

THE TOTAL OXIDANT SCAVENGING CAPACITY (TOSC) ASSAY AND ITS APPLICATION TO EUROPEAN AND UNDER-UTILIZED BRAZILIAN FRUITS

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ABSTRACT

There is evidence that antioxidants in food could play an important role in the prevention of several illnesses like cancer and cardiovascular diseases due to the fact that they could scavenge reactive oxygen species (ROS). ROS can damage biological molecules and are generated as by-products of normal cell aerobic respiration. The total oxidant scavenging capacity (TOSC) assay permits to study the antioxidant capacity against three different ROS with physiological relevance – peroxy and hydroxyl radicals as well as peroxynitrite. The TOSC assay in an optimized and widely automated version was applied for standard antioxidant solutions, fruit juices common in Europe and on the Brazilian fruits açai, camu camu and cashew. They have a considerable commercial potential, but up to now, they are under-utilized. The analyses turned out that the three studied Brazilian fruits present favourable antioxidant features in comparison with the European fruits. Further studies are necessary to elucidate the contributions of the individual food ingredients to the overall antioxidant capacity in more detail and to evaluate their bioavailability.

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1. INTRODUCTION

1.1 Reactive oxygen species and antioxidants in human metabolism

Epidemiological studies demonstrate that food can have beneficial effects on human health in addition to its nutritional value. In recent years, research in this area has focused on the detection of antioxidants in food, because there is evidence that they could play an important role in the prevention of several illnesses such as cancer and cardiovascular disease as well as in the retardation of the aging process. Fruits have received particular attention, because they contain high amounts of known antioxidants such as polyphenols, vitamin C, vitamin E, β -carotene and lycopene [1].

Reactive oxygen species (ROS) are continuously formed in several metabolic pathways of human metabolism such as electron transport chains and active phagocytosis or as intermediates during various enzyme driven reactions [2]. The main ROS resulting from these processes include superoxide anion, hydrogen peroxide, hydroxyl radical, peroxy radical, alkoxyl radical, hypochlorous acid and peroxynitrite. They can cause oxidative damage to macromolecules as DNA, proteins and lipids. Antagonists of these ROS are body's own ROS scavenging antioxidants. Their presence minimizes harmful oxidative reactions. The protective actions may be assisted by antioxidants incorporated by eating food, rich in antioxidants. It is assumed that consumption of food antioxidants is important, in particular when the body's equilibrium between oxidants and antioxidants is disturbed. Such a disequilibrium situation may be caused by malnutrition, illnesses or stress situations. Up to now, the possible role of food antioxidants in those metabolic reactions is far from being understood completely. Therefore, different groups are performing studies on that area. Among them are *in-vitro* studies on the individual contributions of food ingredients to the overall antioxidant capacity, *in-vivo* studies on the rate of absorption (bioavailability) and the influence of antioxidants on prevention of oxidative tissue damages.

1.2 Requirements for reliable in-vitro antioxidant capacity assays

Assays for *in-vitro* assessment of antioxidant capacity against reactive oxygen species should meet the following requirements: assay conditions should correspond as closely as possible to physiological conditions and the ROS used should be relevant for biological systems. The different physiological ROS cover a wide range of reactivity with half-life-times ranging from nanoseconds to some seconds. Therefore, for the assessment of the effectiveness of antioxidants it is necessary to use different ROS with reactivities which are similar to those of the physiological ones [3]. Several *in-vitro* assays to assess the antioxidant capacity are available. However, the above mentioned requirements are not fulfilled in many common assays. Mostly, they are based on only one ROS. Because of the different ROS used in different assays their results are not comparable among each other. Therefore, it is recommended to perform two different assays and merge the different results [4].

2. THE TOTAL OXIDANT SCAVENGING CAPACITY (TOSC) ASSAY

2.1 Method principles

A way out of the above outlined problems could be possible by means of the total oxidant scavenging capacity (TOSC) assay, which was introduced for environment-related studies on marine organisms not so long ago [5]. It is based on the inhibition of the radical-depending formation of ethylene from ketomethiolbutyric acid by antioxidants. The TOSC assay permits to study the antioxidant capacity of samples against three different ROS with physiological relevance – peroxy and hydroxyl radicals as well as peroxyxynitrite.

These three compounds cover the range of reactivity of physiological ROS. The hydroxyl radical is among the most aggressive ones (half-life-time: about 10^{-9} seconds). It is extremely harmful for proteins and membrane lipids. Due to its high reactivity it reacts with almost all molecules which are in the vicinity. The peroxy radical is representative for slow reacting ROS, its half-life-time amounts to several seconds. It is less aggressive but can pass longer distances in biological systems and can affect distant molecules. The reactivity of peroxyxynitrite is between these both extremes. The assay can be accomplished at physiological pH and temperature. Different types of antioxidant reactions (retardant or fast-acting) can be distinguished and even pro-oxidant behaviour of samples is detectable (see Fig. 1).

