Antioxidant Indices: Do They Reflect Animal Health?

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There is increasing evidence that reactive oxygen species (ROS) and their promoted oxidative damage are involved in a large number of pathologies, as well as the aging process (Valdez *et al.,* 2000; Miller *et al.,* 1997; Luukkainen *et al.,* 1999; Cowley *et al.,* 1996). The induced oxidative stress that damages cellular macromolecules results from the difference between the production and removal of potentially damaging ROS. As the rate of removal of ROS is controlled by enzymes and a variety of low molecular weight antioxidants there is great interest in determining tissue antioxidant levels and the way they are related to pathological states.

To assess tissue capacity to remove ROS the concentration of antioxidants present in that tissue must be determined. Due to their different hydrophobic properties, antioxidants will be distributed among different cellular compartments. A comprehensive analysis of all the antioxidants is impractical due to the large number of molecules that can have antioxidant activity, therefore in these types of studies only the more important molecules such as glutathione, uric acid, thiol groups, ascorbic acid, tocopherols, bilirubin and carotenoids are analysed (Crozier *et al.,* 2000). An alternative approach is to evaluate a parameter that can give information regarding the total charge of antioxidants in a given tissue. The index thus obtained is then considered a measure of the tissue's ability to mitigate the damage associated with ROS production (Cao *et al.,* 1998).

The above considerations have lead to a variety of indices aimed at determining the total antioxidant activity present in a complex mixture of compounds. The three most used indices that measure this activity are Total Reactive Antioxidant Potential (TRAP), Trolox Equivalent Antioxidant Capacity (TEAC) and Ferric Reducing Capacity of Plasma (FRAP). A fourth index, Thiobarbituric Acid Reducing Substances (TBARS) measures the result of lipid peroxidation. We chose to determine the TEAC and FRAP indices in our plasma and food specimens because both assays were simple to perform and easily adapted to the Cobas Bio centrifugal analyser. In the future we will determine the TBARS index as a third probe into tissue antioxidant activity.

Materials and Methods

The TEAC Index.

The modified method of Re *et al.,* 1998 was used to determine TEAC in plasma and foods. The method involves the formation of the blue/green free radical cation $ABTS^{\cdot+}$ (2, 2'-azobis (3-ethylbenzothiazoline-6-sulphonic acid) which is reduced to the colourless ABTS by antioxidants. The extent of decolourisation as a percentage inhibition of $ABTS^{\cdot+}$ is a function

of sample antioxidant concentration when compared to the relative activity of Trolox as the standard. Trolox is used as the standard because it is a water soluble molecule with similar properties to the tocopherols. In plasma this index is influenced by endogenous antioxidants such as bilirubin, glutathione, urate the thiol groups of albumin as well as dietary antioxidants such as ascorbic acid, tocopherols, carotenoids and polyphenols. This index measures the free radical quenching capacity of the sample.

The FRAP Index.
The method of Benzie and Strain 1996 was used to determine FRAP in plasma and foods. This method relies on the reduction of ferric ion (Fe^{+++}) to ferrous ion (Fe^{++}) by antioxidants at low pH. The subsequent reaction of Fe^{++} with TPTZ (tripyrdyltriazine) forms an intense blue colour complex that is directly proportional to the Fe^{++} concentration. In plasma this index is influenced by endogenous antioxidants such as bilirubin, glutathione and urate as well as dietary antioxidants such as ascorbic acid, tocopherols, carotenoids and polyphenols. The thiol groups of proteins play a minor role in the FRAP index. This index measures the reducing potential of the sample.

Estimate of individual plasma constituents to antioxidant indices. Modified from Re *et al.*, (1999) and Benzie and Strain (1996)

Plasma Antioxidant	% Contribution to FRAP	% Contribution to TEAC
Ascorbic Acid	15	9
α-tocopherol	5	3
Uric acid	45	23
Bilirubin	5	2
Albumin	8	55
Unmeasured	22	10

Birds

We have on-going investigations into the sources, requirements and metabolism of carotenoids and retinoids in zebra finches (*Taeniopygia guttata*), noisy miners (*Manorina melanocephala*), red and yellow star finches (*Neochmia ruficauda*) and red-brow finches (*Neochmia temporalis*).

Zebra finches.
Ten charcoal, white and 'grey birds' were housed as trios in small cages or collectively in aviaries at the Adelaide Zoo. The experimental protocol was as follows. The birds were converted from a seed diet to a soft food diet (Wombaroo Food Products) that could be manipulated in respect to its composition. Birds were fed a non carotenoid soft food for 6 weeks then a carotenoid based soft food for 30 days. At the end of each dietary period about 120μL of blood was drawn from the jugular vein of each bird using a 0.33mm gauge needle

attached to 1mL syringe previously flushed with heparin. This protocol was restarted to evaluate the next carotenoid.

