UNIVERSITÄT BONN

Institut für Lebensmittelwissenschaft und Lebensmittelchemie

Optimisation of the Total Oxidant Scavenging Capacity Assay and Application on *Euterpe Oleracea* Mart. (Açaí) Pulps and Seeds

von

Ramona Lichtenthäler

The antioxidant capacities of commercial and non-commercial pulps and seeds of the *Euterpe oleracea* Mart., Arecaceae, (açaí) palm were surveyed against peroxyl radicals, peroxynitrite and hydroxyl radicals. Analyses were carried out with the Total Oxidant Scavenging Capacity (TOSC) assay in a modified and automated version. The results were compared to several standard compounds as well as to a number of common European fruit and vegetable juices. Several antioxidants present in açaí pulps and seeds were identified, quantified and their contribution to the overall antioxidant capacities was estimated.

Post address:
Endenicher Allee 11–13
D-53115 Bonn
Germany

Bonn University
December 2004

UNIVERSITÄT BONN
Institut für
Lebensmittelwissenschaft
und Lebensmittelchemie

Optimisation of the Total Oxidant Scavenging Capacity Assay and Application on *Euterpe Oleracea* Mart. (Açaí) Pulps and Seeds

von

Ramona Lichtenthäler

Dieser Forschungsbericht wurde als Dissertation von der Mathematisch - Naturwissenschaftlichen Fakultät der Universität Bonn angenommen und ist auf dem Hochschulschriftenserver der ULB Bonn http://hss.ulb.uni-bonn.de/diss_online elektronisch publiziert.

Angenommen am:	29. April 2004
Prüfung am:	9. Dezember 2004
Referent:	PD Dr. Friedhelm Marx
Korreferent:	Prof. Dr. Gabriele M. König

Contents

List of Figures

List of Tables

Abbreviations and symbols

2C	Catechin or epicatechin dimer
3C	Catechin or epicatechin trimer
4C	Catechin or epicatechin tetramer
5C	Catechin or epicatechin pentamer
ABAP	2,2'-Azobis(2-methylpropionamidine) dichloride
AC	Area of control reaction
ADRC	Area under dose-response curve
AS	Area of sample reaction
AUC	Area under curve
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
BSTFA	N,N-bis-Trimethylsilyltrifluoroacetamide
C	Catechin
CG	Cyanidin-glucoside
Conc.	Concentration
CR	Cyanidin-rutinoside
Cya.	Cyanidin
DT_{50}	1^{st} Derivative of dose-response curve at TOSC of 50%
DTPA	Diethylenetriaminepentaacetic acid

E	Epicatechin
EDTA	Ethylenediaminetetraacetic acid
ESI	Electrospray interface
EtOH	Ethanol
FID	Flame ionisation detector
Fig.	Figure
GC	Gas chromatography
Glu.	Glucoside
HPLC	High performance liquid chromatography
I.D.	Inside diameter
KMBA	α-Keto-γ-methiolbutyric acid
MeOH	Methanol
MS	Mass spectrometry
n.a.	not analysable
NMR	Nuclear magnetic resonance
No.	Number
PCA	Protocatechuic acid
RDA	Retro-Diels-Alder
ROS	Reactive oxygen species
Rut.	Rutinoside
SIN-1	3-Morpholinosydnonimine N-ethylcarbamide
TEAC	Trolox equivalent antioxidant capacity
TOSC	Total oxidant scavenging capacity
Trolox	6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxyl acid
UHQ	Ultra high quality

Chapter 1

Introduction

1.1 General introduction

Reactive oxygen species (ROS) are formed continuously in several metabolic pathways of aerobic organisms such as electron transport chains and active phagocytosis or as intermediates during various enzyme driven reactions [Dip98, Tur99]. The main ROS resulting from these processes include:

- alkoxyl radical (RO·),
- hydrogen peroxide (H_2O_2),
- hydroxyl radical (HO·),
- hypochlorous acid (HOCl),
- peroxyl radical (ROO·)
- peroxynitrite ($ONOO^-$) and
- superoxide anion (O_2^-) [Dip98, Rad96].

Normally, the oxidative damage they can cause to macromolecules such as DNA, proteins and lipids is prevented by a series of specially adapted enzyme systems (e.g. superoxide dismutase and catalase) and several both water and lipid soluble non-protein compounds (e.g. uric acid and tocopherols) [Hall89, Abu99].

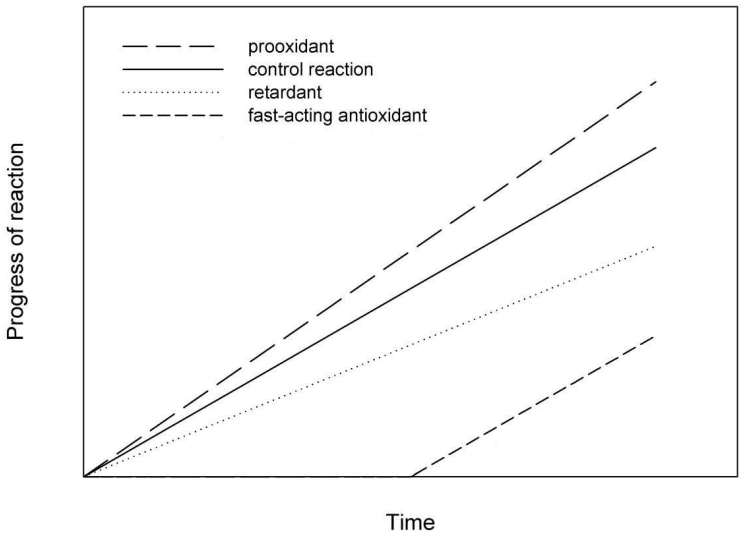

Figure 1.1: Fast-acting antioxidants, retardants and prooxidants

From their reaction mode, two types of ROS scavengers (see fig. 1.1) can be distinguished [Pry93]

- Fast-acting antioxidants are able to delay the formation or to inhibit the reaction of ROS as long as they are present. When they have been exhausted, the reaction returns to its uninhibited rate. Therefore, they affect a lag-time of reaction and its length depends on the concentration of the antioxidant.

- Retardants react too slowly with ROS to cause a lag-time. Depending on their concentration, they decelerate the rate of the reaction more or less effectively but cannot stop it completely.

Prooxidants, in contrast, accelerate the speed of the radical caused reaction (see fig. 1.1).

The protection offered by ROS scavengers is limited and several external factors like exposure to environmental pollutants or cigarette smoke can enforce the internal formation of ROS. If the ROS formation exceeds the antioxidant capacity of the biological system, oxidative stress results [Dip98, Tur99].

There has been considerable evidence that oxidative stress can play an important role in several human illnesses like arteriosclerosis, cancer, Alzheimer's and Parkinson's disease and also in the aging process [Harm01]. On the other

Figure 1.2: Map of Brazil

hand, epidemiological studies have demonstrated that the intake of antioxidants from food could help to maintain health and to prevent illnesses caused by oxidative stress [Blo92, Rim96]. Fruits and vegetables have received special attention in this field, because they contain high amounts of known antioxidants like polyphenols, vitamin C, vitamin E, β-carotene or lycopene [Ame93, Harm01].

Besides from their physiological significance, antioxidants are also important for the protection of food from ROS caused deterioration, in particular from lipid peroxidation [Aruo97]. As some artificial antioxidants like butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) have demonstrated dose depending toxicological effects [Kah93, Stef03], the demand for "natural" antioxidants is growing worldwide [Aruo97].

1.2 The *Euterpe oleracea* MART. (açaí) palm

1.2.1 Botany and occurrence

Euterpe oleracea MART., Arecaceae, is a palm widely distributed in the north of South America. It is one of the most naturally abundant species in the Amazonian estuary floodplains with its greatest quantitative and economic importance in the Pará State of Brazil (see fig. 1.2) [Cav88, Str88].

The occurrence of the palm ranges from growing in low densities to nearly mono-specific stands in homegardens, secondary forest and unman-

Figure 1.3: The *Euterpe oleracea* MART. (açaí) palm

aged woods [Mun96]. The "açaí" tree (pronounced ā-sigh-ee), as it is known to the local inhabitants of the Amazon basin, can reach a height of over 30 m and has pinnate leaves. It is multi-stemmed, sometimes having more than 45 slender trunks in different states of growth and fructification (see fig. 1.3).

As the açaí palm generates additionally a large number of rapidly germinating seeds, it is able to regenerate after the cutting of several stems and has nowadays become the world's main source of palm hearts [Str88, Mun96].

1.2.2 The fruits

Other non-timber products of *E. oleracea* are only of regional relevance, though they have begun recently to get popular in bigger centres like São Paulo and Rio de Janeiro, Brazil. The most important of these goods are the spherical grape-sized fruits that grow in bunches (see fig. 1.4). They are green when young and ripen usually to a dark purple [Str88]. This colour is due to anthocyanins though their exact identification is still discussed con-

Figure 1.4: Açaí fruits

tradictory in literature [Rog00, Bob00, Poz04]. Only some varieties of açaí have fruits that stay green even in their mature stage, and they are locally called "açaí branco" (white açaí) [Str88, Rog00].

Fruits can be collected all over the year [Str88, Mun96] with a main harvesting period during the so-called "dry months" [Smi99, Rog00]. In the area of Belém, Pará - Brazil (see figure 1.2), at the Amazon delta, e.g., this period lasts from August to December. At this time of the year, açaí fruits have a more homogeneous degree of maturation resulting in better organoleptic qualities. The low harvesting season in the rainy months from March to June gives less fruits of lower quality [Rog00].

The fruits are primarily used to prepare a liquid with the consistency of milk-shake by macerating their pulp and mixing it with different amounts of water mostly with simple blenders in special açaí shops [Str88, Rog00]. Depending on the creaminess and the added amount of water, the beverages are divided into different trading qualities. The most important categories are açaí grosso, medio and fino, though this classification is not regulated

Trading qualities	Dry matter content [%]
Açaí grosso	10.67 – 19.86
Açaí medio	8.45 – 15.35
Açaí fino	5.87 – 14.18

Table 1.1: Dry matter range of açaí grosso, medio and fino in the city of Belém, Brazil [Rog00]

.

by law. Huge differences can appear in the composition of commercial açaí samples even if they are from the same trading category. The range of dry matter content is exemplified in table 1.1 for the city of Belém, Brazil, with values varying due to city district and harvesting season.

The açaí juice is consumed pure, sweetened with sugar or thickened with manioc or tapioca flour to form a porridge-like meal. There are also mixtures available with other tropical fruits like acerola (*Malpighia punicifolia*, Malpighiaceae) or guaraná (*Paullinia cupana*, Sapindaceae). Further açaí products are, e.g., ice-cream, milk shake, mousse, chocolate and pie [Str88].

The taste of açaí products is described by the local population as "unusual but savoury" and it is very popular throughout all socio-economic levels [Smi99]. It has been reported, that individual consumption can even reach up to two litres per day by the Indian descent population which suppers açaí habitually [Str88]. On the other hand, flavorists have characterised the taste as similar to beet and carrot, not pleasant, not acid, with a weak odour [Bau00]. Other sources classify it as unique and difficult to describe, metallic, slightly nutty and somewhat creamy with an oily appearance [Str88]. The latter sensation is thereby due to the rather high fat content of the beverage (see table 1.2).

Sales promotions of açaí, especially on the internet, advertise the product to posses high antioxidant capacities and beneficial effects especially for sportsmen. To order açaí juice has nowadays come into fashion in modern Brazilian fitness centres, and it is rumoured that people who consume açaí appear strong and full of energy [Str88]. With the exception of the energy donating properties due to the high fat and protein contents (see table 1.2), only little research has been done in this field. In a study of Pozo-Onsfran et al. [Poz04], açaí pulp had demonstrated relatively high antioxidant capacities compared to other juices. But as this study was based only on a single açaí sample, its significance remains questionable.

Major compounds	[g/100g dry matter]
Fat	45.9 – 50.7
Fibres	32.3 – 34.0
Proteins	8.3 – 18.2
Minerals	2.0 – 3.5
Total carbohydrates	1.5 – 6.7
Glucose	0 – 1.5
Minor compounds	**[mg/100g dry matter]**
Potassium	499 – 932
Calcium	133 – 286
Magnesium	121 – 174
Phosphorus	99 – 124
Sodium	16 – 56.4
Iron	1.5 – 26.0
Zinc	2.0 – 7.0
Copper	1.7 – 2.0

Table 1.2: Composition of the açaí beverage [Rog00]

1.2.3 The seeds

Each açaí fruit contains one light brown seed (see fig. 1.5) that accounts for about 90% of the fruits diameter. The seeds are covered by a layer of rough fibres under the thin violet pulp layer [Str88].

In table 1.3, the composition of açaí seeds is displayed. It is considered

Compounds	Content [g/100g dry matter]
Fibres	63.0 - 81.3
Minerals	1.6 - 6.0
Proteins	5.0 - 5.6
Fat	1.7 - 3.5

Table 1.3: Composition of the açaí seeds [Rog00]

that only in the city of Belém, Brazil, between 100,000 and 120,000 tons of açaí fruits are worked up commercially every year yielding about 300 tons of açaí seeds per day [Str88]. Therefore, shops that have specialised on producing açaí liquids can be easily identified not only by advertisement signs but also by the huge piles of açaí seeds lying in their backyards. Some of the seeds are utilised as pig food or, when rotted, for making a very rich potting soil for plantations or homegardens [Smi99]. Açaí seeds have also become part

Figure 1.5: Açaí seeds

of the local arts and crafts where they are used, e.g., as bracelets or for the decoration of vases and Christmas trees. But most of them are just discarded in the streets and have to be carried away as organic waist by garbage men. As there are no taxes raised for the complete commercial producing chain of açaí, this causes considerable high costs for the local prefectures [Rog00].

A very interesting property of açaí seeds has been observed by local Brazilian traders: the purple açaí beverage decolourises rapidly when it is kept at ambient temperature. But this loss of anthocyanins can be retarded easily by adding halved açaí seeds to the juice. Consequently, there must be compounds present in the seeds that are able to protect the pigments or, in other words, to work as antioxidants. This conclusion is confirmed by a research of Choi et al. [Cho98] about the antioxidant activities of extracts from tropical and oriental medical plants. In their survey, *Euterpe oleracea* seed extracts showed strong antioxidant activities on the oxidation of linoleic acid as well as potent scavenging capacities against DPPH radicals and the superoxide anion. But the compounds that are responsible for these antioxidant capacities have not yet been identified.

1.3 The Total Oxidant Scavenging Capacity (TOSC) assay

Numerous in vitro methodologies have been developed to measure antioxidant capacities. The following demands on this kind of assays can be seen as a consensus in literature:

- At least two different methods should be applied, because it is absolutely possible for an antioxidant to succeed in one assay, to fail in another or to act even prooxidative in a third one [Schl02, Hall95b].
- ROS should be used with relevance to processes in vivo and/or in food [Hall95b].
- Compounds should be assayed at concentrations achievable in the food matrix or in vivo [Hall95b, Reg99].
- The assay should be suitable for pure solutions as well as complex biological tissues.
- Both water- and lipid-soluble compounds should be applicable.

1.3.1 Setup

The Total Oxidant Scavenging Capacity (TOSC) assay developed by the working groups of Regoli and Winston [Reg99, Wins98, Dug00] is a rather new method to measure in vitro antioxidant activities. The assay is based upon the ethylene yielding reaction of the ROS peroxyl radicals, peroxynitrite and hydroxyl radicals with α-keto-γ-methiolbutyric acid (KMBA) (see fig. 1.6).

- Peroxyl radicals are formed by the thermal homolysis of 2,2'-azobis(2-methylpropionamidine) dichloride (ABAP) (see fig. A.1).
- Peroxynitrite is produced by the decomposition of 3-morpholinosydnonimine N-ethylcarbamide (SIN-1) (see fig. A.2).
- Hydroxyl radicals are generated during the iron plus ascorbate driven Fenton reaction (see fig. A.3).

These three ROS cover a broad range of highly different half lives and reactiveness:

- Peroxyl radicals are rather stable molecules with a half-life of several seconds [Dip98].

- Peroxynitrite is already much more reactive with a half-life of 1.9 seconds at a physiological pH. It decomposes rapidly yielding strong oxidants with a reactivity similar to that of hydroxyl radicals [Bec90].

- Hydroxyl radicals are one of the most reactive oxygen species with an estimated half-life of about 10^{-9} seconds [Dip98]. They have so extremely high rate constants that they combine with almost every kind of molecules in their surrounding [Hall84]. Therefore, nearly every compound that is present in food can be seen as a hydroxyl scavenger though they are all not very effective [Reg99]. In addition, different molecules like vitamin C can act as prooxidants for the formation of hydroxyl radicals [Deu98].

The time course of ethylene formation during the TOSC assay is monitored by repeated gas chromatographic analysis of aliquots from the headspace of the reaction vessels. The oxidisable substrate KMBA is kept at a constant concentration and assay conditions are applied which lead to an equivalent ethylene yield by all applied ROS. Thereby, the effectiveness of antioxidants against the three different oxidants can be compared under similar conditions [Reg99, Wins98].

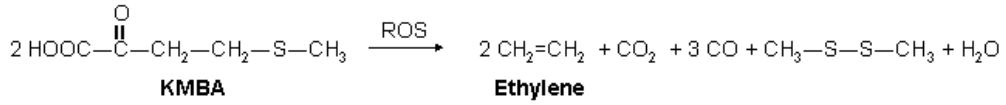

Figure 1.6: Ethylene yielding reaction of KMBA with ROS

1.3.2 Data evaluation

In the TOSC assay, an antioxidant is characterised by its capacity to inhibit the ethylene production by the respective ROS compared to an uninhibited control reaction. Therefore, the kinetic curve that best fits the experimental GC data for ethylene production and the area beneath it are calculated mathematically. TOSC values are quantified by comparing the areas for control (AC) and sample reaction (AS) in accordance to equation 1.1 [Reg99].

$$TOSC[\%] = 100\% - (\frac{\int AS}{\int AC} \cdot 100\%) \tag{1.1}$$

A sample with no ROS scavenging capacity receives a TOSC value of 0%, because it has the same area under the curve (AUC) as the control reaction. A compound that suppresses the ethylene formation entirely possesses an AUC of 0 and thereby a TOSC value of 100% [Reg99, Wins98]. A prooxidant obtains a negative TOSC value because of an AUC greater than that of the control reaction [Leu00].

TOSC values calculated by equation 1.1 give the antioxidant capacity for a specific concentration of a compound. For displaying the results in a more general way, different approaches have been made:

- The **r**TOSC or **relative** TOSC value signifies the slope of the regression line within the linear TOSC to concentration range [Dug00].
- The **c**TOSC or **comparative** TOSC value compares the rTOSC of a compound to that of the water-soluble vitamin E analogue Trolox according to equation 1.2 [Dug00]:

$$cTOSC = \frac{rTOSC(antioxidant)}{rTOSC(Trolox)}. \tag{1.2}$$

- The **s**TOSC or **specific** TOSC value refers to the antioxidant activity of 1 μg [Reg00a] or 1 mg of substance [Reg98].

The first two values presume necessarily the existence of a linear range within the TOSC to concentration curve of a compound. All three have in common that they display only a small section of the complete TOSC to concentration range. Therefore, an improved data evaluation method is necessary.

1.3.3 Application area

The TOSC assay was designed originally to study the oxidative stress of marine organisms like scallops [Reg00a], mussels [Reg98], sponges [Reg00b], penguins [Cor01] or flounders [Winz01] as a biomarker for aquatic environmental pollution.

Outside of this area, only few TOSC publications can be found. In addition, the food concerning surveys are all limited to peroxyl radicals. Examples are articles about the antioxidant activities of flavonoids [Dug00], linoleic acid isomers [Leu00], apples [Ebe00, Wol03a, Wol03b], strawberries [Mey03], raisins [Yeu03] and some other fruits [Sun02].

1.3.4 Advantages

In summary, in can be said that the TOSC assay accomplishes all the experimental claims for in vitro antioxidant testing methods:

- Three different ROS with an important potential to damage biological tissues and a broad range of different reactiveness are used [Reg99, Wins98].
- If desired, even more ROS could be applied in combination with KMBA, e.g., hypochlorous acid [Law85, Kru95] or alkoxyl radicals [Pry94].
- Compounds can be examined down to the lower μM range [Wins98, Leu00].
- The test can be applied to pure antioxidant solutions as well as to complex biological samples like fluids and tissues [Reg99, Wins98].
- It is suitable for detecting both water- and lipid-soluble antioxidants [Wins98, Leu00].

Additional advantages are:

- As the TOSC assay is an AUC technique, it considers the different kinetics of antioxidants and is thereby superior to assays that measure only either an inhibition rate at a fixed time or the lag-phase of a reaction [Pri99].
- In addition, based on these kinetics, fast-acting antioxidants and retardants can be distinguished [Reg99, Wins98].
- Prooxidants are also detectable with this method [Leu00].
- Last but not least, research can be performed with a common GC system.

1.3.5 Limitations

Yet, there are also some drawbacks of the TOSC assay up to now:

- a high time and labour consumption and therefore unsuitability for high throughput surveys because of
  - the need for frequent manual GC injections,

  - thereby, the limitation for analysing samples in parallel,
  - the short shelf life of the the test solutions (SIN-1, e.g., has to be prepared directly before the assay) [Regoli; personal communication] and
  - the low degree of standardisation (antioxidants are added to the assay system in different concentrations and/or volumes and the volumes of the other assay solutions are adjusted accordingly) [Regoli; personal communication],

- the limited data base for TOSC values of food stuffs in literature, especially for peroxynitrite and hydroxyl radicals, and
- the rather complex and time-consuming calculation of TOSC values with common software programs and the limited possibilities for displaying the results.

1.4 Objective and approach of this work

The objective of this work was to analyse the antioxidant capacities of *Euterpe oleracea* MART. (açaí) pulps and seeds against the reactive oxygen species peroxyl and hydroxyl radicals as well as peroxynitrite. In addition, the main compounds should be identified that are responsible for these properties.

To reach these aims, the following steps were taken:

1. Enhancing the TOSC assay conditions by
   - minimising its time and labour consumption and
   - improving its data evaluation.
2. Creating a basis for classifying the açaí results by
   - analysing the antioxidant activities of a number of standard compounds and
   - surveying the antioxidative spectrum of several common European fruit and vegetable juices.
3. Measuring the antioxidant capacities of açaí pulps and seeds by including to the survey
   - non-commercial açaí pulps of different harvesting years and seasons,

- commercial açaí juices of different trading qualities,
- açaí seeds of different harvesting years and seasons and
- pulps and seeds from the purple as well as the white açaí variety.

4. Identifying the main antioxidative compounds of açaí pulps and seeds by
   - separating and fractionating the açaí samples by HPLC and analysing the TOSC values of the fractions,
   - identifying the major compounds of the most active fractions, quantifying them and
   - estimating their contributions to the overall antioxidant capacities of the samples.

Chapter 2

Materials

2.1 Chemicals

2.1.1 Compounds for TOSC assay

- ABAP [2,2'-Azobis(2-methylpropionamidine) dichloride], 98%, no. 40156, Acros Organics (Geel, Belgium),
- Ascorbic acid, no. 900056, Kraemer & Martin (Sankt Augustin, Germany),
- DTPA (Diethylenetriaminepentaacetic acid), purified, no. D-6518, Sigma (Steinheim, Germany),
- EDTA (Ethylenediaminetetraacetic acid), 99%, no. 11843, Acros Organics (Geel, Belgium),
- Ferric chloride ($FeCl_3$) hexahydrate, p.a., no. 21709, Acros Organics (Geel, Belgium),
- KMBA (α-Keto-γ-methiolbutyric acid) sodium salt, no. K-6000, Sigma (Steinheim, Germany),
- tri-Potassium phosphate (K_3PO_4) trihydrate, extra pure, no. 105102, Merck (Darmstadt, Germany),
- SIN-1 (3-Morpholinosydnonimine N-ethylcarbamide) hydrochloride, no. 32885, Sigma (Steinheim, Germany).

2.1.2 Standard compounds

- Ascorbic acid, no. 900056, Kraemer & Martin (Sankt Augustin, Germany),
- Benzoic acid, p.a., no. 100136, Merck (Darmstadt, Germany),
- (+)-Catechin hydrate, 98%, no. C-1251, Sigma (Steinheim, Germany),
- Cyanidin-3-glucoside (Kuromanin) chloride, no. 0915S, Extrasynthese (Genay, France),
- Cyanidin-3-rutinoside (Keracyanin) chloride, no. 0914S, Extrasynthese (Genay, France),
- (-)-Epicatechin, no. E-1753, Sigma (Steinheim, Germany),
- Protocatechuic acid, no. P-5630, Merck (Darmstadt, Germany),
- Trolox (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), 97%, no. 21894, Acros Organics (Geel, Belgium),
- Uric acid, 99+%, no. 17129, Acros Organics (Geel, Belgium).

2.1.3 Further compounds

- BSTFA (N,N-bis-Trimethylsilyltrifluoroacetamide), no. 70122, Macherey-Nagel, (Düren, Germany),
- Ethylene gas, $\geq 99.95\,\%$, no. 00489, Fluka (Buchs, Switzerland),
- Folin & Ciocalteu's Phenol Reagent, 2N, no. F-9252, Sigma (Steinheim, Germany),
- UHQ (Ultra high quality) water, prepared with an UHQ-II system (ELGA, Ubstedt-Weiher, Germany).

2.2 Fruit and vegetable juices

All juices were stored at -28° C.

2.2.1 Commercial samples

The following commercial fruit and vegetable juices were purchased in a local supermarket:

- ACE juice "ACE Vitaminsaft" [vitamin A-, C- and E-rich juice; mixture of orange, apple, grapefruit, passion fruit, acerola, lemon and carrot juice with banana and rosehip pulp and a declared content of 30 mg vitamin C, 5 mg vitamin E and 400 μg vitamin A (added as provitamin A) per 100 mL], WeserGold (Rinteln, Germany),
- Apple juice "Apfelsaft", WeserGarten (Rinteln, Germany),
- Beetroot juices
  - Beetroot juice I "Rote-Bete-Saft" (lactic acid fermented) from Alnatura GmbH (Bickenbach, Germany) and
  - Beetroot juice II "Rote-Bete-Saft" (ca. 0.3% lemon juice added [personal communication of the producer]) from Walther Schoenenberger Pflanzensaft GmbH & Co. (Magstadt, Germany),
- Blueberry juice "Heidelbeersaft Muttersaft", Voelkel GmbH (Höhlbeck, Germany),
- Carrot juices
  - Carrot juice I "Möhrensaft" (lactic acid fermented) from Alnatura GmbH (Bickenbach, Germany) and
  - Carrot juice II "Bio-Möhrensaft" (1 to 2% lemon juice added [personal communication of the producer]) from EDEN-WAREN (Hünfeld, Germany),
- Elderberry juice "Holundersaft Muttersaft", EDEN-WAREN (Hünfeld, Germany),
- Lingonberry juice "Preiselbeersaft Muttersaft", Voelkel GmbH (Höhlbeck, Germany),
- Multivitamin juice "Multi-Vitamin-12-Fruchtsaft" [mixture of apple, orange, pear, pineapple, grape, passion fruit and lemon juice with banana, nectarine, mango, guava and papaya pulp and a declared averaged content of 45 mg vitamin C, 6.5 mg vitamin E and 280 μg vitamin A (added as provitamin A) per 100 mL],

- Sauerkraut juices
  - "Sauerkrautsaft" from Alnatura GmbH (Bickenbach, Germany) and
  - "Bio-Sauerkrautsaft" from EDEN-WAREN (Hünfeld, Germany),
- Sour cherry nectar "Rio Grande Sauerkirsch-Nektar" (mixture of at least 50% cherry juice with water, sugar and glucose-sirup), EUCO GmbH (Hamburg, Germany) and
- Tomato juices
  - "Tomaten-Saft" from Walther Schoenenberger Pflanzensaft GmbH & Co. (Magstadt, Germany) and
  - "Le Jus Bio" from Moulin de Valdonne (Peypin en Provence, France).

The carrot and tomato juices were filtrated through a folded filter (Schleicher & Schuell, Dassel, Germany) to remove insoluble parts.

2.2.2 Self-pressed samples

The following juices were prepared by buying fresh fruits in a local supermarket, pressing them with a common juicer and filtrating them through a folded filter (Schleicher & Schuell):

- Lemon juice,
- Orange juice and
- Pink grapefruit juice.

2.3 Açaí pulps

All pulps were stored at -28° C.

2.3.1 Commercial samples

- One commercial sample of açaí medio (açaí medio I) was bought at a local supermarket in Campinas, São Paulo - Brazil, and transported to Germany by air mail.

- The following commercial samples were obtained for free from the import-export company Klaus Böcker GmbH (Buxtehude, Germany):
  - two samples of açaí grosso (açaí grosso I and II),
  - one sample of açaí medio (açaí medio II) and
  - two samples of açaí fino (açaí fino I and II).

All samples were frozen during transport.

2.3.2 Non-commercial samples

For non-commercial samples, açaí fruits were harvested in the area of the river Aurá near Belém, Pará - Brazil (see fig. 1.2). The samples were gathered from always the same trees in the following harvesting years and seasons:

- Purple açaí:
  - 1998, main harvesting period,
  - 2000, main harvesting period,
  - 2001, low harvesting period and
  - 2002, main harvesting period.
- White açaí:
  - 2002, main harvesting period.

The different harvesting periods of açaí fruits are described in 1.2.2.

The pulps were separated from the seeds in a commercial açaí shop as described in 1.2.2. The samples were freeze-dried in Brazil immediately after preparation and transported to Germany via air mail.

2.4 Açaí seeds

Açaí seeds were gained from the above described self-harvested fruits (see 2.3.2).

- For preliminary tests, an ethanolic extract of seeds from the purple açaí variety of the high harvesting season of 2000 was prepared directly in Brazil by extracting ground seeds exhaustively with a Soxhlet extractor and drying the extract with a vacuum evaporator.

No.	Variety	Harvesting year	Harvesting season	Solvent	Extraction method
1	purple	2000	main	EtOH	Soxhlet
2	purple	2001	low	MeOH	cold
3	purple	2001	low	MeOH	Soxhlet
4	purple	2001	low	EtOH	cold
5	purple	2001	low	EtOH	Soxhlet
6	purple	2002	main	MeOH	cold
7	purple	2002	main	MeOH	Soxhlet
8	purple	2002	main	EtOH	cold
9	purple	2002	main	EtOH	Soxhlet
10	white	2002	main	MeOH	cold
11	white	2002	main	MeOH	Soxhlet
12	white	2002	main	EtOH	cold
13	white	2002	main	EtOH	Soxhlet

Table 2.1: List of prepared açaí seed extracts

- For further analyses, the following seed samples were gathered:
  - 2001, low harvesting seasons, purple açaí variety,
  - 2002, main harvesting season, purple açaí variety as well as
  - 2002, main harvesting season, white açaí variety.

All samples were sent to Germany via air mail. The seeds were extracted in Germany as follows:

- For cold extractions, 100 g of thoroughly crushed açaí seeds were extracted repeatedly with a total of 1 L (2 x 350 mL and 1 x 300 mL) methanol (MeOH) and ethanol (EtOH) at room temperature under occasional stirring during an overall extraction period of three days. The solvents were removed on a sandbed at 30° C.
- For Soxhlet extractions, 40 to 50 g of crushed seeds were extracted with 300 mL methanol and ethanol for 9 hours in a Soxhlet extractor. The solvents were removed in a vacuum drying cabinet at 30° C.

An overview of the prepared açaí seed extracts is given in table 2.1. All seeds and seed extracts were stored at -28° C.

2.5 Analytical equipment

2.5.1 GC system with CombiPAL autosampler

GC system	
Instrument	GC-17A (Shimadzu, Duisburg, Germany)
Detector	Flame ionisation detector (FID)
Software	EZChrom Elite v2.8 (Scientific Software, Pleasanton, USA)
Autosampler	
Instrument	CombiPAL (CTC Analytics, Zwingen, Switzerland)
Syringe	1 mL Headspace syringe
Incubator	Agitator with 6 heatable positions and interval shaking
Software	PAL Cycle Composer v1.5 (CTC Analytics)

Table 2.2: GC system for ethylene quantification within TOSC assay

2.5.2 HPLC-MS system

HPLC system	
Instrument	System Gold (Beckman, Unterschleißheim, Germany)
Degasser	Gastorr 154 (SFD Schambeck, Bad Honnef, Germany)
Solvent module	126
Autosampler	507e
Column oven	Jetstream (W.O.electronics, Langenzersdorf, Austria)
Detector	168 Diode array detector equipped with a micro cell
Software	32Karat Software package v.3 Build 937 (Beckman)
Mass spectrometer	
Instrument	LCQ classic ion-trap (Thermo Finnigan, Egelsbach, Germany)
Ion source	Electrospray interface (ESI) with metal needle kit
Sheath liquid	System Gold Solvent Module 116 (Beckmann)
Software	Xcalibur Software v.1.2 (Thermo Finnigan)

Table 2.3: HPLC-MS system for identification of compounds in açaí samples

2.5.3 HPLC system I

Instrument	600 Multisolvent Delivery System (Waters, Eschborn, Germany)
Degasser	Degasys 1310 (Uniflows, Tokyo, Japan)
Detector	LC 55 B UV-Vis detector (Perkin-Elmer, Norwalk, USA)
Software	EZChrom Elite v2.8 (Scientific Software Inc.)

Table 2.4: HPLC system I for quantification of anthocyanins and fractionating of samples

2.5.4 HPLC system II

Instrument	ESA system (ESA, Chelmsford, USA)
Pumps	Two ESA 580 HPLC pumps
Degasser	Degasys 1210 (Uniflows)
Autosampler	ESA 540
Detector 1	Beckman 168 diode array detector (Beckman)
Detector 2	Coularray model ESA 5600 with six coulometric array cells
Software	Beckman Gold 7.11 (Beckman)

Table 2.5: HPLC system II for quantification of polyphenols in açaí seeds

2.5.5 UV-Vis spectrophotometer

Instrument	554 UV-Vis photometer (Perkin-Elmer, Norwalk, USA)

Table 2.6: UV-Vis photometer for analyses of total phenolic content

2.5.6 GC-MS system

GC system	
Instrument	DI 200 (Delsi-Nermag, Argenteuil, France)
Mass spectrometer	
Instrument	Automass 60 quadrupol (Delsi-Nermag)
Ion source	Electron impact ionisation mode
Software	Lucy v.2.1 (Delsi-Nermag)
Spectrum library	NIST (National Institute of Standards and Technology, Gaithersbury, USA)

Table 2.7: GC-MS system for identification of organic acids in açaí pulps

2.5.7 MALDI-TOF system

Instrument	Voyager DE (PE Biosystems, Weiterstadt, Germany)
Tube	1.2 m flight tube
Laser	LSI nitrogen laser
Recording software	Voyager Instrument Control Software v.950025 REV1
Spectrum processing	PE Data Explorer software v.3.4.0.0

Table 2.8: MALDI-TOF system for analysis of anthocyan degradation products

Chapter 3

Methods

3.1 TOSC assay

All glassware were washed thoroughly with UHQ water before use in the TOSC assay.

