

Bioactive Substances in Tropical Fruits – An Evaluation with TOSC Assay and “Activity Guided” RP-HPLC-Fractionation

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Summary

In order to develop products with added value out of underutilized tropical fruits, açai, blackberry, camu-camu, cashew apple, and tree tomato have been investigated. Firstly, the overall antioxidant capacity of each fruit was determined with the TOSC assay. Furthermore some particular responsible secondary plant compounds could be identified by “activity guided” HPLC separation and HPLC-MSⁿ analyses. By far, camu-camu turned out to be the fruit with the highest antioxidant potential. This could be explained to a certain extend by the extremely high ascorbic acid content of up to 3000 mg / 100 g fresh weight and small amounts of anthocyanins. But also blackberries and açai with low ascorbic acid content showed very respectable results mainly due to anthocyanins and probably other yet unidentified substances.

Introduction

A project aided by the European Union centres the development of products with added values for the human health out of so far underutilized fruits from Latin America. In a cooperation of several European and Latin American research institutions, in total nine promising tropical fruits have been investigated on their nutritional and functional potential. On the other hand economic aspects were taken into account for this project as well. Since all fruits have been cultivated in regions with low income, the development of good agricultural practices and a sustainable and innovative processing infrastructure close to the production site was emphasized. Chosen fruits were blackberries (*Rubus ssp.*), tree tomato (*Cyphomandra betacea*), red pitahaya (*Hylocereus purpusii*), berrycactus (*Myrtillocactus schenkii*), açai (*Euterpe oleracea*), cashew apple (*Anacardium occidentale*), narranjilla (*Solanum quitoense*), palm peach fruits (*Bactris gasipaes*), and camu-camu (*Myrciaria dubia*) of which five have been investigated by us in detail.

In recent years, fruits have received particular attention because they contain high amounts of antioxidants such as polyphenols, vitamin C, vitamin E, β -carotene, and lycopene [1]. These kinds of natural antioxidants are potential scavengers of reactive oxygen species (ROS) that are continuously formed in several metabolic pathways of human metabolism [2]. ROS can cause oxidative damage to macromolecules such as DNA, proteins, and lipids. Beside body's own ROS scavenging antioxidants, protective actions may be assisted by antioxidants from food especially when the body's equilibrium between oxidants and antioxidants is disturbed. Since the possible role of food antioxidants in those metabolic reactions is far from being understood completely, in vitro studies on the individual contributions of food ingredients to the overall antioxidant capacity are performed. Following in vivo studies may be helpful to reveal the rate of absorption and the influence of food antioxidants on prevention of oxidative damages.

The total oxidant scavenging capacity (TOSC) assay is an in vitro method to measure the antioxidant capacity. Originally it was introduced for environmental studies on marine organisms [3] but meanwhile the TOSC assay can also be applied to assess the antioxidant power of water soluble food compounds. It is based on the inhibition of the radical-depending formation of ethylene from α -keto- γ -methiolic butyric acid (KMBA) by antioxidants and permits to study the antioxidant capacity of samples against three different ROS

with physiological relevance – peroxy radicals, hydroxyl radicals and peroxyxynitrite.

For fruits containing high amounts of antioxidant micronutrients, an appropriate in vitro antioxidant activity could be shown [4]. Following the measurement of the in vitro antioxidant capacity of a fruit, an oral application of a corresponding fruit juice on healthy subjects might be useful in order to show whether or not this potential can be exerted in vivo. The so called comet assay based on the method of Singh et al [5] enables the assessment of the bioavailability of plant compounds by determining endogenous oxidative damage in lymphocyte DNA.

Materials and Methods

TOSC Assay

TOSC assay conditions are described in detail in [6]. Briefly, samples and buffered solutions of KMBA are mixed and one of the ROS is formed. Peroxyl radicals are generated by the thermal homolysis of 2, 2'-Azobis(2-methylpropionamide) dichloride. Hydroxyl radicals are formed during the iron plus ascorbate driven Fenton reaction. Peroxyxynitrite is produced by the decomposition of 3-morpholinolonydnonimine-N-ethyl-carbamide. 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 filled with the same amount of water instead of the sample.

