In Vitro Assessment of Antioxidant Properties of Cocoa Extract in Cosmetic Formulation: Comparison between Analysis by Emitted Light (ABEL) & ORAC Antioxidant Capacity Methods

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Introduction

It is difficult to precisely measure the ability of anti-oxidant ingredients and products to scavenge free radicals. This ability is distinct from the concentration of individual ingredients with claimed antioxidant properties, i.e. the sum of the individual antioxidants may not match the total antioxidant activity of the sample [1].

ORAC (Oxygen radical absorbance capacity) method is most commonly used to test cosmetics and foods for anti-oxidant capacity [2,3]. However, the accuracy and precision of the ORAC method, especially for materials with strong antioxidant activity, is very poor and not useful for measuring shelf life or for measuring batch-to-batch consistency.

ABEL (Analysis by emitted light) antioxidant assays use **Pholasin -** a protein that emits light in the presence of free radicals and other non-radical ROS [4] . In the series of assays, test material is challenged by a range of different ROS. The result can be compared to antioxidant standards, or presented as an ABEL relative antioxidant capacity (ABEL-RAC) score [5].

Aim

This study aims to evaluate the novel ABEL-RAC method for quantifying the antioxidant potential of skincare ingredients and products, and to compare the accuracy and reliability of this method to the more established ORAC method.

Material and Methods

Materials

High polyphenol-content Cocoa extract was obtained from *Theobroma cacao* beans using special manufacturing procedure (Barry Callebaut, Belgium). Cream formulation containing propylene glycol, carbomer, sorbitan stearate & sucrose cocoate, isopropyl myristate, dicaprylyl carbonate, triethanolamine and preserved water was formulated as a vehicle for Cocoa extract (placebo cream). Two test creams were prepared, with 0.75% and 3.00% of Cocoa extract (CE), respectively.

ABEL antioxidant test kits with Pholasin using halogenated oxidants, peroxynitrite and enzyme-generated superoxide, respectively, were supplied by Knight Scientific Ltd, UK. For the **ORAC assay** all chemicals were purchased from Sigma, UK. For antioxidant analysis, all reagents and standards were freshly prepared at the beginning of each testing day.

Methods

The ORAC assay was performed according to the Fluorescent Microplate Based ORAC Assay Kit instructions obtained from Oxford Biomedical Research, USA.

The area under the fluorescence curve was used to quantify the antioxidant capacity of test sample by comparison to a standard curve obtained using a range of concentrations of a water soluble vitamin E analogue, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) The Trolox standards used were 12.5, 25, 50, 100, 200 µM (working concentrations). The ORAC score was derived from the blank-corrected linear regression curve for Trolox, and units expressed as µmoles Trolox equivalent units per gram of raw material (µmol TE/g). ***Figure 1*** illustrates a typical response of Trolox standards in the ORAC assay.

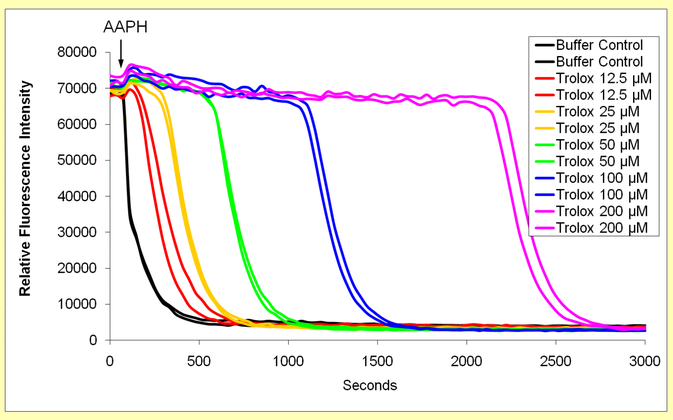

Figure 1. *Typical analysis of Trolox standards in the ORAC assay. The area under the fluorescence curve is used to quantify the antioxidant capacity of a sample by comparison with the Trolox standards*.

The ABEL assays were performed using a BMG Lumistar Galaxy microplate reader, equipped with on-board injectors (BMG Labtech, Aylesbury, UK) and flat-bottomed, white 96-well microplates (Greiner Bio-One Ltd., UK). The final assay volume in each microplate well was always 200 µL.