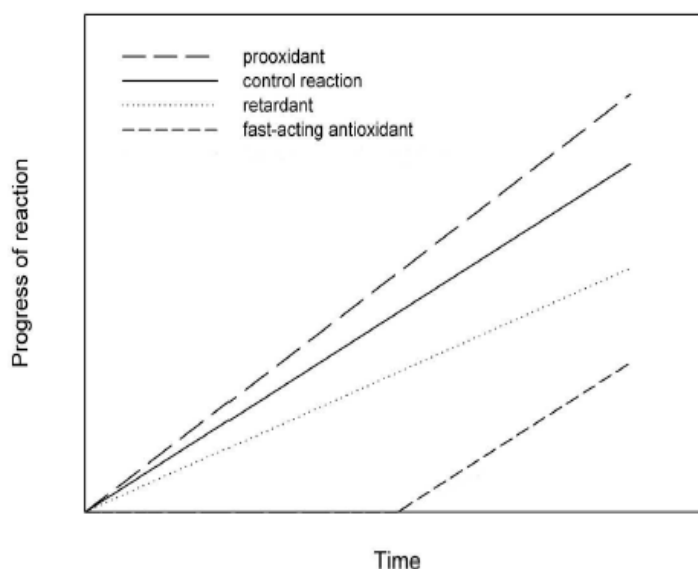


Fig. 1: Time course of the ethylene production during the TOSC assay with fast-acting and retardant antioxidants as well as prooxidants

Recently the assay conditions have been substantially simplified and largely automated. Now the procedure is easier to perform and the results more reliable. Thus, its reliable application on foodstuffs became possible. Assay details are described in [6]. Briefly, samples and buffered solutions of α -ketomethiol butyric acid (KMBA) are mixed and one of the ROS formed. Peroxyl radicals are generated by the thermal homolysis of 2,2'-Azobis(2-methylproprionamide) dichloride (ABAP). Hydroxyl radicals are formed during the iron plus ascorbate driven Fenton reaction. Peroxyxynitrite is produced by the decomposition of 3-morpholinosyndnonimine-N-ethylcarbamide. Due to the presence of the ROS the KMBA molecules are decomposed by liberation of ethylene gas. Presence of antioxidants in the samples decreases the ethylene formation. The time course of ethylene formation is monitored

during one hour by repeated gas chromatographic analyses of 100 μ L aliquots from the headspace in the sample vials and compared to the ethylene formation in control vials (addition of water instead of sample). A typical gas chromatogram is shown in Fig. 2.

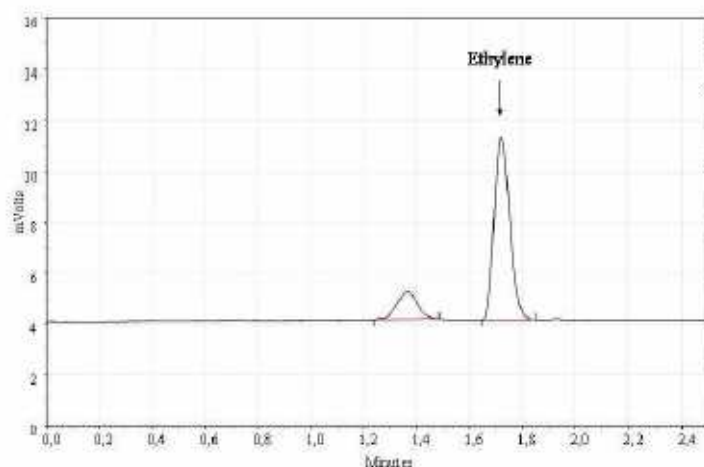


Fig. 2: Headspace gas chromatogram of ethylene (produced by the radical-depending decomposition of ketomethiol butyric acid). The GC column is a 25 m Chrompack Poraplot Q wide bore capillary; column temperature is 80 °C; carrier gas flow is 15 mL.

For the estimation of the TOSC values the kinetic curves that best fit the experimental GC data over a period of 60 min. has to be calculated. Then the corresponding areas under the time curves for samples and controls are determined. TOSC values result from the relation between the area for the uninhibited control and the area for sample reaction. A TOSC value of 0 % characterizes a sample without any antioxidant property. A solution that suppresses the ethylene formation completely achieves a TOSC value of 100%. Fig. 3 shows an example of a TOSC value calculation.

