assume that the consumption of the exotic fruit juice did not influence glutathione levels. It should be, however, mentioned that the broad inter-individual variation of SB *in vivo* and against oxidative challenge *ex vivo* limits the power of the study considering these markers of DNA damage.

In the present study, the time-dependent increase of triglycerides in serum may reflect postprandial changes induced by the standardized meals. Even if postprandial hypertriglyceridemia is suggested to trigger the increase in biomarkers of oxidative stress like malondialdehyde in healthy subjects (Bloomer *et al.*, 2010), 8-iso $PGF_{2\alpha}$ did not change

(**table G.4.2**). Thus, confounding effects on lipid peroxidation *in vivo* by triglycerides in the present study are unlikely.

In conclusion, bolus ingestion of a blended juice of açaí, Andean blackberries and camu camu rich in ascorbic acid and anthocyanins only increased the concentration of plasma ascorbic acid and several unknown substances with reducing properties, but did not further improve the already stable pro-/antioxidative balance in healthy non-smokers. Product-specific preventive effects by consumption of these novel drinks can, thus, not be expected. It cannot be excluded that beneficial effects by this juice blend may rather occur in situations with increased oxidative challenge, e.g., smoking and physical activity.

We declared to have no conflict of interest.

## G.6 Acknowledgment

We thank the Brazilian Agricultural Research Corporation (Embrapa, Brazil) and Centro National de Investigacion en Tecnologia de Alimentos (CITA, Costa Rica) for providing the camu camu pulp and the blackberry juice, respectively.

## G.7 Supporting information description

The study was part of the PAVUC (Producing added value from underutilized tropical fruit crops) project (No. 0015279) and thus, partly supported by a grant of the European Union. The results of this study were presented in parts at the 47$^{th}$ Scientific Conference of the German Nutrition Society in Jena, Germany, (03/2010), and at the Workshop of the Regional Association North-Rhine Westphalia of the Society of Food Chemistry in Münster, Germany (03/2010).

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# H Occurence of phenolic acids in human blood plasma after fruit juice ingestion (manuscript not intended for publication)

## H.1 Introduction

Phenolic compounds are widely distributed in plant-derived food and can be classified into two groups, the flavonoids and the non-flavonoids (Crozier *et al.*, 2009). The flavonoid intake in Germany was estimated at 56 mg/day in a Bavarian subgroup of the national food consumption survey (Lineisen *et al.*, 1997). The same collective was chosen to assess the intake of the non-flavonoid phenolic acids. The amount was estimated at 222 mg/day of which the more abundant part was taken by the hydroxycinnamic acids (211 mg/day) on the contrary to the hydroxybenzoic acids (11 mg/day) (Radtke *et al.*, 1997). Phenolic acids also comprised the dominant group of polyphenols (75% of the total phenolic intake) in a Finnish study that calculated the mean total intake at 863 ± 415 mg/day (Ovaskainen *et al.*, 2008).

Absorption of phenolic compounds from the small intestine is generally more efficient than from the colon and gives rise to higher plasma values (Hollman, 2004). Polyphenols that are absorbed from the small intestine, undergo several metabolization steps like deglycosylation, glucuronidation, sulfatation, and methylation during passage through the gut wall (Crozier *et al.*, 2009). Flavonoids that cannot be absorbed from the small intestine go further to the colon and will be microbially degraded (Hollman, 2004). Several reports are known on the breakdown of anthocyanins into corresponding phenolic acids that are derived from the B-ring of the anthocyanin skeleton (Fleschhut *et al.*, 2006; Keppler and Humpf, 2005; Vitaglione *et al.*, 2007).

Absorption of free phenolic acids takes place in the small intestine. Uptake of hydroxycinnamic acids occurs putatively by sodium-glucose cotransporter (Clifford, 2000). In general, polyphenols have been attributed to be protective agents against degenerative diseases such as cancer, cardiovascular diseases and neurodegenerative disorders (Wang and Ho, 2009). Beneficial effects of phenolic acids are based on the inhibition of initiation and promotion during cancerogenesis (Watzl and Rechkemmer, 2001).

The bioavailability of phenolic acids in human has been shown only in a few reports. Aim of the study was to prove the uptake of individual phenolic acids after ingestion of a tropical fruit juice and to evaluate their generation as metabolites from higher molecular flavonoids or conjugates.

