Structure-function relationship of phenolic antioxidants in topical skin health products

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Abstract

OBJECTIVES: The present work analysed the antioxidative activity of phenol-based antioxidants using an electron spin resonance method to predict the activity and stability of these antioxidants in cosmetic products.

METHODS: The antioxidative power (AP) method was chosen to measure both the capacity and kinetics of an antioxidative reaction by detecting the DPPH (diphenylpicrylhydrazyl) radical. The antioxidative capacity (w_c) relates to the amount of free radicals that can be reduced, whereas the antioxidative reactivity (t_r) relates to the reaction speed and offers a fingerprinting of the redox state of the antioxidant molecules. Fifteen phenolic molecules have been analysed. They differed in the position of the hydroxyl groups and substituents on the aromatic ring. The AP of two distinct formulations containing hydroxytyrosol is presented as well as three phenol-based antioxidants within the same formulation vehicle.

RESULTS: The rate at which phenol (ArOH) reacts with DPPH radicals, defined by the term reactivity (t_r) in this paper, was dependent upon the bond dissociation enthalpy (BDE) of the OH bond. Molecules having weak OH bonds and consequently low BDE values showed high antioxidant reactivity. On the other hand, the capacity factor (w_c), which is the concentration of phenol required to reduce a fixed concentration of DPPH radical, depends on the number and position of hydroxyl groups. The results showed that ortho and para positions of the two hydroxyl groups are important for higher capacity. If one of the two hydroxyl groups is blocked by methylation, both the antioxidative capacity and reactivity are reduced, mainly for ortho disubstituted compounds. The presence of a vinylic side chain improved reactivity and capacity tremendously. AP values may be useful in formulation design when identifying antioxidants that are likely to be physically and chemically stable. The importance of optimization of the formulation vehicle itself for a given antioxidant is also illustrated.

CONCLUSION: Based on the presented findings, it is possible to predict the antioxidative performance of a phenol-based molecule and its stability and oxidation resistance within a cosmetic formulation. This is essential for antioxidant containing dermal formulations designed to combat skin ageing.

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Résumé

OBJECTIFS: Le présent travail a analysé l'activité anti-oxydante des antioxydants à base de phénol en utilisant une méthode de Résonance du Spin Electronique pour prédire l'activité et la stabilité de ces antioxydants dans les produits cosmétiques.

METHODES: La méthode de la puissance anti-oxydante (AP) a été choisie pour mesurer à la fois la capacité et la cinétique d'une réaction anti-oxydante par la détection du radical DPPH (diphénylpicrylhydrazyl). La capacité anti-oxydante (wc) se rapporte à la quantité de radicaux libres qui peuvent être réduits, alors que la réactivité anti-oxydante (tr) se rapporte à la vitesse de réaction et offre une identification ("fingerprint") de l'état des molécules anti-oxydantes redox. Quinze molécules phénoliques ont été analysées. Elles diffèrent par la position des groupes hydroxyle et des substituants sur le noyau aromatique. L'AP de deux formulations distinctes contenant hydroxytyrosol sont présentés ainsi que trois antioxydants à base de phénol dans le même véhicule de formulation.

RESULTATS: La vitesse à laquelle le phénol (ArOH) réagit avec des radicaux DPPH, définie par la réactivité à long terme (tr) dans le présent document, dépendait de l'enthalpie de dissociation de liaison (BDE) de la liaison OH. Les molécules ayant de faibles liaisons OH et par conséquent des valeurs faibles BDE ont montré une réactivité élevée en antioxydants. D'autre part, le facteur de capacité (wc), qui est la concentration de phénol nécessaire pour réduire une concentration fixe de radicaux DPPH, dépend du nombre et de la position des groupes hydroxyle. Les résultats ont montré que les positions ortho et para des deux groupes hydroxyles sont importants pour une plus grande capacité. Si l'un des deux groupes hydroxyle est bloqué par méthylation, à la fois la capacité anti-oxydante et la réactivité sont réduites, principalement pour les ortho- composés di-substitués. La présence d'une chaîne latérale vinylique améliore la réactivité et la capacité considérablement. Les valeurs AP peuvent être utiles dans la conception de la formulation lors de l'identification des antioxydants qui sont susceptibles d'être physiquement et chimiquement stable. L'importance de l'optimisation du véhicule lui-même pour la formulation d'un antioxydant donné est également illustrée.

CONCLUSION: D'après les résultats présentés, il est possible de prédire la performance d'une molécule antioxydante à base de phénol et de sa stabilité et sa résistance à l'oxydation dans une formulation cosmétique. Cela est essentiel pour des formulations dermiques contenant des antioxydants destinés à lutter contre le vieillissement de la peau.