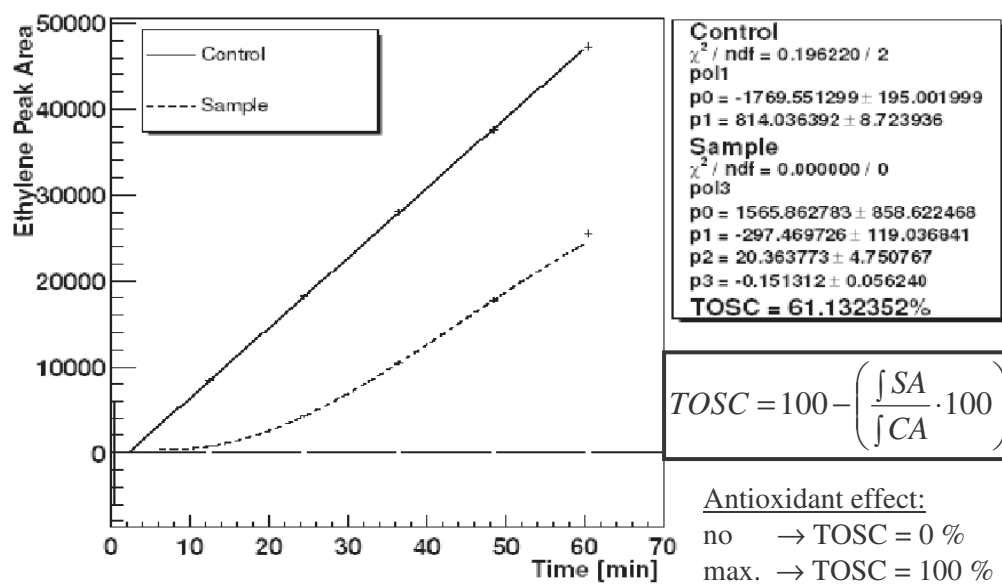


Fig. 3: Calculation of TOSC values from the “areas under the curves” of an uninhibited control reaction and of an antioxidants containing sample reaction resulting in a TOSC value of 61 %.

2.2 Application on standard compounds

The application of the TOSC assay for solutions of pure antioxidants (occurring in plants) demonstrates that their antioxidant capacities are quite different among each other as well as against the three tested ROS. Fig. 4 can be taken as an example where TOSC curves for some antioxidants against peroxy radicals are shown. It is obvious that the curve courses are different and, in some cases, non-linear to a different extent. It illustrates that doubling of the substance concentration does not lead automatically to doubling of the antioxidant capacity of the solution. In the literature, frequently sample concentrations with inhibition rate of 50 % of antioxidants are given for the description of the efficiency.

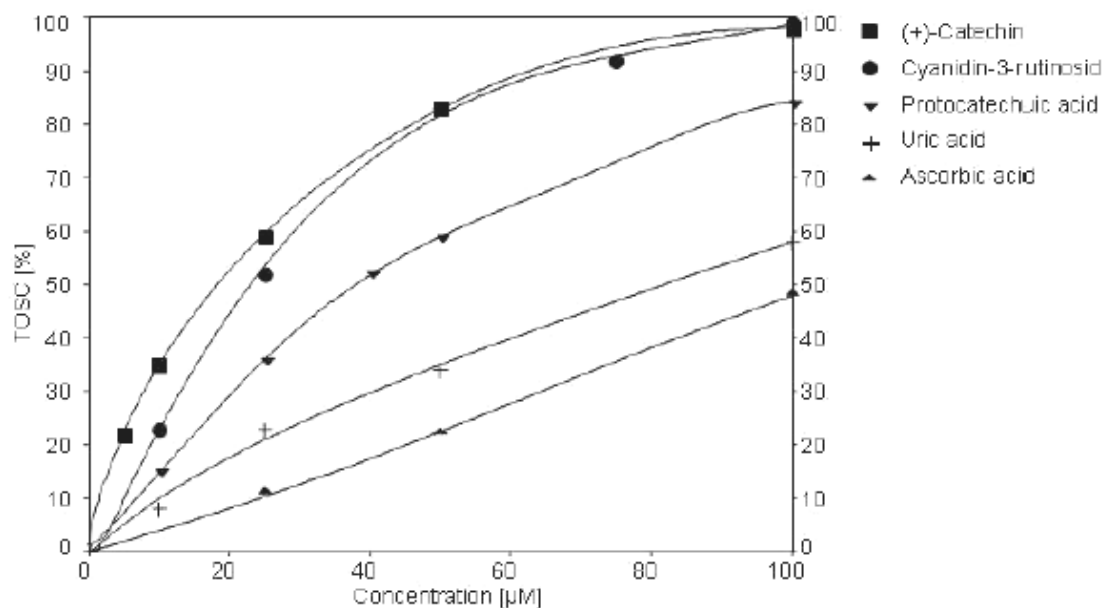


Fig. 4: Scavenging capacities of different antioxidants against peroxy radicals

Because of the non-linear concentration/efficiency correlation of many antioxidants more than one reference point is necessary for a more profound description of antioxidant properties. Therefore, for that purpose it is proposed to calculate the compound concentrations which possess TOSC values of 20 %, 50 % and 80 %, as shown in Table 1.