Star finches.
Four of each red star finches and yellow star finches were housed as pairs in small cages at the Passwell/Wombaroo research facility. The protocol for these birds was the same as for zebra finches.

Red-brow finches.
Twenty birds were mist netted under permit, immediately blood sampled as described above and were as trios in small cages for 3 weeks on a mixed seed diet. The birds were blood sampled at the end of the holding period.

Noisy miners
Eleven birds previously wild caught under permit were housed as groups of 3 or 4 in small aviaries. The experimental protocol was similar to that for the zebra and star finches except that the noisy miners were fed Lorikeet & Honeyeater Food (Wombaroo Food Products).

Other bird species
Several individuals were blood sampled as part of Adelaide Zoos continuing health management programme. Four princess parrots (*Polytelis alexandrae*) treated for megabacteria infection were blood sampled on admission and post treatment. A yellow-tailed black cockatoo (*Calyptorhyncus funereus*) from Gorge Wildlife Park was treated for a bacterial infection and was blood sampled on admission and during the treatment.

Mammals.

There are continuing investigations at the Adelaide Zoo into the proportion and composition of browse in the diets of the yellow-footed rock wallaby (*Petrogale xanthopus*), brush-tailed rock wallaby (*Petrogale penicillata*), tammar wallaby (*Macropus eugenii*) and Malayan tapirs (*Tapirus indicus*).

Yellow-footed rock wallabies.
Seven animals were trapped at Mount Fitton in the north Flinders ranges and blood sampled during a pouch young investigation. These samples were compared to 84 samples of yellow-footed rock wallaby, 40 samples of tammar wallaby and 22 samples of brush-tailed rock wallaby taken at Adelaide Zoo.

Malayan tapirs.
The quantity and quality of browse for these animals was reviewed in respect of their overall health. They were blood sampled on a diet containing fruit and vegetables that was relatively low in browse and then resampled about one month after the proportion of browse was increased by about 50% and the fruit removed from their diet.

Food.

Bird seeds, both green and ripe, vegetables, fruit and browse commonly fed to animals at Adelaide Zoo were assayed for antioxidant activity. The intent was to include antioxidant capacity in with the mix of other nutrients so that animal nutrition was optimised.

Results and Comments.

TEAC and FRAP values for birds fed a carotenoid enriched diet compared to one wild species.

Values expressed as mean (SEM)

		Non carotenoid diet		30 days on lutein diet 30 mg/kg		30 days on zeaxanthin diet 30 mg/kg	
	n	TEAC mM/L	FRAP mM/L	TEAC mM/L	FRAP mM/L	TEAC mM/L	FRAP mM/L
Zebra Finch	30	1.30 (0.04)	1.26 (0.08)	2.68 (0.19)	2.66 (0.10)	2.49 (0.09)	2.44 (0.14)
Red Star Finch	5	1.65 (0.05)	2.48 (0.16)	2.21 (0.16)	2.37 (0.09)	3.62 (0.13)	5.16 (0.29)*
Yellow Star Finch	5	1.53 (0.05)	3.28 (0.21)	2.09 (0.15)	3.34 (0.13)	3.67 (0.13)	5.32 (0.30)*
Noisy Miner	11	1.46 (0.04)	2.30 (0.15)	2.11 (0.15)	2.91 (0.11)	3.53 (0.12)	4.31 (0.24)*
		Wild Birds					
Red-brow Finch	20	2.44 (0.10)	4.56 (0.25)				

Zebra finch circulating TEAC and FRAP values increased significantly after 30 days on lutein and zeaxanthin diets. However their circulating carotenoid (not reported) was lower than that of the star finches and noisy miners which contributed to a significantly lower TEAC and FRAP ($p<0.5$, independent t test).

Star finch and noisy miner circulating TEAC and FRAP values increased significantly after 30 days on lutein and zeaxanthin diets and TEAC and FRAP values differed significantly between the lutein and zeaxanthin diets (* $p<0.5$, independent t test). Structurally zeaxanthin has one more conjugated double bond than lutein which confers additional antioxidant activity on zeaxanthin. During these experiments there was no significant change in plasma proteins or uric acid so the increases in antioxidant indices could be attributed to increased circulating carotenoid.

Princess Parrots
Values expressed as mean (SEM)

Date	Species	n	TEAC mM/L	FRAP mM/L
02-Sep-06	Princess Parrot MB	4	1.64 (0.02)	1.25 (0.10)
29-Nov-06	Princess Parrot MB treated	4	1.97 (0.12)*	1.61 (0.08)*

Princess parrots were admitted with megabacteria infection and treated with amphotericin dispersed over parrot pellets at the rate of 400mg/kg. After 8 weeks circulating TEAC and FRAP values were significantly different to those on admission (* $p<0.5$, independent t test).