Introduction

Antioxidants belong to a heterogeneous group of organic molecules which are able to reduce free radicals and interrupt radical chain reactions. A variety of functional groups are responsible for their anti-radical activity, involving different mechanisms of actions with electron donors and acceptors being a common feature for all mechanisms. Commonly used antioxidants in the cosmetic and food industry are phenolic compounds, defined as molecules with a hydroxyl-substituted aromatic ring. The benefits of delivering antioxidants to the body, and more specifically directly to the skin by topical application, continue to drive significant product research and development activity. When skin is exposed to internal and external stressors (i.e. ultraviolet light, pollution, etc.), free radicals (unstable atoms that have an unpaired electron in their outermost shell) are generated causing oxidative stress and cell damage [1]. Antioxidants may reduce oxidative stress by directly neutralizing free radicals and/or elevating the body's own endogenous antioxidant system [2]. In addition to their potential therapeutic and cosmetic benefits, antioxidants within a formulation can also play an important role in ensuring the safety and quality of the finished (topical) product throughout its shelf life.

Formulating such molecules in topical skin health products presents challenges in terms of retaining their functional activity and aesthetic acceptability of the product [3]. As antioxidants are generally characterized by a high reactivity with oxygen and other organic molecules, the conservation of their activity prior to application on the skin is one of the biggest hurdles to overcome. In addition, this enhanced reactivity often leads to discoloration of the product, which can either be due to oxidation or polymerization [3, 4]. The ability to predict the antioxidant activity (related to potential efficacy) and chemical reactivity (related to product quality and stability) of antioxidant compounds based on their molecular structures would enable product research and development activities to be progressed more rapidly and with less risk.

In order to determine the activity of antioxidants, the method required must be able to quantify both the chemical capacity (the extent to which an antioxidative reaction goes to completion) and the chemical kinetics (the speed at which such a reaction occurs). Common antioxidant tests such as oxygen radical absorbance capacity (ORAC) [5], ferric ion reducing antioxidant power (FRAP) [6] and Rancimate [7] are not compatible for kinetic analyses because they utilize dyes that are in competition with the test sample in the antioxidant reactions [8]. Instead, test methods such as the 2,2-diphenyl-1-picrylhydrazyl radical scavenging capacity assay (DPPH) or the Trolox equivalent antioxidant capacity (TEAC), where the free radical is directly detected, are more suitable as both tests are based on the direct spectrophotometric detection of the used free radicals (DPPH* and ABTS*, respectively).

The present work investigated the antioxidative activities of a range of phenol-based antioxidant molecules using an electron spin resonance (ESR) based methodology called antioxidative power (AP) [9]. This method is able to quantify both the capacity and the kinetics of an antioxidative reaction by directly detecting the free radical DPPH*, which is unaffected by the solvent, the colour or the opacity of the test samples. Even though DPPH is an organic nitro radical and not a reactive oxygen species (ROS), the reactivity and capacity of an antioxidant are still analysed precisely. Using ESR spectroscopy, a quantitative measurement can be obtained.

The antioxidative power method that is presented differs from the standard DPPH* scavenging assay [10, 11] in terms of the differentiation between a static and a dynamic parameter. Additionally, the ESR method discussed here, unlike VIS spectroscopy, can be utilized with coloured, opaque and viscous samples such as creams and ointments.

The DPPH* free radical can be reduced either by the HAT (hydrogen atom transfer) or by the SET (Single Electron Transfer) mechanism. The HAT mechanism occurs when an antioxidant compound quenches free radical species by donating hydrogen atoms. It is influenced by the pH value of the medium, which in turn affects the strength of hydrogen bonding of involved hydroxyl groups. The SET mechanism occurs when an antioxidant transfers a single electron to a target compound (free radical). The resultant radical-cationic antioxidant compound then undergoes a second step and is deprotonated through an interaction with water.

Methods

The AP values of a range of phenolic antioxidants were measured using ESR spectroscopy. A known concentration of DPPH* was allowed to react with different concentrations of the antioxidant. The reaction schematic is shown in Fig. 1 and has been discussed in detail previously [12].

In brief, the measurements of the antioxidant capacity and reactivity were performed using ESR spectroscopy. The measurements discussed in this article were performed with the X-band ESR spectrometer Miniscope MS 300 (Magnettech, Germany) and the following technical parameters: 60 G sweep width, 100 Gain, 1 G modulation amplitude, 7 mW attenuation, 3365 G central field, 0.14 s time constant. The antioxidative power (AP) is a parameter able to quantify both the reaction capacity and velocity of antioxidants. The test radical DPPH (2,2-diphenyl-1-picryl-hydrazyl, Sigma-Aldrich, Munich, Germany) is used as a detector molecule. A stock solution of DPPH at 0.2 mM was prepared in EtOH 99%. Blank solutions of DPPH in EtOH 75% at 0.1 mM were prepared, and the ESR signal intensities (double integration) were determined. At least three concentrations of the test sample were prepared and added to DPPH to obtain an initial radical concentration
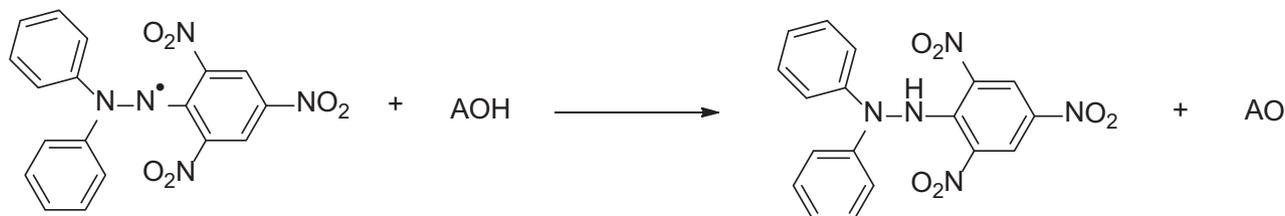
Figure 1 Reaction schematic of the DPPH with antioxidant.