## H.2 Material and methods

### 1. Chemicals

Protocatechuic acid was obtained from Merck (Darmstadt, Germany), gallic acid, from Sigma-Aldrich (Steinheim, Germany), caffeic acid and p-coumaric acid from Serva (Heidelberg, Germany).

### 2. Plasma preparation

A fruit juice blend (400 mL) consisting of açaí, Andean blackberries, and camu camu (in reference to manuscript chapter appendix G.3.1) was ingested by a voluntary healthy non-smoker (30 years, 86 kg, BMI 22.5) after compliance with a 12 h overnight fast and a diet low in polyphenols the day before intervention. Blood samples were prepared for further analysis like described in manuscript chapters of appendix G.3.3 and G.3.5. Briefly, a solid phase extraction was performed to eliminate plasma proteins. The cartridge was loaded with plasma (450 µL) washed and eluated with a solution of methanol, acetonitrile and formic acid (50 + 49.9 + 0.1, v/v/v). The eluate was evaporated under nitrogen steam to dryness and solubilized with 50 µL of methanol, water and trifluoroacetic acid (20 + 79.9 + 0.1, v/v/v).

### 3. Phenolic acid analysis

Identification of phenolic acids was performed following an UPLC/DAD-ESI-MS/MS method. Analysis was carried out using a Waters Acquidity UPLC system (Waters, Milford, MA, USA) consisting of a SDS pump, an automated sample injector, and a PDA detector type UPLC LG 500 nm. Separation was performed by help of an analytical column Acquity HSS-T3 (100 mm x 2.1 mm, 1.8 µm; Waters) kept at 40 °C. Solvents were UHQ water with 0.1% acetic acid (v/v) (mobile phase A) and acetonitrile with 0.1% acetic acid (v/v) (mobile phase B). The flow rate was 0.5 mL/min. The gradient started with 4% B and rose up to 25% B after 10 min. The column was flushed for 2 min at 98% B and re-equilibrated for 3 min with initial conditions. For analysis, 5 µL of each sample were injected. The UPLC was coupled with an electrospray ionization interface mass spectrometer model TQD supplied by Waters. Settings for the negative ionization with MS were as follows: capillary voltage -1.0 kV, cone voltage 30 V, extractor voltage 2 V, RF voltage 1.1 V, collision energy 20 V, source temperature 150 °C, desolvation temperature 450 °C, cone gas (nitrogen) flow 50 L/hr, desolvation gas (nitrogen) flow 800 L/hr, collision gas (argon) flow 0.1 mL/min. Control of the whole system was performed by MassLynx 4.1 software.

Detection was carried out using selected ion recording (SIR) and multiple reaction monitoring (MRM). Following mass traces were taken for SIR: caffeic acid $m/z$ 179, p-coumaric acid $m/z$

163, gallic acid $m/z$ 169, protocatechuic acid $m/z$ 153. Measurements in MRM were performed by choosing the same mass traces and the corresponding mass trace after neutral loss of $CO_2$ for the particular compound. Target compounds were quantified by creation of calibration curves using authentic standard substances.

## H.3 Results and discussion

The occurence of four phenolic acids after bolus ingestion of a tropical juice blend was investigated in this small scale intervention study by UPLC-ESI-MS/MS.

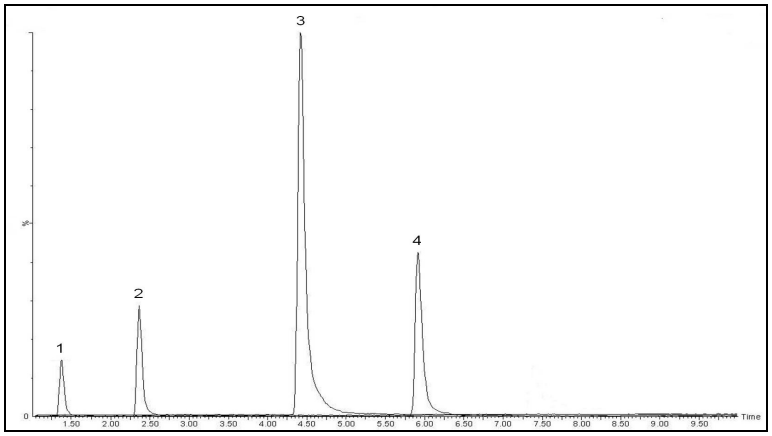


**Figure H.3.1.** Retention behavior of four phenolic acids. Gallic acid (1), protocatechuic acid (2), caffeic acid (3), and p-coumaric acid (4) are detected by UPLC-ESI-MS/MS from a standard mixture according to their particular mass traces.

