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An interlaboratory comparison of methods used to assess antioxidant potentials[1]

J. Buenger*, H. Ackermann*, A. Jentzsch†, A. Mehling‡, I. Pfitzner§, K.-A. Reiffen*, K.-R. Schroeder¶ and U. Wollenweber**

*Merck KGaA, Darmstadt, †BASF AG, Ludwigshafen, ‡Cognis Deutschland GmbH & Co.KG, Duesseldorf, §BioTeSys GmbH, Esslingen, ¶Henkel KGaA, Duesseldorf and **Degussa Goldschmidt Personal Care, Essen, Germany

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Abbreviations: ABAP, 2,2′-azobis(2-aminepropane); ABTS, 2,2′-azino-bis(3-ethyl-benzothiazoline-6-sulfonic acid); BHT, 3,5-di-tert-butyl-4-hydroxytoluene; DCF, 6-carboxy-2′,7′-dichlorodihydrofluorescin diacetate; DPPH, 2,2-diphenyl-1-picrylhydrazyl; PCL, photochemolumiescence; ROS, reactive oxygen species; 4-MBC, 4-methylbrenzcatechin; TBA, thiobarbituric acid; TEAC, trolox equivalent antioxidant capacity assay.

[1]All authors contributed equally to the study.

Synopsis

Many analytical methods are used to measure the antioxidative activity of substances yet little is known about the comparability of the test results between laboratories. After an initial evaluation of a broad range of methods conducted by one laboratory, the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, the trolox equivalent antioxidant capacity (TEAC) assay, the lipid assay (or 2,2′-azobis (2-aminepropane) (ABAP) assay) and the thiobarbituric acid (TBA) assay were selected to be evaluated in the interlaboratory study. The antioxidative potentials of trolox, tocopherol, lipochroman-6, ascorbic acid, 4-methyl-brenzcatechin, and/or 3,5-di-tert-butyl-4-hydroxytoluene (BHT) were assessed using each of the methods. These methods were then evaluated in respect of their reproducibility and classification properties. Based on the results of this study, the DPPH assay followed by the TEAC assay yielded the best results based on reproducibility and sensitivity both within one laboratory and between laboratories. The results of the interlaboratory study were then compared with the single center results obtained from the commercially available photochemolumiescence (PCL) kit. To assess the transferability of chemical data to biological systems, they were also compared with the single center results obtained using the cell-based Dichlorodihydrofluoresceine (DCFH) assay.

Correspondence: Joachim Buenger, Merck KGaA, Frankfurter Straße 250, D-64293 Darmstadt, Germany. Tel.: +49 6151 72 6121; fax: +49 6151 72 8217; e-mail: joachim.buenger@merck.de

Résumé

Beaucoup de méthodes analytiques sont utilisées pour mesurer l'activité anti-oxydante de substances; toutefois peu d'information est disponible quant à la possible comparaison de résultats de tests entre laboratoires. Après une première évaluation d'une large variété de méthodes effectuées par un laboratoire, les méthodes suivantes à savoir le 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, le trolox equivalent antioxidant capacity (TEAC) assay, le lipid assay (or 2,2′-azobis(2-aminepropane) (ABAP) assay) et le thiobarbituric acid (TBA) assay ont été sélectionnées pour être évaluées dans le cadre d'une étude inter-laboratoires. Le pouvoir anti-oxydant de trolox, tocopherol, lipochroman-6, ascorbic acid, 4-methyl-brenzcatechin et/ou BHT a été évalué en utilisant chacune des méthodes. Ces méthodes ont ensuite été évaluées par rapport à leur reproductibilité et leurs

propriétés de classification. Sur la base des résultats de cette étude, les méthodes DPPH et ensuite TEAC ont donné les meilleurs résultats en termes de reproductibilité et sensibilité à la fois au sein d'un même laboratoire et entre laboratoires. Les résultats de l'étude inter-laboratoires ont ensuite été comparés à ceux d'un seul centre obtenus avec le Kit PCL disponible commercialement. Pour évaluer la possibilité de transfert de données chimiques aux systèmes biologiques, ils ont également été comparés aux résultats d'un seul centre obtenus avec l'utilisation du test DCFH sur cellules.

Introduction

Antioxidants are a heterogeneous group of chemical structures that have the ability to slow or block oxidation processes. They are widely used in technical and industrial processes, as agents for food protection or nutritional additives as well as in cosmetics. There are two major foci in the literature of biological systems in which antioxidants are assessed: (1) prevention of food spoilage, whereby usually the destruction of vitamins and the resulting decrease of nutritive value and/or the inhibition of lipid peroxidation which leads to rancidity is stressed. Oxidation of lipids in foods also influence characteristics such as flavor, color, and texture; and (2) prevention of damage incurred to cellular constituents, e.g. proteins, nucleotides, phospholipids, polyunsaturated fats, etc., by free radicals. These types of damage have been implicated as being one of the major causes of aging and as being one of the major players in the progression of diseases.

