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Understanding antioxidants and substantiating claims

Most people today accept without question the importance of vitamins and minerals to health and that extreme deficiency can lead to serious diseases such as scurvy. However, in the 18th Century no one knew what caused scurvy, a disease which had so many symptoms that it was frequently mistaken for asthma, leprosy, syphilis, dysentery and madness. And while physicians speculated as to its cause, people became aware that once victims of scurvy were on shore, they could be cured by eating scurvy grass, wild celery, wood sorrel, nasturtiums, brooklime, Kerguelen cabbage, cabbage trees and fruit and palm wine. In 1753, with the publication of James Lind's Treatise of the Scurvy (recording, perhaps, the first controlled trial ever), there was experimental evidence that citrus had a beneficial effect on prevention of diseases such as scurvy.[1] While these claims to cure, and later prevent scurvy, could not then be substantiated by knowledge of underlying biochemical mechanisms, the value of these anecdotal observations were certainly worthy of consideration.

What is now essential in making claims is that they should be based on acceptable and reproducible experimental evidence and not on suppositions, hopes or expectations.

Among the few words that have migrated from the laboratory to the high street is "antioxidant". Just about everyone seems to be familiar with the word. Nutraceuticals, functional foods, cosmetics, cosmeceuticals and a whole range of personal care products may include the antioxidant word in their promotion and antioxidant materials in their substance. Manufacturers of these products, aware of the current and growing interest in antioxidants, should now be prepared to substantiate, whenever possible, any claims they make for their product from the results of rigorous analysis. And when ethical considerations and practical constraints make it impossible to carry out the otherwise desirable clinical trials, advertisers should not imply they have done so. If you say something is "clinically proven", you will be expected to have carried out clinical trials, designed rigorously to knock down your hypothesis, not designed to prove it. If a complaint should be made to the Advertising Standards Authority, you will be required to provide results of any testing carried out before you made the claim. The message is "be clear and honest, and your customers will have confidence in your products".

Whether or not a product can transfer antioxidant activity to the user is, of course, a very important consideration. However, before a product can be tested for efficacy, it must itself have first been tested for antioxidant content. And if the product is irradiated or treated in any other way to conform to safety requirements, the antioxidant testing must be carried out on the treated product. Irradiation can lead to generation of free radicals, and these are likely to attack the antioxidants in the product.[2]

Knowing that a product contains ingredients with recognised antioxidant activity is not good enough. It is at least prudent, if not essential, to measure the antioxidant activity of individual ingredients in a formulation in order to assess whether there is any positive or negative synergy between ingredients. This information is extremely useful in optimising the formulation and it could save considerable expense. More is not necessarily better![3] It is also essential to know the stability of the antioxidants during storage and use in order to recommend dosage and frequency of application.

What are antioxidants?

The term antioxidant comprises a variety of substances and includes enzymes that neutralise potentially damaging reactive species. Most of these reactive species contain oxygen.[4] Some, but not all of these "reactive oxygen species" (ROS) are free radicals: that is molecules with an excess of negative charge.[3] While free radicals and other non-radical ROS are produced by the body and play an essential part in the body's biochemistry, an excess of them can have dire effects. Antioxidants can neutralise this excess if active and in sufficient amounts at the site of ROS production. However, when ROS production

gets out of control, oxidative stress occurs.[5] When this happens, these reactive chemical species can injure or even kill cells, damage DNA and attack enzymes and other compounds.

Oxidative stress and the skin

Oxidative stress is bad for the skin.[4,6] Free radical damage can cause deterioration of the supportive connective tissues resulting in decreased elasticity and resilience.[7] Exposure of skin to solar ultraviolet radiation starts photochemical reactions in the skin, leading to ROS formation. Sun damage produces both skin cancers as well as photo-ageing which shows itself on the skin as wrinkling, scaling, dryness and mottled pigmentation. Antioxidants have the potential to play an important part in protective and repair mechanisms within the skin.[8,9] The antioxidants of most significance in slowing down free radical damage to the skin are: the vitamins A, C and E, the enzyme superoxide dismutase, the group of chemical compounds known as flavonoids, and the individual chemical substances beta-carotene, glutathione and the co-factors selenium and zinc.[10] But there are others of lesser-known significance.