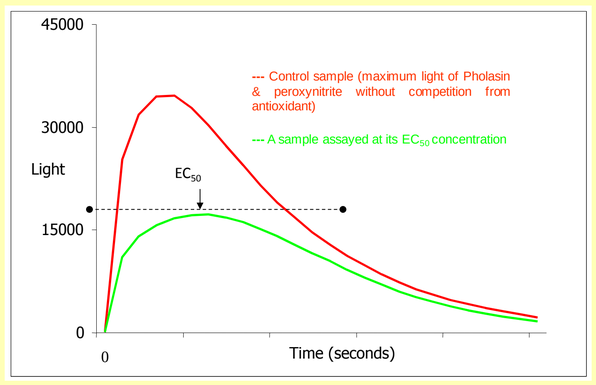

Figure 2. *Effective concentration of the test material (normalized to g/L or mg/mL) that reduces the light produced by Pholasin and ROS by 50% (EC_{50}); EC_{50} is used to calculate ABEL-RAC scores.*

Three types of ABEL antioxidant assays were performed:

- **with halogenated oxidants**
- **with peroxynitrite**
- **with enzyme-generated superoxide**

Calculation of ABEL-RAC scores

Separate light response curves were produced for each concentration of a material tested, as well as for solvent control. The **EC_{50} (effective concentration)** is the concentration (normalized to mg/mL) of a material that reduces the light produced with Pholasin and the free radical or other ROS by 50% (***Figure 2***). The greater the amount of material required to reduce the light by half, the weaker the antioxidant capacity. The EC_{50} values are converted to positive relative antioxidant capacity scores (ABEL-RAC mg scores) for each free radical or oxidant used to challenge the test material. **ABEL-RAC mg scores are the reciprocal of the EC_{50} values multiplied by 100.** Therefore, the higher the ABEL-RAC mg, the higher the antioxidant capacity of the sample.

Results and Discussion

Very high antioxidant capacity scores of the Cocoa extract were measured with both ORAC and ABEL-RAC methods (***Tables I and II***), which is in agreement with its published ORAC values of 11,000 µmol TE/g (Barry Callebaut, Belgium). It is known that the ORAC method uses the net area under the curve (AUC) of one concentration of a test sample to calculate the ORAC score, by comparison to a Trolox standard curve [3]. To assess ORAC intra-assay reproducibility, we calculated ORAC scores using different concentrations of the same sample in the same assay. ***Table I*** shows that the ORAC scores (per gram) of CE were highly dependent on the concentration used for testing. The biggest variation in the ORAC score in a single assay produced a range between 1290 and 11306 µmol TE/g, resulting in coefficient of variation (CV) value of 57.3% for the four different concentrations tested. The differences in ORAC scores may be due to the fact that the net AUC is not linearly related to the sample concentration as it is with Trolox.

ORAC Score (µmol Trolox Equivalent per g)						
	Test Concentration of Cocoa Extract (µg/mL)					
	0.16	**0.31**	**0.63**	**1.25**	**Mean Conc.**	**CV (%)**
Assay 1	1290	11258	11306	9395	**8312**	**57.3**
Assay 2	1896	9532	8715	8653	**7199**	**49.4**
Assay 3	4284	12513	12256	9717	**9692**	**39.4**
Mean of Assays	**2490**	**11101**	**10759**	**9255**		
CV (%)	**63.6**	**13.5**	**17.0**	**5.9**		

Table I. Summary of ORAC scores for Cocoa extract, tested in 3 independent experiments and at 4 concentrations

In contrast to the ORAC method, ABEL-RAC scores are not derived from comparison to an antioxidant standard such as Trolox, thus eliminating the assumption that all samples behave the same as a single standard. Secondly, they are not calculated from a single sample concentration; instead a range of concentrations is tested to get a single score.