Antioxidants are one of the primary defense mechanisms employed to combat the damaging effects of free radicals. A free radical is an atom with at least one unpaired electron in the outermost shell and that is capable of independent existence [1]. The unpaired electron(s) make radicals highly reactive as they seek out other electrons with which to pair, stealing them from the donor molecule in the process which in turn can lead to a chain reaction in the formation of new radicals or damage the molecule involved. Free radicals involving oxygen are referred to as reactive oxygen species (ROS). Among these, superoxide radicals, hydroxyl radicals, hydrogen peroxide, singlet oxygen and nitric oxide are well known examples. Free radicals originate not only from various sources such as exposure to pollutants, cigarette smoke, ozone, UV-irradiation, heavy metals, toxic chemicals, drugs but also as a product of metabolic processes.

As the interface between the body and the environment, the skin is particularly prone to attack by prooxidants originating from both the environment and from metabolic sources. As the skin is continuously exposed to free radicals and others stresses, both physical (e.g. UV-irradiation) and chemical (e.g. pollution, cigarette smoke, etc.), it possesses an intricate network of antioxidant and enzyme systems to combat these radicals and to protect the skin against the damage incurred by them [2, 3]. The delicate balance between antioxidants and prooxidants can be tipped in favor of the prooxidants by events that induce oxidative stress, e.g. by an excess of ROS induced by the exposure to solar UV-irradiation which in turn can lead to a depletion of antioxidants in the skin [4, 5]. Premature cutaneous aging, carcinogenesis, hyperpigmentation and other disorders can subsequently result. The increase in consumer awareness on the role of antioxidants in the prevention of skin aging, explains the strong increase in the use of these substances in cosmetics.

There are many methods used to measure antioxidant capacity. Table I gives a brief overview of some of the main tests used in research and industry. Two general mechanisms are tested: the free radical scavenging capacities of the antioxidant and the protection against the generation of free radicals by antioxidants. There is widespread agreement that the different methodologies used in the evaluation of antioxidant potential can lead to different results [10, 17–20]. This study was therefore initiated to assess the intra- and interlaboratory variances in evaluating antioxidant potential of the major methods used. An initial screening of different methods described in the literature was conducted in one laboratory to evaluate which methods should be tested in the interlaboratory study. For this purpose, the following methods were evaluated (see also Table I): deoxyribose assay [21], 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, superoxide radical assay (xanthine oxidase system; [22, 23]), cis-parinic-acid fluorescence assay [23, 24], trolox equivalent antioxidant capacity (TEAC) assay, r-phycoerythrin assay, lipid assay, protein assay [25] and the DNA assay [26] (data not shown). Because of the lack of reproducibility of the results obtained in our hands when using the DNA assay, the protein assay, r-phycoerythrin assay and the superoxide radical

Table I Methods used to determine the antioxidative potential of substances

Test	Name-giving molecule/abbreviation	Measurement	References (reviews)
ABTS	2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)	Inhibition of the initiation of oxidation	[6]
DCFH-DA	Dichlorofluorescein-diacetate	Analyses the ability of an antioxidant to inhibit the oxidation of DCF	[7–9]
DMPD	*N,N*-dimethyl-*p*-phenylenediamine	Analyses the ability to reduce the radical cation	[6, 10]
DPPH	2,2-diphenyl-1-picryl-hydrazyl	Analyses the ability to reduce the radical cation	[6, 10, 14]
FRAP	Ferric reducing ability of plasma	Uses metal ion to produce oxidation and analyses the ability to reduce ferric ion	[7, 10]
Lipid assay	Linoleic acid	Measures the ratio of the rate differences in the generation of double-bonds in the presence or absence of antioxidants	[11]
ORAC	Oxygen radical absorbance capacity	Measures the inhibition in the loss of fluorescence due to the oxidation by peroxylradicals	[6, 7]
PCL	Luminol photochemiluminescence	Analyses the delay in oxidation; the lag phase is a parameter of antioxidant activity, and the ability to scavenge the radical	[10]
Phycoerythrin assay	r-Phycoerythrin	Measures the inhibition in the loss of fluorescence due to the oxidation of phycoerythrin by peroxylradicals	[7, 12]
TBA	2-Thiobarbituric acid	An indirect fluorometric screening test of total oxidative stress	[13]
TEAC	Trolox-equivalent antioxidant capacity assay	Compares the ability of an antioxidant to scavenge the ABTS+ cation with that of Trolox	[6, 7, 10, 14, 15]
TRAP	Radical-trapping antioxidant capacity	Analyses the delay in oxidation. Compares the ability of an antioxidant to scavenge the ABTS+ cation with that of Trolox	[6, 7, 10, 16]

assay, these methods were not considered for further testing. The greatly varying quality of the reagent used in the cis-parinic-acid fluorescence assay was rejected for testing. The deoxyribose assay and some of the other methods are restricted to the use of either aqueous or organic solvents thus limiting their use. Although these methods have their merits in studying the antioxidative potential of substances, the TEAC, DPPH and lipid assays were identified as being the best methods for the interlaboratory study as they are relatively robust, can be established in a laboratory reasonably easily as a new method, require relatively standard equipment and deliver reproducible results. The TBA assay was a routinely used method used in one of the other laboratories, can be used for screening larger numbers of antioxidants and thus justifying its inclusion in the interlaboratory study.