Personal care products

A great deal of research effort is presently devoted to understanding and measuring the types and functions of antioxidants and their role in the oxidative status of the individual.[5] Many studies are aimed at demonstrating beneficial health effects resulting from topical application of preparations containing antioxidants.[11] While the underlying mechanisms for these effects are not fully understood, enough is known about the distribution, activity and regulation of antioxidants in the skin to enable the development of pharmaceutical and cosmetic strategies involving antioxidant formulations.[12,13] These developments are aimed at reducing the risk of UV-induced cancers, photo-ageing and desquamatory skin disorders.[14,15]

Antioxidant concentration versus antioxidant capacity

Measuring the concentration of specific antioxidants in a sample is one approach to quality assurance. However, this approach can be misleading because the sum of the individual antioxidants present in a sample may not match the total antioxidant activity of the sample.[16] This can be explained in two ways: first the actual ability of the individual antioxidants in the sample to quench free radicals and other reactive species, that is their antioxidant capacity, may vary weight for

weight, and secondly the complex mixture of molecules in natural materials may act in synergy, leading to enhanced, or perhaps diminished, antioxidant capacity, compared to the sum of the individual antioxidants. It is therefore more revealing to use the total relative antioxidant capacity (RAC) of a unit weight of the sample.[17] This is analogous to the specific activity of enzymes in which enzyme activity is expressed per weight of protein.

Factors affecting differences in antioxidant capacity of materials from different sources may be related to conditions of cultivation, storage and transportation prior to, and during, processing. In addition, the treatment of ingredients and finished products before, during and after manufacture may affect their total antioxidant capacity. For example, irradiation of ingredients as well as of the finished product will lead to loss of antioxidant activity because free radicals produced during irradiation may attack some or even all of the antioxidants present in the sample.[18] And if all the antioxidants are attacked, but free radical production continues, then pro-oxidants, molecules that when attacked by free radicals produce even more free radicals, may be generated, sometimes from antioxidants that have lost their own activity.[19,3]

During processing and manufacture of products, free radicals can be produced from: grinding, compacting, exposure to UV light, drying, heating and especially solubilising materials by sonication.[20,21] It is therefore desirable to monitor the RAC of the ingredients, and of the finished product throughout the various stages of manufacture, and if necessary, to change procedures to avoid losses. To obtain valid use-by dates, the manufacturer should also test the finished product during storage. The cost benefit of doing this testing is obvious in relation to manufacturing efficiency, but it will also give added confidence to the discerning customer, and thus, afford a competitive advantage to the product.

Are antioxidants in products good or bad?

During photosynthesis, green plants convert solar energy into redox energy.[22] The high concentrations of polyphenols, carotenoids, tocopherols, tocotrienols, glutathione, ascorbic acid and enzymes with antioxidant activity (redox-active secondary metabolites, or antioxidants) in plants are related to protection afforded to the plant from potential oxidative damage resulting from photosynthesis.[23] Animal cells do not have such an armoury of protection against damaging ROS, and as a consequence, the pathogensis of most chronic degenerative diseases and ageing is considered to involve mechanisms of oxidative stress.[5,24]

Oxidative stress also occurs as a result of inflammation by the production of ROS from the inflammatory leucocytes as well as from tobacco smoke, environmental pollutants, food constituents, drugs, radiation, ethanol. Many epidemiological studies, experimental animal studies and *in vitro* experiments with cell culture support the hypothesis that antioxidants protect against potential damage from oxidative stress. However, caution must be taken when formulating products with the object of providing antioxidant benefit, as there have been some large randomised intervention trials using α-tocopherol or β-carotene that have not been supportive.[25]

In addition, assessing whether the product exhibits hormesis, (that leads to free radical generation at particular concentrations and mixtures of antioxidants) should help to avoid harmful effects of the product while enabling the product to be optimised for its ability to prevent oxidative stress.[2,16-18]

Making claims

If claims are made about:

- The antioxidant activity of a finished product, then the finished product must be tested and the antioxidant activity quantified.
- The antioxidant activity of the ingredients in a finished product, then those ingredients will need to be tested separately.
- The product's shelf life, then the results of stability tests for stated timed intervals need to be stated, or if accelerated shelf-life trials were used, this qualification should be included.
- Possible benefits acquired by using a product on the skin, then being able to demonstrate that the product could be absorbed by the skin could allow you to