Tab. 1: Calculated concentrations of antioxidants (μM) for different TOSC values (20 %, 50 % and 80 %) against peroxy radicals

	TOSC (%)			Trolox-Equivalents at TOSC (%)		
	20	50	80	20	50	80
Trolox	15	71	167	1.00	1.00	1.00
Catechin	4	18	46	3.75	3.94	3.63
Epicatechin	6	20	47	2.5	3.55	3.55
Cyanidin-3-glucoside	9	23	45	1.67	3.09	3.71
Cyanidin-3-rutinoside	9	24	48	1.67	2.96	3.48
Protocatechuic acid	14	40	91	1.07	1.78	1.84
Uric acid	24	81	172	0.63	0.88	0.97
Ascorbic acid	45	105	194	0.33	0.68	0.86

In the literature, frequently Trolox is used to standardize antioxidant capacity values of different compounds. For that purpose concentrations are calculated which are necessary to obtain inhibition rates of 50 %. The presented data demonstrate that considering only one reference point will lead to incorrect correlations. From Table 1 it can be seen that in the case of catechin the course of effectiveness at different concentrations is very similar to that of Trolox (from 20 % to 80 % inhibition in the relation of about 3.7 to 1). However, if the effectiveness of protocatechuic acid is compared with that of Trolox, the values are very similar for 20 % inhibition (1.07 to 1) whereas the efficiency of protocatechuic acid is increased substantially in comparison with Trolox at 50 % inhibition (1.78 to 1).

3. ANTIOXIDANT CAPACITY OF COMMON EUROPEAN JUICES

The application to real samples like naturally turbid juices shows another advantage of the headspace GC based TOSC assay in comparison to methods based on photometrical or fluorimetrical sample analysis. The latter ones presuppose the separation of solid compounds from the sample matrix. That is not necessary in the case of the TOSC assay because the gaseous ethylene is liberated from the matrix prior to the quantification.

As a starting point for a database for the antioxidant capacity in food a series of common European commercial and non-commercial juices were analysed [7] for their antioxidant capacity against peroxy and hydroxyl radicals as well as peroxynitrite. Results for selected juices are compiled in Tab. 2. Some general conclusions can be drawn from that. For apple, carrot and sauerkraut juice the antioxidant capacities against all of the three ROS are very similar. Most of the studied juices are most efficient against peroxy radicals, somewhat less against peroxynitrite and lowest against hydroxyl radicals. In general, the biggest differences between the efficiencies of the analysed juices are observable against peroxy radicals, as well. All of the studied juices rich in anthocyanins and betalains presented outstanding

scavenging properties against peroxy and the comparatively best values against peroxy nitrite. Lingonberry juice is worthy of special highlighting; among the studied juices it has the highest scavenging capacity against the highly reactive hydroxyl radical, by far. An influence of lactic acid fermentation on the scavenging capacity against peroxy radicals and peroxy nitrite is obvious. Corresponding TOSC values for lactic acid fermented beetroot and carrot juices are considerably increased while the values for hydroxyl radicals remain on the level of the nonfermented juices. The observed improvement of the antioxidant capacity after fermentation is in agreement with literature data on sweet potato yoghurt [8].

Table 2: Calculated dilution factors of European juices for TOSC values of 20 %, 50 % and 80 %

	Peroxy radicals			Peroxy nitrite			Hydroxyl radicals		
	Calculated dilution factors for TOSC of								
	20%	50%	80%	20%	50%	80%	20%	50%	80%
Lingonberry juice	1667	556	238	588	106	22	214	90	33
Blueberry juice	833	357	167	909	141	29	141	48	13
Elderberry juice	769	286	137	606	128	26	99	32	13
Beetroot juice (lactic acid fermented)	500	185	100	500	135	34	105	32	10
Beetroot juice (natural)	337	114	57	357	109	29	100	30	9
Orange juice	125	41	20	105	33	13	106	27	8
Carrot juice (lactic acid fermented)	128	41	19	123	31	5	100	36	8
Pink grapefruit juice	133	40	17	115	33	12	145	33	10
Lemon juice	105	38	18	108	34	14	33	11	-
Apple juice	100	35	14	103	22	4	139	36	8
Sauerkraut juice	99	26	12	76	22	7	106	33	9
Tomato juice	81	25	11	64	18	4	64	19	5
Carrot juice (natural)	69	21	9	62	19	3	100	30	8

4. ANTIOXIDANT CAPACITY OF UNDER-UTILIZED TROPICAL FRUITS FROM BRAZIL

4.1 The açai (*Euterpe oleracea* Mart.) fruit pulp

Açai is one of the most naturally occurring palm species in the eastern Amazonian estuary floodplains. Their spherical grape-sized fruits are green when young and ripen usually to a dark purple colour, due to a high content of anthocyanins [9]. Fruits can be harvested throughout the year with higher yields and better organoleptic qualities during the 'dry months' (August – December in the area of the Amazon delta). Sales promotions of açai advertise the product to be rich in antioxidants, and to have several beneficial health effects especially for sportsmen.