The DPPH and TEAC assays measure the scavenging properties of an antioxidant whereas the TBA and lipid assays reflect the protective properties of the antioxidant. TEAC assay evaluates the capacity to scavenge the pre-formed radical monocation of 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) by such hydrogen-donating antioxidants whereby both the concentration of the antioxidant and duration of the reaction on the inhibition of the radical cation absorption are taken into account. The DPPH assay reflects the ability of antioxidants to scavenge the stable radical 2,2-diphenyl-1-picrylhydrazyl. The TBA assay assesses the protective properties of the antioxidant by measuring the inhibition of the formation of one of

the degradation products of lipid peroxidation, malondialdehyde, by the antioxidant. The lipid assay assesses the protective properties of the antioxidant by measuring the inhibition of linoleic acid peroxidation by the stable radical (ABAP). Further studies using the commercially available PCL kit (Analytik Jena AG, Jena, Germany) and the cell-based DCFH assay were performed by a single laboratory to gain insights into the comparability of evaluations obtained using analytical methods and those obtained using '*in vitro*' systems.

Materials and methods

Reagents

DPPH (2,2-diphenyl-1-picrylhydrazyl hydrate 95% free radical), L-α-lecithin: Sigma-Aldrich GmbH (Deisenhofen, Germany); ABTS (2,2′-azino-bis-(3-ethylbenzothiazolin-6-sulfonic acid) diammonium-chloride, trolox: Fluka (Buchs, Switzerland); DL-α-tocopherol, sodium dodecyl sulfate (SDS), linoleic acid: Calbiochem (Schwalbach/Ts., Germany); ABAP (2,2′-azinobis-(2-amidinopropane) hydrochloride): Polysciences Inc. (Warrington, PA, U.S.A.); Lipochroman-6: Lipotec S.A. (Barcelona, Spain); fetal calf serum (FCS), Dulbecco's modified Eagle's medium (DMEM), Hank's balanced salt solution (HBSS), 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (HEPES), penicillin/streptomycin: BioChrom (Berlin, Germany); fibroblast growth medium (FGM), normal human skin fibroblasts (NHDF): PromoCell (Heidelberg, Germany); 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate di(acetoxymethyl) ester (C-H_2DCF-DA/AM): Molecular Probes (Eugene, Oregon, U.S.A.); ascorbic acid, 4-methyl-o-dihydroxybenzene, BHT, all organic solvents were of analytical grade: Merck KGaA (Darmstadt, Germany). All further chemicals were 'pro analysis' from Merck KGaA or from Sigma. Heat Resistant Nunc Plate: Nalge Nunc Int. (Rochester, NY, U.S.A.); PCR Plate 96-well: Eppendorf (Hamburg, Germany).

DPPH assay

The determination of the antioxidative capability of a substance using the DPPH assay is based on the reaction with the stable radical DPPH. A reaction solution with a DPPH concentration of 55–60 μmol L^{-1} is prepared in absolute ethanol (equivalent to $E_{515} = 0.664$); 0.5 mL of the antioxidant reagent in a suitable solvent are added to 2.5 mL of this reaction solution. In order to obtain satisfactory results, the concentration of the antioxidant reagent should be adjusted so that the highest concentration tested yields a decrease in the extinction of approx. 70% during the reaction. Serial dilutions based on this concentration are then used to generate dilution concentrations of 80%, 60%, 40%, 20% and 0% (blank). The extinction is determined at 515 nm (1 cm, 25°C) immediately after the admixture of the antioxidizing agent, after 2 min, and then every 10 min until the reaction has finished or, in other words, when the decrease in the extinction measured in the antioxidant reaction mixture is the same as the extinction decrease of the blank value. For the determination of the blank value a mixture of 2.5 mL of the reaction solution and 0.5 mL of solvent is measured likewise. The antiradical activity is defined as the concentration of the antioxidizing agent that lowers the DPPH concentration (blank value) to 50% of the initial amount (efficient concentration 50 = EC_{50}).