3.1.1 Preparation of assay solutions

- **Potassium phosphate buffer**: A 100 mM (53.264 g/2L) K_3PO_4 buffer was prepared and the pH was adjusted with phosphoric acid to 7.4.
- **Solution 1**: A 0.25 mM KMBA (0.00846 g/200mL) plus 0.1 mM DTPA (0.00786 g/200mL) solution was made by dissolving the compounds jointly in the potassium phosphate buffer.
- **Solution 2**: A joint 2.25 μM $FeCl_3$, 4.5 μM EDTA and 0.25 mM KMBA solution was produced. Therefore, $FeCl_3$ and EDTA were each dissolved separately as a 225 μM $FeCl_3$ solution (0.0061 g/100mL) in UHQ water and a 450 μM EDTA solution (0.0033 g/25mL) in the potassium phosphate buffer, respectively. From each of these solutions, 2 mL were poured in a joint 200 mL graduated flask. 0.00846 g KMBA were added and the flask was filled up with buffer.
- **Solution 3**: A 200 mM ABAP solution (5.424 g/100mL) was made with the potassium phosphate buffer.
- **Solution 4**: A 0.8 mM SIN-1 solution (0.0165 g/100mL) was made with UHQ water.
- **Solution 5**: A 1.8 mM solution of ascorbic acid (0.0316 g/100mL) was prepared in UHQ water.

The solutions 1 and 2 were divided in portions of 800 μL and filled in septum sealed 10 mL headspace vials yielding 250 vials per preparation. The solutions 3, 4 and 5 were each put into septum capped 1.5 mL HPLC vials in portions of 1 mL giving each a stock of 100 vials. All solutions were stable at -28° C for at least 3 months.

3.1.2 Preparation of samples

3.1.2.1 Standard compounds

- Benzoic acid, trolox and uric acid were dissolved in the 100 mM potassium phosphate buffer at pH 7.4.
- Ascorbic acid, (+)-catechin, cyanidin-3-glucoside, cyanidin-3-rutinoside, (-)-epicatechin and protocatechuic acid were dissolved in UHQ water.
- All standard compounds were prepared in at least five different concentrations for each of the ROS to cover the respective range from a low to a high antioxidative capacity as complete as possible.
- Each of these solutions was analysed at least in quadruplicate.

3.1.2.2 Fruit and vegetable juices

- The juices (see 2.2) were thinned with UHQ water to at least five different dilutions for each of the three ROS as described above for the standard compounds.
- The diluting was done in duplicate in all cases and each solution was measured at least twice.

3.1.2.3 Açaí pulps

- 5 g of the freeze-dried non-commercial pulps (see 2.3.2) were suspended with UHQ water to a final volume of 50 mL [i.e. the medium dry matter content of the commercial juices (see table 1.1)] and the suspensions were ultrasonicated for 10 min.
- These suspensions as well as the commercial samples (see 2.3.1) were centrifuged for 10 min at 5000 U/min or 2800 g, resp., with a Heraeus Biofuge stratos (Kendro, Hanau, Germany) and filtrated through a folded filter (Schleicher & Schuell, Dassel, Germany).

TOSC assay solutions (cf. 3.1.1)	Generation of		
	Peroxyl radicals	Peroxynitrite	Hydroxyl radicals
Solution 1	+	+	–
Solution 2	–	–	+
Solution 3	+	–	–
Solution 4	–	+	–
Solution 5	–	–	+

Table 3.1: Used TOSC assay solutions for the generation of the different ROS

- The resulting solutions were diluted with UHQ water as described for the fruit and vegetable juices.

3.1.2.4 Açaí seed extracts

- 0.5 g of the seed extracts (see 2.4) were suspended with UHQ water to a final volume of 10 mL, sonicated for 10 min, centrifuged for 10 min at 5000 U/min or 2800 g, resp., with a Heraeus Biofuge stratos (Kendro) and filtrated through a folded filter (Schleicher & Schuell).

- The solutions were diluted with UHQ water as described for the fruit and vegetable juices.

3.1.3 Assay procedure

- For control reactions, 100 μL of UHQ water were injected manually through the septum of the headspace vials with solution 1 or 2 (see table 3.1).

- For sample reactions, 100 μL of sample solution were used instead.

- Six samples were prepared simultaneously and were tempered at the agitator unit of the CombiPAL autosampler (see table 3.2). One sample set included normally five sample reactions with declining concentrations and one control reaction.

- The ethylene yielding reaction of KMBA and ROS of the first sample was started by injecting manually 100 μL of solution 3, 4 or 5 (see table 3.1) through the septum of the headspace vials.

- Simultaneously, the CombiPAL autosampler was started (see table 3.2).

Instrument	CompiPAL autosampler (see table 2.2)
Incubation temperature Incubation time	37° C 60 min
Sample agitating	Every 55 s for a period of 5 s
Parallel analysed samples	6
Sampling times Sample amount	0, 12, 24, 36, 48 and 60 min 100 μL from sample headspace
Sampling method and macro	See B.1

Table 3.2: Automated TOSC assay procedure by CombiPAL autosampler

- The GC analyses of ethylene were performed according to table 3.3.
- To the next samples, the reaction starter was charged at intervals of 2 min.
- The samples were shaken repeatedly and the further sampling was accomplished automatically by the CombiPAL autosampler in accordance to table 3.2.

Instrument	GC system with CombiPAL autosampler (see table 2.2)
Column	Chrompack PoraPLOT Q column 27.5 m x 0.53 mm x 20 μm (Varian, Darmstadt, Germany)
Carrier gas Carrier gas flow	Nitrogen 15 mL/min
Split	Off
Oven temperature Injector temperature Detector temperature	80° C 100° C 220° C

Table 3.3: GC parameters for analyses of ethylene

3.1.4 Data evaluation

An overview of the evaluated parameters is provided in figure 3.1.

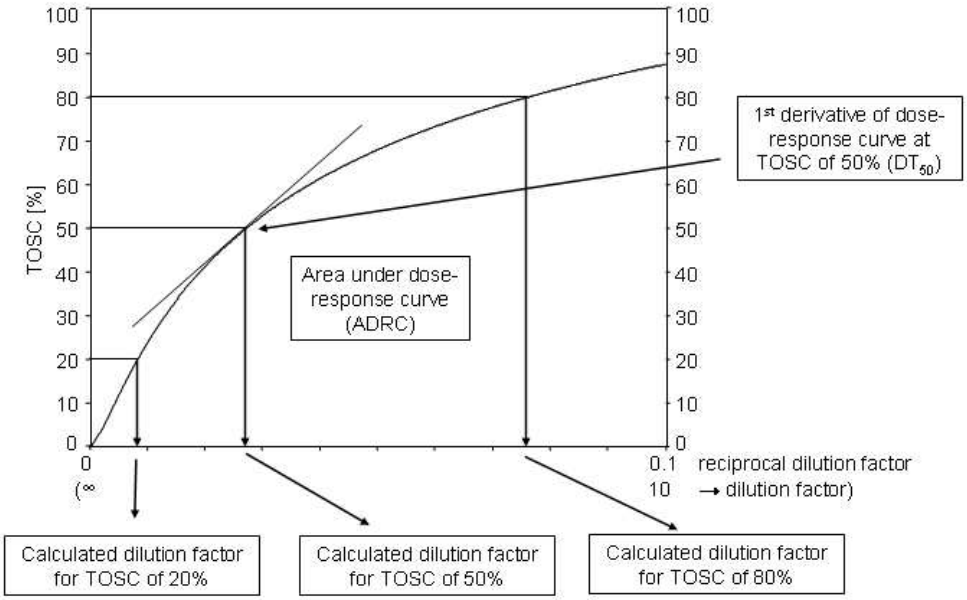

Figure 3.1: Overview of data evaluation

3.1.4.1 Experimental TOSC values

The kinetic curves that best fit the experimental GC data for ethylene production over 60 min and the area beneath them were calculated. Calculations were made using a C-macro specially coded for this purpose in combination with the data analysis software Root v3.02/07 (developed at the CERN particle physics centre, Genève, Switzerland).

The data for control and sample reactions are saved as ".dat"-files with a common text editor as displayed in the tables 3.4 and 3.5 including:

- the kind of ROS that was assayed,
- either "control" or the concentration and kind of sample that was analysed and
- the repeatedly measured peak areas of ethylene for the six sampling times.

Peroxyl radicals					
Control					
0	12	24	36	48	60
0	8592	18301	28186	38058	47988
0	8596	18513	28262	37841	47512
0	7881	17232	26703	35941	45173
0	8462	18442	28527	38084	47825

Table 3.4: Example for a control reaction data file

Peroxyl radicals					
Sample					
0	12	24	36	48	60
0	724	4095	10102	17194	24617
0	701	4311	10343	17402	25214
0	802	4554	11133	19009	27030
0	721	4210	10411	17640	25166

Table 3.5: Example for a sample reaction data file

In the "Root" program shell, the macro "antiox.C" (see B.2) is loaded with **.L antiox.C**. The data evaluation is started with **"AntioxLoad"** in combination with the following information:

- name of control file,
- appropriate order of polynom for control curve,
- start and end of control curve integration,
- name of sample file,
- appropriate order of polynom for sample curve integration and
- start and end of sample curve integration.

For the displayed files, e.g., a reasonable combination would be **AntioxLoad("control.dat","pol1",2,60,"sample.dat","pol3",6,60)**. The macro output file includes a graphic of the fitted control and sample curves together with the TOSC value of the sample (see figure 3.2).

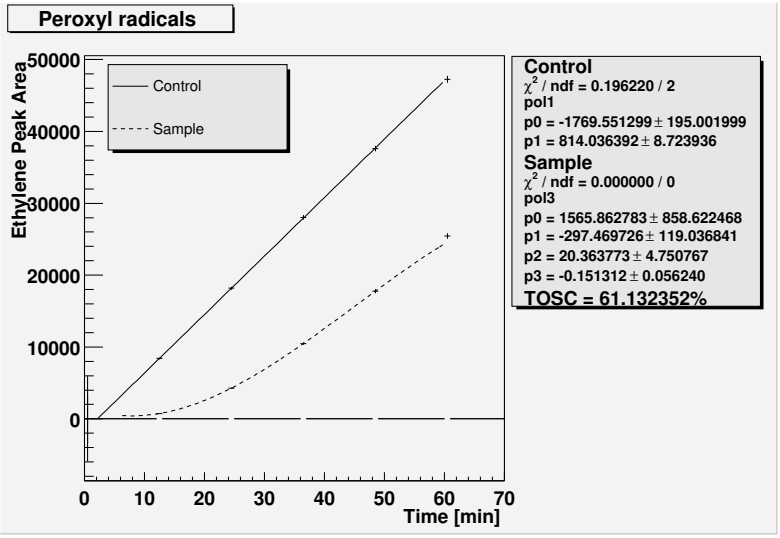

Figure 3.2: Output file from the Root macro

3.1.4.2 Calculated TOSC values

The experimental TOSC values (see 3.1.4.1) were plotted versus

- the corresponding concentrations of the standard compounds and
- the corresponding dilutions of the fruit and vegetable juices, the açaí fruit pulps and the solutions of the açaí seed extracts as described in 2.2, 2.3 and 2.4.

Dose-response curves were fitted that showed the best correlation for the respective data. Based on the resulting equations, the dilution factors of the juices, pulps and seed extracts and the concentrations of the standard compounds were calculated, that matched TOSC values of 20, 50 and 80%, respectively (see figure 3.1). Curve fits and TOSC calculations were accomplished with the software TableCurve 2D v5.1 (SYSTAT Software Inc., Richmond, USA).

3.1.4.3 ADRC and DT_{50}

The areas under the dose-response curves (ADRC) of the juices and the açaí pulps from the zero point of the coordinate system up to a reciprocal dilution of 0.1 (i. e. a dilution of 1:10) were calculated with TableCurve 2D v5.1 from SYSTAT Software Incorporation as well as the 1^{st} derivative of the curves at

a TOSC of 50% (DT_{50}). These values are also visualised in figure 3.1. They were normalised by defining the corresponding area and the 1^{st} derivative of the diagonal of the coordinate system as 1.

3.2 Identification of compounds in açaí samples

The same solutions as prepared for TOSC analyses (see 3.1.2) were also used for the identification of compounds in açaí pulps and seeds. Before HPLC-analyses, the solutions were filtrated additionally through 0.45 μm cellulose membrane filters (Schleicher & Schuell).

Individual polyphenols including anthocyanins were identified by multi step-mass spectrometric fragmentation after high-performance liquid chromatographic separation and UV-Vis diode array detection of açaí samples prepared. The identification of individual sample compounds was based on a UV spectral library setup in the laboratory from standard compounds and the comparison of typical mass fragmentation patterns from the samples and standards.

For GC-MS analyses, the freeze-dried açaí pulps were solved in DMF and trimethylsilylated with BSTFA (N,N-bis-Trimethylsilyltrifluoroacetamide).

3.2.1 Anthocyanins in açaí pulps by HPLC-MS

Instrument	HPLC-MS system (see table 2.3)
HPLC parameters	
Analytical column	Aqua (3 μm C_{18}, 150 mm x 2 mm I.D., 25° C) (Phenomenex, Aschaffenburg, Germany)
Guard column	Security Guard (C_{18}, 4 mm x 2 mm I.D.)
Sample amount	5 μL
Mobile phase A	5% v+v formic acid in distilled water
Mobile phase B	5% v+v acetonitrile in methanol
Flow	200 μL/min
Gradient elution program	
Gradient type	Linear
0 min	10% B
25 min	65% B
Washing step	10 min with 100% B
Re-equilibrating	15 min with the initial conditions
UV scan range	200–595 nm
Comparison with MS data	Track of 520 nm
MS parameters	
Ionisation enhancement	Addition of 100 μL/min methanol
Source voltage	4.0 kV (positive mode)
Sheath gas flow	60
Auxiliary gas flow	25
Capillary voltage	+26 V
Capillary temperature	200° C
First octapole offset	-3 V
Interoctapole lens voltage	-16 V
Second octapole offset	-5 V
Ion trap DC offset	-10 V

Table 3.6: Identification of anthocyanins in açaí pulps by HPLC-MS

3.2.2 Other polyphenols in açaí pulps by HPLC-MS

Instrument	HPLC-MS system (see table 2.3)
HPLC parameters	
Analytical column	Aqua (3 μm C_{18}, 150 mm x 2 mm I.D., 25° C) (Phenomenex, Aschaffenburg, Germany)
Guard column	Security Guard (C_{18}, 4 mm x 2 mm)
Sample amount	5 μL
Mobile phase A	1% v+v acetic acid in distilled water
Mobile phase B	1% v+v acetic acid in acetonitrile
Flow	200 μL/min
Gradient elution program	
Gradient type	Linear
0 min	5% B
45 min	20% B
77 min	60% B
Washing step	10 min with 100% B
Re-equilibrating	15 min with the initial conditions
UV scan range	200–595 nm
Comparison with MS data	Track of 280 nm
MS parameters	
Ionisation enhancement	Addition of 100 μL/min methanol
Source voltage	4.5 kV (negative mode)
Sheath gas flow	60
Capillary voltage	-45 V
Capillary temperature	200° C
First octapole offset	+3 V
Interoctapole lens voltage	+22 V
Second octapole offset	+7 V
Ion trap DC offset	+10 V

Table 3.7: Identification of other polyphenols in açaí pulps by HPLC-MS

3.2.3 Organic acids in açaí pulps by GC-MS

Instrument	GC-MS system (see table 2.7)
GC parameters	
Column	BPX 5 (SGE, Melbourne, Australia) 25 m x 0.25 mm x 0.25 μm
Sample amount	1 μL
Carrier gas Carrier gas flow	Helium 0.5 bar
Split	20 mL/min
Oven temperature	100–300° C at 4° C/min
Injector temperature	300° C
Transfer line temperature	250° C
MS parameters	
Ionisation energy	70 eV
Ion source temperature	250° C

Table 3.8: Identification of organic acids in açaí pulps by GC-MS

3.2.4 Degradation products of anthocyanins by MALDI-TOF

Instrument	MALDI-TOF system (see table 2.8)
Laser beam wavelength Laser pulse width Laser repetition rate	337 nm 3 ns 3 Hz
Polarity Accelerating voltage	Positive 25 kV
Acquisition mass range	1,000 – 25,000 Da
Calibration matrix	Sinapic acid

Table 3.9: Identification of anthocyanin degradation products by MALDI-TOF

3.2.5 Polyphenols in açaí seeds by HPLC-MS

Instrument	HPLC-MS system (see table 2.3)
HPLC parameters	
Solvents	
Mobile phase A	1% v+v acetic acid in distilled water
Mobile phase B	1% v+v acetic acid in acetonitrile
Flow	300 μL/min
Gradient elution program	
Gradient type	Linear
0 min	0% B
80 min	40% B
100 min	100% B
Washing step	10 min with 100% B
Re-equilibrating	10 min with the initial conditions
All other parameters in accordance to table 3.7	
MS parameters	
All parameters in accordance to table 3.7	

Table 3.10: Identification of polyphenols in açaí seeds by HPLC-MS

3.3 Quantification of compounds in açaí samples

3.3.1 Anthocyanins in açaí pulps by HPLC-UV

For quantification of anthocyanins, external standards were used and calibration curves were plotted for each standard compound on the basis of peak area. The HPLC parameters are displayed in table 3.11.

Instrument	HPLC system I (see table 2.4)
Analytical column	MAX-RP 80A (4 μm C_{18}, 150 mm x 4.6 mm) (Phenomenex, Aschaffenburg, Germany)
Guard column	Security Guard, *C_{18}, 4 mm x 3 mm
Sample amount	20 μL
Mobile phase A	2% v+v formic acid in UHQ water
Mobile phase B	2% v+v formic acid in acetonitrile
Flow	800 μL/min
Gradient elution program	
Gradient type	linear
0 min	0% B
40 min	30% B
60 min	90% B
Washing step	10 min with 100% B
Re-equilibrating	20 min with the initial conditions
Wavelength UV detector	525 nm

Table 3.11: Quantification of anthocyanins in açaí pulps

3.3.2 Polyphenols in açaí seeds by HPLC-UV

Instrument	HPLC system II (see table 2.5)
Analytical column	Aqua (3 μm C_{18}, 150 mm x 4.6 mm I.D., 30° C) (Phenomenex, Aschaffenburg, Germany)
Guard column	(Security Guard, C_{18}, 4 mm x 3 mm I.D., 30° C) (Phenomenex)
Sample amount	10 μL
Mobile phase A	0.02 M NaH_2PO, pH 3.4
Mobile phase B	Acetonitrile + 0.05 M NaH_2PO, pH 3.0 (2:1, v:v)

continued on next page

continued from previous page	
Flow	800 µL/min
Gradient elution program	
Gradient type	linear
0 min	0% B
5 min	8% B
25 min	10% B
40 min	21% B
65 min	35% B
80 min	100% B
Washing step	10 min with 100% B
Re-equilibrating	10 min with the initial conditions
Wavelength UV detector	280 nm
Voltage coulometric cells	0, 110, 220, 330, 440 and 550 mV

Table 3.12: Quantification of polyphenols in açaí seeds by HPLC-UV

3.4 Total phenolic content of açaí pulps by UV-Vis spectrometry

The total phenolic content of samples was analysed according to the Folin-Ciocalteu method described by Julkunen-Tiitto [Jul85]:

- 1 g of freeze-dried sample was extracted three times with 80% acetone during a period of 20 h.
- The extracts were concentrated with a vacuum evaporator to a final volume of 30–40 mL, filtrated through a folded filter (Schleicher & Schuell) and filled up with UHQ water to a final volume of 100 mL.
- Depending on the phenolic content, 100–500 µL of the extracts were transferred to a 10 mL flask.
- 2 mL of UHQ water and 1 mL of Folin & Ciocalteu's Phenol Reagent were added and the mixtures were shaken.
- 5 mL of a 20% solution of sodium carbonate were added immediately.
- The resulting solutions were filled up with UHQ water to a final volume of 10 mL and their absorption at 765 nm was analysed after 20 minutes with a UV-Vis spectrophotometer (see table 2.6).

- Gallic acid was used as reference standard.

3.5 Fractionating of samples by HPLC

- For fractionating, samples were separated by HPLC with the same parameters as used for the quantification of anthocyanins in açaí pulps (see table 3.11).
- The sample collecting was started directly after sample injection and the solutions were gathered manually in 6 mL polypropylene scintillation vials (Neolab, Heidelberg, Germany).
- Each fraction spanned a period of 5 min and had thereby a volume of 4 ml due to the HPLC flow of 0.8 mL/min.
- The entire collecting time differed depending on the kind of samples:
  - for açaí pulps and the anthocyanin standard solutions, 45 min of the HPLC run were collected yielding 9 different fraction,
  - for açaí seed extracts, the gathering lasted 60 min giving 12 different fractions.
- Each sample fractionation was carried out twice and also blanks were gathered to take a possible influence from the analytical equipment into account.
- All collected samples were freeze-dried, dissolved in 500 μL of UHQ water, ultrasonicated for 10 min and shaken thoroughly before further analyses.

Chapter 4

Results and discussion

4.1 TOSC assay modifications

The modifications discussed herein have been published at [Lic02].

4.1.1 Incubation temperature

In some TOSC assay publications, samples were incubated during the reaction at 35° C [Reg99, Reg00b] and in others at 39° C [Wins98, Dug00]. Though the rate of radical generation is twofold higher at 39° C than at 35° C, the TOSC values are not affected by this changing [Wins98].

Therefore, the incubation temperature was altered in this work to 37° C, because this brings the assay closer to the conditions present in the human body.

4.1.2 Use of DTPA during peroxyl radical generation

In the original assay procedure, DTPA was applied during the production of peroxynitrite to prevent the potential formation of hydroxyl radicals in sideways of the reaction [Regoli; personal communication].

Foods can contain high amounts of the potential hydroxyl radical precursor ascorbic acid and metal ions like iron and copper. In addition, although a contamination of the assay glassware with traces of metal ions can be diminished by faithful cleaning with UHQ water, it cannot be eliminated completely.

To prevent all possible side reactions, DTPA was added not only to the peroxynitrite but also to the peroxyl assay solutions. For the control reaction, the ethylene production was not influenced by this alteration. In contrast, for a 5 mM solution of ascorbic acid, e.g., the TOSC value against peroxyl

radicals could be increased from 70% to a complete inhibition of the ethylene formation by adding DTPA (see table C.1).

4.1.3 Preparation and handling of assay solutions

Compared to the original method instructions [Regoli; personal communication], the preparation of the TOSC assay solutions was simplified (see 3.1.1). The labour- and time consumption of the assay preparations could be minimised due to the following measures:

- As far as possible, compounds were diluted jointly instead of adding them one after the other:
  - For the formation of peroxyl radicals, KMBA and DTPA were solved together in buffer instead of adding successively to the vials buffer, KMBA and DTPA.
  - The same joint solution was used for the peroxynitrite assay.
  - For the generation of hydroxyl radicals, a combined solution of KMBA, $FeCl_3$ and EDTA in buffer was prepared instead of adding buffer, $FeCl_3$, EDTA and KMBA one after the other to the vials.
- The volumes for control and sample reaction were standardised to make possible the use of the same pool of prepared vials for both kind of reactions.
- The shelf life of the TOSC assay solutions was extended to at least three months redundantising daily preparations by
  - the use of UHQ water for the preparation of all solutions,
  - the thorough washing of glassware with UHQ water and
  - the deep freezing of the solutions in small portions.

4.1.4 Optimisation of GC conditions for ethylene analysis

Ethylene was the only volatile and by FID detector traceable compound arising from the TOSC assay solutions as initial tests showed. Its identity was confirmed by the addition of a pure ethylene standard gas to the headspace vials. Between one and three other small peaks eluting directly after the dead time of the column could be attributed to volatile compounds derived from the ambient laboratory air by blank injections.

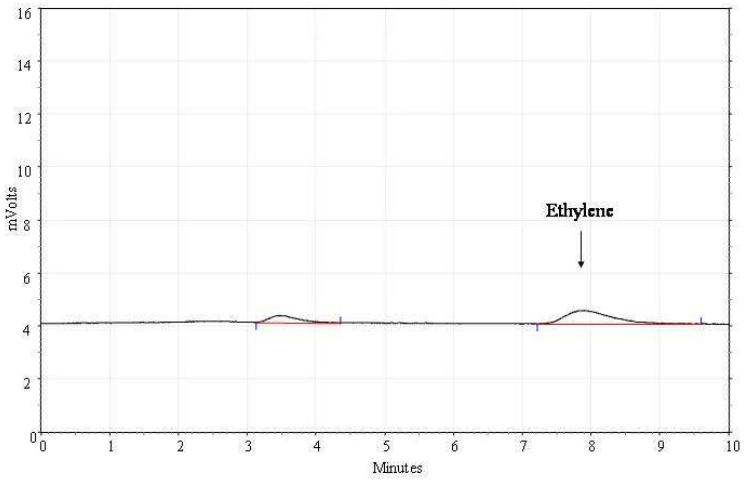

Figure 4.1: Ethylene separation at 50° C column temperature and 5 mL/min carrier gas flow

To ensure a sufficient separation of ethylene from the other volatiles, GC analysis were performed initially under usual separation conditions [Pha00] with a carrier gas flow rate of 5 mL/min and a column temperature of 50° C. Under these conditions, the peaks were well separated (see figure 4.1). On the other hand, ethylene eluted rather late under these conditions with a retention time of approx. 7.9 min. In addition, the ethylene peak had a width of nearly 2.5 min extending the required run time up to 10 min and making the peak integration difficult.

By increasing the column temperature and the carrier gas flow rate to 80° C and 15 mL/min resp., the retention time of ethylene could be shortened to 1.7 min and the peak width was narrowed to approx. 0.2 min without loss of separation efficiency (see figure 4.2). The resulting GC parameters for separation are displayed in table 3.3.

4.1.5 Automation of ethylene measurement

The incubation of samples under repeated shaking as well as the analyses of ethylene was automated by using a CombiPAL autosampler (see table 3.2) giving the following advantages:

- The reproducibility of an autosampler in taking and injecting samples

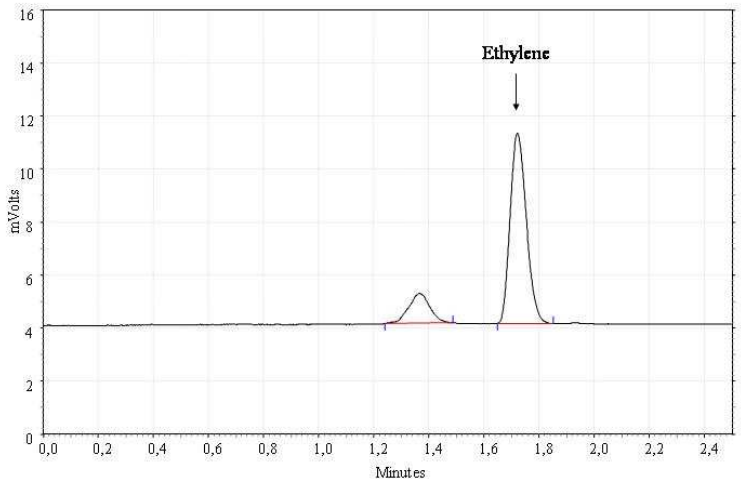

Figure 4.2: Ethylene separation at 80° C column temperature and 15 mL/min carrier gas flow

outmatches the accuracy of manual work.

- As the TOSC assay is a kinetic study, an exact timing of sample taking is extremely important and this can be done much better by an autosampler than by a human.

- After adding the reaction starter to the headspace vials, no more manual work had to be done during the further course of the assay sparing time for other labour.

4.1.6 Data evaluation

The calculation of experimental TOSC values as described in 3.1.4.1 resulted in the following benefits:

- By the use of a specially coded macro, the calculations were simplified and the necessary time exposure was minimised.

- As the output file contains not only the experimental TOSC value of the sample but also its curve progression, it could be used additionally to evaluate the kinetic of the samples.

For displaying the results, new data evaluations were developed. The starting point of considerations was that all samples were analysed in at least five different concentrations/dilutions for each of the radicals. This resulted in at least five different experimental TOSC values for each sample and each ROS. A direct comparison of these values is rather difficult because the applied concentrations differed depending on the activities against the three ROS. As already disussed in 1.3.2, other researchers have tried to solve this problem by extra- or interpolating their experimental data to a single concentration vs. inhibition point limiting the significance of their results . Instead, to reduce on the one hand the experimental data but to give on the other hand a concise review of the complete course, concentrations were calculated for three representative spots of the concentration vs. inhibition rate curve (i.e. a TOSC of 20, 50 and 80%, resp.).

For the fruit and vegetable juices as well as the açaí pulps, two more parameters (see 3.1.4.3) were computed to display the dose-response curve in a even more general way: the area under dose-response curves (ADRC) and the 1^{st} derivative of the curves at a TOSC of 50% (DT_{50}). Both values depend strongly on the initial concentration of samples. For juices and pulps, the "initial concentration" is the pure sample. For the standard compounds and the açaí seed extracts, no real "initial" concentration exists but it had to be chosen depending on the activities towards the different ROS. Therefore, the ADRC and DT_{50} were unemployable for the latter kinds of samples.

4.2 Standard compounds

For TOSC analyses, standard compounds were chosen that

- had already been analysed by Regoli et al. [Reg99, Wins98] to review the influence of the assay enhancements on the TOSC results (i.e. ascorbic acid, benzoic acid, trolox and uric acid) or
- turned out during the further analyses to be present in açaí fruits and/ or seeds to estimate their contribution to the overall antioxidant capacity of the samples [i.e. (+)-catechin, cyanidin-3-glucoside, cyanidin-3-rutinoside, (-)-epicatechin and protocatechuic acid (PCA)].

The results of these analyses have been published at [Lic02].

4.2.1 TOSC against peroxyl radicals

The experimental TOSC values of the different standard compounds against peroxyl radicals are displayed in table C.1. The calculated concentrations for

	Conc. [μM] for TOSC of				Conc. [mg/L] for TOSC of		
	20%	50%	80%	r^2 of fit	20%	50%	80%
(+)-Catechin	4	18	46	0.9999	1.2	5.2	13.4
(-)-Epicatechin	6	20	47	0.9970	1.7	5.8	13.6
Cyanidin-3-glucoside	9	23	45	0.9996	4.4	11.2	21.8
Cyanidin-3-rutinoside	9	24	48	0.9994	5.7	15.1	30.2
Protocatechuic acid	14	40	91	0.9999	2.2	6.2	14.0
Trolox	15	71	167	0.9971	3.8	17.8	41.8
Uric acid	24	81	172	0.9985	4.0	13.6	28.9
Ascorbic acid	45	105	194	0.9999	7.9	18.5	34.2

Table 4.1: Calculated concentrations of standard compounds for TOSC against peroxyl radicals

a TOSC of 20, 50 and 80% and the correlation of the underlying curve fits are displayed in table 4.1. For a comparison of the compounds among each other, the concentrations are given in μM units. Some curves of compounds with high, medium and low antioxidant capacities are visualised additionally in figure 4.3. For a later comparison with the açaí samples, the results are also displayed in mg/L concentrations in table 4.1.

Benzoic acid is excluded from this survey and classified as unsuitable, because even very high concentrations (100,000 μM) showed very low scavenging capacities (TOSC of about 20%). Assaying higher concentrations of this compound would be irrelevant, because they do not appear naturally or as food additives.

The first thing that catches the eye for the other compounds is the nearly throughout non-linear relation between concentration and inhibition. Only ascorbic acid and uric acid show an almost linear progression of the curves at their lower TOSC values.

Secondly, a continuous trend can be observed over the whole concentration range: the compounds with the highest antioxidant capacity at a TOSC of 20% are also the most active ones at a TOSC of 50 and 80%, respectively. This fact is mirrored by a rather similar run of the curves in figure 4.3.

On the basis of μM concentrations, the antioxidant capacity of compounds for peroxyl radicals can be recapitulated as: catechin = epicatechin = cyanidin-3-glucoside = cyanidin-3-rutinoside > protocatechuic acid > trolox = uric acid > ascorbic acid. By quantitative comparison, catechin possesses an antioxidant capacity towards peroxyl radicals about two times

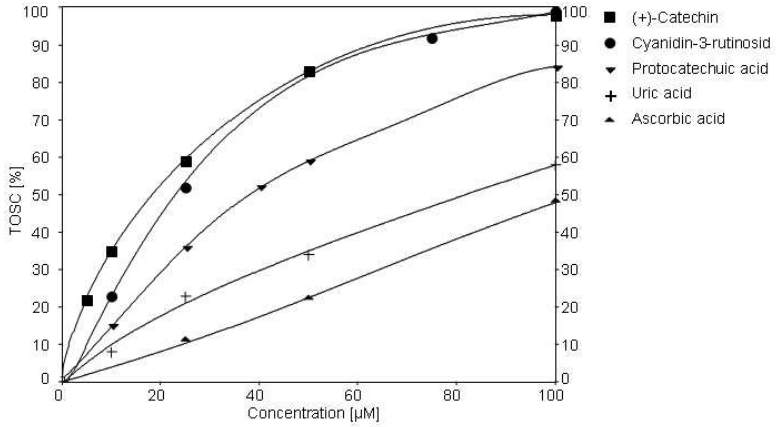

Figure 4.3: TOSC of some standard compounds against peroxyl radicals

higher than protocatechuic acid, four times higher than trolox and uric acid and about five times higher than ascorbic acid.

4.2.2 TOSC against peroxynitrite

The experimental TOSC values of the different standard compounds against peroxynitrite can be found in table C.2. The calculated results for peroxynitrite are shown in table 4.2 and some characteristic proportions are charted in figure 4.4.

Again, benzoic acid is shut out from the comparison, because a very high concentration of 100,000 μM showed a very low effectiveness against peroxynitrite (TOSC less than 50%).

For the other assayed standard compounds, again a distinctive non-linear relation between concentration and inhibition rate emerges from the data. But surprisingly, no persistent trend of antioxidant capacity can be seen over the entire concentration range: the compounds with the highest scavenging capacities at a TOSC of 20% (catechin, epicatechin and protocatechuic acid) are counted among those substances with the lowest scavenging activity at a TOSC of 80%. For trolox, ascorbic acid and uric acid, this trend is inverted, whereas the corresponding concentrations to a TOSC of 50% are rather similar for all compounds. This finding is reflected by the crossing of the dose-response curves in figure 4.4.