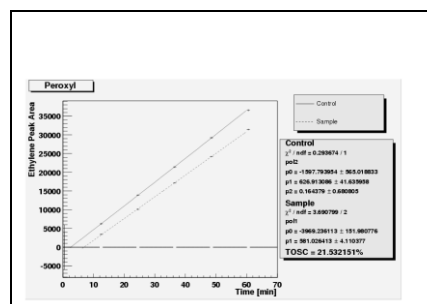


Figure 1: Calculation of a TOSC value from the “areas under the curve” resulting in a TOSC value of 21%.

For the estimation of the TOSC values the kinetic curves that best fit the experimental GC data over a period of 60 min have to be calculated. Then the corresponding areas under the time curves (AUC) for samples and controls are determined. TOSC values result from the relation between the area of the uninhibited control and the area of the sample.

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%. An example for TOSC value calculation is shown in Fig. 1.

“Activity guided” RP-HPLC-Fractionation

In order to clarify the question which substances of a sample do have an antioxidant potential, the water soluble sample compounds are separated with reversed phase HPLC by collecting the eluent in several fractions. A gradient separation permits the detection of substances with different polarities.

The liquid chromatographic system consisted of a pump model 2250 and a central processor model 1152 (Bischoff, Leonberg, Germany), a Degasys 1310 degasser (Uniflows, Tokyo, Japan), a detector LC-55 B (Perkin-Elmer, Waltham, USA) and a column Synergi 4u MAX-RP 80-A (Phenomenex, Aschaffenburg, Germany). The system was controlled by EZChrom Elite v2.8 (Scientific Software, Pleasanton, USA). Solvents for the gradient elution program were 2% formic acid in high-purity water (mobile phase A) and 2% formic acid in acetonitrile (ACN) (mobile phase B). The gradient separation was conducted with a linear program starting either with 0% B to 40% B after 25 min (Fractionation 1) or starting with 0% B to 25% B after 40 min (Fractionation 2) depending on the amount of detected substances. Before starting the program the column was equilibrated with A for 15 min to initial conditions. After separation the column was flushed for 20 min with B. The flow rate was 0.8 mL / min. The chromatogram was recorded at a wavelength of 260 nm. The injection slope had a volume of 20 μ L.

Following the fractionation the antioxidant capacity of the respective fractions was verified. An example is given in Fig. 2.

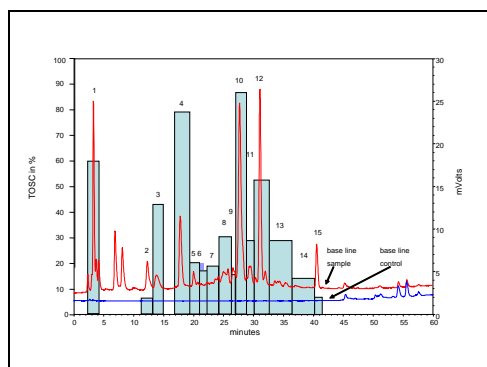


Figure 2: HPLC chromatogram of fractionation 2 of the seed jelly of a red tree tomato recorded at 260 nm combined with particular experimental TOSC values against peroxy radicals.

Identification of the antioxidant compounds

Identification of polyphenolic antioxidant compounds is performed by HPLC/DAD-MSⁿ. The liquid chromatograph was a summit system consisting of a Degasys DG-1310 degasser (Uniflow), a P-580A HPG pump, an ASI-100 T automated sample injector, a STH-585 column oven, and an UVD-340S UV-vis detector equipped with a capillary cell (Dionex, Germering, Germany). The system was controlled using the Chromeleon software package v6.7 SP2 (Dionex). The analytical column (Aqua 3 μ m C 18, 150 mm, 2 mm i.d., Phenomenex) was kept at 25 °C. 5% acetonitrile and 0.2% trifluoroacetic acid (TFA) in high purity water (mobile Phase C) and 45% ACN with 0.2% TFA in high purity water (mobile Phase D) were used as solvents with a flow rate of 0.2 mL / min. A gradient elution program was used starting at 5% C with a linear gradient of 0.5% D / min up to 30% D after 60 min. The column was washed with 100% D for 10 min and re-equilibrated for 15 min with the initial conditions. 10 μ L of each sample was injected for analysis and the chromatogram was monitored at 200 – 595 nm.