A chromatogram of a standard mixture containing the target compounds is shown in **figure H.3.1**. According to their particular mass traces, the determined phenolic acids were detected in the juice blend (**figure H.3.2**) but only gallic acid (8.19 mg/serving), p-coumaric acid (0.4 mg/serving) and protocatechuic acid (1.48 mg/serving) could be quantified. The concentration of caffeic acid was below the level of quantification. **Figure H.3.3** shows the results of the plasma determination. After a 12 h overnight fast, the presence of gallic acid and p-coumaric acid could not be constituted in the plasma sample that was withdrawn immediately before ingestion of the fruit juice. Surprisingly, caffeic acid and protocatechuic acid were detected despite the fasting condition of the participant. Probably, a longer period than a one-day abstinence from polyphenol containing diet with subsequent overnight fasting is needed to assure a proper total clearance of these phenolics from the blood stream. Unfortunately, information on pharmacokinetic studies shedding light on the total clearance of phenolic acids is scarce. Caffeic acid was studied in rabbits by Uang *et al.* (1997). In this study, most of the unchanged caffeic acid was excreted in the urine within 2 h. An investigation in human showed that the maximal urinal excretion of cinnamic acid occurred in the first 4 h of a 48 h survey

(Clifford, 2000). Data about the pharmacokinetic of gallic acid and protocatechuic acid were not found.

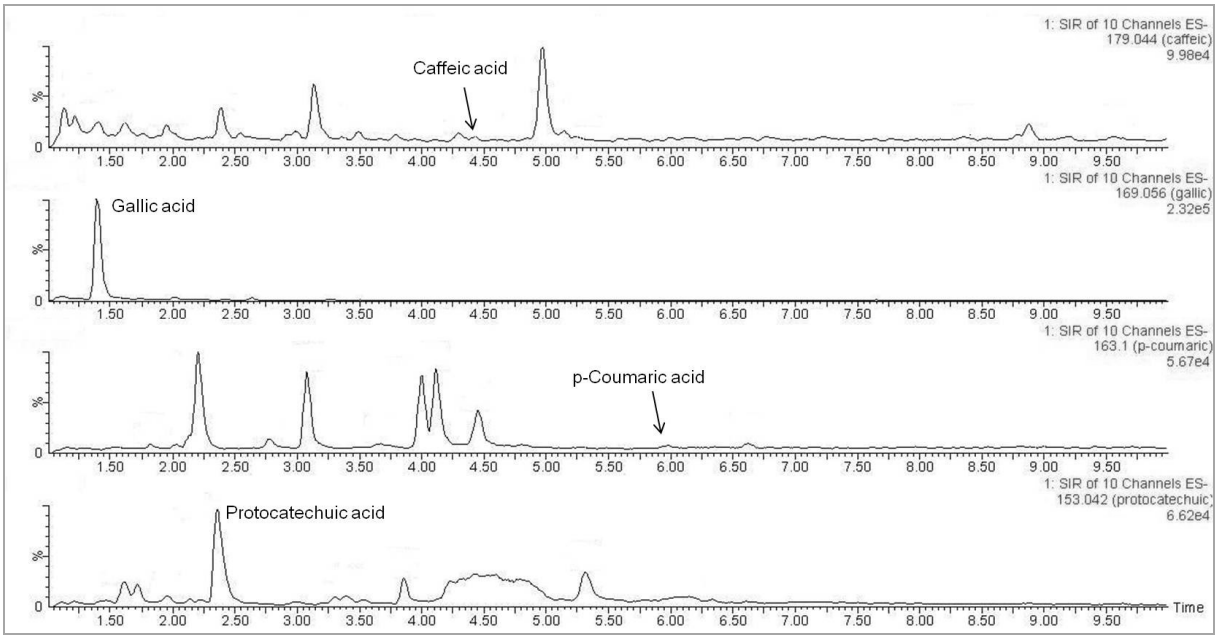


**Figure H.3.2.** Phenolic acids in a tropical fruit juice blend. Target compounds were detected by UPLC-ESI-MS/MS according to their particular mass traces.

Generally, compounds absorbed from the small intestine usually appear in the plasma in maximum concentration within less than 2.5 h (Williamson and Clifford, 2010). Hence, a further blood withdrawal was obtained 2 h after juice intake to proof the bioavailability of the determined phenolic acids.