TEAC assay

The determination of the antioxidative activity of substances using the TEAC assay is based on their capability to reduce the stable radical ABTS [2.2′-azinobis-(3-ethyl-benzothiazolin-6-sulfonic acid)] in comparison with the standard trolox. An ABTS solution (1.7 mmol L^{-1} in water) is mixed in the ratio 5:1 with potassium peroxydisulfate solution (4.3 mmol L^{-1} in water) and incubated for 12–16 h at room temperature in darkness. Immediately prior to measurement, this stock solution is diluted with ethanol or water to an extinction of $E_{734} = 0.700 \pm 0.020$ (=reaction solution; 1 cm, 734 nm). A stock solution of trolox (=standard) was prepared and serially diluted to concentrations ranging from 0 to 252 μmol L^{-1}. The investigated antioxidant solutions were prepared in the same manner, whereby the highest concentration of the antioxidizing agent should reduce the extinction of the reaction solution to 40–60% within 6 min (100% = without sample). Either water or ethanol is used as solvents for both trolox and the samples. For the measurement, 0.1 mL of the samples or standards are mixed with 2.0 mL of the reaction solution and the extinction at 734 nm (1 cm, 25°C) is measured after exactly 6 min against the solvent. The TEAC value denotes the concentra-

tion of trolox that causes the same percentage of absorption inhibition of the radical cation as 1 mmol L^{-1} of the substance to be examined.

Lipid assay

The lipid assay assesses the differences in the rate of oxidation of linoleic acid by radical ABAP 2,2′-azobis(2-amidinopropane) dihydrochloride in relation to the tocopherol standard. Prior to and during the reaction, the temperature of the linoleic acid reaction mixture [70 μL of linoleic acid (2.3 mmol L^{-1})]; 100 mL 0.05 M phosphate buffer (sodium phosphate monohydrate dissolved in water; 2.88 g SDS; pH 7.4) is adjusted and maintained at 40°C. Two microliters of this solution is then mixed with 0.01 mL of ABAP (0.4 mol L^{-1} in 0.05 M sodium phosphate buffer, pH 7.4). After incubation for 2–5 min, 0.02 mL of the antioxidant sample (2.5 μmol L^{-1} in ethanol or water), standard (2.5 μmol L^{-1} tocopherol in ethanol) or adequate sample solvent are added and the ΔE/20 min is determined photometrically at 236 nm (1 cm; 40°C).

TBA assay

The determination of the antioxidative activity using the TBA assay is based on the prevention of the formation of malondialdehyde, a degradation product of lipid peroxidation. For the preparation of a 0.4% liposome solution, 200 mg L-α-lecithin are dissolved in 20 mL potassium phosphate buffer (0.01 M, pH 6.0) and are sonicated for 3 min (50 W, $T < 40$°C). The suspension is stable at 4–8°C at least for 1 week. Before onset of the experiment, the liposome solution is diluted 1:1 with potassium phosphate buffer. Following aqueous solutions are prepared: 10 mmol L^{-1} iron(II) sulfate, 50 mmol L^{-1} ascorbic acid, 20% trichloroacetic acid, 1.4% TBA as well as 5% BHT dissolved in ethanol. Six different concentrations (1:1 dilution) are prepared from each antioxidizing agent. Exactly 200 μL of the diluted liposome solution are transferred to the wells of a heat-resistant Nunc Plate; 4 μL of each test concentration or the solvent control are added to a well. After adding of 10 μL of a mixture of iron(II) sulfate and ascorbic acid (1:1) into all wells with the exception of the wells used to monitor the reagent background, the plate is incubated for 60 min at 37°C. Addition of 8 μL BHT solution into each well and thorough mixing then stops the reaction; 65 μL from each well are transferred into the wells of a 96-well PCR plate, mixed with 60 μL trichloroacetic acid solution and 85 μL TBA solution and developed for 15 min at 95°C. The PCR plate is then centrifuged, 100 μL of the supernatant is transferred into a 96-well flat bottom microtiter plate and the extinction is determined at 540 nm in a suitable ELISA reader.

DCFH assay

This cell culture-based method detects the intracellular oxidation of the nonfluorescent DCFH (the reduced form of DCF) by free radicals to the fluorescent dye DCF. The intracellular antioxidant capacity of a substance can be assessed by the lag in the formation of DCF. Normal human dermal fibroblasts (NHDF; PromoCell) were cultured in FGM:DMEM (1:1) supplemented with penicillin (50 U mL^{-1}), streptomycin (50 μg mL^{-1}) and 5% (v/v) FCS (5% CO_2; 37°C). Cell populations between cumulative population doubling levels 3–20 were used. Prior to the DCFH assay the test substances were investigated concerning cytotoxic potential. Cytotoxicity was assessed by determining the mitochondrial activity via the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay (Cell Proliferation Kit (MTT); Roche Diagnostics, Mannheim, Germany). For the evaluation of the antioxidant potential via the fluoroscan assay, NHDF were seeded in a 48-multiwell plate at a density of 3×10^3 cells per well. After reaching confluency, the cells were supplemented with one concentration (50 μM) of the test solutions for 48 h. In addition, cells are treated with the vehicle (=control). After the supplementation period, the cells were rinsed twice with Hank's balanced salt solution (HBSS) including 20 mM HEPES (HBSS/HEPES solution) and stained with 2.5 μM of C-H_2DCF-DA/AM for 45 min at 37°C. Cells were then washed twice and covered with HBSS/HEPES solution. Subsequently, measurement of the background fluorescence (excitation 485 nm, emission 538 nm) was performed over a period of 15 min in a multiplate fluorometer FL600 (Laboratoriesystems, Helsinki, Finland). Thereafter, extracellular oxidative stress was induced by adding 100 μM H_2O_2 for 120 min and the DCF production was monitored by measuring the fluorescence. A determination of the protein content [27] of each well followed to normalize the values measured.