Table 1: The analysis of grape seed extract using two methods, ABEL-RAC and ORAC

ABEL-RAC score per mg (divided by 1,000)

Concentrations µg/mL	0.63,1.3, 2.5,5,10	0.5,1.0, 2.0,4.0,8.0	0.75,1.5, 3.0,6.0,12.0	Mean	% cv
Day 1 score	545	551	535	544	1.5
Day 2 score	534	551	524	536	2.5
Day 3 score	553	570	536	553	3.1
Mean	544	557	532	544	2.4
% cv	1.8	2.0	1.2	1.5	

ORAC score (divided by 100) as µmol Trolox equivalent units per g

Concentration µg/mL	1	2	4	8	16	Mean	% cv
Day 1 score	412	360	259	173	116	264	47.0
Day 2 score	283	287	234	164	120	218	34.0
Day 3 score	322	303	235	168	126	231	36.5
Mean	339	317	243	168	121	238	39.5
% cv	19.5	12.1	5.7	2.6	4.1	10.0	

make indirect claims to possible benefits of using the product.

- The health benefits of using a product with quantified antioxidant activity, then results of trials need to be presented in order to demonstrate efficacy or the claim qualified by suggesting only the possibility of a health benefit.

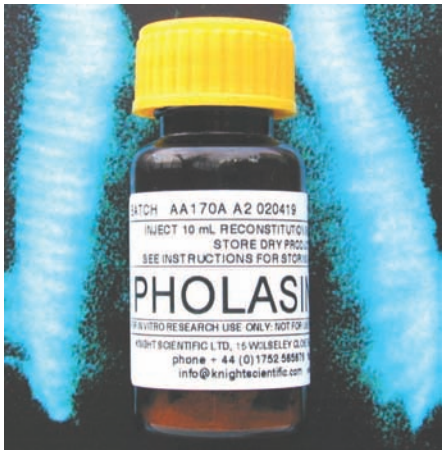
It is essential that any claims made in marketing and advertising literature, as well as on the product package, are to be supported by evidence obtained from testing, and if relevant, clinical trials. Never assume that because a product contains ingredients accepted as having antioxidant properties that the finished product is antioxidant. In order to make such a claim, the finished product would need to tested for antioxidant activity. And in order to claim that the use of a particular antioxidant product led to beneficial effects, you would need to demonstrate efficacy in its recommended use. However, you could make lesser claims if you referred to published clinical trials providing your literature made this point clearly and did not lead the reader to assume that the particular product being promoted had been used in the clinical trial referred to. However, there would be no point in carrying out clinical trials unless the product had first been tested and shown to have high antioxidant capacity which remained stable for the duration of its shelf-life. Similarly, establishing a theoretical dose and frequency of use, could be estimated from *in vitro* tests. While these are no substitute for clinical trials, carrying out rigorous *in vitro* tests could save considerable time and money in designing and carrying out such trials. There is no point in running a clinical trial to test antioxidant efficacy if the product has lost its activity, or even worse, has become pro-oxidant.

Measuring antioxidant activity of products and their ingredients

Many analytical methods exist for the measurement of the concentration of individual ingredients. However, other methods aim to measure the functional capacity – that is the ability of a material to neutralise free radicals. Functional methods are more closely related to the final biochemical function of the material in the body. In most of these methods the sample is exposed to free radicals and/or oxidants and the degree of quenching of the free radicals/oxidants is measured and sometimes quantified against a standard of known concentration.

Such tests include:

- TEAC (Trolox-Equivalent Antioxidant Capacity) involving production of a coloured intermediate which is decolourised on exposure to antioxidants.[26]
- ORAC (Oxygen Radical Absorbance Capacity) involving a fluorescent probe that loses activity when challenged by peroxyl radicals with its decay retarded by antioxidants.[27]
- TRAP (Total Reactive Antioxidant Potential) in which peroxyl radicals are generated and used in the presence of a fluorescent molecule which decays when attacked by the radicals.[28]
- FRAP (Ferric reducing ability of plasma) in which plasma, adjusted to pH 3.6, is used to reduce ferric 2,4,6-tripyridyl triazine complex to ferrous ions; the reduction is measured at an absorbance of 593 nm with the result quantified against the reducing capacity of Trolox used as a standard in the assay.[29]
- Bleaching of crocin (purified from saffron) is another TEAC test using 2,2'-Azobis-(2,-amidinopropare) dihydrochloride as a peroxyl radical donor.[30]
- ABEL (Analysis By Emitted Light) Antioxidant Assays with Pholasin[31,32] a protein that emits light with free radical and other non-radical ROS, in which the material to be tested is challenged by a range of different ROS. The result can be compared to antioxidant standards and/or by an ABEL-RAC score which is the reciprocal of the EC_{50} (the concentration of sample that reduces the light of Pholasin by half) multiplied by 100.[2]