Yellow-tailed Black Cockatoo

Date	α-tocopherol mg/L	TEAC nM/L	FRAP mM/L	Total Carotenoid mg/L
03-Apr-07	2.50	1.90	0.56	0.67
10-Apr-07	32.00	2.50	1.13	3.37
16-Apr-07	13.70	2.66	1.63	7.90

On 3rd April a yellow-tailed black cockatoo (the Eyre Peninsula critically endangered race) was admitted from Gorge Wildlife Park with a bacterial infection. A series of tests on the plasma, including α-tocopherol, TEAC, FRAP and total carotenoid, were performed on admission. The bird was immediately treated with a course of clavulox and then vitamin E injection on 5th April and the tests repeated on the 10th April. All values had increased significantly over those on admission. The large increase in a-Tocopherol was a reflection of the vitamin E injection. The tests were repeated again on the 16th May prior to release.

Mammals

Wallaby plasma TEAC and FRAP values
Values expressed as mean (SEM)

Species	n	α-tocopherol mg/L	TEAC nM/L	FRAP nM/L
		Adelaide Zoo Animals		
Brush tail rock wallaby	22	6.61 (0.78)	2.06 (0.12)	0.49 (0.07)
Tammar wallaby	40	6.68 (0.83)	2.21 (0.08)	0.51 (0.05)
Yellow foot rock wallaby	84	6.17 (0.33)	2.31 (0.08)	0.64 (0.04)
		Mt Fitton Animals		
Yellow foot rock wallaby	7	14.21 (3.11) *	2.56 (0.01) *	1.07 (0.06)*

The wild Mount Fitton yellow-footed rock wallabies had significantly greater circulating TEAC and FRAP values (* $p<0.5$, independent t test) than any of the wallaby species at Adelaide Zoo. The main contributor to this was the significantly higher circulating level of α-tocopherol which probably originated from the amount and type of browse in their diet. This has lead to a reduction of fruit and root vegetables and an increase of native browse in the zoo wallaby diet.

Malayan Tapirs

Date	Animal	α-tocopherol mg/L	TEAC mM/L	FRAP mM/L	Total carotenoid mg/L
15-Feb-07	Mia	6.80	1.18	0.16	0.05
26-Jan-07	Surlong	5.80	1.47	0.06	0.12
30-Apr-07	Jalita	7.30	0.79	0.26	0.22
Mean		6.63	1.15	0.16	0.13
24-Jul-07	Mia	7.90	3.09	0.42	0.34
27-Jul-07	Sulong	9.20	3.29	0.67	0.37
24-Jul-07	Jalita	10.70	3.10	0.42	0.28
Mean		9.27*	3.16*	0.50*	0.33*

The diet of the Malayan tapirs was similar to that of the other herbivores at Adelaide Zoo, relatively high in fruit and vegetables and low in browse.. After the browse component was increased 50% in the diet from June 07 the plasma TEAC, FRAP, α-tocopherol and total carotenoid increased significantly (* $p<0.5$, independent t test).

Antioxidant indices of some browse

Description	α-tocopherol mg/kg	TEAC mM/L	FRAP mM/L	Total Carotenoid mg/kg
Lucerne Hay -old	11.1	68.3	52.1	592.3
Lucerne Hay -fresh	21.6	129.3	104.4	1011.3
Ficus Leaf	150.2	71.0	146.7	494.9
Variegated Ficus Leaf	13.0	25.8	154.9	139.5
Fiddlewood Leaf	44.0	85.8	139.4	307.3
Eucalyptus sp new leaf	16.2	156.3	581.7	140.3
Eucalyptus sp old leaf	32.3	147.1	456.6	493.3
Kikuyu	28.3	54.8	28.7	107.3
Jacaranda Leaf	73.2	158.4	114.4	175.5
Poplar Leaf	79.0	139.4	120.4	375.3
Saltbush Leaf	16.2	45.9	30.8	174.9
Microleana stipoides leaf	28.8	136.6	104.3	883.6
Danthonia sp leaf	30.1	73.4	57.0	556.9
Melichris sp leaf	80.3	375.6	737.7	296.5

The α-tocopherol and total carotenoid content in fresh browse produces their relatively high TEAC and FRAP values. This coupled with a generally higher solids and protein content makes browse a superior food to fruit and vegetables for most herbivores. See the tables of vegetables and fruit for comparison.