PCL assay

The PCL detection method using the Photochem system is used to measure antioxidant activities, especially superoxide anion scavenging activity of test substances. The results can be expressed in equivalents of ascorbic acid (for water soluble antioxidants) or in equivalents of trolox (for lipid soluble antioxidants). For this study the antioxidative potency is expressed as the IC_{50} value, i.e. the concentration of antioxidant needed to reduce the chemiluminescence to 50% of the unattenuated value. The reaction was carried out using the Photochem apparatus from Analytik Jena AG based on the PCL kit according to the methods described by the manufacturer.

Statistical evaluation

A statistical evaluation of the intralaboratory and interlaboratory variation as well as for the ranking of the antioxidant capacity of a substance depending on the method used was conducted. In an initial step, outlier values for each analytical method were identified using graphic-statistical procedures (Dixon and Cochran tests for outliers, IR-Charts, Shewhart control charts according to ISO 5725) and excluded from further statistical analyses. Where possible, the data was evaluated with comprehensive linear statistical modeling. For multiple comparison the Tukey–Kramer HSD test was used that controls for each comparison the test level of $\alpha = 0.05$. Shewhart control charts were used for variance analysis and for defining the upper and lower control limits. The calculation of the actual results occurs, i.e. from whole mean value m, repeating variation σr (intralaboratory) and comparison variation σR (interlaboratory), with the respective coefficients of variation CV1 and CV2. The corresponding limits r and/or R are calculated from the variations and represent the refusal thresholds for calculated differences of two single values from the same and/or from different laboratories with a confidence interval of 95%. The coefficients of variation CV1 and CV2 describe σr and σR in units of m and relate the variations, solely not significant to the position of the mean value. The target corridor in the sense of relative correctness of the values obtained per substance by a method used was defined as the range of variation between the upper and lower control limits derived from the respective Shewhart control charts in respect to the mean values obtained by the different laboratories.

Results

Six laboratories took part in the study. Prior to the initiation of testing, standard operating procedures were distributed to each laboratory and training sessions for each test method were performed. Test substances were distributed by one laboratory to ensure that substances from same lot were used for testing by each laboratory. Test results were collected and subjected to the statistical analyses by one center.

Implementation of the assays: principles and pitfalls

For the determination of the antioxidative capacity using the DPPH assay, the reduction by means of an antioxidizing agent is monitored by measuring the decrease in the extinction at 515 nm at different concentrations over time until a steady-state has been attained. At each concentration of the antioxidizing agent the residual DPPH concentration is determined as percent of the initial extinction and plotted against the molar ratio of antioxidizing agent/DPPH. The maximal concentration of the sample should be $\Delta E \leq 70\%$. The antiradical activity is defined as the concentration of the antioxidizing agent that lowers the DPPH concentration to 50% of the initial amount (efficient concentration = EC_{50}). The smaller this value, the greater is the activity against radicals. Depending on the substance tested, testing can last between 30 s and 24 h. BHT is a very slow reacting antioxidant with the potential to protect lipids for a longer time. BHT reacted very slowly in the DPPH assay and did not achieve a plateau or endpoint in the kinetic after 24 h. Other substances such as α-tocopherol react faster, as evidenced by the quick change in absorption kinetics, yet do not exhibit the long-term effect. Therefore, the kinetic ΔE/time is an important criterion to evaluate results of specific substances. Taking the individual kinetics into account and integrating a time limit into the measurement-scheme should improve the comparability of values.

The lipid assay utilizes the radical starter ABAP [2,2′-azobis (2-amidinopropane) di-hydrochloride] which leads to a constant formation rate of peroxyl radicals. Both ABAP and linoleic acid

(cis,cis-9,12 octadecadienoic acid) are dispersed in SDS-micelles. The radicals cause the oxidation of linoleic acid to the isomer with conjugated double bonds, which is monitored by the increase in the extinction at 236 nm. The capability of the antioxidizing agent to inhibit the rate of formation is determined relative to the tocopherol standard. The temperature, concentration and use of linoleic acid are critical for the lipid assay and should be used as specified in the described procedure. Maintaining the order of the addition of the reagents to the mixture and thorough mixing of the reagents is essential. Changes can lead to problems such as reactions not taking place, etc. Strict adherence to the protocol is also necessary as otherwise large variations in the results can occur. Care should also be taken to prepare the linoleic acid stocks on a daily basis and to protect the linoleic acid from oxidation during storage.