TOSC assays with the açai fruit pulp approve the assumption of high antioxidant capacity of açai [10]. In comparison with the above described results for common European juices the average antioxidant capacity against peroxy radicals of the açai fruit pulp was very high similar to that of lingonberry and blueberry juice. Against peroxy nitrite, all samples demonstrated high antioxidant capacity even though it was not as outstanding. Compared with other juices, the TOSC values are to be considered moderate.

The TOSC assay in the form here utilised covers only water-soluble antioxidants. That means, mainly vitamin C and polyphenols are to be considered responsible for the antioxidant capacity for the antioxidant capacities of the sample. As açai is only a poor source of vitamin C, the main attention has to be drawn on polyphenols as compounds which could be responsible for the antioxidant capacity. Our HPLC-MS analyses prove the presence of two different anthocyanins in appreciable amounts and some others in minor concentrations. The two main anthocyanins were identified as cyanidin-3-glycoside and cyanidin-3-rutinoside, confirming findings of [9], [11]. Additionally, one of the minor anthocyanins was identified as peonidin-rutinoside (see Fig. 5). Furthermore, protocatechuic acid, flavan-3-ols (catechin monomers through tetramers) and quercetin-rutinoside were identified in minute quantities in the açai fruit samples. The summarised anthocyanin contents vary between 13 and 456 mg/100 g DM. It is striking that the sample with the highest anthocyanin content also had the highest TOSC values and the sample with the lowest TOSC value also contained the lowest amount of anthocyanins. In order to estimate the contributions of the individual anthocyanins to the overall antioxidant capacity TOSC values of cyanidin-3-glycoside and cyanidin-3-rutinoside were determined. It turned out that the studied açai samples showed far higher antioxidant capacity against peroxy and peroxy nitrite (10-fold to 200-fold) in comparison with pure anthocyanin solutions.

Consequently, the main part of the antioxidant capacities of açai fruit pulp must be due to other, not yet identified, compounds. An approach to solve that problem could be to separate the soluble sample compounds by reversed phase HPLC, collect the eluent in several fractions, verify the respective antioxidant capacities and identify the included compounds by HPLC-MS. First trials turn out that besides the anthocyanin fraction (25 -30 min.) others contribute considerably to the overall antioxidant capacity. The identification of these compounds is in progress.