ABEL assays, in addition to quantifying antioxidant activity, identify pro-oxidants and/or the formation of pro-oxidants as a function of concentration of antioxidants in the product and any synergy between constituents. Knowledge of the latter is fundamental to a consideration of formulations and dosages and to understanding how to possibly avoid adverse reactions. A precise and accurate way to compare the antioxidant activity of ingredients and finished products, batch to batch and between different products, is with the ABEL-RAC antioxidant scoring system.[2,16-18,33]

ABEL (Analysis By Emitted Light) Antioxidant Assays with Pholasin

There are currently six different antioxidant tests for quantifying antioxidant (and pro-oxidant) capacity based on the use of Pholasin, a substance that emits light in the presence of free radicals and oxidants.[34,31] In these tests, samples containing unknown antioxidants are challenged with defined oxidants:

superoxide (high concentration), superoxide (enzymatically produced), hydroxyl radical, peroxynitrite, peroxyl radical and hypochlorous acid, in the presence of the luminescent material. The ABEL-RAC scores per mg of test material are easy to understand and enable comparisons to be readily made between different materials, batches and other products. And these assays are the only ones that can identify those ingredients that do not follow typical dose/response relationships but are pro-oxidant at some concentrations and antioxidant at others. Such unusual behaviour is known as hormesis.[2,16]

In a sixth assay, the test material is exposed to white blood cells that are stimulated to produce free radicals.[35]

ABEL antioxidant tests are used for QC of materials before, during and after processing and for QA of finished products. They have also been used to determine the total antioxidant capacity (TAC) of blood from people, horses, dogs, rodents, birds and badgers with the advantage that only a small amount (5 µL of serum or plasma) is needed for a test. Such assays have been used to monitor changes in TAC following intervention studies with horses.[35]

Of all the tests discussed above, ORAC is the best known and has been used to score foods for antioxidant capacity. However, the accuracy of the ORAC method, especially for materials with strong antioxidant activity, is extremely poor and not useful when measuring shelf life and measuring batch to batch consistency.

A very careful comparison of the two methods, ORAC and ABEL-RAC was carried out in our laboratory using grape seed extract, a well known strong antioxidant. The results are shown in Table1.

ORAC scores are derived from single concentration measurements, whereas ABEL-RAC scores are derived from the linear regression of 3-5 concentrations of the analyte. The comparison here is between concentration ranges for ABEL-RAC and individual concentrations for ORAC. In carrying out the ORAC method[35] it has been suggested that an assay is run to establish a concentration that is on scale and that the score is determined from taking any sample that is on scale. It is implied that the result will be the same irrespective of what concentration is selected. As can be seen in the table, while the reproducibility, in terms of the coefficient of variation of the results from day to day for one concentration is from 2.6%-19.5%. However, the coefficients of variation for a sixteen-fold range of concentrations range from 34-47 while the range of results for the five concentrations and the three days is from 121 to 412. This is to be compared with a range of 524 to 570 for a similar range of concentrations for ABEL-RAC. And while there is a clear inverse relationship between the ORAC score and the concentration of the grape seed extract, which concentration should be used in quoting the ORAC value?

It would therefore be misleading to use ORAC results for labeling on packages or for shelf life and batch to batch reproducibility. The ABEL-RAC method is suitable for claims of batch to batch reproducibility and shelf life when up to 5% deviation is considered acceptable.

Conclusion

In conclusion the message is to only make claims that can be substantiated by results obtained from rigorous testing. Where this is not possible, other lesser claims can be made but they must be qualified for what they are.

What is important is that the consumer can make informed decisions based on knowledge of the facts, rather than the manufacturer's hopes or expectations. PC

- The following are registered trademarks: ABEL, Pholasin, Trolox. ABEL-RAC is a trademark.

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