The TEAC assay is based on reactions with the stable cation of ABTS. The percentage of the reduced ABTS is determined as a function of concentration and time and it is calculated relatively to the reactivity of trolox (TEAC). The TEAC value denotes the concentration that causes the same percentage of absorption inhibition of the radical cation at 734 nm as 1 mmol L^{-1} of the substance examined. The maximum concentration of the sample should be chosen in that range where the decrease of absorbance ΔE is between 40% and 60%. As some laboratories observed that the baseline value changes in the course of measurements, it is necessary to check this value regularly.

The TBA assay is based on the detection of malondialdehyde, a degradation product of the lipid peroxidation, which forms a red dye with TBA in acidic environment. Increasing concentrations of antioxidative substances prevents the formation of malondialdehyde and reduces the dye formation. In this way the antioxidative potential can be quantified. The temperature in the reaction solution is critical and must be controlled. Water baths proved more dependable than sand baths. It can be envisaged that a suitable rheological modifier could be used to prevent sedimentation of liposomes. Chelating substances are unsuitable for the TBA assay, because the concentration of free ions can be too low to initiate the color reaction. The reaction mixture should not be agitated when adding the $FeSO_4$. Unexpectedly, the color reaction could not be initiated when using Rovisomes, a commercially available liposomal delivery system (ROVI Cosmetics, Schlüchtern) possibly because the perfect spherical form increases their stability against oxidation. In contrast, the liposomes produced by ultrasonic treatment are a heterogeneous mixture of spheres, open tubes, sheets and other forms and may therefore be more susceptible to oxidation processes. The liposomes should be prepared every week.

Repeating standard deviation (σr) or 'intralaboratory' variation

Taking the data obtained from the various laboratories after outlier adjustment into consideration, the variation of the single values within the individual laboratories is dependent on the substance and the methods used. The range of variation of the intralaboratory standard deviations is summarized in Table II. Whereas the CV1 values of the DPPH, lipid and TEAC assays are below 20%, the results obtained by the TBA test exhibited a higher degree of variability with variations ranging from 18% to 102%.

Comparison standard deviation – 'interlaboratory' (σR)

The differences of the mean values between the laboratories exhibited a greater variation over most values (CV2, Table II) than the intralaboratory values. Regarding the DPPH, lipid and TEAC assays, the values exhibited up to 70% variation whereas the CV2 values with the TBA assay exhibited more than 150% variation.

The ratio of the repeating and comparison standard deviation ($\sigma R/\sigma r$) results in a measure of the comparability of the measurement results from laboratory to laboratory. The larger the ratio of $\sigma R/\sigma r$ ($V\sigma\sigma > 1$) is, the less is the comparability of the absolute values of the results from laboratory to laboratory. Regarding the individual tests comparability is disparate (Table II).

Investigation of the ranking in the individual assays

DPPH assay

All six laboratories tested the five substances using the DPPH assay. All values generated by laboratory 6 were much higher than those of the other laboratories. Only two ranks were distinguishable by the values generated by this laboratory.

Table II Coefficient of variation of the repeating standard deviation found in the intra- and interlaboratory results

	Ascorbic acid	BHT	4-MBC	Tocopherol	Trolox
Intralaboratory results (CV1) (%)					
DPPH	7.8	18.0	2.0	6.8	6.2
Lipid	14.0	5.1	2.6	–	5.4
TBA	–	18.0	20.0	102.0	27.0
TEAC	7.5	5.7	7.7	7.6	–
Interlaboratory (CV2) (%)					
DPPH	12	57	6	19	9
Lipid	67	18	35	–	10
TBA	–	87	78	154	92
TEAC	11	40	10	9	–
Ratio of repeating and comparison standard deviation ($V\sigma\sigma$)					
DPPH	1.5093	3.1210	1.7526	2.7771	1.4241
Lipid	4.851	3.596	13.391	–	1.7534
TBA	–	4.8163	3.9846	1.4930	3.3898
TEAC	1.4168	6.6942	1.1717	1.1414	–

Table III Ranks and target corridors

			Target corridor		Observed range of CV3* (%)	
Rank	Substance	Mean	Lower limit	Upper limit	Lower imit	Upper limit
DPPH assay (tested by five laboratories)						
1	4-MBC	0.24	0.22	0.25	1	6
2	Trolox	0.26	0.23	0.30	1	17
2	Tocopherol	0.26	0.25	0.27	2	11
3	Ascorbic acid	0.28	0.23	0.30	3	12
4	BHT	0.87	0.52	1.38	4	15
Lipid assay (tested by three laboratories)						
1	Trolox	0.94	0.91	0.97	1	5
2	BHT	0.76	0.64	0.86	1	11
3	4-MBC	0.53	0.39	0.64	2	5
4	Ascorbic acid	0.32	0.18	0.40	1	38
TEAC assay (tested by six laboratories)						
1	Tocopherol	1.01	0.96	1.12	1	11
1	4-MBC	0.97	0.79	1.05	1	24
1	Ascorbic acid	0.96	0.92	1.08	4	12
2	BHT	0.47	0.10	0.76	2	14
TBA assay (tested by six laboratories)						
1	Lipochroman-6	−0.38	nd	nd	27	46
2	BHT	−0.06	nd	nd	13	54
3	Trolox	0.23	nd	nd	21	43
3	Tocopherol	0.31	nd	nd	37	94
3	4-MBC	0.38	nd	nd	14	62

*Laboratory coefficient of variation for all mean values and every substance. nd, not determined.