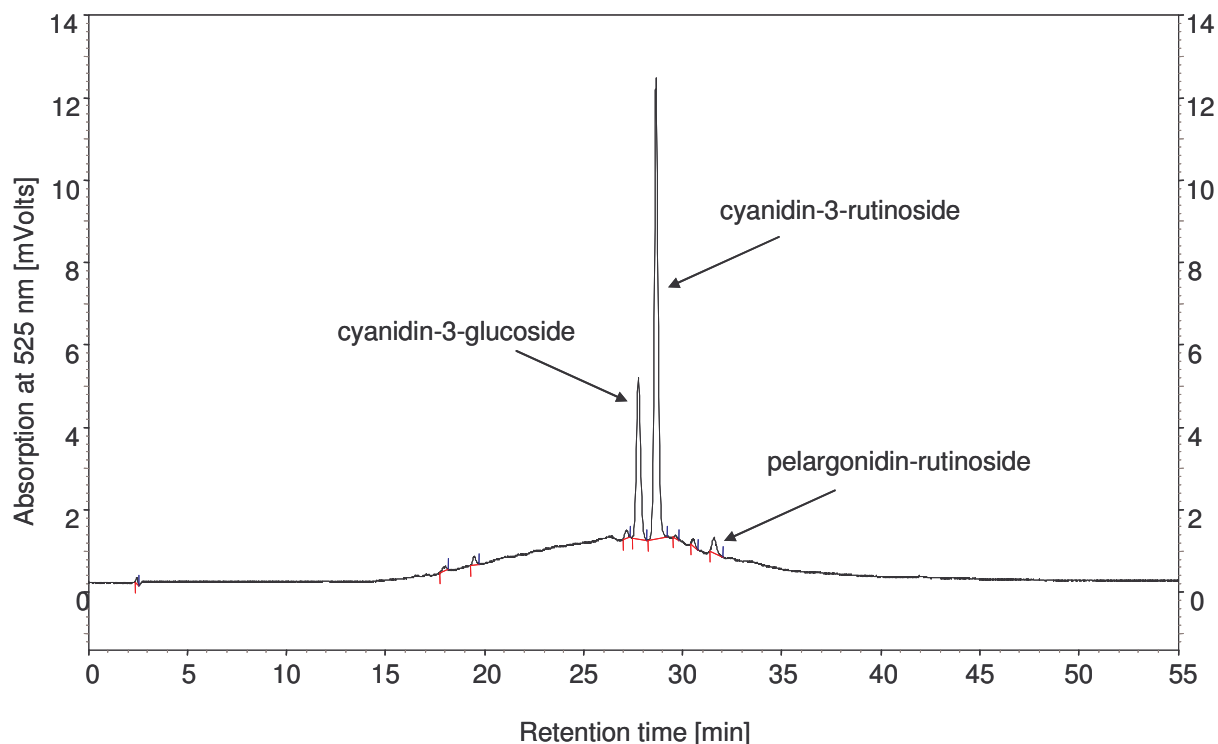


Fig. 5: HPLC separation of anthocyanins from açai pulp, Vis detection at 525 nm

4.2 The camu-camu (*Myrciaria dubia* (H.B.K.) Mc Vaugh) fruit

The camu-camu [*Myrciaria dubia* (H.B.K.) Mc Vaugh], a shrub from the Myrtaceae family, is one of the promising Amazonian fruits, which has obtained increasing attention since the last years. It grows naturally in the Amazonian basin. The fruit has the highest content of natural vitamin C known (about 1000-3000 mg/100g). TOSC assays against peroxy and hydroxyl radicals as well as peroxy nitrite indicate outstanding antioxidant features of the camu camu fruit. In comparison with the European and açai juices, the camu camu juice showed the highest antioxidant capacity against peroxy radicals and peroxy nitrite

To evaluate the contribution of ascorbic acid to the overall antioxidant capacity of camu camu the juice was diluted to achieve the same ascorbic acid concentration that was used for a standard ascorbic acid solution (1.66 mg/100 mL) [12].

The resulting TOSC of the camu camu juice was 68 % and that of the ascorbic acid standard was 49 %. As expected, ascorbic acid contributes to a major part to the total antioxidant capacity. However, the difference in the TOSC values of the camu-camu juice and the ascorbic acid standard solution indicates that other compounds or synergistic effects must be involved, as well.

Considering the fact, that anthocyanin containing fruits like different berries and açai show elevated antioxidant capacity one of the compound classes to be considered is that of the anthocyanins. Recently, in camu-camu fruits cyanidin-3-glucoside and delphinidin-3-glucoside were identified and an overall content of anthocyanins from about 54 mg/100 g was reported [13].

However, only a moderate contribution of anthocyanins to the TOSC value is to be expected, because in comparison for example with the palm fruit açai (up to approx. 450 mg anthocyanins/100 g), its concentration is low. Therefore, we looked for another polyphenol group, the flavonolglycosides, which occurs frequently in fruit.

The combination of multi step-mass spectrometric fragmentation with HPLC separation and UV-Vis diode array detection [14] allowed us to identify a number of flavonol-mono-glycosides with quercetin and myricetin as aglycones and different carbohydrates attached [12]. Further investigations are necessary that aims at the structural characterisation of the other flavonolglycosides present, the analysis of the type of attached sugars, and the quantification of these compounds as well as a correlation to their impact on the overall antioxidant capacity.

4.3 The cashew (*Anacardium occidentale* L.) apple

The cashew (*Anacardium occidentale* L.) is native to Brazil. It is cultivated on a big scale in tropical regions with dry seasons, among others in the northeast of Brazil. The cashew apple consists of a small nut and a yellow to reddish apple like pseudo fruit, representing about 90 % of the complete “apple”. In Brazil, up to now most of the harvested pseudo fruits (approximately 2 millions of tons) are discarded though they are rich in vitamin C (ca. 200 mg/100 mL juice) and have an exotic aroma [15]. The cashew apple juice shows a good antioxidant capacity against peroxy radicals, but less than camu camu, açai and lingonberry. In case of peroxy nitrite its antioxidant capacity is in the range of açai and lingonberry. Its scavenging capacity against hydroxyl radicals is remarkably high, higher than that of almost all other studied fruit juices; only lingonberry compares with it [16]. Certainly, ascorbic acid contributes considerably to the overall antioxidant capacity of the juice. However, comparison of the antioxidant capacities of the fruit juice with those of equal concentrated ascorbic acid solution turns out that other ingredients play a decisive role, as well. It is assumed that the identified phenolic compounds gallic acid (major compound), a series of galloylated proanthocyanidins and quercetin and myricetin glycosides are the responsible compounds.