Possibly, the different compounds feature varying reaction mechanisms towards peroxynitrite, e.g. interfering at different stages of the multilevel formation of this ROS (see figure A.2) or its reaction with the KMBA molecule

	Conc. [μM] for TOSC of				Conc. [mg/L] for TOSC of		
	20%	50%	80%	r^2 of fit	20%	50%	80%
(+)-Catechin	6	54	554	1.0000	1.7	15.7	160.8
(-)-Epicatechin	7	47	567	0.9997	2.0	13.6	164.6
Cyanidin-3-glucoside	24	105	534	1.0000	11.6	50.9	259
Cyanidin-3-rutinoside	25	92	574	0.9999	15.8	58.1	362
Protocatechuic acid	13	108	780	0.9997	2.0	16.6	120.2
Trolox	38	97	277	0.9984	9.5	24.3	69.3
Uric acid	37	83	223	0.9999	6.2	14.0	37.5
Ascorbic acid	39	106	329	1.0000	6.9	18.7	57.9

Table 4.2: Calculated concentrations of standard compounds for TOSC against peroxynitrite

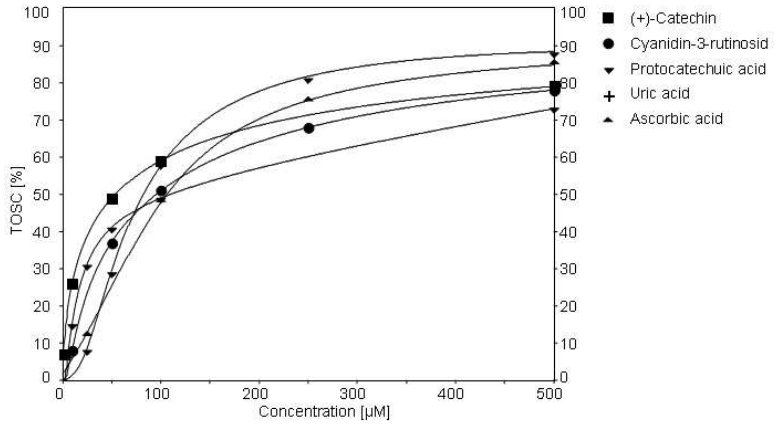

Figure 4.4: TOSC of some standard compounds against peroxynitrite

	Concentration [μM] for TOSC of				Concentration [mg/L] for TOSC of		
	20%	50%	80%	r^2 of fit	20%	50%	80%
Trolox	622	2,599	11,510	0.9999	156	651	2,881
Benzoic acid	1,040	6,230	22,600	1.0000	127	761	2,759

Table 4.3: Calculated concentrations of standard compounds for TOSC against hydroxyl radicals

(see figure 1.6). Therefore, no absolute ranking order of scavenging activities for peroxynitrite can be stated. But it can be said at least that the necessary concentrations of the different compounds are much more alike for an inhibition of peroxynitrite than they are for peroxyl radicals.

Figure 4.4 reveals another interesting observation: throughout all compounds, the antioxidant capacity approaches at high concentrations a certain plateau of inhibition that can not be surmounted even when much higher concentrations of the antioxidant are added. A strong increase of the concentration of trolox, e.g., from 1,000 to 100,000 μM enhances its scavenging capacity for peroxynitrite from 90 to only 99%, offering still no complete protection against this ROS.

4.2.3 TOSC against hydroxyl radicals

The experimental TOSC values of the different standard compounds against hydroxyl radicals can be found in table C.3.

Only for benzoic acid, a certain activity against this ROS could be detected, even though at the upper limit of concentrations representative for an occurrence in food. None of the other analysed compounds showed significant antioxidant capacities towards hydroxyl radicals in a relevant concentration range. In figure 4.5, the tested compounds are shown at a concentration of 500 μM revealing no big differences between their, low, activities.

Ascorbic acid, in contrast, has even prooxidative features at this concentration level, not very surprising as it is also used as the starter for the formation of hydroxyl radicals (see 1.3.1). But by adding higher amounts, the prooxidative effect of ascorbic acid can be changed to an at least low inhibition capacity for hydroxyl radicals (see table C.3).

For trolox, no natural occurrence or food additive concentration level exists as it is the artificial water-soluble analogue of vitamin E. Therefore, though the activity against hydroxyl radicals was many times lower than against peroxyl radicals or peroxynitrite, this compound was also analysed

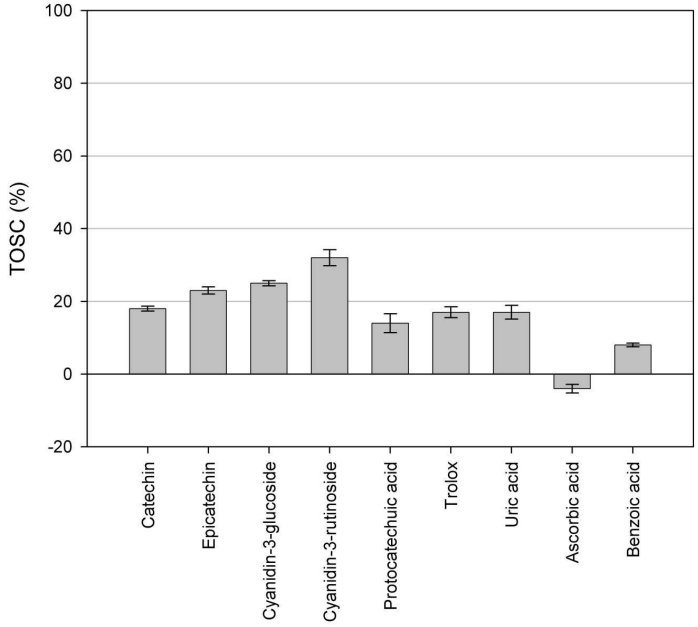

Figure 4.5: TOSC of 500 μM standard compounds against hydroxyl radicals

over the complete TOSC range of this ROS. In table 4.3, the calculated concentrations for trolox and benzoic acid are shown for a TOSC of 20, 50 and 80%, respectively.

Again, no linear relation between TOSC and concentration can be found from the results. In addition, the same plateau effect as described for peroxynitrite exists also at the upper end of the concentration range for hydroxyl radicals.

4.2.4 Comparison of the three ROS

All three tested ROS have in common that the relation between their inhibition and the necessary concentration of the tested standard compounds is nearly throughout non-linear. This finding underlines clearly the importance of analysing different concentration levels of a compound to get a well-founded overview of its inhibition capacities against an ROS.

Though benzoic acid is a weak scavenger of all three ROS, it features one noteworthiness: in contrast to all other tested compounds, it demonstrates its best scavenging capacities towards hydroxyl radicals, followed by peroxynitrite and peroxyl radicals being the least inhibited ROS.

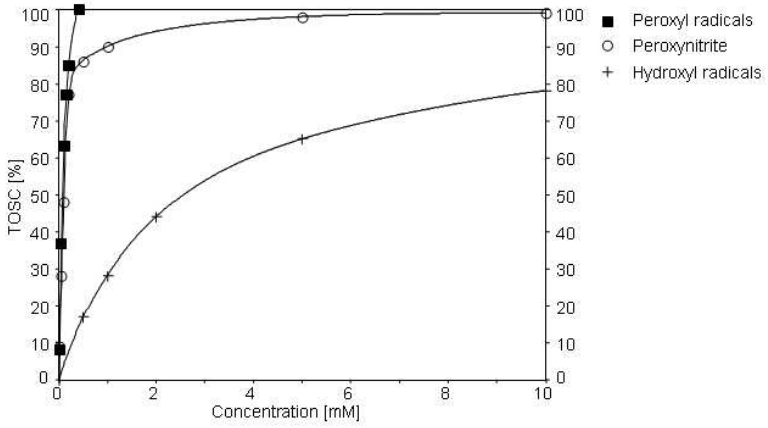

Figure 4.6: TOSC of trolox for the three ROS

Apart from benzoic acid, the scavenging capacity for peroxyl radicals and peroxynitrite is relatively alike for all compounds at low inhibition rates (TOSC of 20%). For the group of trolox, uric acid and ascorbic acid it is also comparable for both ROS at a TOSC of 50%. All other compounds require the twice to fivefold concentration for peroxynitrite than they need for peroxyl radicals. Differences grow even larger at a TOSC of 80%: most compounds need concentrations at an average ten times higher level for peroxynitrite than for peroxyl radicals; for trolox, ascorbic and uric acid it is just twice the concentration. The scavenging capacities of nearly all analysed compounds against hydroxyl radicals are even so low that the necessary concentrations for a sufficient inhibition of this ROS are out of a usual concentration range. In figure 4.6, the varying behaviour towards the three ROS is displayed taking trolox as an example.

The difference in the scavenging capacities of the compounds against the three assayed ROS can be explained by the highly different half lives and reactiveness of the ROS [Hall95b, Reg99]. Peroxyl radicals are the most stable ones with the lowest reactivity. Therefore, they can be simplest scavenged requiring the lowest amounts of antioxidants. Peroxynitrite is already much more reactive, but it is easily beaten by the highly aggressive hydroxyl radicals. They are so reactive that they combine with almost every molecule they can reach. So, nearly every compound can be seen as a hydroxyl scavenger though they are all not very effective [Reg99]. These facts explain also, why the results vary so much for peroxyl radicals, less for peroxynitrite and are so close together for hydroxyl radicals.

In addition, it is the most likely explanation, why it is so difficult to reach

a complete protection against peroxynitrite and especially against hydroxyl radicals (see figure 4.6). At least a few of the highly reactive ROS seem to be always able to react rapidly enough with the KMBA molecules before they can be scavenged by antioxidants causing the described plateau effect of inhibition.

4.2.5 Comparison with original TOSC assay results

To make sure that the TOSC assay modifications as described in 4.1 did not affect negatively its results, the data were compared to those for the original method instructions by Regoli et al. [Reg99].

First conclusions for a comparison can be drawn from a ranking of activities for the three ROS given by Regoli as:

- **Ascorbic acid**[1]: peroxynitrite > peroxyl radicals
- **Benzoic acid**: hydroxyl radicals $\gg$ peroxyl radicals > peroxynitrite
- **Trolox**: peroxyl radicals > peroxynitrite $\gg$ hydroxyl radicals
- **Uric acid**: peroxynitrite $\simeq$ peroxyl radicals $\gg$ hydroxyl radicals

The results of this ranking for trolox, the relative high scavenging capacities of benzoic acid against hydroxyl radicals and also the low inhibition activity of uric acid against hydroxyl radicals are in entire accordance to those for the modified assay conditions. A ranking of the further results of benzoic acid is more problematic. As the results of the other analysed standard compounds demonstrate, the scavenging activity against especially peroxynitrite is highly concentration dependent (see 4.2.2). Benzoic acid showed too low activities against peroxyl radicals and peroxynitrite in relevant concentration ranges and was because of this not further analysed. The data for a final classification of its scavenging capacities against these two ROS are therefore insufficient.

The inhibition capacity for uric acid against peroxynitrite is stated by Regoli as comparable to the one for peroxyl radicals [specific TOSC values (see 1.3.2) of 4.4 against peroxynitrite and 3.1 for the peroxyl radicals, respectively]. For ascorbic acid, the capacity for peroxynitrite is classified as higher than for peroxyl radicals, though the specific TOSC values are also rather alike with 3.4 against peroxynitrite and 2.0 for the peroxyl radicals, respectively. Therefore, it seems to be more reasonable to classify both compounds as comparable in scavenging these two ROS with a slight superiority for the

[1] Hydroxyl radicals not assayed

inhibition of peroxynitrite. As already discussed in 4.2.4, the scavenging capacities of these two compounds are under the modified assay conditions also widely alike for peroxyl radicals and peroxynitrite at the lower concentration range (calculated TOSC of 20 and 50%).

But at higher concentrations (calculated TOSC of 80%), the tendency turns to a higher inhibition activity for peroxyl radicals in contrast to the findings of Regoli. This might be due to the addition of DTPA to not only the peroxynitrite but also to the peroxyl radical assay (see 4.1.2), because ascorbic acid and uric acid can be not only antioxidants but also prooxidants [Deu98, Pat03]. As it was shown for ascorbic acid (see 4.1.2), the adding of DTPA enhances its antioxidant capacity against peroxyl radicals. Probably, the antioxidant capacity against peroxyl radicals in the absence of DTPA is overlaid by the simultaneously production of hydroxyl radicals caused by the presence of metal traces. Eliminating this prooxidant effect could cause the described change in the activity order for the two ROS. For uric acid, a similar scenario is imaginable.

A quantitative comparison of the data is rather difficult as most results from Regoli were published as figures with rather low quality and not as numbers. But from the graphics [Reg99] it can at least be estimated that the results are similar to those for the modified assay conditions.

In summary, the results for the modified assay conditions are widely in accordance with those published by Regoli et al. with smaller differences due to improvements in the prevention of side reactions in the peroxyl assay.

4.2.6 Comparison with TEAC values from literature

The TEAC (Trolox equivalent antioxidant capacity) assay is one of the most commonly used in vitro antioxidant methods. Therefore, it is of interest to compare the results of the TOSC assay with TEAC values from the literature.

But, a quantitative comparison with TEAC results is rather difficult, because at least four modifications of the assay are currently in use and they yield different results [Schl02]. In addition, different researchers measured different concentrations (e.g. 1 mM or 100 mg/L solutions) [Mil97, Rec97]. A direct comparison of these results would assume a linear correlation between concentration and inhibition rate over the whole concentration range. For the TOSC assay, the results for all three ROS indicate clearly, that such a presumption is rather unrealistic. Furthermore, it was recently proven, that also TEAC values of compounds like the flavonoids depend as well significantly on the measured concentration [Kim02]. In addition, it could be shown that the TEAC assay results depend also on the measuring time that was used [Mir99] making the comparability of the results from different researchers

Peroxyl radicals	Fast-acting antioxidant	Retardant
Cyanidin-3-glucoside	+	+
Cyanidin-3-rutinoside	+	+
Ascorbic acid	+	–
Trolox	+	–
Uric acid	+	–
(+)-Catechin	–	+
(-)-Epicatechin	–	+
Protocatechuic acid	–	+
Benzoic acid	–	Low activity

Table 4.4: Reaction mode of standard compounds against peroxyl radicals

even more uncertain.

A blue sheet of TEAC values from different scientists is therefore unapplicable. But at least one researching group can be found that analysed most standard compounds that were also assayed in this work with the TOSC assay with the same TEAC version [Mil97, Ric96]. Their results can be simplified to the following order of antioxidant capacities: cyanidin-3-rutinoside > cyanidin 3-glucoside = epicatechin = catechin > protocatechuic acid > ascorbic acid = trolox. That finding is mostly in accordance to the results for peroxyl radicals (see 4.2.1) due to the fact that the used ROS have a rather similar reactivity. Admittedly, other authors [Rec97, Kim02] indicate TEAC activity sequences varying from that one shown above.

A comparison to the results for hydroxyl radicals is not possible, because none of the analysed standard compound shows sufficient scavenging capacities in the TOSC assay towards this ROS in a relevant concentration range (see 4.2.3). A comparison with peroxynitrite is neither feasible, since the sequence of antioxidant capacities for this ROS is largely concentration depending (see 4.2.2).

4.2.7 Classification of compounds by reaction mode

As the TOSC assay is based upon kinetic studies, it is not only possible to determine concentration depending inhibition rates, but also to distinguish between different antioxidant types by their time course of ethylene formation (cf. figure 1.1). The results of this classification are displayed in the tables 4.4, 4.5 and 4.6.

The survey reveals, that only few fast-acting antioxidants are among the considered compounds. Furthermore, this behaviour can only be observed

Peroxynitrite	Fast-acting antioxidant	Retardant
Ascorbic acid	–	+
(+)-Catechin	–	+
Cyanidin-3-glucoside	–	+
Cyanidin-3-rutinoside	–	+
(-)-Epicatechin	–	+
Protocatechuic acid	–	+
Trolox	–	+
Uric acid	–	+
Benzoic acid	–	Low activity

Table 4.5: Reaction mode of standard compounds against peroxynitrite

Hydroxyl radicals	Fast-acting antioxidant	Retardant
Benzoic acid	–	Low activity
(+)-Catechin	–	Low activity
Cyanidin-3-glucoside	–	Low activity
Cyanidin-3-rutinoside	–	Low activity
(-)-Epicatechin	–	Low activity
Protocatechuic acid	–	Low activity
Trolox	–	Low activity
Uric acid	–	Low activity
Ascorbic acid	–	Low activity / Prooxidant

Table 4.6: Reaction mode of standard compounds against hydroxyl radicals

towards peroxyl radicals. Most compounds act as retardants against the three ROS, and their scavenging capacity against hydroxyl radicals is even very low. Depending on the concentration, ascorbic acid acts as a prooxidant or a retardant of hydroxyl radicals. Benzoic acid has rather low inhibition capacities for all three ROS, at least in a relevant concentration range.

One remarkable observation can me made for the two assayed anthocyanins: they turned out to be not only fast-acting antioxidants for peroxyl radicals but also, at a later period of the reaction, retardants (see fig. 4.7). Such a phenomenon has not yet been described in literature. Possibly, a decomposition product or a second functional group of the anthocyanins comes into play as soon as the initial molecules have been exhausted.

In summary, none of the herein presented compounds offers a complete

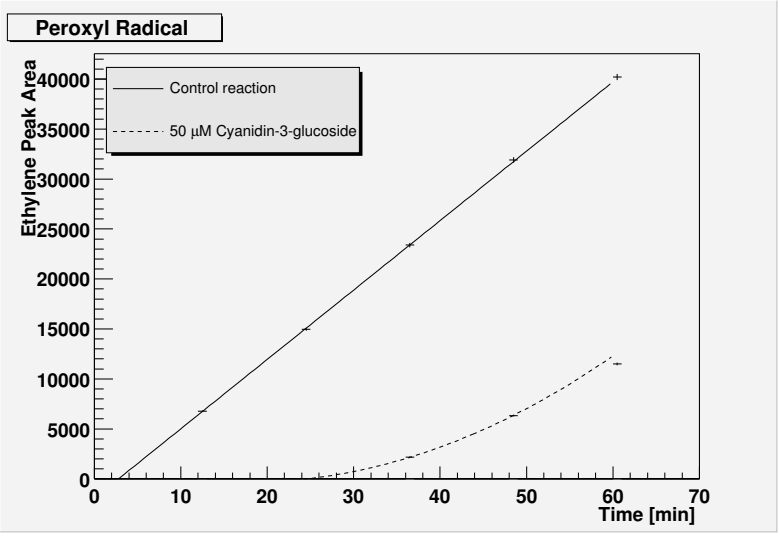

Figure 4.7: Cyanidin-3-glucoside: fast-acting antioxidant plus retardant

protection against hydroxyl radicals and peroxynitrite in a relevant concentration range. Again, the lack of fast-acting antioxidants for these two ROS might be due to their extremely reactivity and their very high rate constants [Hall95b]. Only the much slower reacting peroxyl radicals [Hall95b] can be scavenged completely by a couple of compounds (ascorbic acid, cyanidin-3-glucoside, cyanidin-3-rutinoside, trolox and uric acid).

4.3 Fruit and vegetable juices

For a comparison with the TOSC values of açaí fruit pulps, several common European fruit and vegetable juices were analysed that contain high amounts of known antioxidants like:

- anthocyanins (e.g. lingonberry juice and sour cherry nectar),
- betalains (beetroot juice),
- carotenoids (e.g. carrot and tomato juice),
- flavanones (e.g. orange and pink grapefruit juice),
- hydroxycinnamates (e.g. apple and tomato juice) and
- vitamin C (e.g. ACE, lemon and multivitamin juice) [Pro02, Kau01, Eis95].

The results of these analyses have been accepted for press at [Lic03].

4.3.1 Limitation of juice analyses due to sample pH

The pH of a solution can have an important influence on the antioxidant capacity of compounds. Protonating or deprotonating a molecule can affect its ability to donate an electron or a hydrogen radical and, by this, its efficiency to work as an antioxidant. For some flavonoids, it was shown that their antioxidant activity could be increased significantly by increasing the pH [Lem01], the capacity of some hydroxybenzoates was only affected at high pH and trolox was not influenced over the whole tested pH range [Tyr99]. Therefore, results for the antioxidant activity of solutions should only be compared if they have the same pH or when it is proven that they consist only of pH-independent antioxidants.

	pH	Lowest analysable dilution level for TOSC assay
Beetroot juice Carrot juice Tomato juice	> 4	pure
ACE juice Apple juice Blueberry juice Elderberry juice Lingonberry juice Multivitamin juice Orange juice Pink grapefruit juice Sauerkraut juice Sour cherry nectar	2.5 – 4	1:5
Lemon juice	<2.5	1:10

Table 4.7: pH of juice samples and lowest analysable dilution level for TOSC assay

To make sure that all juices were analysed under comparable conditions, the pH of the samples was at first analysed. In further experiments, it was surveyed, how much the juices had to be diluted to not exceed the capacity of the buffer in the reaction vessels (see 3.1.1). An overview of this interrelation is given in table 4.7.

	Calculated dilution factors for TOSC of					
	20%	50%	80%	DT_{50}	ADRC	r^2
Diagonal defined as	–	–	–	1.0	1.0	–
Peroxyl radicals						
Carrot juice I filtrated	128	41	19	1.5	1.4	1.0000
Carrot juice I unfiltrated	119	45	20	1.7	1.4	1.0000
Carrot juice II filtrated	69	21	9	0.8	1.0	1.0000
Carrot juice II unfiltrated	73	24	10	0.9	1.0	1.0000
Peroxynitrite						
Carrot juice I filtrated	123	31	5	0.6	1.0	1.0000
Carrot juice I unfiltrated	145	31	5	0.6	1.0	0.9998
Carrot juice II filtrated	62	19	3	0.5	0.8	0.9998
Carrot juice II unfiltrated	78	20	4	0.5	0.9	0.9995
Hydroxyl radicals						
Carrot juice I filtrated	100	36	8	0.9	1.1	0.9998
Carrot juice I unfiltrated	105	32	9	0.9	1.1	1.0000
Carrot juice II filtrated	100	30	8	0.8	1.1	1.0000
Carrot juice II unfiltrated	111	29	9	0.8	1.1	0.9997

Table 4.8: Influence of filtration on TOSC of carrot juice

4.3.2 Influence of filtration on TOSC

The TOSC assay in the herein presented form covers only water-soluble antioxidants. Filtrating insoluble parts out of juices before TOSC analyses should therefore not influence directly their antioxidant capacities. An indirect influence of the filtration process can on the other hand not be excluded from the first due to, e.g., a longer exposure of the samples to ambient temperature during the filtration process or an interaction of the samples with the filter material.

To review this assumption, the TOSC values of two different carrot juices (the analysed juices with the highest content of insoluble parts) were determined for the filtrated and unfiltrated samples. In table 4.8, the results for these experiments are opposed for the three assayed ROS.

It can be seen that no significant difference exists between the antioxidant capacities of filtrated and unfiltrated samples. Some smaller differences at certain points of the dose-response curves are levelled when a broader range of data is taken into account (i.e. the value for the ADRC).

Because of these findings together with a better homogeneity and an easier

handling of filtrated samples, the other juices with high pulp content (tomato, lemon, orange and pink grapefruit juice) were also analysed filtrated.

4.3.3 TOSC against peroxyl radicals

The experimental TOSC values of the juices against peroxyl radicals are displayed in table D.1. In table 4.9, the calculated dilutions corresponding to TOSC values of 20, 50 and 80%, the ADRC and the DT_{50} of the samples are shown. Dose-response curves of juices that are representative for the best, middle and worst results of the antioxidant spectrum are displayed additionally in figure 4.8.

	Calculated dilution factor for TOSC of					
	20%	50%	80%	DT_{50}	ADRC	r^2
Diagonal defined as	–	–	–	1.0	1.0	–
Lingonberry juice	1667	556	238	19.0	>2.0	1.0000
Blueberry juice	833	357	167	14.3	>1.9	0.9995
Elderberry juice	769	286	137	11.4	>1.9	1.0000
Beetroot juice I	500	185	100	8.1	>1.9	1.0000
Sour cherry nectar	526	185	79	6.7	>1.9	0.9991
Beetroot juice II	337	114	57	4.5	>1.8	1.0000
ACE juice	213	69	30	2.4	1.6	0.9990
Multivitamin juice	217	68	28	2.2	1.6	1.0000
Orange juice	125	41	20	1.5	1.4	1.0000
Carrot juice I	128	41	19	1.5	1.4	1.0000
Pink grapefruit juice	133	40	17	1.4	1.3	1.0000
Lemon juice	105	38	18	1.5	1.3	1.0000
Apple juice	100	35	14	1.2	1.2	0.9996
Sauerkraut juice I	99	26	12	0.8	1.1	1.0000
Tomato juice I	81	25	11	0.8	1.0	0.9997
Tomato juice II	71	23	10	0.8	1.0	0.9989
Carrot juice II	69	21	9	0.8	1.0	1.0000
Sauerkraut juice II	66	21	9	0.7	0.9	0.9999

Table 4.9: Calculated dilution factors, DT_{50} and ADRC of fruit and vegetable juices for TOSC against peroxyl radicals

In all cases, a non-linear correlation between sample concentrations and antioxidant capacities is observed. The highest antioxidant capacities against peroxyl radicals occur in the group of the red juices (i.e. the berry juices rich

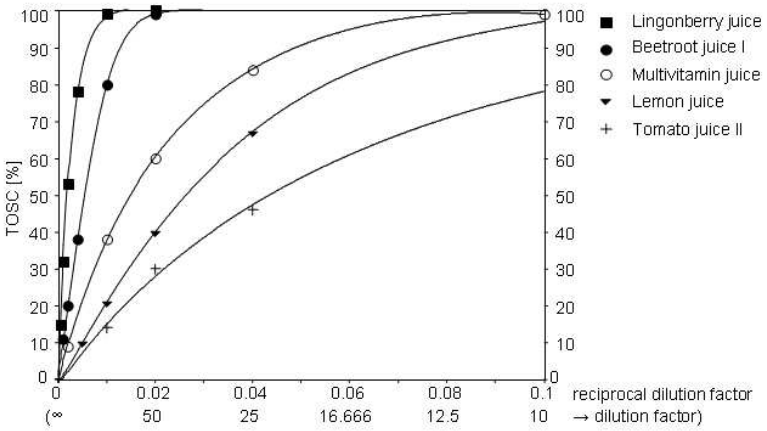

Figure 4.8: TOSC of some juices against peroxyl radicals

in anthocyanins and the beetroot juices containing betalains) with the lingonberry juice being the most effective one and the beetroot juice II arranging at the lower end of activities in this group. The results of these juices for the concentrations corresponding to the different TOSC values as well as the DT_{50} are noticeably higher than for all other analysed samples.

Their activities are even so high that it is only possible to estimate values for their ADRC. This is because ADRC values are only comparable if the same range of curve is used for the integration. For all other juices, it is appropriate to integrate a range up to a dilution of 1:10 for the three assayed ROS. For the red juices, solutions diluted to 1:50 are in all cases already concentrated enough to stop the formation of ethylene by peroxyl radicals completely or at least nearly completely. For the less diluted samples, the inhibition capacity would be even higher. But experimental TOSC values higher than 100% are impossible per definition because they would correspond to negative ethylene peak areas. In most cases, a mathematical extrapolation to TOSC values higher than 100% is also not realisable. This is because the non-linear relation between concentration and antioxidant capacity prohibits an extrapolating of curves much further than the last analysed experimental point. In this setting, it would mean to extend the curves five times further than they are documented by experiment. To get at least approximate values for these ADRC, TOSC values of 100% are presumed for all reciprocal dilutions between 0.02 and 0.1. Because this means an underestimation of reality, “>” symbols are added to the calculated values.

The red juices are followed in activity by the vitamin-added juices (ACE and multivitamin juice) with results very close to each other. The next group

with very similar antioxidant capacities are carrot juice I together with the citrus juices rich in flavanones (orange, pink grapefruit and lemon juice) and the apple juice being close behind. Both sauerkraut and tomato juices as well as carrot juice II belong to the class with the lowest antioxidant capacities against peroxyl radicals.

4.3.4 TOSC against peroxynitrite

The experimental TOSC values of the juices against peroxynitrite are displayed in table D.2. In table 4.10, the calculated dilutions corresponding to TOSC values of 20, 50 and 80%, the ADRC and the DT_{50} are shown. Some characteristic dose-response curves from the spectrum of juice activities are displayed in figure 4.9.

	Calculated dilution factor for TOSC of			DT_{50}	ADRC	r^2
	20%	50%	80%			
Diagonal defined as	–	–	–	1.0	1.0	–
Beetroot juice I	500	135	34	3.7	1.6	0.9998
Blueberry juice	909	141	29	2.8	1.6	1.0000
Elderberry juice	606	128	26	2.7	1.5	0.9994
Beetroot juice II	357	109	29	2.8	1.5	1.0000
Lingonberry juice	588	106	22	1.7	1.5	1.0000
Sour cherry nectar	435	82	16	1.7	1.4	1.0000
Multivitamin juice	161	40	14	1.1	1.3	0.9999
ACE juice	143	37	13	1.0	1.2	1.0000
Lemon juice	108	34	14	1.1	1.2	1.0000
Orange juice	105	33	13	1.1	1.2	0.9991
Pink grapefruit juice	115	33	12	0.9	1.2	0.9999
Carrot juice I	123	31	5	0.6	1.0	1.0000
Sauerkraut juice I	76	22	7	0.6	1.0	1.0000
Apple juice	103	22	4	0.4	0.9	0.9994
Tomato juice I	64	18	4	0.4	0.8	0.9999
Carrot juice II	62	19	3	0.5	0.8	0.9998
Sauerkraut juice II	56	17	6	0.5	0.8	0.9997
Tomato juice II	55	17	4	0.4	0.8	0.9997

Table 4.10: Calculated dilution factors, DT_{50} and ADRC of fruit and vegetable juices for TOSC against peroxynitrite

Again, all juices show a very distinctive non-linear correlation between

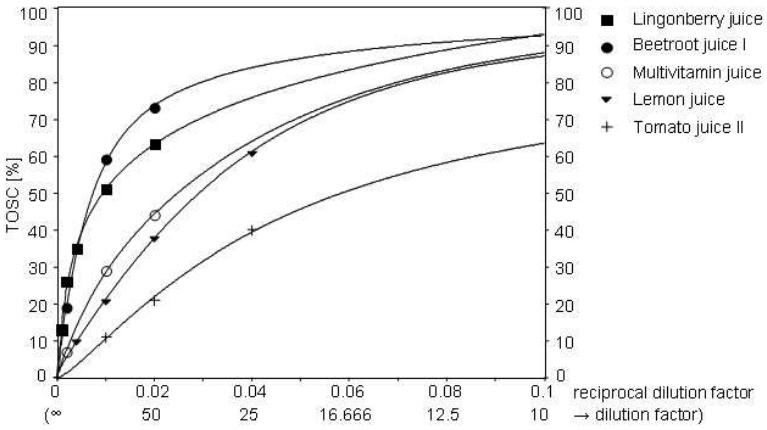

Figure 4.9: TOSC of some juices against peroxynitrite

sample concentrations and antioxidant capacities.

The highest antioxidant capacities for this ROS can also be found in the group of the juices rich in anthocyanins or betalains, though the distance to the other juices gets smaller. Interestingly, the course of the dose-response curves for anthocyan and betalain juices seems to be different for the reaction with peroxynitrite: to reach a TOSC of 20%, the anthocyanin juices can be used much more diluted than the beetroot juices. For a TOSC of 80% it is the opposite way around, while the results for a TOSC of 50% are more similar (see figure 4.9). This is also mirrored by the comparatively high value for the DT_{50} of beetroot juice I. Possibly, the two types of juices feature different reaction mechanisms towards peroxynitrite like it was discussed for some of the analysed standard compounds (see 4.2.2).

The red juices are followed by the fused groups of vitamin-added and citrus juices. The antioxidative capacities of carrot juice I as well as the apple juice are not as good as against peroxyl radicals and the two juices are therefore arranged in a new group together with sauerkraut juice I. In this group, the phenomenon of juices with different slopes for the curves but similar values for a TOSC of 50% emerges also (i.e. for sauerkraut juice I and apple juice). Like for the anthocyanin and betalain juices, a different kind of reaction mechanism can be suggested herein. The two tomato juices together with carrot juice II and sauerkraut juice II form again the class with the lowest antioxidant capacities.

4.3.5 TOSC against hydroxyl radicals

The experimental TOSC values of the juices against hydroxyl radicals are displayed in table D.3. In table 4.11, the calculated dilutions corresponding to TOSC values of 20, 50 and 80%, the ADRC and the DT_{50} are given. In figure 4.10, some juices representing the antioxidative spectrum for this ROS are shown.

	Calculated dilution factor for TOSC of			DT_{50}	ADRC	r^2
	20%	50%	80%			
Diagonal defined as	–	–	–	1.0	1.0	–
Lingonberry juice	214	90	33	3.4	1.6	1.0000
Sour cherry nectar	161	45	15	1.3	1.3	0.9999
Blueberry juice	141	48	13	1.5	1.3	1.0000
ACE juice	123	34	13	1.1	1.2	1.0000
Elderberry juice	99	32	13	1.1	1.2	1.0000
Multivitamin juice	154	36	10	0.9	1.2	1.0000
Beetroot juice I	105	32	10	0.9	1.1	1.0000
Pink grapefruit juice	145	33	10	0.8	1.1	0.9999
Apple juice	139	36	8	0.9	1.1	1.0000
Carrot juice I	100	36	8	0.9	1.1	0.9998
Sauerkraut juice I	106	33	9	0.7	1.1	1.0000
Beetroot juice II	100	30	9	0.8	1.1	1.0000
Carrot juice II	100	30	8	0.8	1.1	1.0000
Sauerkraut juice II	112	28	8	0.7	1.1	1.0000
Orange juice	106	27	8	0.7	1.0	0.9992
Tomato juice I	64	19	5	0.5	0.9	0.9995
Tomato juice II	52	16	5	0.5	0.8	0.9998
Lemon juice	36	11	n.a.[a]	0.3	0.6	1.0000

[a]not analysable due to low sample pH

Table 4.11: Calculated dilution factors, DT_{50} and ADRC of fruit and vegetable juices for TOSC against hydroxyl radicals

As for the other two ROS, the relation between juice dilution and TOSC is for all samples clearly non-linear. Only lingonberry juice, sour cherry nectar and blueberry juice demonstrate superior antioxidant properties against hydroxyl radicals compared to the majority of juices that build a large midfield with nearly equal antioxidant activities. The two tomato juices and also the lemon juice are even less antioxidative.