Therefore, the results of laboratory 6 were not used for the further calculations. The values for BHT were the lowest when measured in laboratory 4, which is in contrast to all other laboratories, where they were the highest. Therefore, the BHT-value of this laboratory was not used for further evaluations. The analyzed ranks and target corridors are listed in Table III.

Lipid assay

Because of the high degree of variation in the results, no clear ranking of the substances was possible when evaluating the results from laboratories 5 and 6. The results of laboratory 3 were not included in the calculation as only two substances had been examined. Therefore, only the results from three laboratories were used to assess rankings and target corridors (Table III). A ranking of the substances into two groups was only possible and an assignment was not unambiguously possible in all cases.

TEAC assay

Homogeneous ranking about all laboratories was found to a large extent when using the TEAC assay. Two ranks were defined in which tocopherol, 4-MBC and ascorbic acid were allocated to one rank and BHT, which had statistically significantly lower values than the others, was ranked differently. The magnitude of the difference between substances was strongly dependent on the laboratory. The analyzed ranks and target corridors are listed in Table III.

TBA assay

To a large extent, a homogeneous ranking of the substances was possible with the data generated by all laboratories. However, this was only possible by using logarithms and the subsequent elimination of the negative values in order to create a certain homogeneity of variances, thus allowing unambiguous ranking. Because of the great variations no target corridor was determined. The ranking based on the results of the statistical analyses can be found in Table III.

PCL assay

The PCL assay was not a part of the interlaboratory study and was performed for comparison reasons only as it is a commercially available method. The results are shown graphically in Fig. 1. For all antioxidants a concentration-dependent inhibition of the UV-induced chemiluminescence was observed. Although tocopherol and 4-MBC reacted very similarly, all other antioxidants showed less activity. Based on the results obtained, the antioxidants investigated can be ranked as shown in Table IV. Further statistical analysis including target corridor and observed range of CV was not performed, because of the fact that only one laboratory performed this assay.

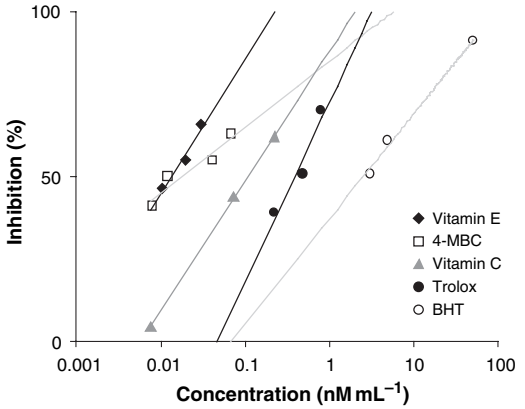

Figure 1 Concentration-dependent inhibition of the UV-induced chemiluminescence by tocopherol, 4-MBC, ascorbic acid, trolox and BHT measured with the PCL assay.

Table IV IC_{50} values and ranking of the substances when using the PCL assay

Rank	Substance	IC_{50} (nM mL^{-1})
1	Tocopherol	0.012
2	4-MBC	0.02
3	Ascorbic acid	0.083
4	Trolox	0.24
5	BHT	1.72

DCFH assay

The DCFH assay is the link between the chemical-reaction-based and the *in vivo* assays. The six substances used for the other assays were tested in this *in-vitro* cell-culture-based system. In the cytotoxicity assay the maximum concentration of the substance which was still well tolerated by the fibroblasts was determined. A concentration of 50 μmol L^{-1} of each substance was then tested. The assay was performed eight times in triplicates. As only one laboratory of the six laboratories included in the trial had the means and experience to perform this assay, no interlaboratory data was compiled and subjected to statistical analyses. No clear ranking of the substances was statistically possible although it was evident that tocopherol had the highest increase at 29% and ascorbic acid had the slightest increase at 8%. Therefore, in principle the results of the other assays were more or less confirmed. The results are depicted in Table V.

Table V Mean values and intralaboratory variation coefficients obtained when using the DCFH assay

				Target range of means		Observed range of CV	
Rank	Substance	Mean	CV	Upper limit	Lower limit	Upper limit	Lower limit
1	Tocopherol	29	3	na	na	na	na
2	4-MBC	22	8	na	na	na	na
3	Lipochroman	16	5	na	na	na	na
3	Trolox	16	3	na	na	na	na
4	BHT	13	5	na	na	na	na
5	Ascorbic acid	8	5	na	na	na	na

na, not analyzed.