The dose response curves of the antioxidant capacity from açai, camu-camu and cashew in juice in comparison with some other fruit juices against peroxy radical, peroxy nitrite and hydroxyl radical are presented in Figures 6-8.

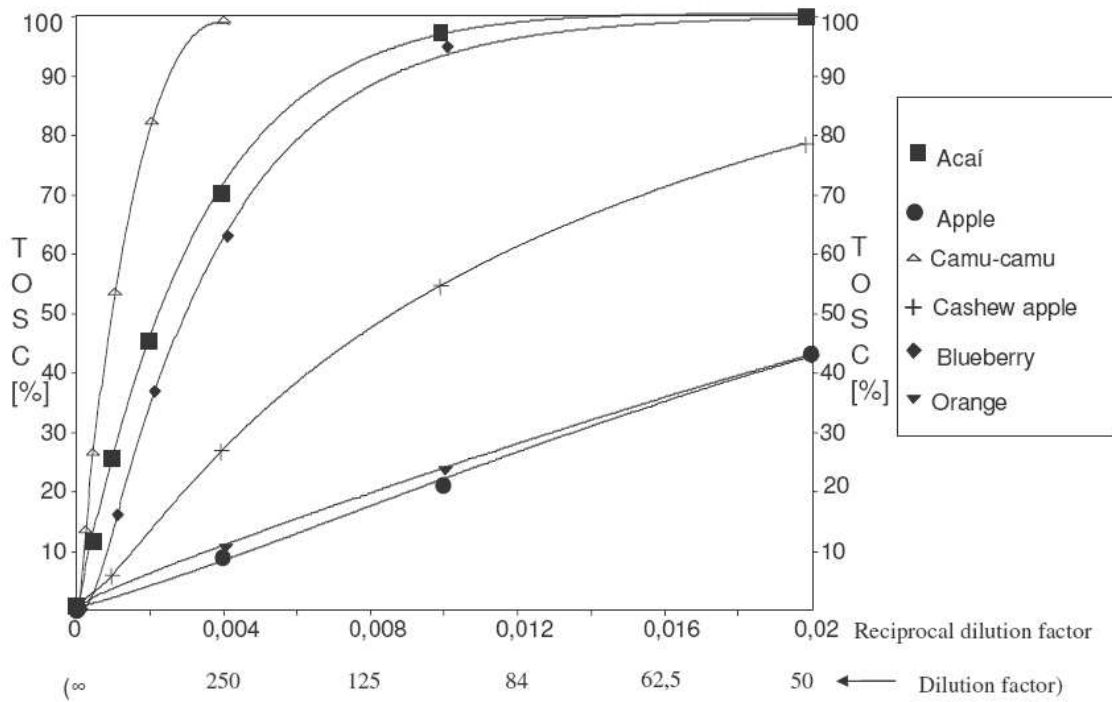


Figure 6: TOSC of açaí, camu-camu and cashew juice in comparison with some other fruit juices against peroxy radical.