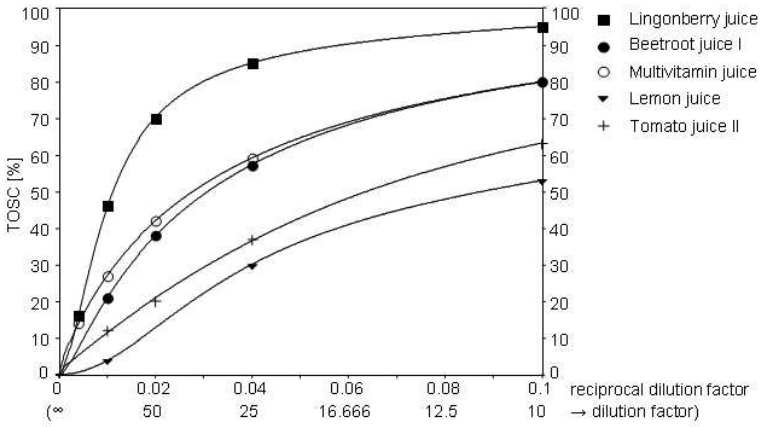

Figure 4.10: TOSC of some juices against hydroxyl radicals

The, at first sight, astounding low antioxidative capacity of the lemon juice against hydroxyl radicals is most likely due to its high vitamin C content associated with possible prooxidant effects [Deu98]. Therefore, it is more astounding that not also the other juices rich in vitamin C (ACE, multivitamin, orange and pink grapefruit) show a similar low inhibition against hydroxyl radicals as the lemon juice. It suggests, that there are compounds with antioxidant properties present in these juices that are more effective in overriding the prooxidant features of vitamin C than there are in lemon juice. Due to the low pH of the lemon juice, only corresponding TOSC value of 20 and 50% are calculable for this sample (cf. 4.3.1).

4.3.6 Comparison of the three ROS

The biggest differences between the analysed juices appear against peroxyl radicals. For peroxynitrite, the variations get much smaller. And for hydroxyl radicals, the borders between the different groups of juices have nearly vanished. In addition, nearly all juices are most efficient against peroxyl radicals, much less against peroxynitrite and worst in the inhibition of hydroxyl radicals.

As explained for the analysed standard compounds (see 4.2.4), this is due to the highly different reactivities of the three assayed ROS [Hall95b, Reg99]. The rather stable peroxyl radicals can be simplest scavenged. And if different antioxidants are present in a complex mixture like a juice, they have time enough to compete for the reaction with the peroxyl radicals and the most active ones can dominate. The scavenging of peroxynitrite and especially hydroxyl radicals is already much more difficult causing higher required con-

centrations of antioxidants and making less differences between the different types of antioxidants. For hydroxyl radicals, the situation is additionally complicated as foods can contain possible prooxidants of this ROS like vitamin C [Deu98]. A complete protection from peroxynitrite and hydroxyl radicals is nearly impossible causing the same plateau effect of inhibition at higher concentrations of the juices as described for some of the analysed standards (see 4.2.4).

Therefore, the juices rich in anthocyanins and betalains are worth of special highlighting: they were not only the best ones in scavenging peroxyl radicals but showed also superior antioxidant features against the highly reactive ROS peroxynitrite as also, at least weakly, against hydroxyl radicals.

4.3.7 Comparison of juices from different companies

Though the statistics are rather poor for a comparison of juices from only two different producers, some interesting first hints can be taken from their TOSC results.

For the two tomato juices, no significant differences can be found between the antioxidant activities against all three assayed ROS. For the two sauerkraut juices, just minor variations are detectable.

The only bigger differences appear among the two beetroot juices and also among the two carrot juices. This could of course result from, e.g., different origins of the beetroots and carrots, different varieties or different stages of maturity before the producing of the juices. But, in both cases the lactic-acid-fermented juice not blended with lemon juice (samples with no. I) shows significant higher antioxidant capacities against peroxyl radicals and peroxynitrite than the non-fermented sample that was blended with lemon juice (samples with no. II); for hydroxyl radical the results are nearly equal. An influence of the added lemon juice can be excluded widely. The added amounts of lemon juice are on the one hand rather small with about 0.3 and 1 to 2%, respectively [personal communication with producer]. In addition, an influence of the lemon juice should be seen especially for the hydroxyl radicals by making the results for the juices with number II worse than for those with number I. But, as already stated, no significant difference can be found for this ROS.

Instead, it seems more reasonable to suspect an influence from the lactic-acid-fermentation. As it was shown in literature, lactic-acid-fermentation was able to improve the antioxidant capacity of sweet potato yoghurt and milk [Kud00, Kud01]. Fermentation enhanced also the antioxidant properties of a lot of other kinds of food like pomegranate juice [Schu99], soybean [McC03] or a special Asian antioxidant cocktail called EM-X (derived from ferment of

unpolished rice, papaya and sea weeds) [Aruo02].

Therefore, a positive influence of fermentation on the antioxidant capacities of the analysed juices stands at least to reason.

4.3.8 Comparison with data from the literature

Only few TOSC articles concerning foods are up to now published and they are all restricted to peroxyl radicals. A comparison with the herein presented data is therefore limited. One article can be found [Sun02] that deals with at least some of the fruits that were also surveyed in this work. In this study, the best activities were found for cranberries that are from the same genus Vaccinium as the smaller relative lingonberry. Apples had the second best results followed by lemons and oranges with rather similar activities. These results are largely in accordance to the results presented herein. Only the ranking of the apple juice does not fit to the results of Sun et al. [Sun02].

To get a broader basis for the classification of the TOSC results of juices, they are compared additionally in the following paragraph to results of other antioxidant assays.

Much research has been done, e.g., about the antioxidant activity of blueberries [Con02, Kal99]. Most articles concerning antioxidant berries from the genus Vaccinium are published about cranberries, but some surveys deal also with lingonberries [Käh01, Zhe03]. Analyses have also been done in the field of the antioxidant capacities of sour cherries [Halv02, Rec97] and elderberries [Lug03]. In accordance to the TOSC findings presented herein, all researchers detected high antioxidant capacities for the analysed berry samples, especially when compared with other kinds of fruits [Pro02, Wan96, Sun02].

The good antioxidant properties of beetroot have also been stated in a couple of articles [Kan01, Esc98]. But, they have not yet been compared directly to results for fruits or especially berry juices. So, it is not possible to confirm the promising results for beetroot juice by the literature.

Henn et al. [Hen98] found for multivitamin and ACE juices antioxidant capacities worse than those of anthocyanin rich juices but in the same range as those of orange and grapefruit juices. This corresponds to the results for peroxynitrite in the TOSC assay. Proteggente et al. [Pro02] specified also fruits rich in flavanones (e.g. oranges and grape fruits) to be second best after fruits rich in anthocyanins.

The results for apple juices in literature are not so uniform: some results were rather poor [Pro02, Wan96, Hen98] whereas others researchers detected rather good antioxidant activities [Sun02, Vin01]. Anyhow, this is not surprising because there are more than 20,000 different varieties of apples grown worldwide [Fra97], even though not all of them are important for the com-

mercial manufacturing of apple juice. This is maybe also the explanation for the differences between the TOSC results of apple juice presented herein compared to those of Sun et al. [Sun02].

For tomatoes, there are also contradictory results in the literature, but those with weak results [Pro02, Leo02] dominate the few articles with more promising results [Wan96] clearly. The antioxidant activities of carrots are mostly compared to vegetables and not to fruits, so it is difficult to rank their results. It can be said that carrots showed better antioxidant capacities than tomatoes and worse than white cabbage in the surveys of Gazzani et al. [Gaz98a, Gaz98b]. This aligns partly with the TOSC findings for peroxyl radicals and peroxynitrite of carrot, tomato and sauerkraut juices.

A comparison with the TOSC results for hydroxyl radicals is more problematic. Though a lot of research has been done about the antioxidant capacities of different fruits and, less, about vegetables, most studies are conducted with rather stable ROS, even if different assays are used within the same survey. And if studies are carried out with more reactive ROS, they concentrate predominately to special groups of fruits or vegetables. In literature, it can be found that, e.g., berry crops [Wan00] as well as white cabbage [Rac02] showed antioxidant capacities against hydroxyl radicals. But as the results are based on different concentrations they can not be compared directly. Therefore, it is not possible to line up the results for hydroxyl radicals in the TOSC assay with other surveys.

In summary, some of the TOSC results are in good accordance with the literature and some in not so good. Obviously, this is because most other surveys are done with rather stable ROS, whereas the TOSC assay uses three different ROS with a broad spectrum of reactiveness.

4.3.9 Comparison of juices with trolox

It is very common for in vitro antioxidant assays, to express the results for a sample as equivalents of the water-soluble vitamin E analogue trolox. The most prominent example for this proceeding is the TEAC assay (cf. 4.2.6).

Hence, the significance of such equivalents is rather questionable. Keeping in mind the ascertained non-linear relation between concentration and antioxidant capacities of trolox as well as the juice samples for the TOSC assay, it is only legitimate to compare the results for selected points of the respective dose-response curves.

Possible starting-points for such considerations could be “I want to obtain a TOSC value of X. Which concentration of trolox or dilution of juice do I have to use to get this?” or “I have an X mM solution of trolox that yields a TOSC value of Y. Which dilution of juice do I have to use to get

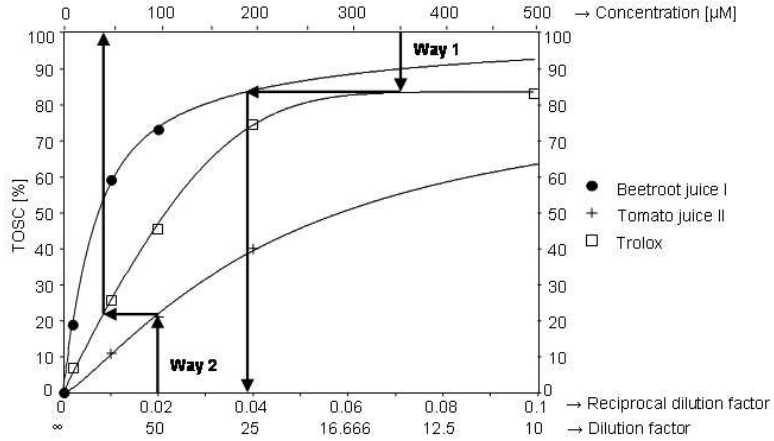

Figure 4.11: Comparison of TOSC of trolox and some juices

the same result?" or "My juice is diluted 1:X and gives a TOSC values of Y. How concentrated has the trolox solution to be to achieve the same inhibition?". The answers to these questions can be taken mathematically from the fitted respective dose-response curves like it was done for calculating concentrations/dilutions corresponding to TOSC values of 20, 50 and 80%.

In figure 4.11, these considerations are demonstrated graphically. Way 1, for example, starts at a 350 μM trolox solution. Following the arrows it can be seen, that the same inhibition as for the trolox solution can also be obtained by a 1:27 diluted beetroot juice 1. Way 2 demonstrates, that a 1:50 diluted tomato juice II has the same antioxidant capacity as a 40 μM trolox solution.

Though ADRC and DT_{50} are helpful for the comparison of the juices among each other, they can not contribute to a comparison with trolox. This is because they depend highly on the units that are chosen for the axes of the coordinate system. Though the y-axis is the same for all samples (i.e. TOSC between 0 and 100%), it is not possible to choose the same x-axis as well for the juices as for a standard compound like trolox. Juices can not be expressed in concentration of molar units, only if it would have been ascertained which compounds in the juice are responsible for its antioxidant activity and in which concentrations they are present. But then it would be no longer a comparison of trolox and a juice but of trolox and (at least) one other standard compound. Trolox could also be quoted in units of weight per volume (like g/L, e.g.), what seems to be, at first sight, the better choice. For the juices, their content of dry matter could be analysed (e.g. by freeze-drying) to display them in the same unit. But this would assume that all

the compounds that contribute to the dry matter contribute equally to the antioxidant activity of the juice. On the other hand, one could also think of illustrating trolox in terms of dilutions based on a special starting concentration. But then the results would depend strongly on which concentration is chosen for such a starting point. As figure 4.6 demonstrates, there is no ideal trolox concentration level to start with, because the dose-response curves for the three assayed ROS differ too much making a general solution for this problem impossible.

4.4 Açaí pulps

To get an overview of the antioxidant spectrum of açaí pulps, a broad variety of samples was analysed including

- commercial açaí samples of the three trading qualities (cf. 1.2.2)
  - açaí grosso,
  - açaí medio and
  - açaí fino
- and non-commercial samples from
  - different harvesting years,
  - different harvesting seasons (see 1.2.2) and
  - both açaí varieties with purple and white fruit pulps.

Parts of these results have been submitted for publication at [Lic04].

4.4.1 TOSC against peroxyl radicals

The experimental TOSC values of the açaí pulps against peroxyl radicals can be found in table E.2. In table 4.12, the calculated dilutions and concentrations corresponding to TOSC values of 20, 50 and 80%, the ADRC and the DT_{50} are shown. Figure 4.12 displays some typical dose-response curves for purple açaí samples with good, medium and low antioxidant capacities as well as for the white açaí (açaí branco).

All açaí samples demonstrate very good antioxidant capacities against peroxyl radicals with the exception of açaí branco. Like the fruit juices rich in betalains and anthocyanins (see 4.3.3), their activities are even so high, that their ADRC values can only be estimated. Since this means an undervaluation, a “>” symbol is added to all calculated values.

	Calculated dilution factor for TOSC of			DT_{50}	ADRC	r^2
	20%	50%	80%			
Diagonal defined as	–	–	–	1.0	1.0	–
Açaí grosso I	1250	435	189	15.9	>1.9	0.9999
Açaí medio I	1111	385	175	14.3	>1.9	1.0000
Açaí grosso II	833	286	128	10.4	>1.9	0.9998
Açaí pulp 2002	769	256	120	9.7	>1.9	1.0000
Açaí pulp 1998	588	238	106	9.2	>1.9	0.9998
Açaí medio II	714	227	98	7.8	>1.9	0.9998
Açaí fino I	667	217	103	8.0	>1.9	0.9999
Açaí pulp 2000	641	210	104	7.6	>1.9	0.9997
Açaí pulp 2001	606	182	87	6.4	>1.9	0.9999
Açaí fino II	500	145	61	4.8	>1.8	0.9998
White açaí	76	30	15	1.3	1.2	1.0000
	Calculated conc. [mg/L] for TOSC of			DT_{50}	ADRC	r^2
	20%	50%	80%			
Cyanidin-3-glucoside	4.4	11.2	21.8	–	–	0.9996
Cyanidin-3-rutinoside	5.7	15.1	30.2	–	–	0.9994

Table 4.12: Calculated dilution factors, DT_{50} and ADRC of açaí pulps for TOSC against peroxyl radicals

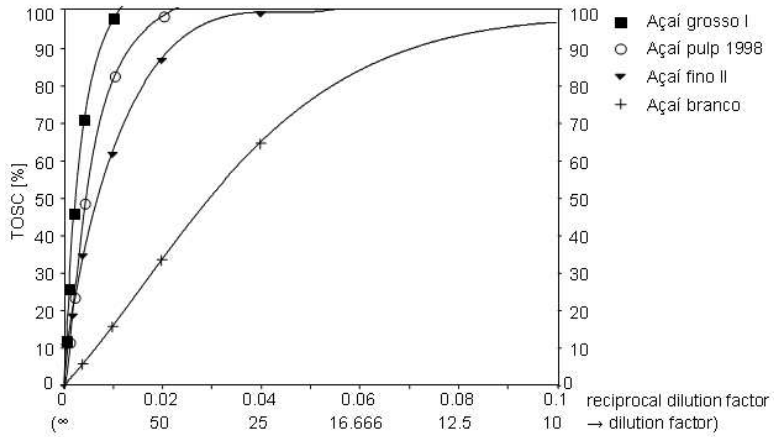

Figure 4.12: TOSC of some açaí pulps against peroxyl radicals

	Calculated dilution factor for TOSC of					
	20%	50%	80%	DT_{50}	ADRC	r^2
Diagonal defined as	–	–	–	1.0	1.0	–
Açaí grosso I	526	99	19	2.0	1.5	1.0000
Açaí medio I	588	100	13	1.7	1.4	1.0000
Açaí pulp 2002	455	85	12	1.4	1.4	0.9999
Açaí grosso II	435	63	12	1.2	1.3	0.9998
Açaí medio II	400	67	10	1.2	1.3	1.0000
Açaí pulp 1998	313	64	10	1.2	1.3	0.9999
Açaí fino I	357	59	10	1.0	1.3	1.0000
Açaí pulp 2000	278	58	9	1.3	1.1	1.0000
Açaí pulp 2001	208	45	8	1.2	1.0	1.0000
Açaí fino II	175	35	6	0.7	1.1	0.9999
White açaí	86	17	3	0.3	0.8	0.9996
	Calculated conc. [mg/L] for TOSC of					
	20%	50%	80%	DT_{50}	ADRC	r^2
Cyanidin-3-glucoside	11.6	50.9	259	–	–	1.0000
Cyanidin-3-rutinoside	15.8	58.1	362	–	–	0.9999

Table 4.13: Calculated dilution factors, DT_{50} and ADRC of açaí pulps for TOSC against peroxynitrite

Açaí grosso I and açaí medio I have the best results followed by a large midfield with similar outcomes and the açaí pulp from the low harvesting season of 2001 together with açaí fino II showing the worst results. Compared to the fruit and vegetable juices (see 4.3.3), all purple açaí liquids are in the top class of peroxyl radical scavengers together with, e.g., lingonberries and beetroot. Açaí branco, in contrast, ranks among the samples with the lowest antioxidant capacities like tomato and sauerkraut juices.

4.4.2 TOSC against peroxynitrite

The experimental TOSC values of the açaí pulps against peroxynitrite are given in table E.1. In table 4.13, the calculated dilutions and concentrations corresponding to TOSC values of 20, 50 and 80%, the ADRC and the DT_{50} are shown. Some of the results are displayed in figure 4.13.

Against peroxynitrite, all purple açaí samples demonstrate good antioxidant capacities even though not as outstanding as against peroxyl radicals.

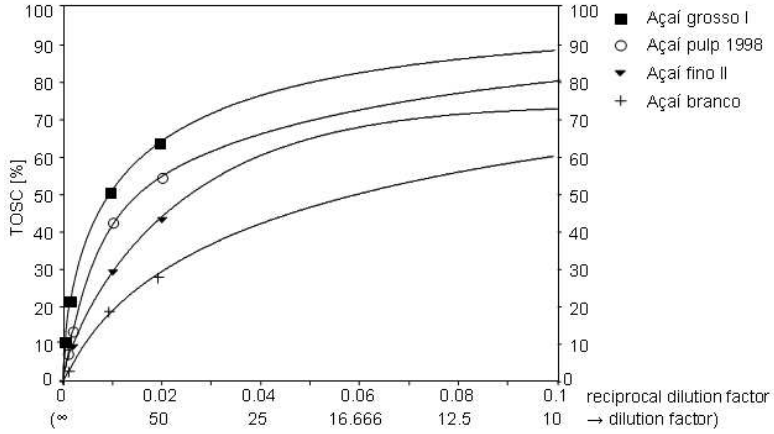

Figure 4.13: TOSC of some açaí pulps against peroxynitrite

Again, açaí grosso I and açaí medio I have the highest antioxidant activities and the lowest activities are detected for the açaí pulp of 2001 and açaí fino II. Though the purple açaí samples show better results against peroxynitrite than most other analysed fruit and vegetable juices, they are not as good as, e.g., lingonberry or beetroot juice (see 4.3.4). The antioxidant capacities of açaí branco are again rather poor and among the juices with the worst results.

4.4.3 TOSC against hydroxyl radicals

The experimental TOSC values of the açaí pulps against hydroxyl radicals are shown in table E.3. In table 4.14, the calculated dilutions and concentrations corresponding to TOSC values of 20, 50 and 80%, the ADRC and DT_{50} are given. Figure 4.14 displays some of the dose-response curves for this ROS.

For hydroxyl radicals, no real ranking of samples can be detected. All purple açaí samples have very similar and at the same time low antioxidant capacities against this ROS. Açaí branco is even less effective than the other samples. Compared to the other juices (see 4.3.5), the results are at the lower end of the ranking for hydroxyl scavenging capacities.

4.4.4 Comparison of the three ROS

All purple açaí pulps show very good antioxidant capacities against peroxyl radicals, rather good inhibition results for peroxynitrite and very low antioxidant activities against hydroxyl radicals. These results correspond partly to those of Pozo-Insfran et al. [Poz04] for one analysed açaí pulp sample.

	Calculated dilution factor for TOSC of			DT_{50}	ADRC	r^2
	20%	50%	80%			
Diagonal defined as	–	–	–	1.0	1.0	–
Açaí pulp 2001	111	43	7	1.2	1.0	0.9997
Açaí grosso II	161	39	9	1.3	1.1	0.9997
Açaí fino II	83	29	9	0.9	1.1	1.0000
Açaí pulp 2000	94	29	5	0.5	1.0	0.9998
Açaí medio I	59	24	10	1.0	1.0	0.9994
Açaí medio II	84	26	6	0.7	1.0	0.9993
Açaí grosso I	57	23	10	0.9	1.0	1.0000
Açaí fino I	53	21	9	0.9	0.9	0.9994
Açaí pulp 2002	57	22	8	0.8	0.9	0.9998
Açaí pulp 1998	50	18	7	0.7	0.9	1.0000
White açaí	27	7	3	0.2	0.5	0.9998

Table 4.14: Calculated dilution factors, DT_{50} and ADRC of açaí pulps for TOSC against hydroxyl radicals

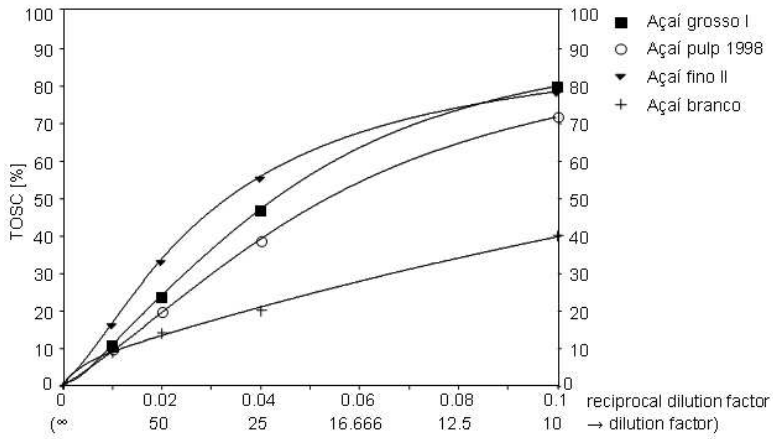

Figure 4.14: TOSC of some açaí pulps against hydroxyl radicals

	Dry matter content [%]
Açaí grosso I	13.9
Açaí grosso II	13.4
Açaí medio I	11.5
Açaí medio II	10.0
Açaí fino I	7.7
Açaí fino II	6.5

Table 4.15: Dry matter content of analysed commercial açaí beverages

The worst results for a purple sample in this work are found for the low harvesting quality of 2001. As all non-commercial samples were taken from the same açaí trees, this finding indicates that the low organoleptic quality during this period (see 1.2.2) could also correspond to low antioxidant activities. The pulp of the white açaí demonstrates only very low inhibition capacities. This implies that the anthocyanins present in the samples might have an influence on their antioxidant properties.

While the activity ranking of all samples was very similar for peroxyl radicals and peroxynitrite, no real order of activities could be detected for hydroxyl radicals. As already discussed for the standard compounds and also the fruit and vegetable juices (cf. 4.2.4 and 4.3.6), the highly reactive hydroxyl radicals combine with nearly every compound they can reach. But as açaí pulps have only very low contents of e.g. sugars (see table 1.2) and organic acids (see table E.4; GC-MS parameters given in table 3.8), the possible targets for the hydroxyl radicals are rather limited in the açaí pulps. This might cause the low inhibition capacity of the pulps against this ROS.

4.4.5 Influence of dry matter content on TOSC

The dry matter content of commercial açaí samples varies not only a lot between but also among the three trading qualities, because they are up to now not regulated by law (see table 1.1). This variation could be one explanation for the differing antioxidant capacities of the analysed samples. To review the influence of this parameter, the dry matter content of the commercial samples was analysed by freeze-drying. The results of these analyses are given in table 4.15. For the non-commercial samples, the dry matter content was adjusted to 10% (cf. 2.3.2).

For peroxyl radicals and peroxynitrite, the best antioxidant capacity of a commercial samples is found for a grosso quality; the worst is a fino quality. Like their content of dry matter (13.9 and 6.5%, resp.), their antioxidant

capacities differ about factor 2. But, açaí medio I (11.5% dry matter), e.g., has better results than açaí grosso II (13.4% dry matter) and the pulps of 2000 and 2001 (dry matter adjusted to 10%) are worse than açaí fino I (7.7% dry matter). For hydroxyl radicals, no ranking can be detected matching the contents of dry matter.

Therefore, differences in the antioxidant capacities of the açaí samples can not be explained alone by different dilutions of the base material during the manufacturing process. There must be also significant differences in the base material itself.

4.4.6 Identification of phenolic compounds

As açaí is only a poor source of vitamin C [Rog00] and the TOSC assay covers only water-soluble compounds in the form presented herein [Reg99, Wins98], polyphenols are the most reasonable compounds for its antioxidant properties. Though there has already been some research in this field, the results are contradictory: Rogez [Rog00] and Iaderoza et al. [Iad92] detected cyanidin-3-glucoside and -rutinoside in açaí fruits whereas Bobbio et al. [Bob00, Bob02] found cyanidin-3-arabinoside and cyanidin-3-arabinosylarabinoside. In the study of Pozo-Insfran et al. [Poz04], cyanidin-3-glucoside was the only anthocyanin in bigger amounts in açaí pulps.

By HPLC-MS analyses (see table 3.6), the presence of two different anthocyanins in açaí fruits could be proven in appreciable amounts and some others in minor concentrations. The system settings used for the analysis of anthocyanins allowed a good ionisation and selective MS-detection of the molecular ions in the oxonium form, revealing their molecular weight directly. Subsequent MS-fragmentation experiments showed the dissociation of the aglycon and the glycoside, delivering their molecular masses. In table E.5, the MS data for the identified compounds are shown. For procyanidins, beside the structural informative fragment ions, the masses of the three most abundant fragment ions are given.

Thus, the two main anthocyanins were identified as cyanidin-3-glucoside and cyanidin-3-rutinoside in accordance with the findings of Rogez [Rog00] and Iaderoza et al. [Iad92] and also partly with those of Pozo-Insfran et al. [Poz04]. One of the minor anthocyanins could be identified as peonidin-rutinoside (see figure 4.15). Due to their low amount in the samples, the others could be assigned only tentatively to anthocyanins with pelargonidin, peonidin, delphinidin, petunidin and malvidin as aglycon.

Other non-anthocyanin polyphenols were identified with the MS parameters given in table 3.7. Thereby, protocatechuic acid, flavan-3-ols (catechin mono- through tetramers) and quercetin-rutinoside could be identified in

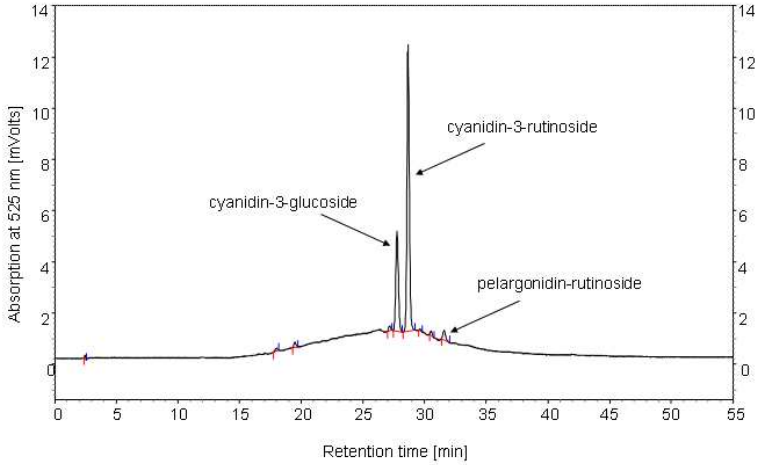

Figure 4.15: HPLC separation of anthocyanins in açaí pulps

minute quantities in the açaí pulp samples.

4.4.7 Anthocyanin content and comparison with TOSC

The concentrations of the two main anthocyanins in the analysed açaí samples are shown in table 4.16.

Cyanidin-3-rutinoside is the dominating compound in all samples with concentrations ranging from 1 mg/L in açaí branco up to 456 mg/L in açaí grosso I. The concentration of cyanidin-3-glucoside varies from 0 mg/L in açaí branco and 1 mg/L in açaí medio I to 54 mg/L in the pulp of 2002. Summing up the amounts of both anthocyanins to a total anthocyanin content, the concentrations varies between 1 mg/L for açaí branco and 463 mg/L for açaí grosso I. The ratio of the two compounds lies between 1.6 for the pulp of 2001 and 65 for açaí grosso I in favour of cyanidin-3-rutinoside. This finding is in contrast to the study of Pozo-Insfran et al. [Poz04] for a single açaí pulp who found cyanidin-3-glucoside as the predominant anthocyanin with 1040 mg/L while cyanidin-3-rutinoside was not detected. But, besides the limited data basis in this article, the anthocyanin identification was not confirmed by more unequivocal means like HPLC-MS but was based only on spectral interpretations. Therefore, the significance of this finding remains questionable.

No significant correlation can be detected between the different trading qualities and the anthocyanin content of the samples. Though a grosso quality has the highest anthocyanin content of all analysed samples (açaí grosso I) and a fino sample has the lowest concentration (açaí fino II) of the com-

	Anthocyanin content [mg/L]			Total phenolic content [mg/L]
	Cya.-3-glu.	Cya.-3-rut.	$\sum$	
Açaí grosso I	7	456	463	4600
Açaí pulp 2002	54	157	211	Not analysed
Açaí fino I	5	106	111	1900
Açaí medio I	1	99	100	4100
Açaí pulp 1998	19	79	98	2500
Açaí grosso II	19	76	95	3200
Açaí pulp 2000	27	61	88	2200
Açaí medio II	7	67	74	2700
Açaí fino II	6	24	30	Not analysed
Açaí pulp 2001	5	8	13	2000
White açaí	0	1	1	Not analysed

Table 4.16: Anthocyanin and total phenolic content of açaí pulps

mercials, the second best commercial result is found for the other fino sample (açaí fino I) being ahead of the other grosso and medio juices.

The pulp of the low harvesting season of 2001 has a noticeably low anthocyanin content compared to all other pulps. This underlines the above described quality differences between the two main harvesting periods (see 1.2.2). The very low anthocyanin concentrations of the açaí branco sample confirms its classification as an anthocyanin-free variety of *Euterpe oleracea*. The large spectrum of anthocyanin content detected in this survey is in accordance with the findings of Rogez [Rog00], who stated anthocyanin contents between 71 and 1022 mg/kg of fruits for 60 purple açaí samples.

At first sight, there seems to be a correlation between anthocyanin content and antioxidant capacities of samples at least for peroxyl radicals and peroxynitrite. The samples with the highest anthocyanin contents (açaí grosso I, açaí pulp of 2002 and açaí medio I) are also the samples with the highest antioxidant activities against these two ROS (cf. tables 4.12 and 4.13). The samples with the lowest anthocyanin content (açaí fino II, açaí pulp of 2001 and açaí branco) have at the same time the worst ROS scavenging capacities against peroxyl radicals and peroxynitrite. But though açaí grosso I has a 36 times higher total anthocyanin content than the açaí pulp of 2001, its calculated dilution factor for a TOSC of 50% against peroxyl radicals is only about 2 times higher than that of the pulp. The same ratio can also be found for a 50% inhibition of peroxynitrite.

In the figures 4.16, 4.17 and 4.18 the correlation between the anthocyanin content and the TOSC for the three ROS is reviewed mathematically. The

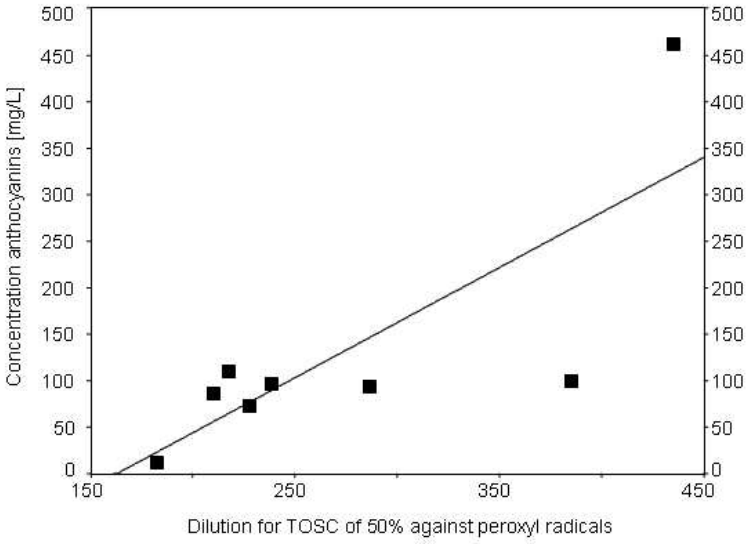

Figure 4.16: Correlation between anthocyanin content and TOSC against peroxyl radicals ($r^2 = 0.6061$)

low values for a correlation with an r^2 of 0.6061, 0.4670 and 0.1390, respectively, underline the missing interrelation between these parameters.

The facts get even more confusing when the antioxidant activities of the açaí samples are compared directly to those of the pure anthocyanin standard compounds. In the tables 4.12 and 4.13, the calculated concentrations for a TOSC of 20, 50 and 80% of cyanidin-3-glucoside and cyanidin-3-rutinoside for peroxyl radicals and peroxynitrite are opposed directly to those of the açaí pulps. For hydroxyl radicals, the antioxidant activities of these anthocyanins are in relevant concentration levels too low to allow calculations (cf. 4.2.3).