An LCQ classic ion-trap mass spectrometer (MS) equipped with an electrospray interface (ESI) and a metal needle kit was coupled to the HPLC and controlled with Xcalibur software v1.2 (Thermo Fisher Scientific, Dreieich, Germany). A flow of 100 μ L / min methanol delivered by a System Gold programmable solvent module 116 (Beckman, Unterschleissheim, Germany) was added through a T-union before the HPLC eluent entered the ion source to enhance ionization of very polar compounds. The settings for the MS were as follows: source voltage, 4.5 kV (negative mode); sheath gas flow, 60; auxiliary gas flow, 0; capillary voltage, -45 V; capillary temperature, 200 °C; first octapole offset, +3.0 V; interoctapole lens voltage, +22.0 V; second octapole offset, +7.0 V; ion trap DC offset, +10.2 V. [7].

Results

Açaí

Açaí is one of the most common naturally occurring palm species in the eastern Amazonian estuary floodplains. Their spherical grape-sized fruits usually ripen to a dark purple colour due to a high content of anthocyanins [8]. TOSC assays with the açaí fruit pulp show a low radical scavenging activity against hydroxyl radicals (data not shown) but approve the assumption of a high antioxidant capacity at least against peroxy radicals (Fig. 3). As the TOSC assay covers only water soluble antioxidants, mainly vitamin C and polyphenols are to be considered responsible for the antioxidant capacity of the sample. Because of the negligible vitamin C content in açaí the main attention has to be drawn to the polyphenols. The HPLC-MS analysis proves 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. The sample with the highest anthocyanin content also had the highest TOSC values. However, an estimation of the contributions of the two main anthocyanins to the overall antioxidant capacity showed a far higher antioxidant capacity against peroxy radicals and peroxy nitrite for the natural açaí samples in comparison with the pure anthocyanin standard solution.

Camu-camu

Camu-camu grows naturally in the Amazonian basin and has the highest content of natural vitamin C known in a fruit (up to 3000 mg / 100 g). TOSC assays against peroxy and hydroxyl radicals as well as peroxy nitrite indicate outstanding antioxidant features of the camu-camu fruit as can be seen in Fig. 3-5. 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) [4]. The resulting TOSC of the camu-camu juice was 68% compared to 49% of the ascorbic acid standard. Consequently, ascorbic acid contributes to a major part to the antioxidant capacity. Due to a relatively low overall anthocyanin content of about 54 mg / 100 g [9], the difference in the TOSC values indicates that other compounds or synergistic effects must be involved.

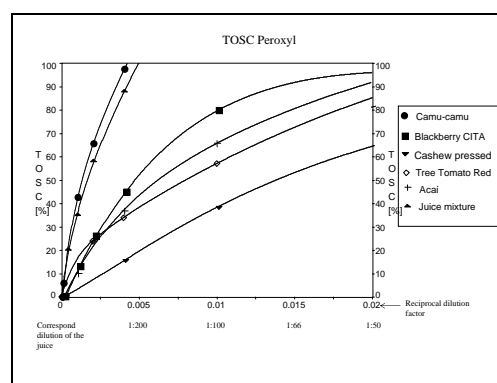


Figure 3: TOSC of açaí, blackberry, camu-camu, cashew, tree tomato and a juice mixture against peroxy radicals.

Blackberry

In Latin America blackberries are commercially cultivated from the southern highlands of Mexico to the northern Andes and contain reasonable amounts of antioxidants like ascorbic acid and polyphenols among others anthocyanins [10]. Mentionable TOSC values were found against all determined ROS. Noteworthy is the antioxidant potential against hydroxyl radicals (Fig. 5). Fruits from different origins in Latin America and Germany were studied and showed a similar behaviour whereas TOSC values for samples from Latin America were somewhat higher than that from Germany.

Cashew apple

The cashew tree originates from the north east of Brazil, a tropic region with pronounced droughts. It is cultivated on a big scale for the production of cashew nuts that are located within the bean-shaped fruit. The peduncle is thickened to a juicy and due to high amounts of glucose and fructose relatively sweet yellow to reddish pseudo fruit, representing about 90% of the complete "apple". Up to now only 2% of these pseudo fruits are further processed although they are rich in vitamin C (ca. 200 mg / 100 mL juice) and have an exotic aroma [11]. A juice of the cashew apple shows its highest antioxidant capacity against peroxy radicals, followed by peroxynitrite and hydroxyl radicals. However, compared to juices rich in anthocyanins TOSC values are lower (Fig. 3-5). Half of its antioxidant capacity against peroxy radicals can be attributed to ascorbic acid. Moreover, the glucose and fructose content contributes to the antioxidant potential particularly against hydroxyl radicals. Phenolic anacardic acid could also be identified in cashew apple juice but showed no antioxidant activity.