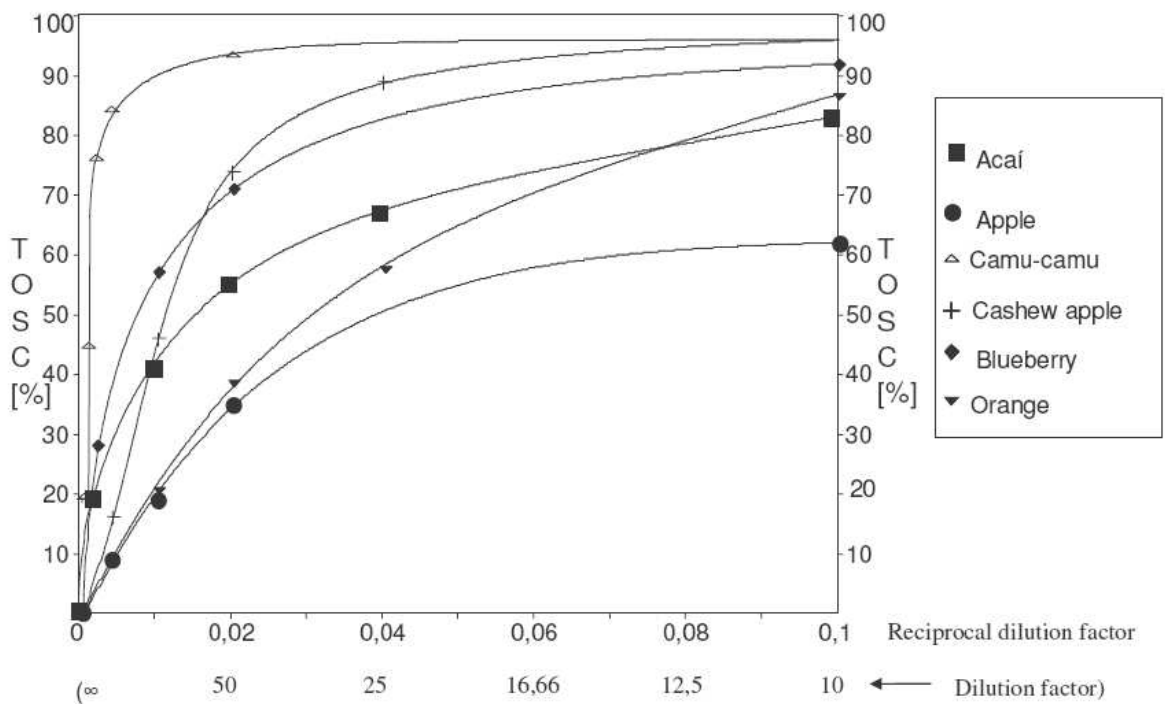


Figure 7: TOSC of açaí, camu-camu and cashew juice in comparison with some other fruit juices against peroxynitrite.

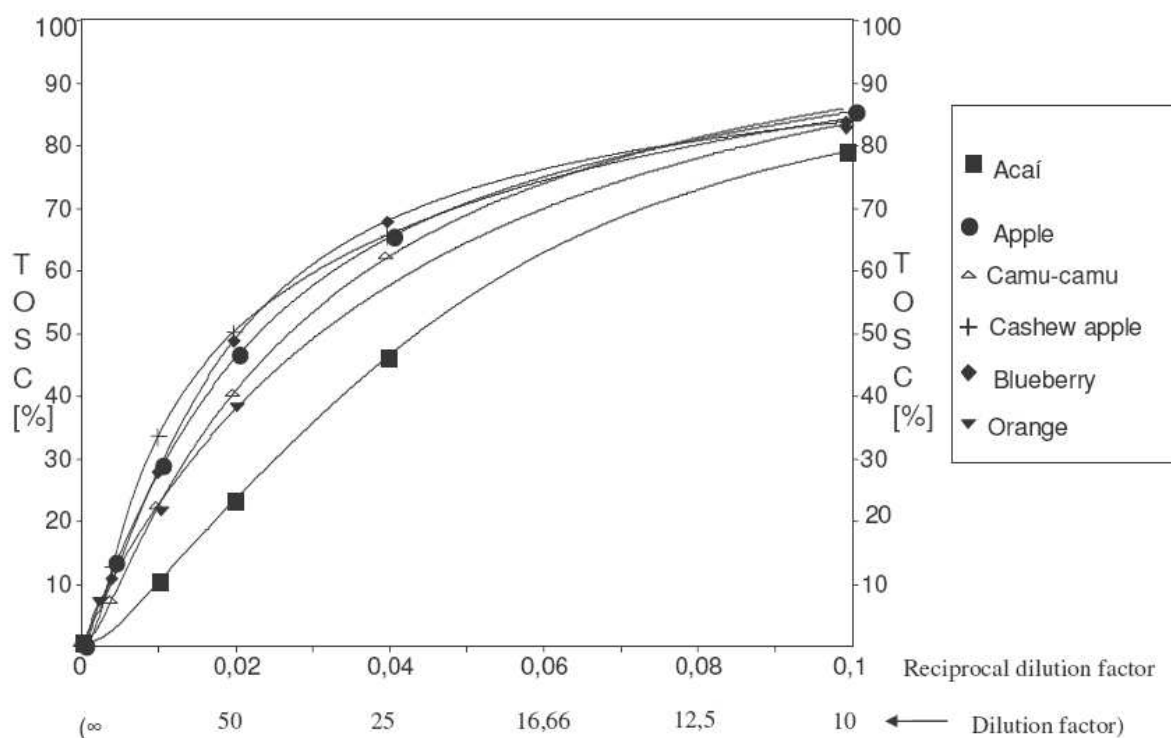


Figure 8: TOSC of açai, camu-camu and cashew juice in comparison with some other fruit juices against Hydroxyl radical.

4. Conclusion

All in all, the three studied under-utilized tropical fruits from Brazil açai, camu-camu and cashew present favourable antioxidant features in comparison with common European fruits. Obviously, considerable parts of the measured antioxidant capacities are not only due to the “established” known antioxidants. It is evident that further studies are necessary to understand the contributions of their ingredients to the overall antioxidant capacities and occurring interrelations and to evaluate the bioavailability of that parameter. Recent clinical trials using supplements of vitamin C, vitamin E, or carotenoids have provided inconsistent results [17]. In terms of disease prevention, clinical trials with whole fruit and vegetable products are more likely to give positive results [18].

Against that background these Brazilian fruits are promising candidates for use in functional food with health benefits. They do have a considerable commercial potential once their in-vivo effectiveness will be proved in detail.

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