Açaí grosso I, for example, has to be diluted 1:435 to reach a TOSC value of 50% for peroxyl radicals. Starting from a total anthocyanin content of 463 mg/L, this means a concentration in the diluted solution of about 1 mg/L. To get the same inhibition from the pure anthocyanins, an eleven- to fifteen-fold higher concentration has to be used. For peroxynitrite, the ratio lies for a TOSC of 50% at about 11 to 12 times in favour of açaí grosso I. Taking the same considerations into account, the açaí pulp of 1998 is 27 to 37 times more effective for a 50% inhibition of peroxyl radicals and 33 to 38 times for peroxynitrite than it can be explained by the anthocyanin content. For the açaí pulp from 2001, the differences spread to a ratio of 157 to 211 for peroxyl radicals and 176 to 201 for peroxynitrite. And for hydroxyl radicals, the antioxidant capacities of all samples are so close together that

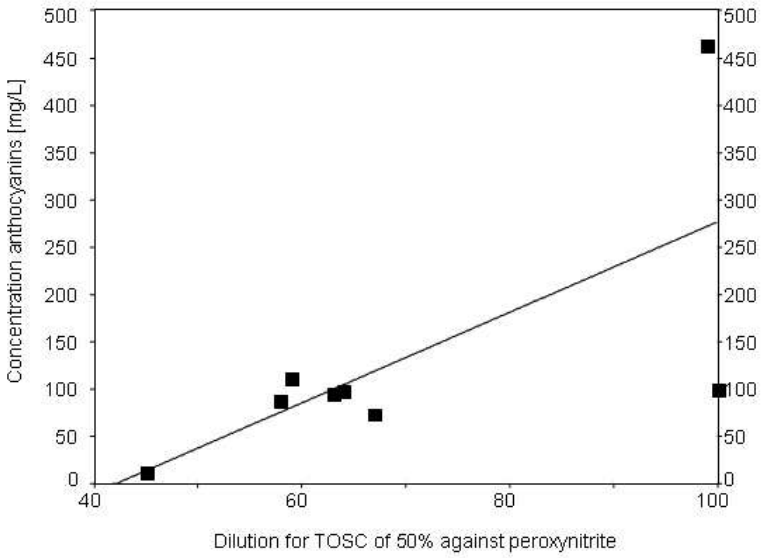

Figure 4.17: Correlation between anthocyanin content and TOSC against peroxynitrite ($r^2 = 0.4670$)

an influence of the anthocyanin content can be excluded completely.

Therefore, no correlation can be seen between the anthocyanin content and the antioxidant capacity of the analysed açaí pulps. This is in contrast to the findings of Pozo-Insfran at al. [Poz04], who came to the conclusion that the anthocyanins of açaí pulps correlated to their antioxidant capacity. But, as already mentioned, their results were only for a single sample making their statement very daring.

Based on the results for more samples with varying concentrations of anthocyanins, it can be concluded that the main part of the antioxidant capacities of açaí pulps must be due to other, yet not identified, compounds. This consideration is supported by the results of different groups of researchers for other samples rich in anthocyanins. Miller et al. [Mil97] found out that there must be a significant unidentified antioxidant in blackcurrant drink, because the good results of this beverage could not be explained only by its vitamin C and anthocyanin content. Their research confirms the earlier results from Harper et al. [Harp69] who concluded that it were not the anthocyanins that stabilised the ascorbic acid in blackcurrant juice. A survey of Wang et al. about the total antioxidant capacity of fruits [Wan96] revealed also that there may be some unknown antioxidants present in fruits that need to be identified.

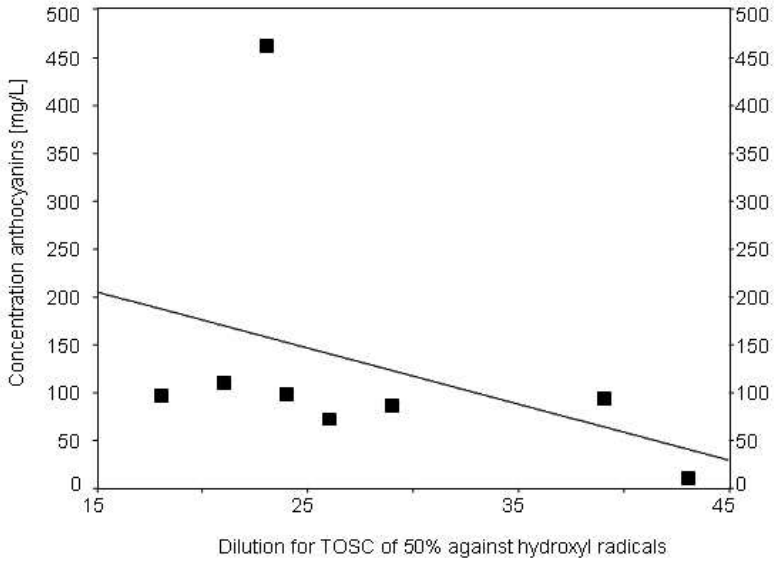

Figure 4.18: Correlation between anthocyanin content and TOSC against hydroxyl radicals ($r^2 = 0.1390$)

4.4.8 Total phenolic content and comparison with anthocyanin content and TOSC

Analysing the total phenolic content by the Folin-Ciocalteu assay is a rather old but still often performed method to estimate the antioxidant capacities of samples [Jul85]. While some researchers found a correlation between these two parameters [Zhe03, Kal99] other groups demonstrated the opposite [Hei98, Käh01]. To find out the relevance of the total phenolic content for the antioxidant capacities of açaí fruits in the TOSC assay, most samples were analysed with the Folin-Ciocalteu method [Jul85]. The results are shown in table 4.16.

For the antioxidant capacities of samples against peroxyl radicals and peroxynitrite, the total phenolic content seems to give at least a hint. The samples with the highest total phenolic content (açaí grosso I and açaí medio I) have also the best scavenging capacities for these two ROS whereas the samples with rather poor antioxidant properties (açaí pulps of 2000 and 2001) have at the same time the lowest total phenolic content. In the figures 4.19, 4.20 and 4.21, the interrelation between TOSC and total phenolic content of the samples is reviewed mathematically. For peroxyl radicals, the correlation is rather good, for peroxynitrite already less strong while for hydroxyl radicals not even a hinted correlation exists.

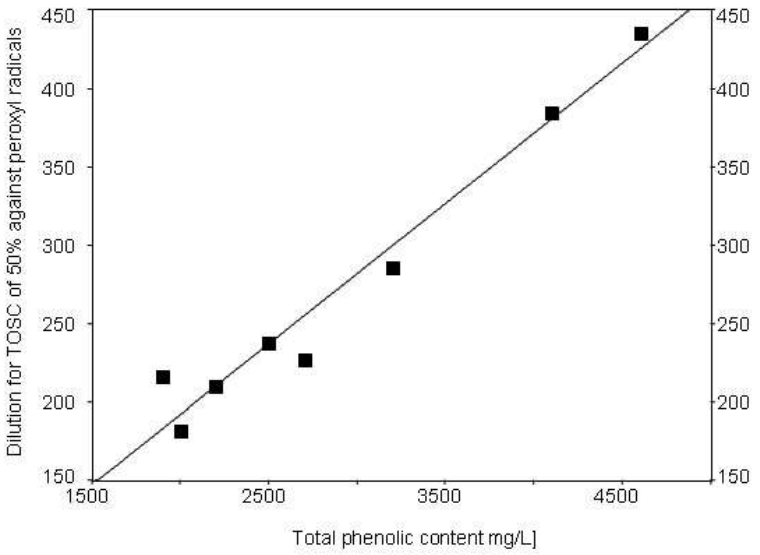

Figure 4.19: Correlation between total phenolic content and TOSC against peroxyl radicals ($r^2 = 0.9596$)

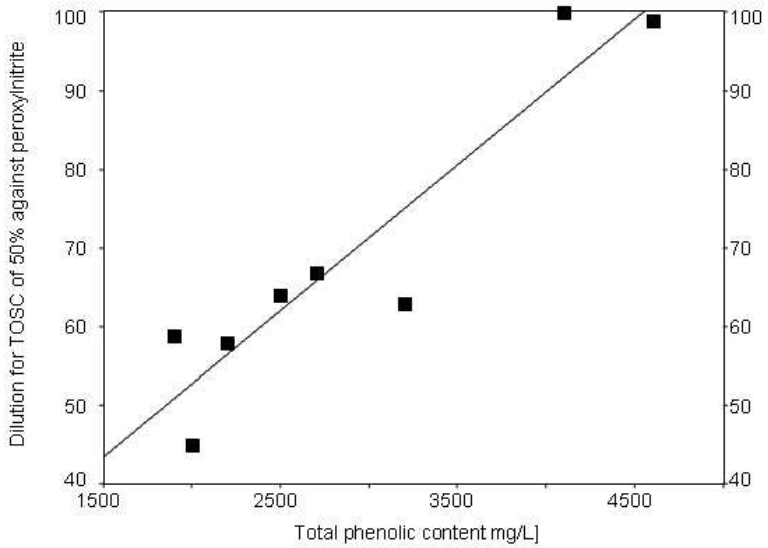

Figure 4.20: Correlation between total phenolic content and TOSC against peroxynitrite ($r^2 = 0.8712$)

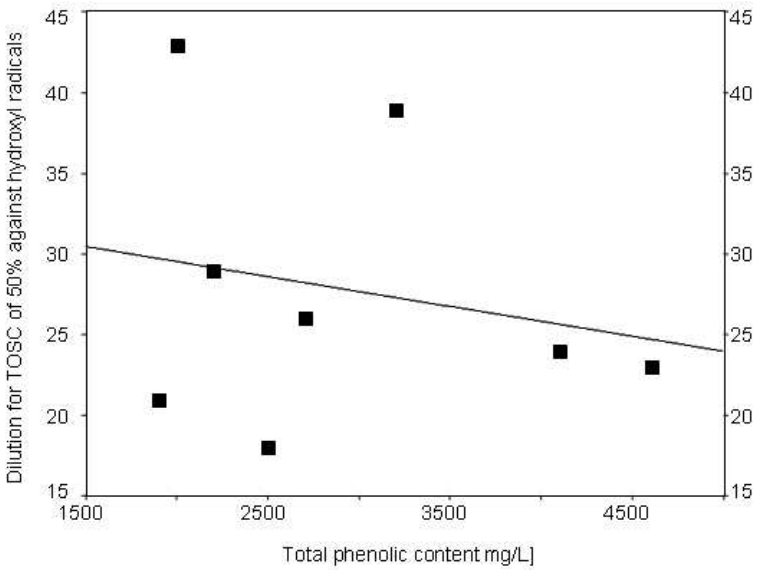

Figure 4.21: Correlation between total phenolic content and TOSC against hydroxyl radicals ($r^2 = 0.0438$)

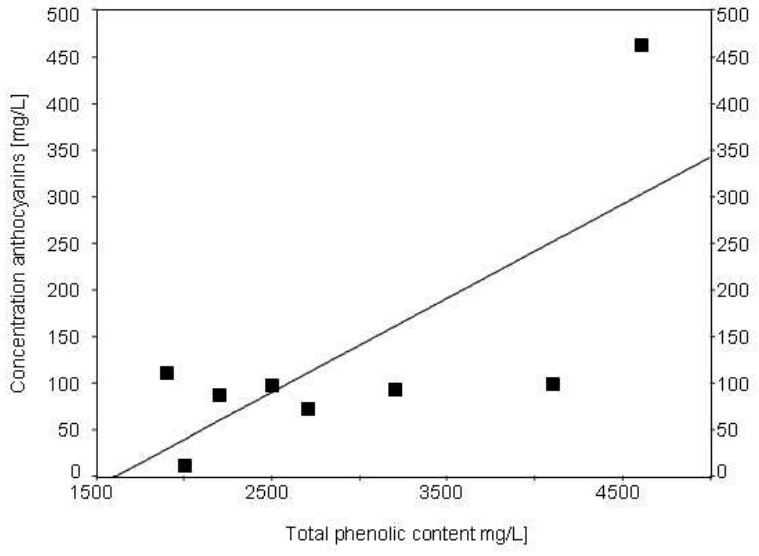

Figure 4.22: Correlation between anthocyanin content and total phenolic content ($r^2 = 0.5284$)

As other phenolic compounds are only detected in minor quantities (see 4.4.6), the anthocyanins should make up the largest part of the total phenolic content of the açaí samples. But, no correlation between these two parameters emerges (see figure 4.22). Though the anthocyanin content of the samples varies in a great scale, only minor differences can be found for their total phenolic content (see table 4.16). In addition, the anthocyanin content makes up only about 10% or even less of the total phenolic content.

As Singleton et al. [Sin74] demonstrated, anthocyanins respond only poor to the Folin-Ciocalteu method in contrast to other phenolic compounds and are therefore not mirrored validly by this parameter. So, other, yet not identified compounds must cover most part of the total phenolic content of the samples.

4.4.9 Fractionating of samples by HPLC and TOSC

To identify the compounds that are responsible for the antioxidant activities of açaí pulps, three samples were fractionated by HPLC as described in 3.5. The samples were such chosen that they covered high (açaí grosso I), medium (açaí pulp of 2002) and low (açaí fino II) TOSC results. These samples included also the pulps with the highest detected content of cyanidin-3-rutinoside (açaí grosso I) and cyanidin-3-glucoside (açaí pulp of 2002). The antioxidant capacities of the fractions were analysed against peroxyl radicals. Peroxyl radicals were chosen, because all pulps demonstrated their highest antioxidant activities against this ROS revealing at the same time the biggest differences between the samples (see 4.4.1).

The TOSC values of the HPLC fractions are shown in table 4.17. For the two samples representing the extremes of the survey (açaí grosso I and açaí fino II), the results of the fractions are additionally opposed to the simultaneously recorded HPLC chromatograms at 525 nm in the figures 4.23 and 4.24.

For all three samples, a similar distribution of antioxidant capacities over the HPLC run time is found:

- In the fractions from 0–15 min, rather low antioxidant activities are observed.
- Starting with the fraction from 15–20 min, the activity of all samples rises.
- The climax of activity is reached with similar high activities of the two fractions from 25–30 min and 30–35 min.

HPLC fraction [min]	TOSC against peroxyl radicals [%]		
	Açaí grosso I	Açaí pulp 2002	Açaí fino II
0–5	18	6	9
5–10	14	3	5
10–15	7	2	3
15–20	30	18	20
20–25	76	50	46
25–30	96	87	81
30–35	97	92	82
35–40	79	64	52
40–45	39	24	23

Table 4.17: TOSC of açaí pulp HPLC fractions

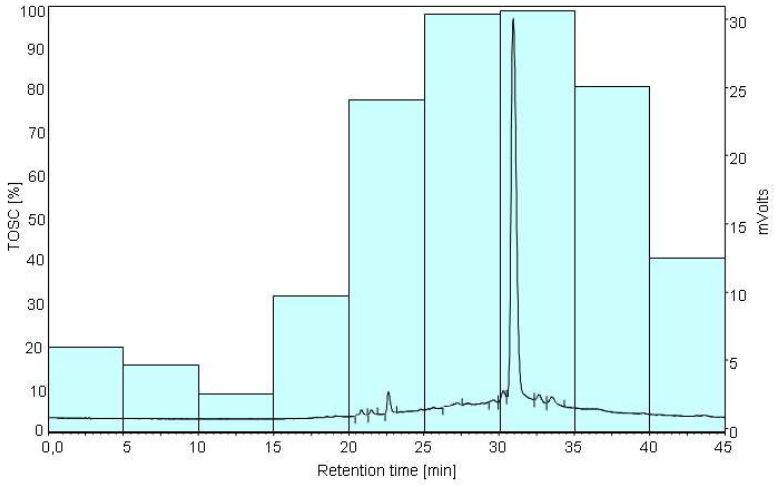

Figure 4.23: TOSC and absorption at 525 nm of açaí grosso I HPLC fractions

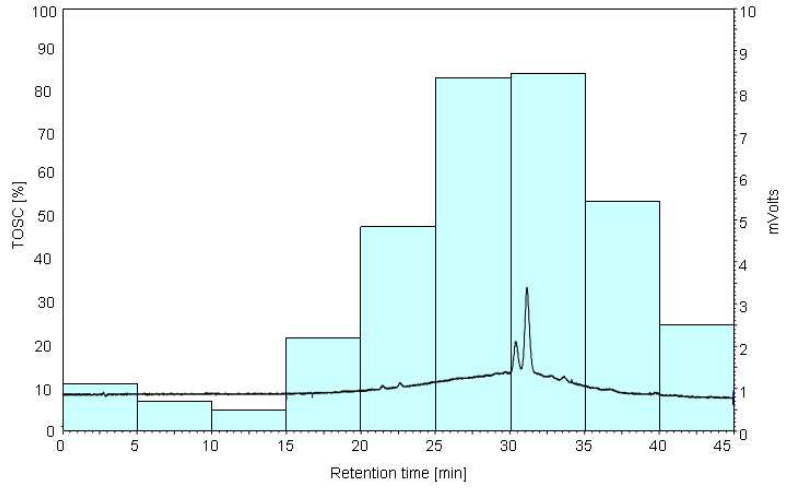

Figure 4.24: TOSC and absorption at 525 nm of açaí fino II HPLC fractions

- From the fraction from 35–40 min on, the activity of all samples declines again.

First interesting hints and hypotheses can be taken from a comparison of these findings with the simultaneously recorded HPLC signals of the samples:

1. One of the highest activities is found for the fraction containing the two major anthocyanins suggesting an influence of these compounds.

2. The rise and fall of the activity between 15 and 45 min is mirrored by a simultaneous course of the baseline of the chromatograms. Together with an absorption not only at the less specific wavelength of 210 nm but also at 525 nm this could direct to one or more coloured compounds that are not well separated by the column. Their late retention window indicates that these molecules might have rather big molecular weights and/or low polarities.

For hypothesis no. 1, a direct influence of the anthocyanins on the antioxidant capacity can be excluded in advance. As it was already shown in 4.4.7, there is no correlation between the anthocyanin content and the TOSC of açaí pulps. This assumption is confirmed when the absolute amount of anthocyanin present in the prepared HPLC fractions is calculated. For açaí grosso I, e.g., 20 μL of an about 460 mg/L anthocyan containing solution are injected to the HPLC. This is equivalent to an absolute anthocyan amount of 9.2 μg. After freeze-drying, the residue is solved in 500 μL UHQ water yielding a concentration of 18.4 mg/L. This solution possesses a TOSC of 97%

what is more than double as high as it was found for the standard solutions of the pure anthocyanins (see table 4.1). For the two other samples with a much lower anthocyanin content, the gap between the result of the fractions and the standard solutions gets even bigger.

Therefore, though the highest antioxidant capacity appears in the anthocyanin fraction, its activity should be due to other compounds eluting at the same retention time window as the anthocyanins. For the anthocyanins, an only indirect influence is imaginable, e.g., by having synergistic effects in combination with other antioxidative compounds present in the same fraction.

To find out which other antioxidants are present in the anthocyanin fraction and to review also hypothesis no. 2, the fractions that had demonstrated interesting aspects were analysed by HPLC-MS in accordance to the method parameters given in table 3.10. To exclude the possibility of signals caused by the fractionation process, one fractionated blank run was also measured completely. The results of these analyses can be resumed as follows:

- In the fractions of the blank run, no MS-signals were found. Therefore, the signals detected in the further analyses are really due to the respective samples but not to the fractionation process.

- From 0–15 min, only few and very small signals were detected fitting together with the rather low antioxidant activities of these fractions.

- For the fractions from 15 to 45 min, a mountain-like rise and fall of the baseline of the HPLC chromatogram had been observed before (see figures 4.23 and 4.24). After being re-injected to the HPLC-MS system, the fractions eluted still at the original retention times. Consequently, more than one compound must be responsible for the baseline course. This consideration is confirmed by a large number of different and rather small signals that were detected by HPLC-MS in the fractions in addition to the already known major anthocyanins (see figure 4.25).

Due to the many different compounds present in small amounts in the relevant fractions, it was not possible to identify the decisive antioxidants in açaí pulps alone by means of sample fractionation.

4.4.10 Accelerated degradation and influence on TOSC

To make the distinction easier, which of the compounds in açaí pulps possess antioxidant properties, the three samples were subjected to an accelerated

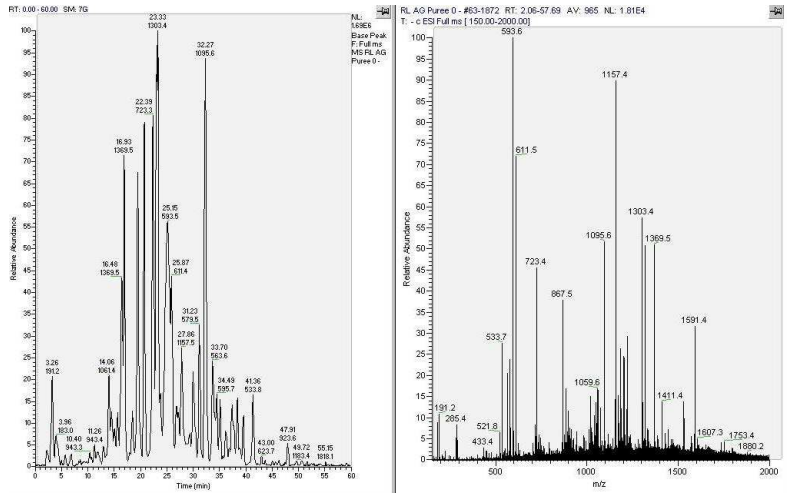

Figure 4.25: Base peak chromatogram and mass spectrum over run time of açaí grosso I

degradation. For this purpose, the pulps were stored in a drying cabinet at 37° C. In regular intervals, the TOSC of the samples against peroxyl radicals was tested in a dilution of 1:50. Peroxyl radicals were chosen as the ROS with the highest and also most different antioxidant capacities of the samples (cf. 4.4.1). The dilution of 1:50 was selected, because the three samples had shown a TOSC close to 100% in this concentration before degradation providing the biggest possible scope for an observation of a decrease of antioxidant capacities. In parallel, the anthocyanin concentration of the samples was monitored as a parameter for the proceeding of the degradation. The storage of samples was maintained until all anthocyanins in the samples had vanished. The results of these analyses are shown in table 4.18.

It can be observed that the loss of anthocyanins in the samples progressed the faster the higher the concentration was in the beginning. While the anthocyanin content of açaí grosso I and the pulp of 2002 decreased already during the first week of storage to 20% and even only 6%, resp., of the original concentrations, the much lower concentrated açaí fino II sample kept during the same time 63% of its anthocyanins. After this strong reduction in the beginning, the anthocyanin loss of the two higher concentrated samples slowed down also. After four weeks of storage, no more anthocyanins were detectable in açaí fino II; the complete anthocyanin degradation for açaí grosso I and the pulp of 2002 lasted six weeks.

In contrast, the TOSC of the samples was much less influenced by the storage. The antioxidant capacities of açaí grosso I and the pulp of 2002 were only minimal lower after six weeks of storage with 99% compared to 100% in

Açaí grosso I				
	Concentration [mg/L]			TOSC [%] against peroxyl radicals in dilution 1:50
	Cya.-3-rut.	Cya.-3-glu.	∑ Antho.	
Day 0	456	7	463	100
1 Week	94	1	95	100
2 Weeks	38	1	39	99
3 Weeks	24	0	24	99
4 Weeks	9	0	9	99
5 Weeks	1	0	1	98
6 Weeks	0	0	0	99
Açaí pulp 2002				
	Concentration [mg/L]			TOSC [%] against peroxyl radicals in dilution 1:50
	Cya.-3-rut.	Cya.-3-glu.	∑ Antho.	
Day 0	157	54	211	99
1 Week	12	1	13	99
2 Weeks	5	0	5	98
3 Weeks	3	0	3	98
4 Weeks	1	0	1	98
5 Weeks	1	0	1	96
6 Weeks	0	0	0	96
Açaí fino II				
	Concentration [mg/L]			TOSC [%] against peroxyl radicals in dilution 1:50
	Cya.-3-rut.	Cya.-3-glu.	∑ Antho.	
Day 0	24	6	30	94
1 Week	9	2	11	92
2 Weeks	3	1	4	91
3 Weeks	1	0	1	87
4 Weeks	0	0	0	82
5 Weeks	0	0	0	75
6 Weeks	0	0	0	66

Table 4.18: Anthocyanin content and TOSC of açaí pulps during storage at 37° C

HPLC fraction [min]	TOSC [%] against peroxyl radicals					
	Açaí grosso I		Açaí pulp 2002		Açaí fino II	
	Day 0	6 Weeks	Day 0	6 Weeks	Day 0	6 Weeks
0–5	18	15	6	14	9	19
5–10	14	5	3	12	5	6
10–15	7	1	2	10	3	5
15–20	30	17	18	24	20	6
20–25	76	42	50	23	46	17
25–30	96	76	87	49	81	31
30–35	97	91	92	70	82	39
35–40	79	90	64	64	52	36
40–45	39	62	24	50	23	21
$\sum$ absolute	456	399	346	316	321	180
$\sum$ relative	100%	87%	100%	91%	100%	56%

Table 4.19: TOSC of açaí pulp HPLC fractions before and after storage at 37° C

the beginning and 96% to 99%, respectively. Solely açaí fino II lost about a third of its original antioxidant activity during the storage. This finding furnishes one more clear proof of no direct participation of the anthocyanins to the TOSC of the açaí pulps. The high stability of the antioxidant capacities of anthocyanin containing samples is confirmed by a study of Garcia-Alonso et al. [Gar03] who had monitored the antioxidant activity of an anthocyanin rich dessert made from grape, cherry, raspberry, blackberry and blackcurrant during storage. In their trial, the antioxidant activity of the sample remained more or less constant after a one year storage at 8°, 21° and even 30° C while antioxidant compounds such as anthocyanins and vitamin C decreased.

To identify the compounds with really important antioxidant activities in the açaí pulps, the aged samples were again fractionated and the TOSC of the fractions against peroxyl radicals was analysed as described in 4.4.9. For an easier comparison, the TOSC results are contrasted directly in table 4.19 to those already reported for the fresh samples. In the figures 4.26 and 4.27, the TOSC results of the two extreme samples (açaí grosso I and açaí fino II) are opposed to the absorption of the aged samples at 525 nm.

- Summing up the activities of all fractions, it can be seen that the fractions register a similar low decline in activity for açaí grosso I and the pulp of 2002 and a likely high decrease for açaí fino II as it was reported for the un-fractionated samples (see table 4.18). This indicates, that the decisive antioxidants of the açaí pulps are included in the analysed

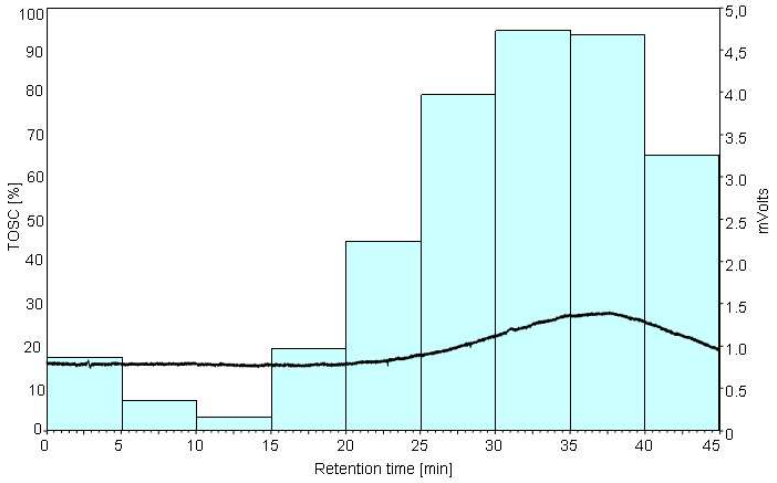

Figure 4.26: TOSC and absorption at 525 nm of degraded açaí grosso I HPLC fractions

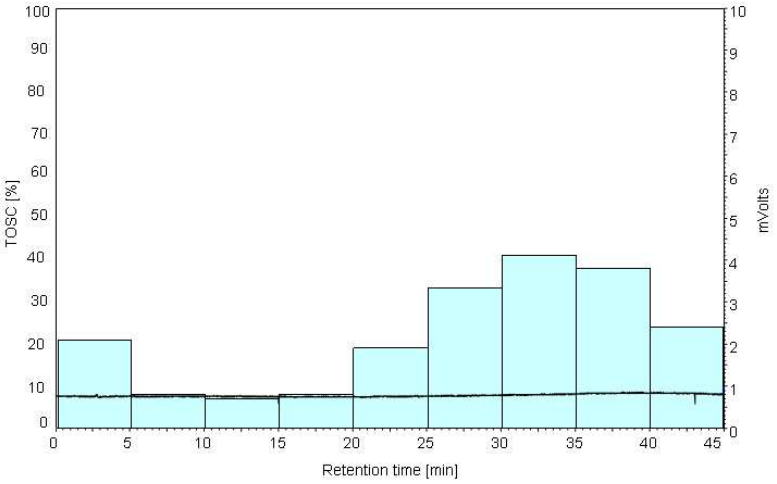

Figure 4.27: TOSC and absorption at 525 nm of degraded açaí fino II HPLC fractions

fractions. Minor differences could be due to no longer possible synergistic effects after the compounds are separated into different fractions.

- The activity of the fraction from 0–5 min increased during the storage for açaí fino II and the pulp of 2002 while it stayed nearly the same for açaí grosso I.

- The activities of the fractions between 5 and 15 min were for açaí grosso I and açaí fino II similar low as in the beginning and declined in the fraction from 15–20 min.

- Only for the açaí pulp of 2002, an increase in the activities of the fractions from 5–20 min could be observed.

- The mountain-like rise and fall of the chromatogram baselines as described before for the fresh pulps was also found for the degraded samples. But in contrast to the fresh samples, the climax of the baseline as well as the TOSC results had shifted 5 min to the fractions from 30–35 min and 35–40 min.

The fractions with interesting findings in the TOSC assay were analysed by HPLC-MS as described in 4.4.9. Again, a large number of different signals was detected in the interesting fractions and their identification was rather complicated due to their low concentration. In addition, most compounds showed no common parent ion or MS-MS fragmentation pattern (see figure 4.28). But, as all pulp samples lost their anthocyanins completely during the storage, it can be suggested that at least some of the signals belong to decomposition products of the original anthocyanin molecules. And, as it was shown in 4.2.7, it is also very likely, that these degradation products possess antioxidant activities.

To corroborate this hypothesis experimentally, a 100 μM solution of cyanidin-3-rutinoside was stored at 37° C in a drying cabinet. After 3 weeks, no more anthocyanin was present while the TOSC against peroxyl radicals went only down from 99% to 95% (see table 4.20). And even the TOSC of a 75 μM solution of cyanidin-3-rutinoside decreased after 11 months storage at 4° C only from 92% to 75%; the anthocyanins in this standard solution had also vanished completely.

With a TOSC close to 100% in the beginning, these solutions were ideally concentrated for a monitoring of the antioxidant activities during storage. But their concentration was too low for an unequivocal identification of the decomposition products. Therefore, standard solutions with a concentration of 1 g/L were prepared additionally from cyanidin-3-rutinoside as well

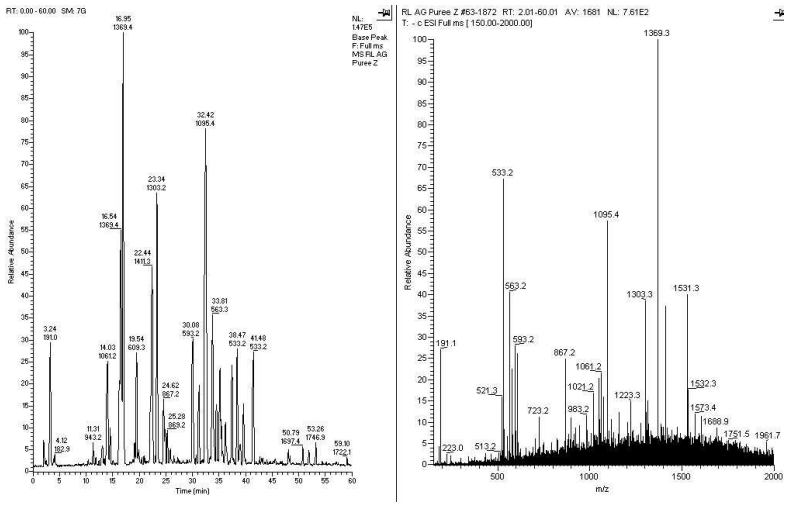

Figure 4.28: Base peak chromatogram and mass spectrum over run time of aged açaí grosso I

Time of storage at 37° C [days]	TOSC against peroxyl radicals [%]	Concentration [μM/L]
0	99	100
21	95	0
Time of storage at 4° C [months]	TOSC against peroxyl radicals [%]	Concentration [μM/L]
0	92	75
11	72	0

Table 4.20: Influence of storage on TOSC of cyanidin-3-rutinoside

as -glucoside and they were also stored at 37° C. In regular intervals, the anthocyanin content of the solutions was quantified as described in table 3.11. The results of these analyses are displayed in table 4.21. The storage of the solutions was continued until less than 0.5% of the original concentrations were left.

The first interesting finding is, that the decrease of the anthocyanin concentration lasted much more longer than expected from the degradation of the açaí pulps and from the low stability that has been reported for anthocyanins in the literature [Cab00]. While the solutions were analysed in the beginning every week, the intervals were extended later to two and in the end to four weeks due to the slow progress of the degradation. Cyanidin-3-rutinoside was even a bit more stable than cyanidin-3-glucoside. This fits to the results of Ichiyanagi et al. [Ich01] who found that the degradation of

Time of storage at 37° C [days]	Concentration [mg/L]	
	Cyanidin-3-glucoside	Cyanidin-3-rutinoside
0	1000	1000
7	705	844
15	565	812
21	361	547
29	270	481
42	146	382
57	80	273
71	49	193
84	32	126
98	18	77
112	17	58
141	7	17

Table 4.21: Degradation of anthocyanin standard solutions during storage at 37° C

anthocyanins is more dependent on the conjugated sugar unit than on the aglycon structure. More than 24 weeks of storage were necessary to eliminate the anthocyanins in both samples nearly completely. The high stability of the two anthocyanins could, on the one hand, be due to the thorough use of UHQ water for the used glassware and the preparation of the solutions. But, on the other hand, is also likely, that the anthocyanins are stabilised as soon as first decomposition products with antioxidant activities are formed. For the açaí samples, on the contrary, the very much lower stability of their anthocyanins compared to the pure standard compounds implies the presence of anthocyanin degrading enzymes and/or prooxidants in the pulps. Prooxidative effects could be caused, e.g., by the trace metals iron and copper that were found in açaí pulps in amounts of up to 26 and 2 mg/100g dry matter, respectively (see table 1.2).