The presence of caffeic acid could only be tentatively constituted as the retention time in the plasma samples deviated from that of a standard compound. The untimely retention time might be caused by matrix effects of the sample or the presence of a caffeic acid isomer. However, the molecular ion $[M–H]^-$ at $m/z$ 179 from SIR measurements and fragmentation pattern from MRM measurements were in accordance to that of an authentic caffeic acid standard. The concentration of caffeic acid before and after fruit juice ingestion could not be quantified, but the ion current at $m/z$ 179 suggests a concentration decline after juice intake due to its lower intensity (from $3.00 * 10^5$ to $2.10 * 10^5$, **figure H.3.2**). This may be explained by an ongoing clearance of caffeic acid when compared to the fasting condition. Simultaneously, a negligible supply with free caffeic acid and its conjugates from the study drink as well as caffeic acid as a metabolite derived from higher polyphenols is obvious which would result in higher concentrations of free caffeic acid in the blood stream when compared to the fasting condition. The qualitative bioavailability of caffeic acid was mentioned in a previous study. This compound was not present in plasma prior to the intervention with red wine after a diet low in

polyphenols for 3 days. Plasma level rose to maximum concentration 60 min after wine consumption (Simonetti *et al.*, 2001).

A quantification of protocatechuic acid from the plasma samples could not be performed as it was below the limit of quantification. However, an unchanged concentration of protocatechuic acid in plasma before fruit juice ingestion compared to fasting condition can be assumed as the ion current at $m/z$ 153 shows a similar intensity ($4.80 * 10^3$ before juice intake and $4.83 * 10^3$ after juice intake, **figure H.3.2**). A possible decrease of the concentration as it was observed for caffeic acid might be compensated by free protocatechuic acid provided by the juice itself. Furthermore, protocatechuic acid can also be derived as microbial degradation product of the gut flora from cyanidin-3-O-glucoside (Vitaglione *et al.*, 2007), which is provided as ingredient by all fruits present in the juice (Lichtenthäler *et al.*, 2005; Mertz *et al.*, 2007; Rodrigues and Marx, 2006). Results from a bioavailability study, in which participants obtained a moderate amount of berries over 8 weeks, showed an increase of protocatechuic acid in plasma. The increase accounted for 21% in the berry group compared to the control group (Koli *et al.*, 2010).

Gallic acid showed the highest concentration (120.36 μmol/L) of the target compounds in our study drink. The concentration after ingestion of the study drink in plasma was 5.88 μmol/L. Free gallic acid was previously reported to be well absorbed compared to other polyphenols (Manach *et al.*, 2005), what is in agreement with our findings. As shown in a bioavailability study in healthy humans, peak plasma levels of gallic acid were reached after 1.3 h and 1.4 h subsequent to administration as tablets and as black tea drink, respectively. An oral dose consisting of 50 mg gallic acid gave rise to plasma concentrations of 1.83 μmol/L for the tablets and 2.09 μmol/L for the tea (Shahrzad *et al.*, 2001).

The most interesting aspect of this study is the occurence of a p-coumaric acid isomer in the plasma after fruit juice ingestion. The presence of p-coumaric acid could not be constituted as the retention time of the compound found in the plasma is not in accordance to that of a p-coumaric acid standard. Thus, matrix effects may affect the detection of p-coumaric acid or the presence of isomers is possible as ion currents from SIR and MRM measurements of the compound found in the plasma are in accordance to that of a p-coumaric acid standard. The concentration of the p-coumaric acid isomer in the plasma after fruit juice intake is 0.13 μmol/L. If this compound were not derived in its free form from the fruit juice, it might be seen as a metabolite from other polyphenols. The mass trace of p-coumaric acid shown in **figure H.3.2** suggests that p-coumaric acid and possible isomers may be preferably present as conjugates in the fruit juice assuming the formation of a daughter ion at $m/z$ 163 as a fragment from its conjugates during ionization. As stated by Clifford *et al.* (2000) conjugates of phenolic

acids are not absorbed as such, but they are cleaved by esterases of the gut flora prior to absorption. A study in rats also constituted the occurrence of p-coumaric acid as a metabolite from conjugated derivatives (Gonthier *et al.*, 2003). Furthermore, p-coumaric acid was found to be a metabolite of other polyphenolic compounds like rosmarinic acid (Baba *et al.*, 2005) and chlorogenic acid (Monteiro *et al.*, 2007) in human. As a consequence of the monohydroxylation on the B-ring, p-coumaric acid is reported to be less susceptible to glucuronidation than other polyphenols. Thus, the aglycone occurs in relatively high amounts in plasma after intestinal absorption (Spencer *et al.*, 1999).