Discussion

Redox mechanisms are suspected to play a role in many physiological processes. These include processes that can lead to various skin alterations, e.g. photoaging, dyspigmentation, etc. This makes antioxidants an interesting family of molecules not only for nutrition but also for the cosmetic applications. A plethora of analytical methods are used to measure the antioxidative activity of substances yet little is known about the comparability of the results obtained by using these different methods within one laboratory and even less is known about the comparability of results obtained by different laboratories. In this study, four assays used to assess the antioxidant potential of molecules, namely the DPPH, TEAC, lipid and TBA assay, were evaluated in both an intra- and interlaboratory study.

Each of the procedures has its own advantages and disadvantages which need to be taken into account when assessing the antioxidative potential of a substance [10, 14, 28]. To add to the complexity in assessing the procedures themselves, the problem of establishing a method within a laboratory was evident. The results after having implemented the methods within the different laboratories revealed that reproducibility in intralaboratory (standard deviation in repeated trials) and especially interlaboratory (high standard deviations of the results from different laboratories) was poor making comparisons problematical. Training, equipment and the scale of measurement (e.g. microtiter plates vs. cuvette) proved to be very important and changes led to varying results. Various aspects in performing the experiment also need to be considered, e.g. measurements (e.g. BHT in the DPPH assay) can be necessary over a long time-span and thus the evaporation of solvents has to be controlled to prevent changes in the concentration. In general, a thorough implementation, strict adherence to the protocol and repeated use of methods in each laboratory improved the comparability of the results which in turn led to definable target corridors. As was to be expected, the comparability of absolute values depended on the assay and test substances used. This may in part be the result of the differing suitability of the tests for testing hydrophilic and/or lipophilic samples, e.g. the lipid assay is somewhat better for testing lipophilic substances whereas the DPPH and TEAC assays can be used for both.

Statistical analyses of the data was performed in order to assess the intralaboratory and interlaboratory variations and to define target corridors. The statistical evaluation occurred in two steps. In a first step the robustness of the method was described by determining the intralaboratory and interlaboratory variations. The second step of the statistical evaluation was used to determine the ranking of the test substances per laboratory and method used. Based on the results of these evaluations, a ranking of the substances by the different laboratories was ascertained. Furthermore, the possibility of determining target corridors to define the acceptable quality of a measurement was assessed.

Intralaboratory standard deviation for TEAC (5.7–7.7%), DPPH (2–18%), and lipid (2.6–14%) assay was relatively low thus indicating these methods have a good degree of reproducibility within one laboratory. The TBA assay has an intralaboratory standard deviation of 18–102% indicating a much higher degree of interlaboratory

variation. Laboratories with a low standard deviation in intralaboratory results and more than two definable ranks were able to classify the potentially different substances related to the antioxidative potential without objections. Each of the six laboratories was able to differentiate between at least two rankings, one for strong and one for weak antioxidants. The differences in the ranking of test substances may be related to the various degrees of experience with the individual method and method limitations.

After elimination of the outliers and taking the results of the laboratories into consideration, the interlaboratory variations were dependent on test substance and method used. Although not unexpected, interlaboratory variations were much higher than intralaboratory variations. The TEAC assay exhibited the least amount of variation (9–40%), followed by the DPPH assay (6–57%), lipid assay (10–67%) reflecting the robustness of the methods found in the intralaboratory comparisons. Once again, the TBA assay exhibited a high degree of variation (78–154%). Only two to three statistically different ranks were definable depending on the method used. Interlaboratory rankings revealed that no major deviations in the ranking categories in an individual test were made by the laboratories. The data obtained by the various laboratories using the TEAC, DPPH and lipid assays were used to define target corridors in which the values obtained by a measurement can be considered plausible. The use of these corridors can be considered a measure of quality of the values obtained when using a test method. This can be of considerable importance when setting up a new assay in a laboratory.

In addition to the multicenter study, the PCL assay (a commercially available assay) and the DCFH assay (a cell-based method) were each performed at one laboratory in order to gain a further understanding on how the results obtained by the analytical methods compare to a commercial and cell-based system. The DCFH is a robust method available to prove the transferability of chemically based techniques to living cells. The test set-up was reproducible and suitable to determine the antioxidative capacity *in vitro*.

Conclusions

Many analytical methods are used to evaluate the antioxidant potential of a substance. It is of importance to understand the mechanisms underlying the analytical method or substrate used in order to allow a valid interpretation of the results. Although self-evident, it needs to be emphasized that practice is needed to obtain reproducible results and it is often the seemingly trivial points that decide on the outcome of the experiments. Because of the limitations of each method, more than one method should be used to evaluate the antioxidative capacities of a product. When choosing a method one also needs to take into account whether the substances to be tested are hydrophilic or lipophilic and whether the test system is suitable to assess the antioxidative potential of the substance. Kinetics can also provide additional information for application areas of a substance. Data obtained from substances tested in the DPPH, TEAC and lipid assays allowed the calculation of target corridors to define the acceptable quality of a measurement. Based on the results obtained by this study, the DPPH and TEAC assays are the easiest to implement and yield the most reproducible results.

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