In addition to the anthocyanin quantification, the solutions were also analysed by HPLC-MS in accordance to table 3.10 to monitor the loss and formation of compounds during the decomposition. The MS data of the detected compounds are displayed in the tables E.6 and E.7. In the figures 4.29 and 4.30, the formation of the major degradation products is visualised and they are marked with their retention times. As a quantification of most compounds was not possible due to the lack of pure standard compounds, the results are displayed in peak area units.

Two of the detected degradation products were present in both aged anthocyanin solutions. Their MS data are displayed in table 4.22.

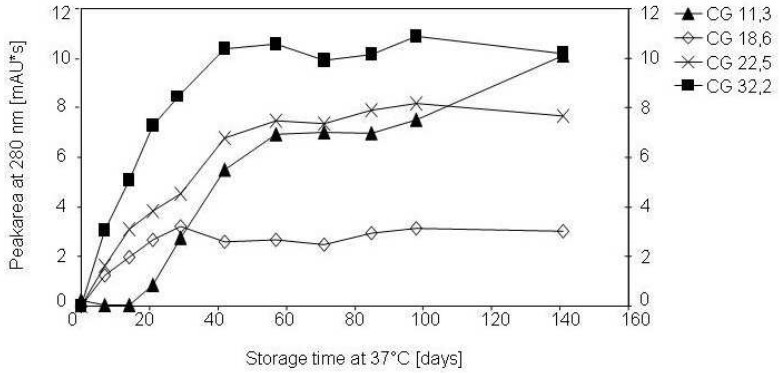

Figure 4.29: Formation of major compounds during the storage of cyanidin-3-glucoside at 37° C

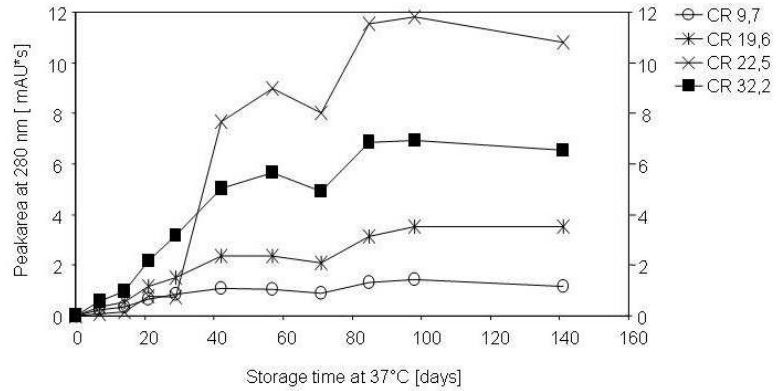

Figure 4.30: Formation of major compounds during the storage of cyanidin-3-rutinoside at 37° C

Retention time [min]	Identity	Parent Ion [m/z (polarity)]	MS/MS Fragments [m/z]
22.5	Cyanidin aglycon with modified structure?	289(-)	no detectable
32.2	?	341(-)	323, 165

Table 4.22: MS data of compounds detected in aged cyanidin-3-glucoside as well as -rutinoside standard solutions

- The first compound might be a modified isomer of the cyanidin aglycon. Cyanidin itself has a parent ion mass of 287 and should elute after the anthocyanins due to its lower polarity. As the parent ion of the detected compound is two mass units higher than the original structure, one of its double bound could be hydrogenated, giving the molecule a higher polarity and causing also the shift in its retention time.
- The identity of the second compound is more difficult to assign. Neither the parent ion is known nor its MS-MS fragments show a common pattern. As the component is present in both aged standard solutions it can only be concluded that it must be a somehow modified cyanidin molecule. From its late retention time it can be presumed that it has a lower polarity than the two anthocyanins.

The other detected compounds were either identified only for the degraded cyanidin-3-glucoside (see table E.6) or for the -rutinoside solution (see table E.7) implying an influence of the conjugated sugar units. Though, there were also some things in common:

- Most of the detected compounds had molecular masses 14 or 16 units higher than the original anthocyanins indicating the presence of an additional keto- or hydroxyl-group.
- As these compounds eluted at different retention times before as well as after the anthocyanins, it can be concluded that they posses isomeric structures with different polarities.

In the degraded cyanidin-3-glucoside solution, one more compounds was detected and could be identified unequivocally as protocatechuic acid. In table 4.26 its formation during the storage is given. Protocatechuic acid was also found as the predominant anthocyanin degradation product from cyanidin glycosides by Seeram et al. [See01]. The other two main degradation compounds from their study, 2,4-dihydroxybenzoic acid and 2,4,6-trihydroxybenzoic acid, could not be detected in this work. But, as Seeram

Time of storage at 37° C [days]	Concentration [mg/L]
0	0.7
7	0.1
15	0.1
21	2.4
29	7.7
42	15.3
57	19.4
71	19.6
84	19.5
98	21.0
141	28.3

Table 4.23: Formation of protocatechuic acid from cyanidin-3-glucoside during storage at 37° C

et al. showed, the kind of molecules that is formed during the degradation of anthocyanins depends largely on the applied storage conditions like temperature and pH. In addition, apart from protocatechuic acid that was also analysed by NMR, the identity of the other compounds was only confirmed in this study by their retention time compared to those of the respective standard compounds.

In studies of Burns et al. [Bur01] and Ichiyanagi et al. [Ich01] it was shown, that anthocyanins can polymerise during storage producing molecules with much higher molecular weights and also antioxidant activities. With the used HPLC-MS system, only molecular masses up to 2,000 Da can be detected. To see if also compounds with higher molecular weights were formed in the degraded anthocyanin standard solutions, the aged cyanidin-3-rutinoside sample was analysed additionally by MALDI-TOF (upper detection limit of 25,000 Da). The applied method parameters are displayed in table 3.9. But, as figure 4.31 shows, no signals with higher molecular masses were detectable. Possibly, the used storage parameters supported more the decomposition of the anthocyanin molecules than their condensation.

The mountain-like rise and fall of the baseline of the açaí pulp HPLC chromatogram (see figures 4.23 and 4.26, e.g.) could not be caused by degrading the anthocyanin standards. Thus, this phenomenon must be due to other compounds or to the combination of the anthocyanins with other compounds that were not present in the pure anthocyanin standard solutions.

To find out which of the anthocyanin decomposition products possess antioxidant activities, the standard solutions were also fractionated by HPLC

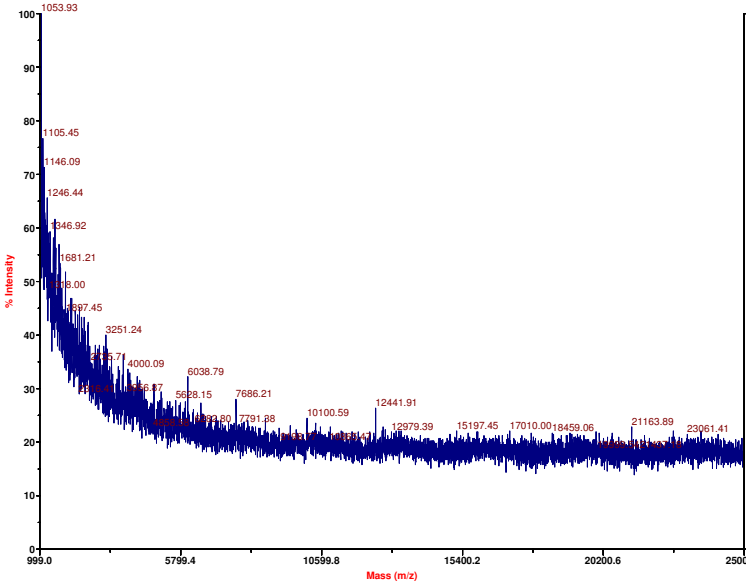

Figure 4.31: MALDI-TOF analysis of a degraded cyanidin-3-rutinoside standard solution

as described in 4.4.9. Fractions were collected at the beginning of the degradation at day 0 and again after 6 and 8 weeks of storage. The results are given in table 4.24.

- In the fresh samples, only the anthocyanin fractions posses antioxidant activities.
- During the storage, the other fractions gain also inhibition capacities.
- In the beginning, this effect is limited to the fractions next to the anthocyanin fraction pointing out to the formation of molecules with similar structures to the original anthocyanins.
- Later, the antioxidant activity spreads also to the rest of the fractions indicating that a large number of compounds with different molecule structures is formed.
- For the glucoside, the activity spread to the non-anthocyanin fractions is slightly higher than for the rutinoside. This fits to the fact that the glucoside concentration declines also faster than that of the rutinoside (see table 4.21).

Combining these findings with the above described detection of compounds in the aged standard solutions, it can be concluded that a large number of

HPLC fraction [min]	TOSC [%] against peroxyl radicals					
	Cyanidin-3-rutinoside			Cyanidin-3-glucoside		
	Day 0	Day 42	Day 57	Day 0	Day 42	Day 57
0–5	0	0	9	0	0	14
5–10	0	0	15	0	0	18
10–15	0	0	10	0	0	12
15–20	0	0	14	1	8	17
20–25	0	0	17	0	5	18
25–30	41	58	43	64	40	44
30–35	0	14	29	0	25	28
35–40	0	0	12	0	0	14
40–45	0	0	3	0	0	13

Table 4.24: TOSC of anthocyanin standard solution HPLC fractions during storage at 37° C

different compounds is formed during the decomposition of anthocyanins and that they have indeed antioxidant capacities.

With this knowledge gained by the degradation of the anthocyanin standard solutions, the HPLC-MS analyses of the fresh and the aged açaí pulps were reviewed again for the un-fractionated samples and also their HPLC fractions. In table 4.25, the compounds are displayed that could be found in the degraded standard solutions as well as in the açaí pulps. The results for the fractions and the un-fractionated pulps were rather alike. Only the identification of compounds in the fractions was more difficult due to much lower concentrations.

Interestingly, the degradation products were more detected in the fresh açaí pulps than in the aged ones. Consequently, the decomposition of the anthocyanins must have started in these samples already before they were subjected to an accelerated aging, maybe during the ripening of the fruits or also the processing of the pulps and including probably also other pathways of anthocyanin degradation that could not be simulated by the pure standard solutions.

Only protocatechuic acid (PCA) was present in both kinds of samples. Interestingly, its content decreased in açaí grosso I and stayed nearly the same in açaí fino II while it doubled in the pulp of 2002 (see table 4.26). But, this pulp was also the only one that contained cyanidin-glucoside in appreciable amounts before the storage (see table 4.18). Therefore, solely herein PCA could be formed in higher concentrations due to the composition of the glucoside. And it could also be the reason why only this pulp gained

Ret. time [*min*]	Identity	Detected in fresh açaí pulps	Detected in aged açaí pulps
11.3	Protocatechuic acid	+	+
16.7	Oxidation product of cyanidin-glucoside	+	–
18.5	Oxidation product of cyanidin-rutinoside	+	–
18.6	Isomeric oxidation products of cyanidin-glucoside	+	–
19.6	Isomeric oxidation products of cyanidin-rutinoside	+	–
23.6	Cyanidin-glucoside	+	–
25.1	Cyanidin-rutinoside	+	–

Table 4.25: Compounds detected in aged anthocyanin standard solution as well as açaí pulps

antioxidant activities in the early HPLC fractions during the storage (see table 4.19) as PCA elutes in the fraction from 15–20 min.

To calculate the absolute protocatechuic acid concentration in this fraction, the following considerations are made: the aged açaí pulp injected to the HPLC system for separation has a PCA concentration of about 19 mg/L. As 20 μL of solution are used, this corresponds to an absolute amount of ca. 0.4 μg. After fractionating and freeze-drying the resulting fraction, the residue is dissolved in 500 μL UHQ water yielding a concentration of 0.8 mg/L. Comparing these value with the TOSC results obtained for a pure standard solution of protocatechuic acid (see table C.1), it can be said that at least a part of the activity gain could be due to the rise in the PCA concentration. As also the inhibition capacities of the fractions near the protocatechuic acid fraction of the pulp of 2002 increased during the aging, it can be at least presumed that there are maybe other decomposition products present with structures similar to the PCA.

	Concentration of PCA [mg/L]	
	Day 0	6 Weeks
Acai grosso I	12.1	5.7
Acai pulp 2002	9.2	18.7
Acai fino II	3.3	4.1

Table 4.26: Concentration of protocatechuic acid in açaí pulps during storage at 37° C

4.5 Açaí seeds

For TOSC analyses, açaí seed samples were chosen that covered

- different harvesting years,
- different harvesting seasons and
- both açaí varieties with purple and white fruits.

4.5.1 Optimisation of extraction procedure

In the survey of Choi et al [Cho98], the highest antioxidant capacities were found for açaí seed extracts prepared with methanol at room temperature; less polar solvents resulted in lower antioxidant activities. These conclusions are in accordance with several other publications for the extraction of antioxidants from plant materials with different solvents [Prz98, Azi99]. Other researcher found similar good results for methanol and ethanol extracts concerning total phenolic content [Nep02] or antioxidant capacities [Sid03]. And the use of a Soxhlet extractor is an also often recommended method to get a high yield of antioxidants [Prz98, Azi99, Bra03].

Taking these considerations into account, different extraction methods (Soxhlet extractor and cold extraction at room temperature) as well as different solvents (methanol and ethanol) were applied in preliminary tests to find the best conditions for the extraction of açaí seeds. A list of the different extracts is given in table 2.1.

To review the influence of the extraction methods on the antioxidant capacities of the seed extracts, all extracts were tested first in a medium concentrated dilution of 1:500 against peroxyl and hydroxyl radicals as well as peroxynitrite. As table F.1 shows, neither the application of a Soxhlet extractor nor the use of ethanol as solvent was followed by significant higher antioxidant capacities. Therefore, the following TOSC research steps were

Extract no.	Calculated dilution factor for TOSC of 20%	50%	80%	r^2
10	9,833	2,729	891	0.9996
6	10,163	2,222	662	1.0000
2	9,524	2,151	589	1.0000
1	2,545	695	267	1.0000

Table 4.27: Calculated dilution factors of açaí seed extracts for TOSC against peroxyl radicals

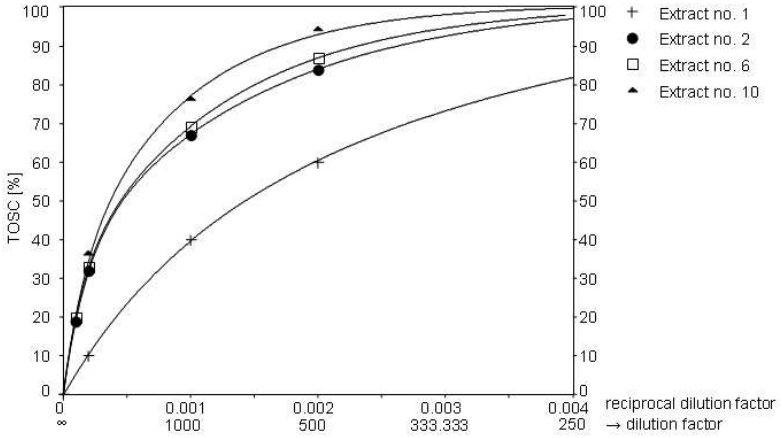

Figure 4.32: TOSC of some açaí seed extracts against peroxyl radicals

focused on the seed extracts obtained by the extraction conditions of Choi et al. [Cho98] (i.e. extraction with methanol at room temperature). The ethanol extract from 2000 obtained with a Soxhlet extractor was also included to the survey because no more seeds were available of this year for preparing other kinds of extracts.

4.5.2 TOSC against peroxyl radicals

The experimental TOSC values of the açaí seed extracts against peroxyl radicals are shown in table F.2. The calculated dilution factors for TOSC values of 20, 50 and 80% are given in table 4.27. The relation between the dilution of the extracts and their antioxidant capacities is visualised additionally in figure 4.32.

For the inhibition of peroxyl radicals, extract dilutions from 1:100 to 1:10,000 were used to cover a TOSC range from a low to a nearly complete

Extract no.	Calculated dilution factor for TOSC of 20%	50%	80%	r^2
10	9,107	1,099	116	1.0000
6	5,473	665	92	1.0000
2	4,525	634	74	1.0000
1	1,808	194	27	1.0000

Table 4.28: Calculated dilution factors of açaí seed extracts for TOSC against peroxynitrite

suppression of the ethylene production. The relation between dilution and TOSC was for all analysed seed extracts clearly non-linear (see figure 4.32). The highest antioxidant capacities against peroxyl radicals were found for the seeds of the white açaí variety (extract no. 10) followed closely by the two seed batches of the purple variety from the high harvesting season of 2002 and the low harvesting season of 2001 (extracts no. 6 and 2) with very similar results. Only the extract from the purple variety of 2000 (extract no. 1), prepared with ethanol in a Soxhlet extractor, showed significant lower antioxidant capacities against peroxyl radicals.

4.5.3 TOSC against peroxynitrite

The experimental TOSC values of the four açaí seed extracts against peroxynitrite are shown in table F.3. In addition, they are displayed in figure 4.33. The calculated dilution factors for TOSC values of 20, 50 and 80% are given in table 4.28.

Though the activity ranking for peroxynitrite was in the same as for peroxyl radicals, the antioxidant capacities of all extracts were lower than for peroxyl radicals. In addition, a broader dilution span from 1:10 to 1:10,000 had to be applied for this ROS to cover a similar inhibition range. In figure 4.33, it can be seen that at higher concentrations a plateau-like flattening of the dose-response curve emerges as it has already been reported for some of the tested standard compounds and juice and vegetable juices (see 4.2.4 and 4.3.6).

4.5.4 TOSC against hydroxyl radicals

The experimental TOSC values of the açaí seed extracts against hydroxyl radicals are shown in table F.4 and are visualised in figure 4.34. The cal-

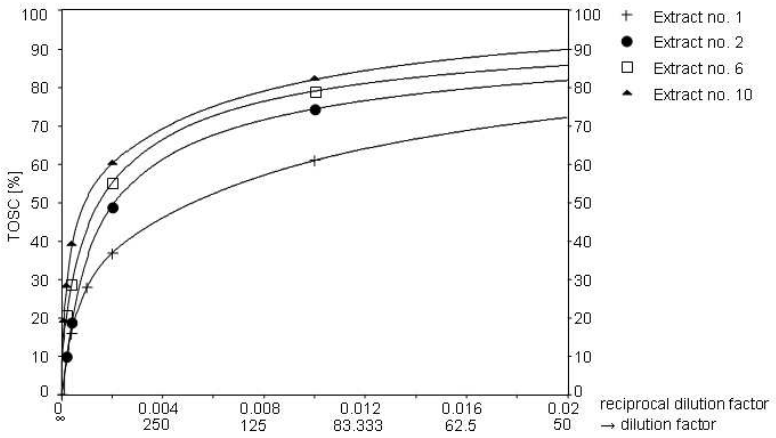

Figure 4.33: TOSC of some açaí seed extracts against peroxynitrite

Extract no.	Calculated dilution factor for TOSC of 20%	50%	80%	r^2
10	1,880	954	176	0.9991
6	1,588	758	203	0.9998
2	1,692	705	36	0.9998
1	366	87	4	0.9999

Table 4.29: Calculated dilution factors of açaí seed extracts for TOSC against hydroxy radicals

culated dilution factors for TOSC values of 20, 50 and 80% are provided in table 4.29.

For hydroxyl radicals, the non-linear relation between extract concentration and antioxidant capacity was even more complex (see fig. 4.34). In the area of the turning points of the dose-response curves, different dilutions of the extracts showed very similar antioxidant activities. Therefore, eight different extract concentrations from undiluted solution up to a dilution of 1:2,500 had to be analysed to describe the curve progression as close as possible. The antioxidant capacities of the extracts no. 10, 6 and 2 are for most part of the dilution range comparable to those for peroxynitrite; only the higher dilutions show a deviant behaviour. On contrast, extract no. 1 has significant lower antioxidant capacities against hydroxyl radicals than against peroxynitrite.

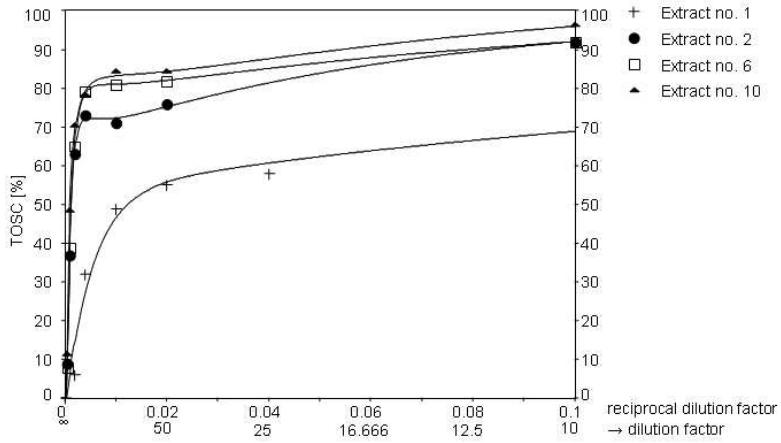

Figure 4.34: TOSC of some açaí seed extracts against hydroxyl radicals

4.5.5 Comparison of the three ROS

Again, the different behaviour of the extracts towards the three ROS can be explained by their highly different reactivities and half lives [Reg99, Hall95b]. Peroxyl radicals can be scavenged rather easy with lower amounts of antioxidants. For the more reactive peroxynitrite and hydroxyl molecules, higher amounts or more effective antioxidants are necessary for a comparable inhibition rate. But from a certain range of the dose-response curves on, even adding much higher amounts of a compound does not offer much higher protection from the ROS, thereby causing a plateau-like flattening of the chart.

The very similar results of the extracts from the purple açaí variety with no. 6 (high harvesting season of 2002) and no. 2 (low harvesting season of 2001) are especially remarkable. It suggests that the antioxidant capacity of açaí seeds is less influenced by the harvesting season than that of the açaí pulps (cf. 4.4). If this could be proven by more data, it would represent a very interesting economic aspect for the utilisation of the açaí palm: while the pulps are of a high organoleptic quality only during some months of the year (see 1.2.2), it could be possible to process the seeds throughout the year in a constant quality. And also the even more promising results for the seeds of the white açaí variety are of financial interest as these fruits are up to now more or less neglected. Of course, this finding should also be confirmed by a larger number of data.

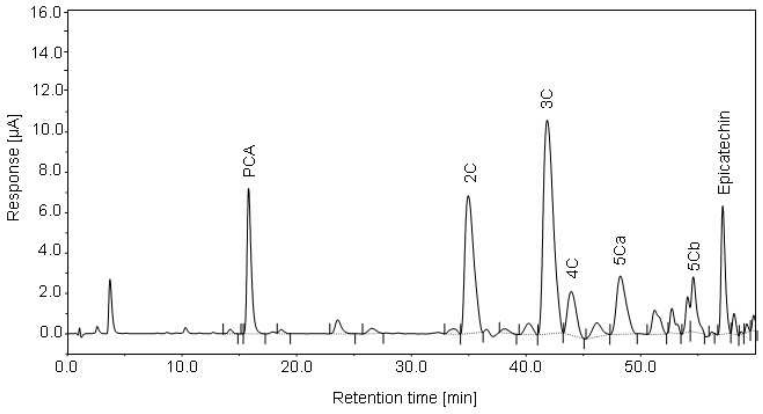

Figure 4.35: HPLC-chromatogram of açaí seed extract no. 10 (coulometric electrochemical detector, 220 mV channel

4.5.6 Identification of phenolic compounds

Though there has already been evidence that açaí seeds have high antioxidant capacities [Cho98], the compounds responsible for these properties have not yet been identified.

The combination of multi step-mass spectrometric fragmentation after HPLC separation, UV-Vis diode array detection and electrodynamic voltammograms allowed the identification of two monomeric [epicatechin and protocatechuic acid (PCA)] and five oligomeric polyphenols [one dimer (2C), one trimer (3C), one tetramer (4C) and two different pentamers (5Ca and 5Cb) of catechin and/or epicatechin] in açaí seeds in appreciable amounts. The method parameters are displayed in the tables 3.10 and 3.12. In table F.5, the MS data for the identified polyphenols are shown. For procyanidins, beside the structural informative fragment ions, the masses of the three most abundant fragment ions are given. A characteristic HPLC-chromatogram of the identified compounds is displayed in figure 4.35.

As some of the identified proanthocyanidins are also present in the açaí pulps in small amounts (see table E.5), it can at least be suggested that these compounds are carried over from the seeds to the pulps during the separating process of the fruits (see 1.2.2).

4.5.7 Polyphenol content and comparison with TOSC

The identified polyphenols in the açaí seed extracts were quantified by UV signal. For the quantification of the proanthocyanidins, catechin was used as external standard [Pap02, Zim01]. A quantification of epicatechin was

not possible because it co-eluted together with too many other compounds to allow a proper peak integration. The identity of the compounds was confirmed by UV spectra and electrodynamic voltammograms. The method parameters are displayed in table 3.12. The concentrations of the identified polyphenols in the seed extracts are presented in table F.7.

In all seed extracts, large amounts of the identified polyphenols were detected. The highest contents of nearly all mono- and oligomeric polyphenols were found in the açaí branco seed extracts with a total of about 3,800 mg/L followed by the extracts from the high harvesting season of 2002 with circa 2,400 mg/L and the extracts of the low harvesting season of 2001 with about 1,600 mg/L. The lowest amounts with circa 600 mg/L appeared in the ethanolic Soxhlet extract of the year 2000. These findings fit together with the ranking of antioxidant activities of the extracts for all three analysed ROS (see subsections 4.5.2 to 4.5.4). It indicates that the identified polyphenols could be the decisive antioxidants of açaí seeds. The fact that the TOSC results for the years 2001 and 2002 were a bit closer together than their polyphenol contents could be due to the non-linear relation between concentration and activity (cf. 4.5.2 to 4.5.4).

The results for the different extraction methods were rather alike. Only the combination of ethanol with a cold extraction at room temperature resulted in slightly lower amounts of all detected compounds. As also the TOSC results were rather close together for the different extraction methods (see 4.5.1), this outcome indicates that the identified polyphenols could be the decisive antioxidants in açaí seeds.

From the high amounts of mono- and oligomeric polyphenols in açaí seeds together with their very promising antioxidant capacities it is likely that açaí seeds could possess similar benefits as, e.g. grape seeds. These by-products of the wine making process are rich in polyphenolic compounds like monomeric flavanols and oligomeric procyanidins and have demonstrated not only in vitro radical scavenging capacities [Ahn02, Lau03] but also, e.g., cataract preventing [Yam02] and antibacterial properties [Jay03].

In table 4.30, the pattern of the single polyphenols in the different extracts is demonstrated. With the exception of extract no. 1, the distribution of the different compounds is very similar for all extracts and, like the total amount of polyphenols, it is not influenced by the extraction parameters:

- The tetrameric proanthocyanidin makes up about one fourth of the total amount of polyphenols.
- The dimer, the trimer and one of the pentamers (5Ca) are each contained in circa one fifth of the polyphenol sum.

Extract no.	Relative concentration [%]					
	PCA	2C	3C	4C	5Ca	5Cb
1	12	36	18	21	9	3
2	2	22	14	25	19	18
3	2	24	16	24	18	15
4	2	26	13	25	21	12
5	2	23	19	24	17	15
6	1	19	19	25	19	18
7	1	19	22	26	18	14
8	1	19	20	27	21	12
9	1	20	23	24	16	16
10	0.3	19	19	25	19	17
11	0.3	19	23	24	18	16
12	0.3	21	20	27	20	12
13	0.4	21	24	26	17	11

Table 4.30: Polyphenol pattern in the different açaí seed extracts

- The second pentamer (5Cb) amounts to about one sixth of the total polyphenols.
- Protocatechuic acid (PCA) is only contained in very small concentrations.

For extract no. 1, the pattern is completely different:

- More than one third of the total polyphenol amount is due to the dimeric proanthocyanidin.
- The trimer and the tetramer come each to about one fifth of the polyphenol sum.
- The pentamer 5Ca and protocatechuic acid are each contained in circa one tenth of the total amount.
- The amount of the second pentamer (5Cb) is negligibly low.

As no seeds but only the extract prepared in Brazil were at disposal of the year 2000 (extract no. 1), it can only be suggested were this different pattern was caused by. As the seeds for this extract as well as those of the year 2001 and 2002 were harvested from the same trees, it is not very likely that the differences come from a natural spread. Bigger variations would have been much more probable to appear between the purple and the white

açaí variety or also between the high and the low harvesting season. But for these samples, the results showed a very similar distribution of compounds. Therefore, it is more likely that the original pattern of extract no. 1 was altered during the extraction procedure, e.g. because of too high temperatures during the concentration process. This assumption is supported by the fact that compounds with a lower polymerisation degree gained ground in this extract compared to those with higher molecular weight. Probably some of the molecule junctions of the higher condensed compounds were broken yielding the smaller molecules. For the high amount of protocatechuic acid it can be suggested that most of it comes from an even further degradation of the proanthocyanidins. In a survey of Arunachalam et al. [Arun03] it was shown that catechin can be degraded to protocatechuic acid by the enzymes of bacteria. Therefore, it is at least imaginable that also other ways of catechin degradation exist that yield protocatechuic acid.

Apart from its much lower total polyphenol amount, the different compound pattern of extract no. 1 could be one additional explanation for its lower antioxidant capacities. In different surveys it was shown that the antioxidant capacity of proanthocyanidins depends largely on their degree of polymerisation. Additional influences come from the kind of molecules that are polymerised (catechin and/or epicatechin), their interflavan linkage type, the analysed concentrations and the medium in which the survey is carried out.

Zhao et al. [Zha99] came to the conclusion that the higher the degree of polymerisation the higher is also the antioxidant capacity of the proanthocyanidins. And in their survey, interflavan 4-6 linkage isomers showed stronger inhibition capacities than the 4-8 types. Another group of researchers [Lei03] came to the same results concerning the influence of the polymerisation degree. But they found out, that the interflavan 4-6 linkage type possesses the higher antioxidant capacities only in aqueous systems; in the lipid phase, the 4-8 isomers were more effective. Stevens et al. [Stev02], in contrast, found out that at a concentration of 1 μg/mL the antioxidant capacities of mono- through trimers were the same; at 0.1 μg/mL, the highest inhibition was detected for one of the analysed dimers.

While the first three authors dealed only with polymerisation degrees up to trimers, Plumb et al.[Plu98] analysed molecules up to tetramers. They discovered that the antioxidant activity in the lipid phase decreased with the polymerisation while it increased in the aqueous phase up to trimers and declined again slightly for tetramers. Counet and Collin [Cou03] found out, that the antioxidant activity of proanthocyanidins increased significantly and non-linear with the degree of polymerisation from the analysed monomers up to the decamer. In another survey [Sai99], the antioxidant activity went up

from the monomers to the trimers and was rather the same for the tetramers. From the pentamers up to the undecamer, it decreased again so that the pentamers were already less effective than the monomers. Epicatechin units were found in this research to be more efficient than catechin units and the 4-6 isomers were more antioxidative than the 4-8 type that got in some concentrations even prooxidative.

The açaí seed proanthocyanidins were not at disposal as pure standard compounds, so it was not possible to determine their antioxidant capacities by experiment. And as the results in literature were too contradictory, the antioxidant activities of the identified proanthocyanidins could also not be calculated on the basis of the results for the monomers catechin and epicatechin. Only for protocatechuic acid an estimation could be done, but in most extracts this compound was present in less than 2% of the total polyphenol amount. Therefore, the previous data are not sufficient to decide whether the identified proanthocyanidins are the decisive antioxidants in açaí seed extracts or not.

4.5.8 Fractionating of samples by HPLC and TOSC

To find out if the antioxidant activities of açaí seeds are due to the identified proanthocyanidins, two seed extracts were fractionated by HPLC as described in 3.5. The samples included one extract with very high antioxidant activities (extract no. 10) and one extract with very low capacities (extract no. 4). Extract no. 1 with the lowest analysed activity was not chosen because its polyphenol pattern differed too much from the other samples (see table 4.30). The antioxidant capacities of the HPLC fractions were analysed against peroxyl radicals. This ROS was chosen for the same reasons as discussed for the açaí pulp fractions (cf. 4.4.9)

The TOSC values of the HPLC fractions are shown in table 4.31. For the fractions of the extract no. 10, the activities were partly so high that the fraction had to be diluted 1:10 before TOSC analyses. As the relation between concentration and antioxidant activity of the seed extracts was proven to be non-linear (see subsections 4.5.2 to 4.5.4), the dilution caused a distortion of the real values. Therefore, the results for the concerning fractions are stated as "ca." numbers. The results of the fractions are additionally opposed to the simultaneously recorded HPLC chromatograms at 210 nm in the figures 4.36 and 4.37.

Though the absolute TOSC values were very different for the two fractionated samples, their activity pattern was the same:

- In the first three fractions up to 15 min, no appreciable activities are

HPLC fraction	TOSC [%] against peroxyl radicals	
	Extract no. 4	Extract no. 10
0-5	5	8
5-10	8	2
10-15	6	5
15-20	21	30
20-25	63	100
25-30	99	ca. 970
30-35	99	ca. 860
35-40	90	ca. 570
40-45	58	87
45-50	24	39
50-55	17	22
55-60	5	3

Table 4.31: TOSC of açaí seed extract HPLC fractions

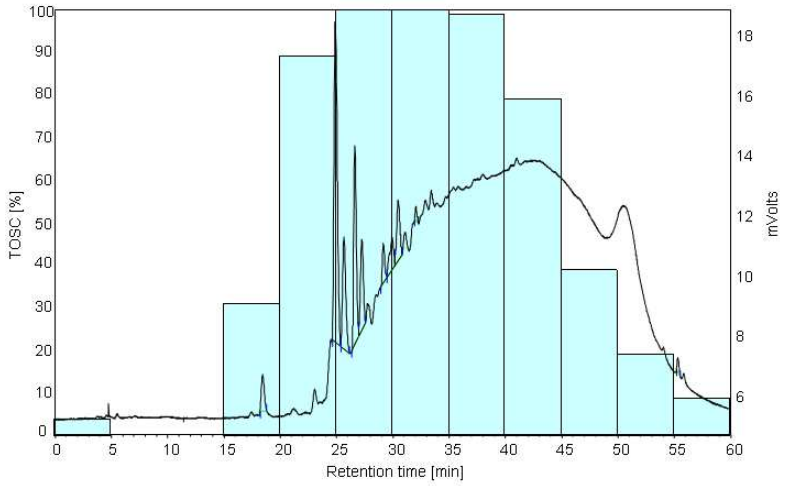

Figure 4.36: TOSC and absorption at 210 nm of HPLC fraction of açaí seed extract no. 4

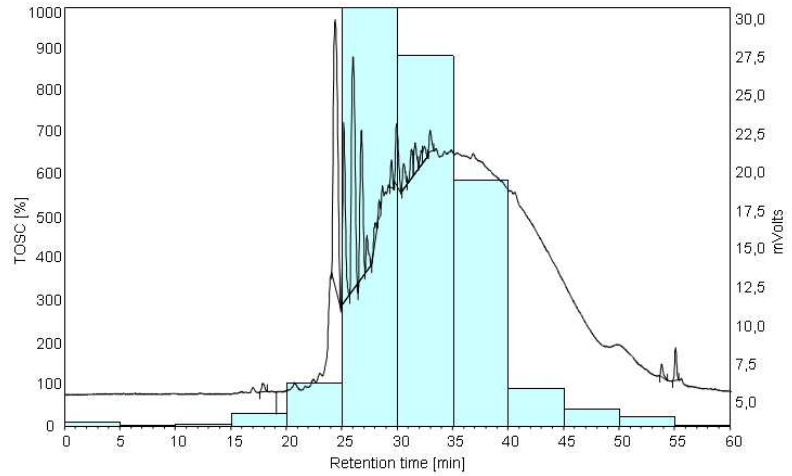

Figure 4.37: TOSC and absorption at 210 nm of HPLC fraction of açaí seed extract no. 10

found.