Antioxidant Indices of Some Vegetables

Description	α-tocopherol mg/kg	TEAC mM/L	FRAP mM/L	Total Carotenoid mg/kg
Yellow Squash	8.6	13.6	32.5	10.1
Corn	7.3	11.1	21.3	22.6
Cos Lettuce	7.4	8.9	15.6	68.0
Red Lettuce	5.4	13.8	18.3	165.7
Green Lettuce	3.5	12.5	16.1	162.0
Cauliflower	1.8	28.4	5.0	3.3
Swede Turnip	9.8	9.1	4.8	6.3
Carrot	7.7	21.6	14.8	108.3
Broccoli	3.0	27.4	16.7	8.3
Brussel Sprout	1.2	22.3	31.0	4.4
Yellow Capsicum	12.3	14.5	29.9	57.1
Orange Capsicum	9.5	20.2	27.8	77.5
Red Capsicum	18.3	10.5	41.8	86.2
Red Potato	0.9	3.8	4.9	1.8
White Potato	1.3	4.2	3.5	0.3
Sweet Potato	2.0	10.5	12.4	37.5

Antioxidant Indices of Some Fruit

Description	α-tocopherol mg/kg	TEAC mM/L	FRAP mM/L	Total Carotenoid mg/kg
Tomato	1.1	21.3	45.4	20.3
Orange	1.8	18.6	62.4	11.3
Mango	8.6	19.5	75.2	15.0
Paw paw	6.2	17.8	82.7	22.9
Peach	1.2	14.3	53.3	11.4
Red Rhagodia	8.1	32.6	98.3	13.8
Ruby Salt Bush	7.5	41.8	102.6	19.3
Persimmon	1.1	16.4	52.6	9.1
Paddy Melon	5.1	56.4	68.4	11.6
Duchess Pear	0.8	10.5	22.2	2.1
Hawthorn Berry	28.8	25.9	99.1	35.8
Packham Pear	0.5	15.9	9.9	0.7
Green Apple	0.7	12.6	32.4	1.0

Antioxidant Indices of Some Green Seed

Description	α-tocopherol mg/kg	TEAC mM/L	FRAP mM/L	Total Carotenoid mg/kg
Ehrharta erecta-Veldt grass	24.4	132.6	78.3	158.4
Red Millet	17.4	128.2	61.9	171.6
Jap Millet	11.8	119.3	58.2	72.3
Setaria palmifolia-Palm grass	20.8	138.5	85.3	42.6
Plain Canary Seed	15.3	119.9	67.7	112.1
French White Millet	10.9	108.3	52.4	115.7
Oats	13.8	116.8	51.6	53.2

Antioxidant Indices of Some Ripe Seed

Description	α-tocopherol mg/kg	TEAC mM/L	FRAP mM/L	Total Carotenoid mg/kg
AHRC Seed Mix 1	9.9	10.1	6.4	6.6
Ehrharta erecta-Veldt grass	18.0	82.6	61.2	5.1
Finch Mix	4.8	18.6	9.8	4.4
French White Millet	3.3	9.5	5.5	6.7
Grey Stripe Sunflower Seed	14.8	129.0	69.2	8.6
Hulled Oats	6.1	12.7	4.7	5.7
Jap Millet	4.1	21.2	13.4	3.7
Maw Seed	5.8	21.9	10.4	6.2
Niger Seed	15.2	143.1	82.6	3.2
Panicum	7.6	7.3	10.5	8.4
Plain Canary Seed	9.0	23.6	12.0	17.5
Red Millet	4.9	10.5	19.4	10.3
Wheat	4.6	15.2	5.7	3.8

Green seed has significantly higher TEAC and FRAP values than ripe seed. This is due to the higher α –tocopherol and total carotenoid content of green seed. The green seed values would account for the high circulating TEAC and FRAP found in the wild red-brow finches. This perhaps confirms that some avicultural practices like feeding green seed during the breeding season when it would be naturally available are correct and why they have persisted over time.

The higher TEAC and FRAP values for the ripe oil-seeds, niger and sunflower, are the product of higher α –tocopherol, protein and unsaturated fat content.

Summary

Over the past 9 months we have performed some 1000 TEAC and FRAP assays on animals associated with either controlled experiments or clinical examinations at Adelaide Zoo and the data certainly suggests that TEAC and FRAP assays reflect nutritional status and the capacity to control oxidative stress in animals. Therefore it could be argued that antioxidant indices do reflect animal health. TEAC and FRAP values in addition to PCV, haemoglobin and total solids tell more about the animal and its environment which makes the information more useful to ecologists and veterinarians giving health certificates.

Over time we intend to establish some norms for the species that we regularly test because although low FRAP indices probably reflect low nutritional status and poor control over oxidative stress there is some evidence that high levels may indicate possible toxicity. All molecules that have reducing power are not necessarily nutrients eg cyanide.

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