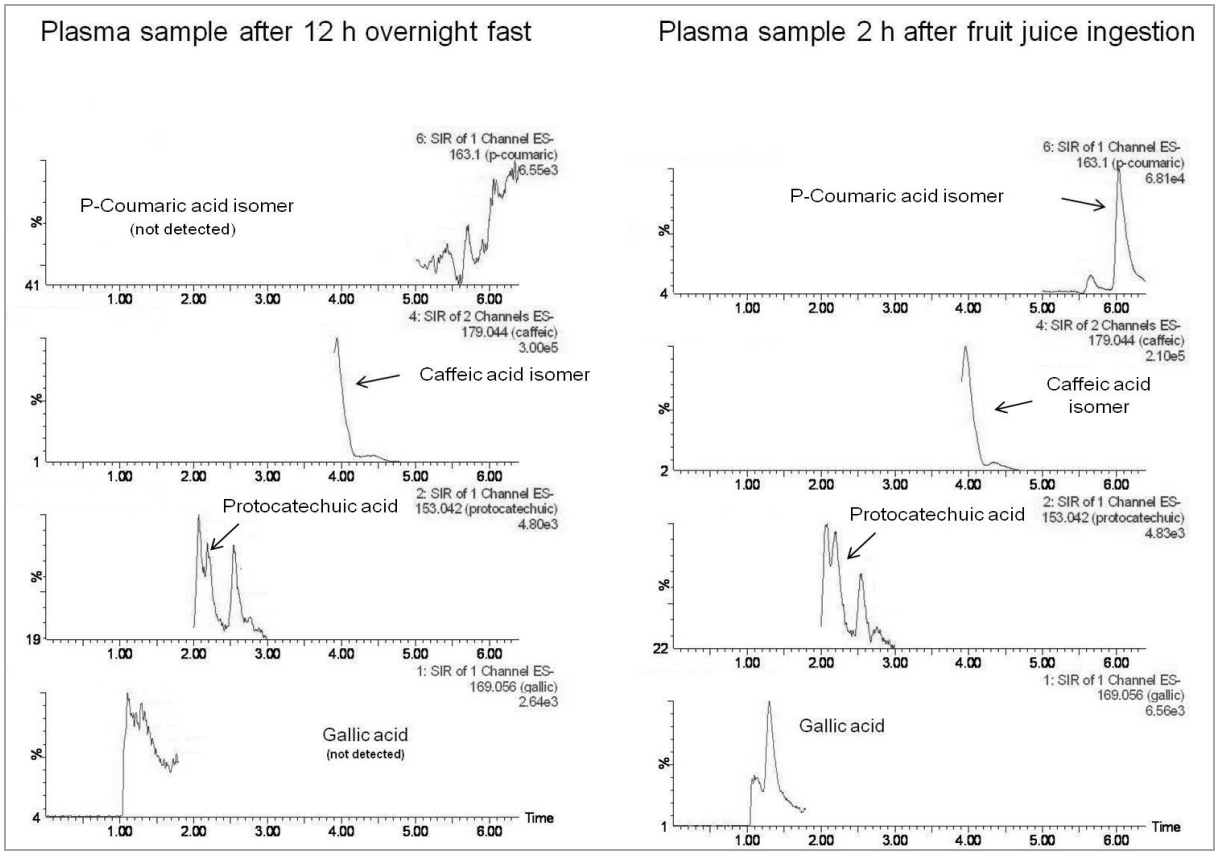


**Figure H.3.3.** Bioavailability of phenolic acids. Mass traces of the four target compounds in plasma are shown immediately before (left) and 2 h after ingestion of a tropical fruit juice.

Conclusively, the bioavailability of in total four phenolic acids after fruit juice ingestion was estimated in this study. As the presence of a caffeic acid isomer and protocatechuic acid was already constituted in the plasma in fasting condition of the subject, their bioavailability could not be assessed. The bioavailability of gallic acid from the fruit juice was calculated. A p-coumaric acid isomer turned out to be a metabolite of other phenolic compounds from the juice drink.

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