	ABEL-RAC Peroxynitrite Assay per mg Cocoa Extract				
	Dilution Ranges: µg/mL				
	1,2,4,8	**0.75,1.5,3,6**	**1.25,2.5,1,10**		
	1	2	3	**Mean**	**CV (%)**
Assay 1	581434	595830	609219	**595494**	**2.3**
Assay 2	601339	596354	583463	**593719**	**1.6**
Assay 3	569583	592036	598951	**586857**	**2.6**
Mean of Assays	**584119**	**594740**	**597211**		
CV (%)	**2.7**	**0.4**	**2.2**		

Table II. Summary of ABEL-RAC scores for Cocoa extract, tested in 3 independent experiments in 4 ranges of concentrations

This eliminates variability that may be introduced when calculating scores using a single point calculation, as shown in the ORAC assay. It also means that even when different concentration ranges are used, ABEL-RAC scores are highly reproducible, with CV values < 3% between ranges (***Table II***). Simalar findings were obtained when day-to-day reproducibility of ORAC and ABEL-RAC scores were assessed (***Tables I and II***).

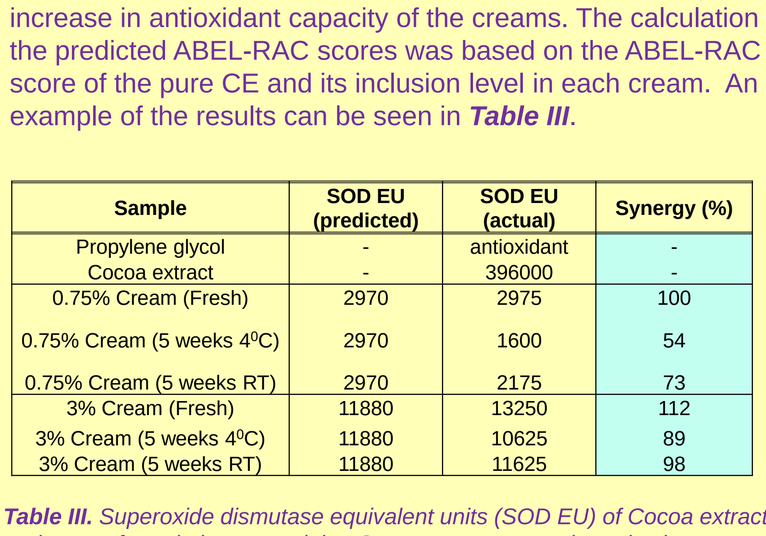

Figure 3. Light response curves for a range of concentrations of Cocoa extract (dissolved in propylene glycol) tested in the ABEL antioxidant assay using peroxynitrite. Peroxynitrite is formed in the assay by the reaction between superoxide anion and nitric oxide, released simultaneously and continually from 3-morpholino-sydnonimine hydrochloride (SIN-1), injected at time zero.

In the second part of the study, a range of cosmetic formulations and ingredients were tested in three ABEL antioxidant assays to monitor the effect of CE incorporation (at different levels) in cosmetic creams, and to monitor their shelf life over 5 weeks. For all three ROS challenges, the addition of CE (0.75 and 3 %) to the emulsion cream resulted in a measurable dose-response increase in antioxidant capacity of the creams. The calculation of the predicted ABEL-RAC scores was based on the ABEL-RAC score of the pure CE and its inclusion level in each cream. An example of the results can be seen in ***Table III***.

Sample	**SOD EU (predicted)**	**SOD EU (actual)**	**Synergy (%)**
Propylene glycol	-	antioxidant	-
Cocoa extract	-	396000	-
0.75% Cream (Fresh)	2970	2975	100
0.75% Cream (5 weeks 4^{0}C)	2970	1600	54
0.75% Cream (5 weeks RT)	2970	2175	73
3% Cream (Fresh)	11880	13250	112
3% Cream (5 weeks 4^{0}C)	11880	10625	89
3% Cream (5 weeks RT)	11880	11625	98

Table III. *Superoxide dismutase equivalent units (SOD EU) of Cocoa extract and cream formulations containing Cocoa extract as evaluated using superoxide (enzyme-generated). Predicted SOD EU values are based on the SOD EU score of the pure Cocoa extract and the inclusion level (w/w) of the Cocoa extract in the cream formulations. The % synergy is the actual SOD EU score represented as a percentage of the predicted SOD EU score.*

Conclusion

ABEL antioxidant method has proven to be of great value to the antioxidant testing of cosmetic ingredients and products. It generates a wide range of relevant data and shows superior reproducibility in comparison with the ORAC method. 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. The method is particularly useful in testing ingredients of natural origin and resulting formulations.

Acknowledgement

Pholasin and ABEL are registered trademarks of Knight Scientific Ltd.

References

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