- Starting with the fraction from 15–20 min, the inhibition capacity of both extracts increases.
- The climax of activities is reached with similar high values for the fractions from 25–30 min and 30–35 min.
- With the fraction from 35–40 min, the antioxidant capacities declines again.
- In the fraction from 55–60 min, the activity is back to negligible low values.

When the results are compared to the recorded HPLC chromatograms the following conclusion can be drawn:

- The climax of activities corresponds to the eluting of the identified proanthocyanidins indicating an influence of these compounds.
- But, the rise and fall of the activities is also mirrored by a simultaneous move of the baseline of the chromatogram. And, not only the total amount of proanthocyanidins but also the height of this mountain-like move is bigger for extract no. 10 than for no. 4. Therefore, it stands at least to reason that there are also other compounds than the proanthocyanidins involved in the inhibition activity of the extracts.

The described course of the baseline can not only be observed at the displayed 210 nm but also at higher wavelengths like 525 nm. All açaí seed extracts had a very strong red colour that resembled grape juice when solved in water. In contrast to anthocyanins [Cab00], this colour could not be changed by altering the pH. Consequently, another group of compounds must be responsible for this colour and maybe also for the mentioned rise and fall of the chromatogram baseline. From the late retention window it can be suggested that rather big and/or un-polar compounds are concerned, similar as it was discussed for the mountain-like shape of the açaí pulp baseline (cf. 4.4.9).

To find out which compounds are present in the fractions, they were analysed by HPLC-MS in accordance to table 3.10. The MS data are given in table F.6.

- In the first three fractions, no bigger signals were detected.
- The fraction from 15–20 min contained the protocatechuic acid.
- Between 20 and 25 min, the first of the identified proanthocyanidins eluted including di- to tetramers with a trimer being the most abundant compound.
- In the fraction from 25–30 min, the highest amount of the procyanidins was found with signals for di- through pentamers and a tetramer being the most important signal.
- Between 30 and 35 min, more procyanidin signals are detected including again di- to pentamers and a dimeric procyanidin having the highest MS signal.
- As all proanthocyanidins were found in more than one fraction it can be concluded that there are different isomers of each compound present in the açaí seeds with maybe a different interflavan linkage type or a varying content of catechin and epicatechin.
- For the fraction from 35–40 min with still very high antioxidant activities, no bigger single signals but a large number of different small signals is detected (see figure 4.38).
- The same could be seen for the later fractions up to 60 min.
- After being re-injected to the HPLC system, the fractions eluted still at the original retention times. Therefore, more than one compound must be responsible for the mountain-like baseline course.

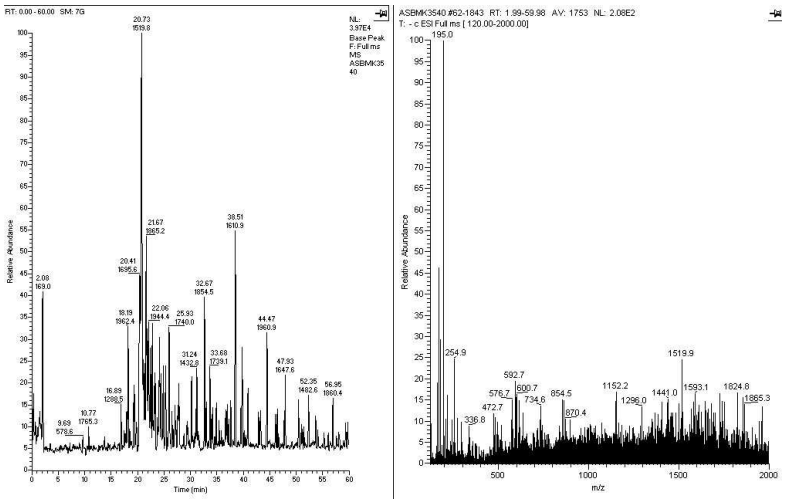

Figure 4.38: Base peak chromatogram and mass spectrum over run time of HPLC fraction 35–40 min of açaí seed extract no. 10

Consequently, the identified proanthocyanidins make up at least one part of the antioxidant activities. But, there seem to be also other yet not identified compounds involved. Counet and Collin [Cou03] came to a similar conclusion for the procyanidin fractions isolated from chocolate. Though they were able to identify several proanthocyanidins up to decamers in their samples, most part of the extract compounds remained unidentified but contributed greatly to the antioxidant activity.

Chapter 5

Summary and outlook

The time- and labour-consumption of the TOSC assay was minimised and the throughput of samples was increased by simplifying the preparation and handling of the assay solutions, optimising the GC separation of ethylene, automating the assay procedure as far as possible and enhancing the data evaluation. Further analytical improvements could be possible by using the next generation of autosamplers, e.g., the so-called "TwinPAL" from Axel Semrau, Sprockhövel, Germany. This instrument would be able to take over also the last, up to now, manual steps of the assay procedure and to double simultaneously the possible number of parallel analyses.

Taking nine food relevant substances from different compound classes as examples, the varying activities of antioxidants towards the three analysed ROS peroxyl radicals, peroxynitrite and hydroxyl radicals could be demonstrated. The results indicate clearly, that antioxidant capacities are most often non-linear related to the respective concentrations. This finding underlines the importance of analysing different concentration levels of a compound to get a well-founded overview of its inhibition capacities. In addition, the analysed compounds could be classified in different antioxidant reaction types. For the anthocyanins, it could be reported for the first time that they possess not only fast-acting but also retarding antioxidant activities against peroxyl radicals. A further useful extension of the assay applications could be, e.g., the simultaneous detection of lipid-soluble antioxidative compounds, for example by employing emulsifiers to the assay solutions. Rudimentary, this problem has been approached in literature, but either the assay results were influenced by the used emulsifier or the handling of the compounds was rather complicated.

The data for fourteen different samples presented herein show that there is a large spectrum of antioxidant properties within the common European fruit and vegetable juices. The results varied not only greatly among the analysed

juices but also between the different ROS that were used. It was shown that a lactic acid fermentation of samples might have a positive influence on their antioxidant capacities. Because the assayed ROS are relevant for natural occurring oxidative processes and cover a large scale of different reactiveness and half lives, it can be confirmed that the TOSC assay is a well-founded method to survey the antioxidant capacities of foods. As this work is the first application of the TOSC assay that uses all three ROS, it creates a broad basis for the classification of up to now unknown food samples.

The ten analysed purple açaí pulp samples from different harvesting years and seasons as well as varying commercial trading qualities showed very good antioxidant properties against peroxyl radicals, good scavenging capacities for peroxynitrite and low inhibition capacities against hydroxyl radicals. Differences in the antioxidant activities of especially the commercial samples could not be explained alone by different dilutions of the base material but must be due to differences in the respective base materials. The pulp from the white açaí variety had much lower antioxidant activities against all three assayed ROS than the purple pulps. Two major anthocyanins (cyanidin-3-glucoside and cyanidin-3-rutinoside) and some minor anthocyanins as well as other minor polyphenols could be identified in açaí pulps. No correlation was detected between the anthocyanin content and the antioxidant activities of the açaí pulp samples. In addition, no correlation could be found between the total phenolic content and anthocyanin content of samples. The total phenolic content provided at least a hint for the scavenging capacities against peroxyl radicals and peroxynitrite, but was not correlated to the inhibition of hydroxyl radicals. By an accelerated aging of açaí pulps and anthocyanin standard compounds it could be shown that the anthocyanin degradation products might be the key to the antioxidant capacities of açaí pulps and maybe also other anthocyanin containing foods. For a further identification of these compounds, a preparative isolation of the interesting compounds, e.g., with the help of new enrichment technologies like membrane filtration with ultra- and nano-filtration materials, and NMR analyses of the isolated pure substances could be of help.

Açaí seed extracts from different harvesting years and seasons as well as the purple and the white variety showed excellent antioxidant capacities against all three analysed ROS. The best results were found for the seeds of the white fruits while only mean differences were detected between the results for the high and the low harvesting season of the purple fruits. Therefore, açaí seeds could be an interesting new product for the worldwide growing demand for antioxidants from natural sources. As most of the seeds are up to now thrown away as organic waist, this could provide a new kind of income to the local inhabitants of the Amazon basin. Of especial economic interest are the

good results for the white açaí fruits and the low harvesting season of the purple fruits as these goods are up to now more or less neglected by the market. Two monomeric (protocatechuic acid and epicatechin) and five oligomeric polyphenols (one di-, tri- and tetramer and two different pentamers of catechin and/or epicatechin) were detected in açaí seeds in appreciable amounts. It could be shown that these compounds make up at least one part of the antioxidant capacities of the açaí seeds. For a more detailed classification of their antioxidant activities and also further information about their structure like the linkage type or the presence of catechin and/or epicatechin in the polymers, preparative isolations and NMR analyses could be similar useful as discussed for the interesting compounds in açaí pulps.

Chapter 6

Acknowledgements

With special thanks to:

- Dr. Friedhelm Marx for the excellent supervision and the great working atmosphere during my doctorate
- Prof. Dr. Rudolf Galensa for the support of this work and his helpful suggestions
- Prof. Dr. Gabriele König for her interest in this work and the acceptance of the co-lecture
- Prof. Dr. José G.S. Maia and his co-workers Ossalin Almeida, Chemistry Department of Federal University of Pará, Belém, Brazil, and Ferdinando C. do Nascimento, Museu Paraense Emilio Goeldi, Belém, Brazil, for the first-class supply with açaí pulps and seeds and the helpful suggestions for this work
- Oliver M. Kind for writing the brilliant ROOT macro,
- Heinz Fabricius for the help with the HPLC and GC-MS analyses, extract preparations and the analyses of total phenolic content,
- Dr. Roberta Belandrino Rodrigues, Marco Haupt, Joachim Schulz and Sonja Brachmann for their assistance with the TOSC analyses,
- Christoph Henke and especially Menelaos Papagiannopoulos for the numerous HPLC-MS analyses
- Benno Zimmermann for the quantification of proanthocyanidins

- Dr. Letzel, Universität Bielefeld, Fakultät für Chemie, Abteilung Massenspektrometrie (OC-I), Bielefeld, Germany, for the MALDI-TOF analyses of the anthocyanin degradation products,
- the Klaus Böcker GmbH, Buxtehude, Germany, for the friendly supply of commercial açaí juices and
- the Unilever Bestfoods Deutschland GmbH, Hamburg, Germany, for the financial support of this work.

Bibliography

[Abu99] Abuja PM (1999): Ascorbate prevents prooxidant effects of urate in oxidation of human low density lipoprotein. *FEBS Lett.* 446 (2-3): 305-308

[Ame93] Ames BN, Shigenaga MK, Hagen TM (1993): Oxidants, antioxidants, and the degenerative diseases of aging. *Proc. Natl. Acad. Sci. USA* 90 (17): 7915-7922

[Ahn02] Ahn J, Grun IU, Fernando LN (2002): Antioxidant properties of natural plant extracts containing polyphenolic compounds in cooked ground beef. *J. Food Sci.* 67 (4): 1364-1369

[Aruo97] Aruoma OI, Spencer JPE, Warren D, Jenner P, Butler J, Halliwell B (1997): Characterization of food antioxidants, illustrated using commercial garlic and ginger preparations. *Food Chem.* 60 (2): 149-156

[Arun03] Arunachalam M, Mohan N, Sugadev R, Chellappan P, Mahadevan A (2003): Degradation of (+)-catechin by Acinetobacter calcoaceticus MTC 127. *Biochimica et Biophysica acta - general subjects* 1621 (3): 261-265

[Aruo02] Aruoma OI, Deiana M, Rosa A, Casu V, Piga R, Peccagnini S, Dessi MA, Ke B, Liang YF, Higa T (2002): Assessment of the ability of the antioxidant cocktail-derived from fermentation of plants with effective microorganisms (EM-X) to modulate oxidative damage in the kidney and liver of rats in vivo; studies upon the profile of poly- and monounsaturated fatty acids. *Toxikol. Lett.* 135 (3): 209-217

[Azi99] Azizah AH, Nik Ruslawati NM, Swee Tee T (1999): Extraction and characterization of antioxidants from cocoa by-products. *Food Chem.* 64: 199-202

[Bau00] Bauer K (2000): Tropical Fruit Flavors: A flavorist's perspective. *Cer. Food World* 45 (5): 204-207

[Bec90] Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA (1990): Apparent hydroxyl radical production by peroxynitrite: implication for endothelial injury from nitric oxide and superoxide. *Proc. Natl. Acad. Sci.* 87: 1620-1624

[Blo92] Block G, Patterson B, Subar A (1992): Fruit, vegetables and cancer prevention: a review of the epidemiological evidence. *Nutr. Cancer* 18: 1-29

[Bob00] Bobbio FO, Druzian JI, Abrao PA, Bobbio PA, Fadelli S (2000): Identification and quantification of the anthocyanins from the fruit of açaí (*Euterpe oleracea*) Mart. *Cienc. Tecnol. Aliment.* 20 (3): 388-390

[Bob02] Bobbio FO, Bobbio PA, Oliveira PA, Fadelli S (2002): Stability and stabilization of the anthocyanins from *Euterpe oleracea* Mart. *Acta Aliment. Hung.* 31 (4): 371-377

[Bra03] Braga MEM, Leal PF, Carvalho JE, Meireles MAA (2003): Comparison of yield, composition, and antioxidant activity of tumeric (*Curcuma longa* L.) extracts using various techniques. *J. Agric. Food Chem.* 51 (22): 6604-6611

[Bur01] Burns J, Gardner PT, Matthews D, Duthie GG, Lean ME, Crozier A (2001): Extraction of phenolics and changes in antioxidant activities of red wines during vinification. *J. Agric. Food Chem.* 49 (12): 5798-5808

[Cab00] Cabrita L, Fossen T, Andersen OM (2000): Colour and stability of the six common anthocyanidin 3-glucosides in aequeous solutions. *Food Chem.* 68: 101-107

[Cav88] Cavalcante PB (1988): Frutas comestíveis da amazônia, 4$^{\text{th}}$ edn. Coleção Adolpho Drucke, Belém, 279 pp.

[Cho98] Choi WS, Lee SE, Lee HS, Lee YH, Park BS (1998): Antioxidative activities of methanol extracts of tropical and oriental medicinal plants *Agric. Chem. Biotech.* 41(7): 556-559

[Con02] Connor AM, Luby JJ, Tong CBS (2002): Variability in antioxidant activity in blueberry and correlations among different antioxidant activity assays. *J. Am. Soc. Hortic. Sci.* 127 (2): 238-244

[Cor01] Corsolini S, Nigro M, Olmastroni S, Focardi S, Regoli F (2001): Susceptibility to oxidative stress in Adélie and emperor penguin. *Polar Biol.* 24: 365-368

[Cou03] Counet C, Collin S (2003): Effect of number of flavanol units on the antioxidant activity of procyanidin fractiosn isolated from chocolate. *J. Agric. Food Chem.* 51 (23): 6816-6822

[Deu98] Deutsch JC (1998): Ascorbic acid possesses labile oxygen atoms in aqueous solution. *J. Chromatogr. A* 802 (2): 385-390

[Dip98] Diplock AT, Charleux JL, Crozier-Willi G, Kok FJ, Rice-Evans C, Roberfroid M, Stahl W, Vina-Ribes J (1998): Functional food science and defence against reactive oxidative species. *Brit. J. Nutr.* 80 (Suppl. 1): S77-S112

[Dug00] Dugas AJ, Castañeda-Acosta J, Bonin GC, Price KL, Fischer NH, Winston GW (2000): Evaluation of the total peroxyl radical-scavenging capacity of flavanoids: structure-activity relationships. *J. Nat. Prod.* 63: 327-331

[Ebe00] Eberhardt MV, Lee CY, Liu RH (2000): Nutrition - antioxidant activity of fresh apples. *Nature* 405: 903-904

[Eis95] Eisenbrand G, Schreier P (1995): *Römpp Lexikon Lebensmittelchemie* Georg Thieme Verlag: Stuttgart (Germany), 993 pp.

[Esc98] Escribano J, Pedreno MA, Garcia-Carmona F, Munoz R (1998): Characterization of the antiradical activity of betalains from *Beta vulgaris* L. roots. *Phytochem. Analysis* 9 (3): 124-127

[Fra97] Franke F (1997): *Nutzpflanzenkunde* 6^{th} edn. Georg Thieme Verlag: Stuttgart (Germany), 509 pp.

[Gar03] Garcia-Alonso FJ, Periago MJ, Vidal-Guevara ML, Cantos E, Ros G, Ferreres F, Abellan P (2003): Assessment of the antioxidant properties during storage of a dessert made from grape, cherry, and berries. *J. Food Sci.* 68 (4): 1525-1530

[Gaz98a] Gazzani G, Papetti A, Massolini G, Daglia M (1998): Anti- and prooxidant activity of water soluble components of some common diet vegetables and the effect of thermal treatment. *J. Agric. Food Chem.* 46: 4118-4122

[Gaz98b] Gazzani G, Papetti A, Daglia M, Berte F, Gregotti C (1998): Protective activity of water soluble components of some common diet vegetables on rat liver microsome and the effect of thermal treatment. *J. Agric. Food Chem.* 46: 4123-4127

[Gut90] Gutteridge JMC, Maidt L, Poyer L (1990): Superoxide dimutase and Fenton chemistry. *Biochem. J.* 269: 169-174

[Hall84] Halliwell B, Gutteridge JMC (1984): Oxygen toxicity, oxygen radicals, transition metals and disease. *Biochem. J.* 219: 1-14

[Hall89] Halliwell B, Gutteridge JMC (1989): Free radicals in biology and medicine, 2nd edn. Oxford: Clarendon Press (England?), 936 pp.

[Hall95a] Halliwell B (1995): Antioxidant characterization - methodology and mechanism. *Biochem. Pharmacol* 49 (10): 1341-1348

[Hall95b] Halliwell B, Aeschbach J, Löliger J, Aruoma OI (1995): The characterization of antioxidants. *Fd. Chem. Toxic.* 33 (7): 601-617

[Halv02] Halvorsen BL, Holte K, Myhrstad MCW, Barikmo I, Hvattum E, Remberg SF, Wold AB, Haffner K, Baugerod H, Andersen LF, Moskaug JO, Jacobs DR, Blomhoff RA (2002): Systematic screening of total antioxidants in dietary plants. *J. Nutr.* 132 (3): 461-471

[Harm01] Harman D. (2001): Free radicals, aging, and disease. *Antioxidants and life style - On line Journal* http://www.antioxidants.com.ar/12/Art147.htm

[Harp69] Harper KA, Morton AD, Rolfe EJ (1969): The phenolic compounds of blackcurrant juice and their protective effect on ascorbic acid, III, The mechanism of ascorbic acid oxidation and its inhibition by flavonoids. *J. Food Technol.* 4: 255-267

[Hei98] Heinonen IM, Lehtonen PJ, Hopia AI (1998): Antioxidant activity of berry and fruit wines and liquors. *J. Agric. Food Chem.* 46: 25-31

[Hen98] Henn T, Stehle P (1998): Gesamtphenolgehalt und antioxidative Kapazität handelsüblicher Getränke. *Ernahrungs-Umschau* 45 (9): 308-313

[Iad92] Iaderoza M, Baldini VLS, dos Santos Draetta I, Bovi MLA (1992): Anthocyanins from fruits of açaí (*Euterpe oleracea* Mart.) and juçara (*Euterpe edulis* Mart.) *Trop. Sci.* 32: 41-46

[Ich01] Ichiyanagi T, Oikawa K, Tateyama C, Konishi T (2001): Acid mediated hydrolysis of blueberry anthocyanins. *Chem. Pharm. Bull.* 49 (1): 114-117

[Jay03] Jayaprakasha GK, Selvi T, Sakariah KK (2003): Antibacterial and antioxidant activities of grape (*Vitis vinifera*) seed extracts. *Food Res. Int.* 36 (2): 117-122

[Jul85] Julkunen-Tiitto R. (1985): Phenolic constituents in the leaves of northern willows: Methods for the analysis of certain phenolics.*J. Agric. Food Chem.* 33: 213-217

[Käh01] Kähkönen MP, Hopia AI, Heinonen M (2001): Berry phenolics and their antioxidant activity. *J. Agric. Food Chem.* 49: 4076-4082

[Kah93] Kahl R, Kappus H (1993): Toxicology of the synthetic antioxidants BHA and BHT in comparison with the natural antioxidant vitamin-E. *Z. Lebensm. Unters. For.* 196 (4): 329-338

[Kal99] Kalt W, Forney CF, Martin A, Prior RL (1999): Antioxidant capacity, vitamin C, phenolics, and anthocyanins after fresh storage of small fruits. *J. Agric. Food Chem.* 47: 4638-4644

[Kan01] Kanner J, Harel S, Granit R (2001): Betalains - a new class of dietary cationized antioxidants. *J. Agric. Food Chem.* 49: 5178-5185

[Kau01] Kaur C, Kapoor H (2001): Antioxidants in fruits and vegetables - the milennium's health. *Int. J. Food Sci. Technol.* 36: 703-725

[Kim02] Kim DO, Lee KW, Lee, HJ, Lee CY (2002): Vitamin C equivalent antioxidant capacity (VCEAC) of phenolic phytochemicals. *J. Agric. Food Chem.* 50: 3713-3717

[Kra01] Krasowska A, Rosiak D, Szkapiak K, Oswiecimska M, Witek S, Lukaszewics M (2001): The antioxidant activity of BHT and new phenolic compounds PYA and PPA measured by chemiluminescence. *Cell. Mol. Biol. Lett.* 6: 71-81

[Kru95] Kruedener S, Schempp H, Elstner EF (1995): Gas-chromatographic differentiation between myeloperoxidase activity and Fenton-type oxidants. *Free Rad. Biol. Med.* 19 (2): 141-146

[Kud00] Kudoh Y, Matsuda S (2000): Effect of lactic acid bacteria on antioxidative activity of sweet potato yogurt. *J. Jpn. Soc. Food Sci.* 47 (9): 727-730

[Kud01] Kudoh Y, Matsuda S, Igoshi K, Oki T (2001): Antioxidative peptide from milk fermented with Lactobacillus delbrueckii subsp. bulgaricus IFO13953. *J. Jpn. Soc. Food Sci.* 48 (1): 44-50

[Lac96] Lachance PA (1996): Future vitamin and antioxidant RDAs for health promotion. *Prev. Med.* 25 (1): 46-47

[Lau03] Lau DW, King AJ (2003): Pre- and post-mortem use of grape seed extracts in dark poultry meat to inhibit development of thiobarbituric acid reactive substances. *J. Agric. Food Chem.* 51 (6): 1602-1607

[Law85] Lawrence G (1985) in Greenwald RA, Handbook of methods for oxygen radical research, Boca Raton, CRC Press, (USA?), 447 pp.

[Lei03] Leite da Silva Porto PA, Nave Laranjinha JA, Pereira de Freitas VA (2003): Antioxidant protection of low density lipoproteins by procyanidins: structure/avtivity relationships. *Biochem. Pharmacol.* 66: 947-954

[Lem01] Lemanska K, Szymusiak H, Tyrakowska B, Zielinski R, Soffers AEMF, Rietjens IMCM (2001): The influence of pH on antioxidant properties and the mechanism of antioxidant action of hydroxyflavones. *Free Rad. Bio. Med.* 31 (7): 869-881

[Leo02] Leong LP, Shui G (2002): An investigation of antioxidant capacity of fruits in Singapore markets. *Food Chem.* 76: 69-75

[Leu00] Leung YH, Liu RH (2000): *Trans*-10,*cis*-12-conjugated linoleic acid isomer exhibits stronger oxyradical scavenging capacity than *cis*-9,*trans*-11-conjugated linoleic acid isomers. *J. Agric. Food Chem.* 48: 5469-5475

[Lic02] Lichtenthäler R, Marx F, Kind OM (2002): Determination of antioxidative capacities using an enhanced total oxidant scavenging capacity (tosc) assay *Eur. Food Res. Technol.* 216 (2): 166-173

[Lic03] Lichtenthäler R, Marx F (2003): Total oxidant scavenging capacities of common European fruit and vegetable juices. *J. Agric. Food Chem.* accepted for press

[Lic04] Lichtenthäler R, Marx F, Maia JGS, Fabricius H, Papagiannopoulos M (2004): Total oxidant scavenging capacities of *Euterpe oleracea* Mart. (açaí) fruits. *Int. J. Food Sci. Nutr.* submitted

[Lug03] Lugasi A, Hovari J (2003): Antioxidant properties of commercial alcoholic and non-alcoholic beverages. *Food* 47 (2): 79-86

[McC03] McCue P, Shetty K (2003): Role of carbohydrate-cleaving enzymes in phenolic antioxidant mobilization from whole soybean fermented with Rhizopus oligosporus. *Food Biotechnol.* 17 (1): 27-37

[Mey03] Meyers KJ, Watkins CB, Pritts M, Liu RH (2003): Antioxidant and antiproliferative activities of strawberries. *J. Agric. Food Chem.* 51 (23): 6887-6892

[Mil97] Miller NJ, Rice-Evans CA (1997): The relative contributions of ascorbic acid and phenolic antioxidants to the total antioxidant activity of orange and apple fruit juices and blackcurrant drink. *Food Chem.* 60 (3): 331-337

[Mir99] Mira L, Silva M, Rocha R, Manso CF (1999): Measurement of relative antioxidant activity of compounds: a methodological note. *Redox Rep.* 4 (1-2): 69-74

[Mun96] Muñiz-Miret N, Vamos R, Hiraoka M, Montagnini F, Mendelsohn RO (1996): The economic value of managing the açaí palm (*Euterpe oleracea* Mart.) in the floodplains of the Amazonian estuary, Pará, Brazil. *Forest Ecol. Manag.* 87 (1-3): 163-173

[Nep02] Nepote V, Grosso NR, Guzman CA (2002): Extraction of antioxidant components from peanut skins. *Grasas y aceites* 53 (4): 391-395

[Pap02] Papagiannopoulos M, Zimmermann B, Mellenthin A, Krappe M, Maio G, Galensa R (2002): Online coupling of pressurized liquid extraction, solid-phase extraction and high-performance liquid chromatography for automated analysis of proanthocyanidins in malt. *J. Chromatogr. A* 958: 9-16

[Pat03] Patterson RA, Horsley ETM, Leake DS (2003): Prooxidant and antioxidant properties of human serum ultrafiltrates towards LDL: important role of uric acid. *J. Lipid Res.* 44 (3): 512-521

[Pha00] Pham-Tuan H, Vercammen J, Devos C, Pat S (2000): Automated capillary gas chromatographic system to monitor ethylene emitted from biological materials *J. Chromatogr. A* 868: 249-259

[Plu98] Plumb GW, De Pascual-Teresa S, Santos-Buelga C, Cheynier V, Williamson G (1998): Antioxidant properties of catechins and proanthocyanidins: effect of polymerisation, galloylation and glycosylation. *Free Rad. Res.* 29: 351-358

[Poz04] Pozo-Insfran DD, Brenes CH, Talcott ST (2004): Phytochemical composition and pigment stability of açaí (*Euterpe oleracea* Mart.). *J. Agric. Food Chem.* 52 (6): 1539-1545

[Pri99] Prior RL, Cao G (1999): In vivo total antioxidant capacity: comparison of different analytical methods. *Free Radical Biol. Med.* 27 (11-12): 1173-1181

[Pro02] Proteggente AR, Pannala AS, Paganga G, Van Buren L, Wagner E, Wiseman S, Van De Put F, Dacombe C, Rice-Evans CA (2002): The antioxidant activity of regularly consumed fruit and vegetables reflects their phenolic and vitamin C composition. *Free Radical Res.* 36 (2): 217-233

[Pry93] Pryor WA, Cornicelli JA, Devall LJ, Tait B, Trivedi BK, Witiak DT, Wu M (1993): A rapid screening test to determine the antioxidants of natural and synthetic antioxidants. *J. Org. Chem.* 58: 3521-3532

[Pry94] Pryor W, Jin X, Squadrito G (1994): One-electron and 2-electron oxidations of methionine by peroxynitrite. *Proc. Natl. Acad. Sci.* 91: 11173-11177

[Prz98] Przybylski R, Lee YC, Eskin NAM (1998): Antioxidant and radical-scavenging activities of buckwheat seed components. *J. Am. Oil Chem. Soc.* 75 (11): 1595-1601

[Rac02] Racchi M, Daglia M, Lanni C, Papetti A, Govoni S, Gazzani G (2002): Antiradical activity of water soluble components in common diet vegetables. *J. Agric. Food Chem.* 50: 1272-1277

[Rad96] Radi R (1996): Kinetic analysis of reactivity of peroxynitrite with biomolecules. *Methods Enzymol.* 269: 354-366

[Rec97] Rechner A, Patz CD, Dietrich H (1997): Beitrag zur Bewertung der antioxidativen Kapazität verschiedener Getränke. *Flüss. Obst* 64 (2): 62-65

[Reg98] Regoli F, Winston GW, Mastrangelo V, Principato G, Bompadre S (1998): Total oxyradical scavenging capacity in mussel *Mytilus* sp. as a new index of biological resistance to oxidative stress. *Chemosphere* 37 (14): 2773-2783

[Reg99] Regoli F, Winston W (1999): Quantification of total oxidant scavenging capacity of antioxidants for peroxynitrite, peroxyl radicals, and hydroxyl radicals. *Toxicol. Appl. Pharm.* 156: 96-105

[Reg00a] Regoli F, Nigro M, Bompadre S, Winston GW (2000): Total oxidant scavenging capacity (TOSC) of microsomal and cytosolic fractions

from Antarctic, Arctiv and Mediterranean scallops: differentiation between three potent oxidants. *Aquat. Toxicol.* 49 (1-2): 13-25

[Reg00b] Regoli F, Cerrano C, Chierici E, Bompadre S, Bavestrello G (2000): Susceptibility to oxidative stress of the Mediterranean demosponge *Petrosia ficiformis*: role of endosymbionts and solar irradiance. *Mar. Biol.* 137: 453-461

[Rim96] Rimm EB, Ascherio A, Giovanucci E, Speigelman D, Stampfer MJ, Willet DC (1996): Vegetable, fruit and cereal fiber intake and risk of coronary heart disease among men. *J. Am. Med. Assoc.* 275: 447-451

[Ric96] Rice-Evans CA, Miller NJ, Paganga G (1996): Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Rad. Biol. Med.* 20 (7): 933-956

[Rog00] Rogez H (2000): Açaí: Preparo, composição e melhoramento da conservação. EDUFPA, Belém (Brazil), 313 pp.

[Sai99] Saint-Cricq de Gaulejac N, Vivas N, de Freitas V, Burgeois G (1999): The influence of various phenolic compounds on scavenging activity assessed by an enzymatic method. *J. Sci. Food Agric.* 79: 1081-1090

[Schl02] Schlesier K, Harwat M, Böhm V, Bitsch R (2002): Assessment of antioxidant activity by using different *in vitro* methods. *Free Radic. Res.* 36 (2): 177-187

[Schö99] Schönafinger K (1999): Heterocyclic NO prodrugs. *Il Farmaco.* 54:316-320

[Schu99] Schubert SY, Lansky EP, Neeman I (1999): Antioxidant and eicosanoid enzyme inhibition properties of pomegranate seed oil and fermented juice flavonoids. *J. Ethnopharmacol.* 66 (1): 11-17

[See01] Seeram NP, Bourquin LD, Nair MG (2001): Degradation products of cyanidin glycosides from tart cherries and their bioactivities. *J. Agric. Food Chem.* 49 (10): 4924-4929

[Sid03] Siddhuraju P, Becker K (2003): Antioxidant properties of various solvent extracts of total phenolic constituents from three different agroclimatic origins of drumstick tree (*Moringia oleifera* Lam.) leaves. *J. Agric. Food Chem.* 51(8): 2144-2155

[Sin74] Singleton VL (1974): Analytical fractionation of the phenolic substances of grapes and wine and some practical uses of such analyses. In: Chemistry of Winemaking., Webb AD, Ed., American Chemical Society: Washington, D.C. (USA), 391 pp.

[Smi99] Smith NJH (1999): The Amazon river forest. Oxford University Press, New York, 208 pp.