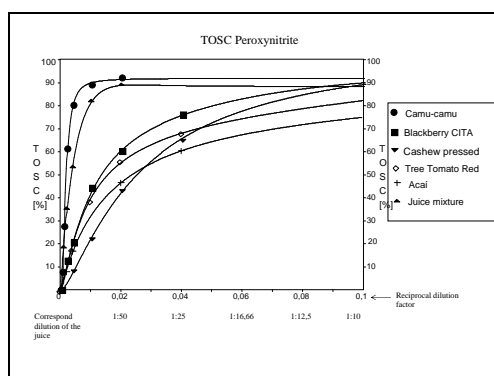


Figure 4: TOSC of açai, blackberry, camu-camu, cashew, tree tomato and a juice mixture against peroxynitrite.

Tree tomato

Tree tomatoes were originally domesticated by Indians from the tropical Andes in South America. In the meantime they are cultivated in tropical and subtropical areas all over the world. Especially in New Zealand tree tomatoes are grown with commercial success [12]. These fruits have an ascorbic acid content of 21.9 – 30 mg / 100 g fresh weight [13]. Additionally relatively high amounts of carotinoids with provitamin A activity e.g. β -carotene and cryptoxanthine were found [14].

In opposite to yellow varieties in red tree tomatoes anthocyanins are present which were mainly identified as rutinosides of pelargonidin, cyanidin and delphinidin. Overall, red tree tomatoes have shown intermediate TOSC values against peroxy radicals and peroxynitrite (Fig 3-4). Probably due to the anthocyanins concentrated in the seed jelly this part performs better against these ROS than the pulp. Only a moderate activity was observed against hydroxyl radicals (Fig. 5).

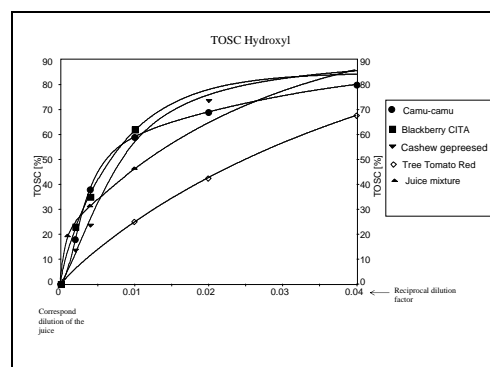


Figure 5: TOSC of blackberry, camu-camu, cashew, tree tomato and a juice mixture against hydroxyl radicals.

Conclusion

TOSC assays on five tropical fruits resulted in a ranking as follows regarding to their effectiveness to scavenge different ROS:

Peroxy radicals:

Camu-camu > açai > blackberry > cashew apple > tree tomato red.

Peroxynitrite:

Camu-camu > blackberry > açai > cashew apple > tree tomato red.

Hydroxyl radicals:

Blackberry > camu-camu > cashew apple > tree tomato red > açai.

It turned out that camu-camu is a fruit with outstanding properties against peroxy radicals and peroxynitrite. Also the potential to scavenge hydroxyl radicals is worth to mention.

These characteristics would make camu-camu a favourable candidate for bioavailability studies with the comet assay. But given the recommendation of the German Nutrition's Association for vitamin C of 100 mg / d a juice of camu-camu could only be administered highly diluted or in small quantities. This suggests mixing up camu-camu with some other investigated fruits of high antioxidant activity that is not only attributed to ascorbic acid. Beside camu-camu two other fruits can be favoured. Açai and blackberries are relatively poor sources for ascorbic acid but both have respectable antioxidant potential. Indeed, TOSC analyses of a mixture of these fruits in proportion of 12, 44 and 44% for camu-camu, açai and blackberry, respectively, showed that high TOSC values could be maintained (Fig 3-5). Despite the low addition of camu-camu the antioxidant power against peroxy radicals and peroxynitrite remained outstandingly high and was kept above levels of blackberry or açai. Only against hydroxyl radicals TOSC values were distinctly lower, possibly in consequence of the weak scavenging activity of açai.

If our juice mixture can give rise to a positive result in vivo after oral application by healthy subjects remains to be seen. In a study accomplished by Weisel et al [15] reduced oxidative DNA damages after drinking of an anthocyanin / polyphenolic-rich fruit juice has already been shown.

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