[Stef03] Stefanidou M, Alevisopoulos G, Chatziioannou A, Koutselinis A (2003): Assessing food additive toxicity using a cell model. *Vet. Hum. Toxicol.* 45 (2): 103-105

[Stev02] Stevens JF, Miranda CL, Wolthers KR, Schimerlik M, Deinzer ML, Buhler DR (2002): Identification and in vitro biological activities of hop proanthocyanidins: inhibition of nNOS activity and scavenging of reactive nitrogen species. *J. Agric. Food Chem.* 50 (12): 3435-3443

[Str88] Strudwick J, Sobel GL (1988): Uses of Euterpe oleracea Mart. in the Amazon Estuary, Brazil. *Adv. Econ. Bot.* 6: 225-253

[Sun02] Sun J, Chu YF, Wu X, Liu RH (2002): Antioxidant and antiproliferative activities of common fruits. *J. Agric. Food Chem.* 50 (25): 7449-7454

[Tur99] Turrens JF (1999): Intracellular sources of reactive oxygen species under normal and pathologic conditions. *Antioxidants and life style - On line Journal* http://www.antioxidants.com.ar/12/Art005.htm

[Tyr99] Tyrakowska B, Soffers AEMF, Szymusiak H, Boeren S, Boersma MG, Lemanska K, Vervoort J, Rietjens IMCM (1999): TEAC antioxidant activity of 4-hydroxybenzoates. *Free Rad. Bio. Med.* 27: 1427-1436

[Vin01] Vinson JA, Su X, Zubik L, Bose P (2001): Phenol antioxidant quantity and quality in foods: fruits. *J. Agric. Food Chem.* 49: 5315-5321

[Wan96] Wang H, Cao G, Prior RL (1996): Total antioxidant capacity of fruits. *J. Agric. Food Chem.* 44: 701-705

[Wan00] Wang SY, Jiao H (2000): Scavenging capacity of berry crops on superoxide radicals, hydrogen peroxide, hydroxyl radicals, and singlet oxygen. *J. Agric. Food Chem.* 48: 5677-5684

[Wins98] Winston GW, Regoli F, Dugas AJ Jr., Fong JH, Blanchard KA (1998): A rapid gas chromatographic assay for determining oxyradical

scavenging capacity of antioxidants and biological fluids. *Free Radical. Biol. Med.* 24 (3): 480-493

[Winz01] Winzer K, Winston GW, Becker W, Van Noorden CJF, Köehler A (2001): Sex-related responses to oxidative stress in primary cultured hepatocytes of European flounder (*Platichys flesus* L.) *Aquat. Toxicol.* 52 (2): 143-155

[Wol03a] Wolfe K, Wu X, Liu RH (2003): Antioxidant activity of apple peels. *J. Agric. Food Chem.* 51 (3): 609-614

[Wol03b] Wolfe K, Liu RH (2003): Apple peels as value-added food ingredient. *J. Agric. Food Chem.* 51 (6): 1676-1683

[Yam02] Yamakoshi J, Saito M, Kataoka S, Tokutake S (2002): Procyanidin-rich extracts from grape seeds prevents cataract formation in hereditary cataractous (ICR/f) Rats. *J. Agric. Food Chem.* 50 (17): 4983-4988

[Yan67] Yang SF (1967): Further Studies on ethylene formation from α-keto-γ-methylthiobutyric acid or β-methylthiopropionaldehyde by peroxidase in the presence of sulfite and oxygen. *J. Biol. Chem.* 244 (16): 4360-4365

[Yeu03] Yeung CK, Glahn RP, Wu X, Liu RH, Miller DD (2003): In vitro iron bioavailability and antioxidant activity of raisins. *J. Food Sci* 68 (2): 701-705

[Zha99] Zhao J, Wang J, Chen Y, Agarwal R (1999): Anti-tumor-promoting activity of a polyphenolic fraction isolated from grape seeds in the mouse skin two-stage initiation-promotion protocol and identification of procyanidin B5-3‘-gallate as the most effective antioxidant constituent. *Carcinogenesis* 20 (9): 1737-1745

[Zhe03] Zheng W, Wang SY (2003): Oxygen radical absorbing capacity of phenolics in blueberries, cranberries, chokeberries, and lingonberries. *J. Agric. Food Chem.* 51: 502-509

[Zim01] Zimmermann B, Friedrich W, Galensa R (2001): Proanthocyanidine in Braugerste und Malz: Analytik einer Polyphenolklasse mittels ASE, SPE und HPLC-CEAD. *Lebensmittelchemie* 55: 158-158

Appendix A

Formation of ROS in the TOSC assay

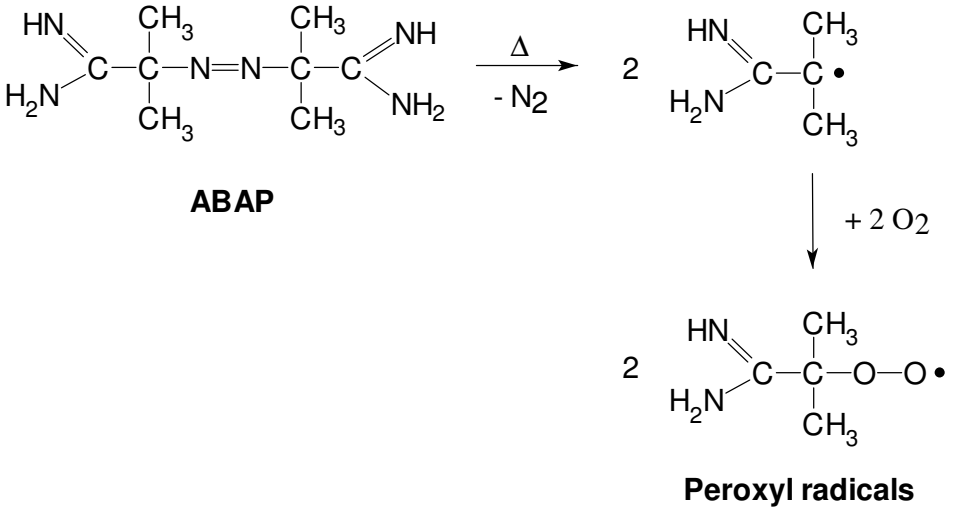

Figure A.1: Formation of peroxyl radicals in the TOSC assay [Kra01]

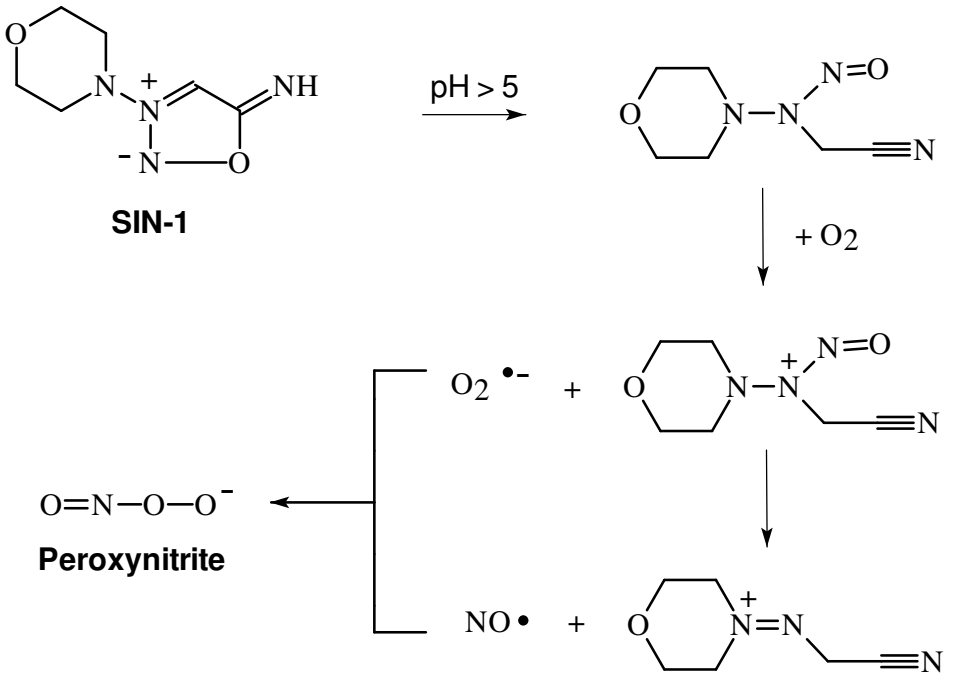

Figure A.2: Formation of peroxynitrite in the TOSC assay [Schö99, Yan67]

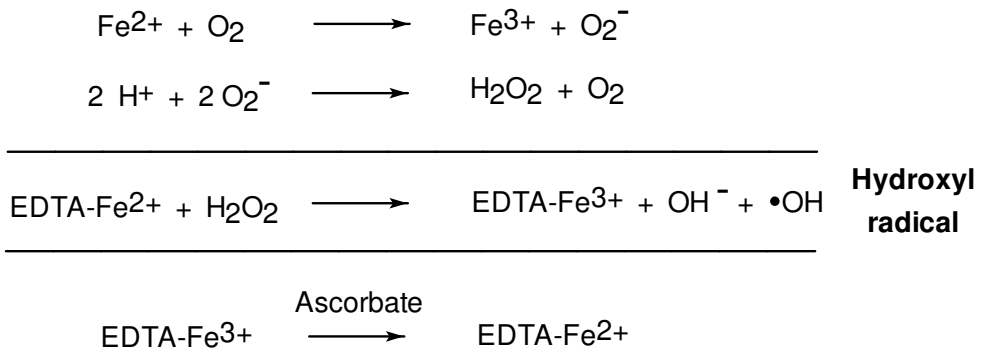

Figure A.3: Formation of hydroxyl radicals in the TOSC assay (Fenton reaction) [Gut90]

Appendix B

TOSC assay modifications

B.1 CombiPAL autosampler method and macro

Parameters of autosampler method:

- Syringe = 1.0 mL-HS
- Incubation Temperature (°C) = 37
- Incubation Time (s) = 720
- Syringe Temperature (°C) = 37
- Agitator Speed (rpm) = 500
- Fill Speed (μL/s) = 500
- Fill Strokes = 0
- Pullup Delay (ms) = 100
- Inject to = Injector
- Injection Speed (μL/s) = 1000
- Flush Time (s) = 10
- Mehrfachinjektionen = 6

Parameters of autosampler macro:

- Incubation Temperature (°C);37;30;200
- Incubation Time (s);720;30;86400

- Syringe Temperature (°C);37;30;150
- Agitator Speed (rpm);500;250;750
- Fill Speed (μl/s);SYR.Fill Speed;SYR.Min Speed;SYR.Max Speed
- Fill Strokes ();SYR.Fill Strokes;0;99
- Pullup Delay (ms);SYR.Pullup Del;0;10000
- Inject to;INJECTOR;
- Injection Speed (μl/s);SYR.Inject Speed;SYR.Min Speed;SYR.Max Speed
- Flush Time (s);10;0;600
- Mehrfachinjektion ();6;0;99

Macro steps:

1. SET_TEMP (Object name = SYR,Temperature = Syringe Temperature, Accuracy +– = 0,)
2. SET_TEMP (Object name = Agitator, Temperature = Incubation Temperature, Accuracy +– = 0)
3. SET_AGI (Agitator = Agitator, State = On, Speed = Agitator Speed, On Time = 5, Off Time = 55)
4. REPEAT (Count = Mehrfachinjektion)
5. START_TIMER (Timer = 1)
6. GET_SAMPLE (Source = Agitator, Index = 1, Sample Volume = SL.volume, Penetration = 0, Fill Speed = Fill Speed, Pullup Delay = Pullup Delay, Fill Strokes = Fill Strokes, Needle Blocking = Off)
7. INJ_SAMPLE (Injector = Inject to, Sync Signal = Inject, Injected Signal = Injected,Inject Speed = Injection Speed, Post Inject Delay = 5000)
8. MOVETO OBJECT (Object Name = Home)
9. SET_AGI (Agitator = Agitator, State = On, Speed = Agitator Speed, On Time = 5, Off Time = 55)
10. START_FLUSH (Flush Time = Flush Time)

11. WAIT (Time = Flush Time)
12. STOP_FLUSH
13. WAIT_TIMER (Timer = 1,Time = Incubation Time–600)
14. GET_SAMPLE (Source = Agitator, Index = 2, Sample Volume = SL.volume, Penetration = 0, Fill Speed = Fill Speed, Pullup Delay = Pullup Delay, Fill Strokes = Fill Strokes, Needle Blocking = Off)
15. INJ_SAMPLE (Injector = Inject to, Sync Signal = Inject, Injected Signal = Injected,Inject Speed = Injection Speed, Post Inject Delay = 5000)
16. MOVETO_OBJECT (Object Name = Home)
17. SET_AGI (Agitator = Agitator, State = On, Speed = Agitator Speed, On Time = 5, Off Time = 55)
18. START_FLUSH (Flush Time = Flush Time)
19. WAIT (Time = Flush Time)
20. STOP_FLUSH
21. WAIT_TIMER(Timer = 1, Time = Incubation Time–480)
22. GET_SAMPLE (Source = Agitator, Index = 3, Sample Volume = SL.volume, Penetration = 0, Fill Speed = Fill Speed, Pullup Delay = Pullup Delay, Fill Strokes = Fill Strokes, Needle Blocking = Off)
23. INJ_SAMPLE (Injector = Inject to, Sync Signal = Inject, Injected Signal = Injected,Inject Speed = Injection Speed, Post Inject Delay = 5000)
24. MOVETO_OBJECT (Object Name = Home)
25. SET_AGI (Agitator = Agitator, State = On, Speed = Agitator Speed, On Time = 5, Off Time = 55)
26. START_FLUSH (Flush Time = Flush Time)
27. WAIT (Time = Flush Time)
28. STOP_FLUSH
29. WAIT_TIMER(Timer = 1, Time = Incubation Time–360)

30. GET_SAMPLE (Source = Agitator, Index = 4, Sample Volume = SL.volume, Penetration = 0, Fill Speed = Fill Speed, Pullup Delay = Pullup Delay, Fill Strokes = Fill Strokes, Needle Blocking = Off)

31. INJ_SAMPLE (Injector = Inject to, Sync Signal = Inject, Injected Signal = Injected,Inject Speed = Injection Speed, Post Inject Delay = 5000)

32. MOVETO_OBJECT (Object Name = Home)

33. SET_AGI (Agitator = Agitator, State = On, Speed = Agitator Speed, On Time = 5, Off Time = 55)

34. START_FLUSH (Flush Time = Flush Time)

35. WAIT (Time = Flush Time)

36. STOP_FLUSH

37. WAIT_TIMER(Timer = 1, Time = Incubation Time–240)

38. GET_SAMPLE (Source = Agitator, Index = 5, Sample Volume = SL.volume, Penetration = 0, Fill Speed = Fill Speed, Pullup Delay = Pullup Delay, Fill Strokes = Fill Strokes, Needle Blocking = Off)

39. INJ_SAMPLE (Injector = Inject to, Sync Signal = Inject, Injected Signal = Injected,Inject Speed = Injection Speed, Post Inject Delay = 5000)

40. MOVETO_OBJECT (Object Name = Home)

41. SET_AGI (Agitator = Agitator, State = On, Speed = Agitator Speed, On Time = 5, Off Time = 55)

42. START_FLUSH (Flush Time = Flush Time)

43. WAIT (Time = Flush Time)

44. STOP_FLUSH

45. WAIT_TIMER(Timer = 1, Time = Incubation Time–120)

46. GET_SAMPLE (Source = Agitator, Index = 6, Sample Volume = SL.volume, Penetration = 0, Fill Speed = Fill Speed, Pullup Delay = Pullup Delay, Fill Strokes = Fill Strokes, Needle Blocking = Off)

47. INJ_SAMPLE (Injector = Inject to, Sync Signal = Inject, Injected Signal = Injected,Inject Speed = Injection Speed, Post Inject Delay = 5000)

48. MOVETO_OBJECT (Object Name = Home)

49. SET_AGI (Agitator = Agitator, State = On, Speed = Agitator Speed, On Time = 5, Off Time = 55)

50. START_FLUSH (Flush Time = Flush Time)

51. WAIT (Time = Flush Time)

52. STOP_FLUSH

53. WAIT_TIMER(Timer = 1,Time = Incubation Time)

54. END

55. TRANSP_VIAL (From Tray = Agitator, From Index = 1, To Tray = SL.tray, To Index = SL.index)

56. TRANSP_VIAL (From Tray = Agitator, From Index = 2, To Tray = SL.tray, To Index = SL.index+1)

57. TRANSP_VIAL (From Tray = Agitator, From Index = 3, To Tray = SL.tray, To Index = SL.index+2)

58. TRANSP_VIAL (From Tray = Agitator, From Index = 4, To Tray = SL.tray, To Index = SL.index+3)

59. TRANSP_VIAL (From Tray = Agitator, From Index = 5, To Tray = SL.tray, To Index = SL.index+4)

60. TRANSP_VIAL (From Tray = Agitator, From Index = 6, To Tray = SL.tray, To Index = SL.index+5)

B.2 Root macro for TOSC calculations

```
void antiox(const char* filename) {

  // Read in both the data and the control file
  ifstream file;

  Float_t t[6], e[6];
  Int_t ncols, maxcols, nlines, l;
  TF1 *uf = new TF1("user","[0]*x+[1]*x^2+[2]*x^3");
  TText *tl[100];

  // Open file
  cout << "Reading file " << filename << " ...";
  file.open(filename, ios::binary|ios::in);

  // Create profile
  TProfile *hprof = new TProfile("prof", "prof", 62, 0, 62);

  // Read time markers
  file >> t[0] >> t[1] >> t[2] >> t[3] >> t[4] >> t[5];

  // Read concentrations and fill profile
  Int_t maxcols = 6;
  Int_t nlines = 0;
  while ( !file.eof() ) {
    file >> e[0] >> e[1] >> e[2] >> e[3] >> e[4] >> e[5];
    for (Int_t j = 0; j < maxcols ; j++ ) {
      if ( e[j] > 0 ) { hprof->Fill(t[j], e[j], 1); }
    }
    nlines++;
  }

  hprof->Draw();

  // Some layout
  hprof->SetXTitle("Time [min]");
  hprof->SetYTitle("Ethylene Peak Area");

  // Fitting
  hprof->Fit(uf);
}
```

Appendix C

Results of standard compounds

C.1 Experimental TOSC values of standard compounds

	TOSC [%] for concentration [μM] of						
Peroxyl radicals	5	10	25	40	50	75	100
Ascorbic acid			11		22		48
(+)-Catechin	22	35	59		83		98
Cyanidin-3-glu.		24	52		85	94	98
Cyanidin-3-rut.		23	52		83	92	99
(-)-Epicatechin	14	33	55		83		98
Protocatechuic acid		14	35	51	58		83
Trolox		18			37		63
Uric acid		8	23		34		58

	TOSC [%] for concentration [μM] of					
Peroxyl radicals	150	200	250	300	5,000	100,000
Ascorbic acid		81		96	100	
Benzoic acid						21
Trolox	77	85				
Uric acid			96			

Table C.1: Experimental TOSC values of standard compounds against peroxyl radicals

	TOSC [%] for concentration [μM] of					
Peroxynitrite	1	5	10	25	50	100
Ascorbic acid		6		12	13	48
(+)-Catechin	7		26		49	59
Cyanidin-3-glucoside			6		35	49
Cyanidin-3-rutinoside			8		37	51
Epicatechin		12	26		51	60
Protocatechuic acid			15	31	41	49
Trolox			9		28	48
Uric acid				8	29	58
	TOSC [%] for concentration [μM] of					
Peroxynitrite	200	250	500	1,000	5,000	100,000
Ascorbic acid		75	85	89	92	
Benzoic acid						45
(+)-Catechin			79			
Cyanidin-3-glucoside		67	79			
Cyanidin-3-rutinoside		68	78			
Epicatechin			79			
Protocatechuic acid			73			
Trolox	77		86	90	98	99
Uric acid		81	88	90		

Table C.2: Experimental TOSC values of standard compounds against peroxynitrite

	TOSC [%] for concentration [μM] of			
Hydroxyl radicals	500	1,000	2,000	2,500
Ascorbic acid	-3			
Benzoic acid	6			36
(+)-Catechin	18			
Cyanidin 3-glucoside	25			
Cyanidin 3-rutinoside	30			
Epicatechin	23			
Protocatechuic acid	13			
Trolox	17	28	44	
Uric acid	16			
	TOSC [%] for concentration [μM] of			
Hydroxyl radicals	5,000	10,000	50,000	100,000
Ascorbic acid	25			
Benzoic acid	46	61	89	95
Trolox	65	78		

Table C.3: Experimental TOSC values of standard compounds against hydroxyl radicals

Appendix D

Results for fruit and vegetable juices

D.1 Experimental TOSC values of fruit and vegetable juices

Peroxyl radicals	TOSC [%] for dilution of				
	1:2500	1:1000	1:500	1:250	1:200
ACE juice			11		
Apple juice				8	
Beetroot juice I		11	20	38	
Beetroot juice II		8	14	26	
Blueberry juice		16	37	63	
Carrot juice I				9	
Carrot juice II				4	
Elderberry juice	6	16	31	55	
Lemon juice					10
Lingonberry juice	15	32	53	78	
Multivitamin juice			9		
Orange juice				11	
Pink grapefruit juice				12	
Sour cherry nectar		8	23	39	

continued on next page

continued from previous page						
	TOSC [%] for dilution of					
Peroxyl radicals	1:100	1:50	1:25	1:10	1:5	pure
ACE juice	36	62	86	99		
Apple juice	19	39	62	88		
Beetroot juice I	80	99				
Beetroot juice II	55	86	100			
Blueberry juice	95	99				
Carrot juice I	25	43	69	96		100
Carrot juice II	14	26	44	77		99
Elderberry juice	91	99				
Lemon juice	21	40	67	97		
Lingonberry juice	99	100				
Multivitamin juice	38	60	84	99		
Orange juice	24	43	70	98		
Pink grapefruit juice	25	43	67	96		
Sauerkraut juice I	20	32	52	85	99	100
Sauerkraut juice II	13	26	44	76	94	
Sour cherry nectar	72	94				
Tomato juice I	16	31	49	82	97	100
Tomato juice II	14	30	46	78	95	99

Table D.1: Experimental TOSC values of fruit and vegetable juices for peroxyl radicals

Peroxynitrite	TOSC [%] for dilution of								
	1:1000	1:500	1:250	1:100	1:50	1:25	1:10	1:5	pure
ACE juice		7		26	42		85	94	
Apple juice			9	19	35		62	74	94
Beetroot juice I		19		59	73		93	96	100
Beetroot juice		14		52	68		91	96	99
Blueberry juice	19	28		57	71		92		
Carrot juice I				24	40	54	70	80	96
Carrot juice II				12	24	43	63	75	94
Elderberry juice	15	23		55	67		92		
Lemon juice			10	21	38	61	87		
Lingonberry juice	13	26	35	51	63		93		
Multivitamin juice		7		29	44		88	95	
Orange juice				21	39	58	87	94	
Pink grapefruit juice			9	23	38	58	85		
Sauerkraut juice I				16	28	46	72	88	99
Sauerkraut juice II				12	21	40	66	82	99
Sour cherry nectar		18		46	60		86	93	
Tomato juice I				11	25	42	63	78	96
Tomato juice II				11	21	40	63	76	96

Table D.2: Experimental TOSC values of fruit and vegetable juices for peroxynitrite

Hydroxyl radicals	TOSC [%] for dilution of							
	1:500	1:250	1:100	1:50	1:25	1:10	1:5	pure
ACE juice		12	23	39	60		92	
Apple juice		12	26	42	59	77	88	
Beetroot juice I			21	38	56	80	91	100
Beetroot juice			20	38	54	77	91	100
Blueberry juice		11	28	49	68	84		
Carrot juice I			21	40	58	77	88	97
Carrot juice II			20	37	55	77	88	97
Elderberry juice		8	20	36	59	85		
Lemon juice			4		30	53		
Lingonberry juice		16	46	70	85	95		
Multivitamin juice		14	27	42	59		91	
Orange juice	8		20	35		75	87	
Pink grapefruit juice		14	25	40		79	90	99
Sauerkraut juice I			22	40	56	77	88	97
Sauerkraut juice II			22	36	53	76	88	98
Sour cherry nectar		13	30	67		89	96	
Tomato juice I			13	24	44	66	81	96
Tomato juice II			12	20	37	63	79	95

Table D.3: Experimental TOSC values of fruit and vegetable juices for hydroxyl radicals

Appendix E

Results for açaí pulps

E.1 Experimental TOSC values of açaí pulps

	TOSC [%] for dilution of							
Peroxynitrite	1:1000	1:500	1:100	1:50	1:25	1:10	1:5	pure
Açaí grosso I	10	21	50	63		88	93	100
Açaí grosso II		19	41	55	67	83	91	
Açaí medio I		22	50	61		84	91	
Açaí medio II		17	43	55		80	88	
Açaí fino I		15	41	53		80	88	98
Açaí fino II		9	29	43		72	82	95
Açaí pulp 1998	7	13	42	54		80	88	98
Açaí pulp 2000		11	39	53		79	87	
Açaí pulp 2001		10	33	48		77	85	
Açaí pulp 2002		18	48	58		83	90	98
White açaí		3	19	28		61	73	93

Table E.1: Experimental TOSC values of açaí pulps against peroxynitrite

	TOSC [%] for dilution of				
Peroxyl radicals	1:2000	1:1000	1:500	1:400	1:250
Açaí grosso I	11	25	45		70
Açaí grosso II		17		39	54
Açaí medio I		22	40		67
Açaí medio II		15	26		47
Açaí fino I		12	26		45
Açaí fino II		11	19		35
Açaí pulp 1998		11	23		48
Açaí pulp 2000	7	13	26		43
Açaí pulp 2001	5	12	23		40
Açaí pulp 2002		15	29		51
White açaí					6
	TOSC [%] for dilution of				
Peroxyl radicals	1:200	1:100	1:50	1:25	1:10
Açaí grosso I		97	100		
Açaí grosso II	64	88	99		
Açaí medio I		94	99		
Açaí medio II		79	96		
Açaí fino I		81	97		
Açaí fino II		62	87	99	
Açaí pulp 1998		82	98		
Açaí pulp 2000		82	98		
Açaí pulp 2001		74	97		
Açaí pulp 2002		87	99		
White açaí		15	31	59	88

Table E.2: Experimental TOSC values of açaí pulps against peroxyl radicals

	TOSC [%] for dilution of							
Hydroxyl radicals	1:250	1:100	1:50	1:25	1:10	1:5	1:2	pure
Açaí grosso I		10	23	46	79	90		100
Açaí grosso II	12	28	49	66	86	98		
Açaí medio I		11	23	50	80	93		
Açaí medio II		17	31	52	71	82		
Açaí fino I		10	23	41	79	87	90	97
Açaí fino II		16	33	55	78	87		99
Açaí pulp 1998		10	20	39	72	87		98
Açaí pulp 2000		20	45	65	80	90		
Açaí pulp 2001		24	45	62	75	88		
Açaí pulp 2002		10	24	44	75	86		99
White açaí		9	14	20	40	61		95

Table E.3: Experimental TOSC values of açaí pulps against hydroxyl radicals

E.2 Organic acids in açaí pulps

Compound	Content [mg/100g dry matter]
Citric acid	27
Malic acid	7
Ascorbic acid	not detectable

Table E.4: Concentration of organic acids in açaí pulps

E.3 MS data of compounds in açaí fruits

Identity	Retention time [min]	Parent ion [m/z] (polarity)]	MS/MS Fragments [m/z] (neutral loss)]
Cyanidin-glucoside	7.4	449(+)	287 (-162 Hexose-H_2O)
Cyanidin-rutinoside	8.3	595(+)	449 (-146 Hexose-H_2O) 287 (-308 Hexose+ Desoxyhexose-2*H_2O)
Peonidin-rutinoside	10.6	609(+)	463 (-146 Hexose-H_2O) 301 (-308 Hexose+ Desoxyhexose-2*H_2O)
Protocatechuic acid	11.1	153(-)	109 (-44 CO_2)
Procyanidin dimer	18.5	577(-)	425 (RDA[1]) 451 (-$C_6H_6O_3$[2]) 407, 289, 559
Procyanidin trimer	20.2	865(-)	713 (RDA) 739 (-$C_6H_6O_3$) 695, 577, 407
Catechin	20.6	289(-)	245, 205, 179
Procyanidin tetramer	20.8	1153(-)	1001 (RDA) 1027 (-$C_6H_6O_3$) 984, 575, 865
Quercetin-rutinoside (Rutin)	37.2	609(-)	300/301 (-308/309 Rutinose-H_2O)

Table E.5: MS data of identified polyphenols in açaí pulps

[1] Retro-Diels-Alder reaction
[2] $C_6H_6O_3$ = Phloroglucinol

E.4 MS data of compounds in aged anthocyan standard solutions

Retention time [min]	Identity	Parent Ion [m/z] (polarity)]	MS/MS Fragments [m/z]
6.2	Oxidation product of cyanidin-glucoside	465(-)	no detectable
8.0	Oxidation product of cyanidin-glucoside	465(-)	no detectable
11.3	Protocatechuic acid	153(-)	109
16.7	Oxidation product of cyanidin-glucoside	463(-)	283
18.6	Isomeric oxidation products of cyanidin-glucoside	463/465(-)	no detectable
22.5	Cyanidin aglycon with modified structure?	289(-)	no detectable
23.6	Cyanidin-glucoside	449(+)	287
32.2	?	341(-)	323, 165

Table E.6: MS data of compounds in aged cyanidin-3-glucoside standard solution

Retention time [min]	Identity	Parent Ion [m/z] (polarity)]	MS/MS Fragments [m/z]
7.0	Oxidation product of cyanidin-rutinoside	611(-)	no detectable
9.7	Oxidation product of cyanidin-rutinoside	611(-)	285
18.5	Oxidation product of cyanidin-rutinoside	609(-)	283
19.6	Isomeric oxidation products of cyanidin-rutinoside	609/611(-)	283
22.5	Cyanidin aglycon with modified structure?	289(-)	no detectable
25.1	Cyanidin-rutinoside	595(+)	449, 287
32.2	?	341(-)	323, 165

Table E.7: MS data of compounds in aged cyanidin-3-rutinoside standard solution

Appendix F

Results for açaí seeds

F.1 Experimental TOSC values of açaí seed extracts

Extract no.	TOSC [%] for dilution of 1:500		
	Peroxyl radicals	Peroxynitrite	Hydroxyl radicals
1	60	37	6
2	84	54	63
3	78	49	38
4	76	47	51
5	77	50	50
6	87	55	65
7	79	50	63
8	84	56	74
9	83	51	46
10	94	60	70
11	90	57	66
12	91	58	73
13	88	58	70

Table F.1: Influence of extraction procedure on TOSC of açaí seed extracts

Peroxyl radicals	TOSC [%] for dilution of					
Extract no.	1:10,000	1:5,000	1:1,000	1:500	1:250	1:100
1		10	40	60	82	99
2	19	32	67	84	97	
6	20	33	69	87	98	
10	19	36	76	94	99	

Table F.2: Experimental TOSC values of açaí seed extracts against peroxyl radicals

Peroxynitrite	TOSC [%] for dilution of						
Extract no.	1:10,000	1:5,000	1:2,500	1:1000	1:500	1:100	1:10
1			16	28	37	61	88
2	10	19	27		54	77	94
6		21	29		55	79	94
10	19	28	39		60	82	95

Table F.3: Experimental TOSC values of açaí seed extracts against peroxynitrite

Hydroxyl radicals	TOSC [%] for dilution of				
Extract no.	1:2,500	1:1,000	1:500	1:250	1:100
1			6	32	49
2	9	37	63	73	71
6	8	39	65	79	81
10	11	48	70	78	84
Hydroxyl radicals	TOSC [%] for dilution of				
Extract no.	1:50	1:25	1:10	1:2	Pure
1	55	58	71	87	95
2	76		92	99	
6	82		92	100	
10	84		96	100	

Table F.4: Experimental TOSC values of açaí seed extracts against hydroxyl radicals

F.2 MS data of compounds in açaí seeds

Identity	Retention time [min]	Parent Ion [m/z] (polarity)]	MS/MS Fragments [m/z] (neutral loss)]
Protocatechuic acid	11.1	153(-)	109 (-44 CO_2)
Procyanidin dimer	18.5	577(-)	425 (RDA) 451 (-$C_6H_6O_3$) 407, 289, 559
Procyanidin trimer	20.2	865(-)	713 (RDA) 739 (-$C_6H_6O_3$) 695, 577, 407
Procyanidin tetramer	20.8	1153(-)	1001 (RDA) 1027 (-$C_6H_6O_3$) 984, 575, 865
Procyanidin pentamer	21.1	1441(-)	1289 (RDA) 1315 (-$C_6H_6O_3$) 1272, 863, 1153
Procyanidin pentamer	22.3	1441(-)	1289 (RDA) 1315 (-$C_6H_6O_3$) 1272, 863, 1153
Epicatechin	26.5	289(-)	245, 205, 179

Table F.5: MS data of identified polyphenols in açaí seeds

HPLC fraction [*min*]	Parent Ion [m/z] (polarity)]	MS/MS Fragments [m/z]	Identity
15–20	153(-)	109	Protocatechuic acid
20–25	577(-)	407, 289, 559	Procyanidin dimer
	865(-)	695, 577, 407	Procyanidin trimer
	1153(-)	984, 575, 865	Procyanidin tetramer
25–30	577(-)	407, 289, 559	Procyanidin dimer
	865(-)	695, 577, 407	Procyanidin trimer
	1153(-)	984, 575, 865	Procyanidin tetramer
	1441(-)	1272, 863, 1153	Procyanidin pentamer
	1441(-)	1272, 863, 1153	Procyanidin pentamer
30–35	577(-)	407, 289, 559	Procyanidin dimer
	865(-)	695, 577, 407	Procyanidin trimer
	1153(-)	984, 575, 865	Procyanidin tetramer
	1441(-)	1272, 863, 1153	Procyanidin pentamer
	1441(-)	1272, 863, 1153	Procyanidin pentamer

Table F.6: MS data of identified compounds in açaí seed extract HPLC fractions

F.3 Concentrations of identified polyphenols in açaí seed extracts

Extract no.	Variety	Year	Harvesting season	Solvent	Extraction method
1	Purple	2000	High	EtOH	Soxhlet
2	Purple	2001	Low	MeOH	Cold
3	Purple	2001	Low	MeOH	Soxhlet
4	Purple	2001	Low	EtOH	Cold
5	Purple	2001	Low	EtOH	Soxhlet
6	Purple	2002	High	MeOH	Cold
7	Purple	2002	High	MeOH	Soxhlet
8	Purple	2002	High	EtOH	Cold
9	Purple	2002	High	EtOH	Soxhlet
10	White	2002	High	MeOH	Cold
11	White	2002	High	MeOH	Soxhlet
12	White	2002	High	EtOH	Cold
13	White	2002	High	EtOH	Soxhlet

Extract no.	Concentration of polyphenols [mg/L]						
	PCA	2C	3C	4C	5Ca	5Cb	$\sum$
1	84.3	247.4	122.2	144.7	64.6	19.7	683
2	31.5	420.2	275.7	484.3	366.9	342.6	1,921
3	39.1	426.5	284.1	427.7	318.4	268.0	1,764
4	26.7	314.0	160.3	294.1	247.9	145.0	1,188
5	40.5	390.8	322.1	400.4	278.9	250.1	1,683
6	13.9	485.2	471.8	638.3	476.3	446.9	2,532
7	16.2	421.6	508.6	583.9	410.6	320.9	2,262
8	12.5	416.7	437.4	594.8	457.7	270.6	2,190
9	21.6	513.1	602.7	627.2	412.2	404.1	2,581
10	10.6	775.2	768.5	1002.3	766.0	665.3	3,988
11	11.2	790.7	929.4	1009.3	739.8	640.3	4,121
12	9.1	661.2	628.5	836.8	631.6	385.0	3,152
13	15.4	789.8	929.3	978.7	661.6	420.8	3,796

Table F.7: Concentrations of polyphenols in açaí seed extracts