of 0.1 mM. The signal intensity decay of each concentration of the test samples is recorded at different time intervals during the reaction until saturation is reached, and all antioxidant active molecules have reacted with the test radical.

From these intensities, a first-order kinetic term is obtained for each concentration set. The kinetic parameters of the monoexponential fits $y = y0 + Ae^{(-x/t)}$ are used to calculate the reaction time t_r, and the static parameters are used to calculate the characteristic weight w_c. Both parameters are used to calculate the AP by means of the following equation:

$$AP = RA.N(DPPH)/t_r.w_c \tag{1}$$

where $RA = (1/e^2)$, $N = 6.022 \times 10^{16}$ spins per mL, and $t_r = t_r[1] + t_r[2] + t_r[3]/3 *(1/e)$

The ESR signal intensity decay of the DPPH free radical was monitored over a period of 40 min for each antioxidant concentration. The extent of the antioxidative reaction was quantified by determining the concentration of an antioxidant that is able to reduce a defined amount of DPPH* radical. This parameter is termed as *capacity* (w_c). The lower the value for w_c is, the lower the concentration of the antioxidant required for radical reduction, and the stronger the antioxidant.

For a direct comparison of different antioxidants, the AP method is standardized to the activity of vitamin C (ascorbic acid, supplied by Sigma-Aldrich, Munich, Germany at the highest grade of purity). The antioxidative activity of a solution of 1 ppm vitamin C is defined as 1 antioxidative unit (1 AU).

The kinetic parameter, the speed at which the reaction occurs, was quantified by measuring the time necessary to achieve the reduction of a defined amount of DPPH* radicals. This parameter is referred to as *reactivity* (t_r). The shorter t_r is, the faster the reaction occurs.

The antioxidant *activity* or antioxidant power (AP) is calculated taking into account both capacity and reactivity:

$$AP = \frac{[DPPH] \text{ reduced}}{mg \text{ min}}$$

AP = [DPPH radical] reduced

$AOX_{concentration} \times t_r$

The AP value is expressed in n° spins per mg*min.

The structural features of phenolic antioxidants investigated in this work included (a) hydroxyl-substituted phenols, (b) methoxylated phenols, (c) catechols with different side chains and (d) methoxylated tri-substituted phenols (Table I). A fundamental understanding of the structural skeleton, the type of side chains and the polarity of the antioxidant in relation to activity could therefore be elucidated from this systematic evaluation.
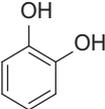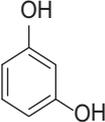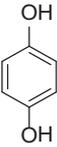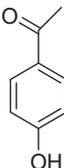
Results and discussion

In the first instance, a series of hydroxyl-substituted phenols were tested, differing in the position(s) of OH groups as shown in Table II. These molecules had different antioxidant capacities and reactivities, with catechol showing the highest activity. The rate at which phenol (ArOH) reacts with DPPH radicals, defined by the term reactivity (t_r) in this paper, depends upon the bond dissociation enthalpy (BDE) of the OH bond. When the BDE of an antioxidant is low, meaning that the hydrogen atom is held weakly to the hydroxyl oxygen atom substituent, the more active and faster the

Table I Structural features/antioxidant molecules investigated for *activity* by ESR

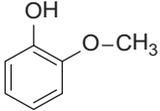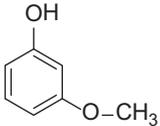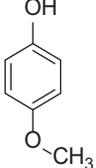| Hydroxyl-substituted phenols	Methoxylated phenols	Catechols with side chains	Methoxylated tri-substituted phenols
Catechol	2-Methoxyphenol	Hydroxytyrosol	Vanillyl alcohol
Resorcinol	3-methoxyphenol	Hydroxytyrosol acetate	Homovanillyl alcohol
Hydroquinone	4-Methoxyphenol	Caffeic acid	Coniferyl alcohol
Hydroxyacetaphenone			Ferulic acid
			2-methoxy-4-vinyl phenol

Table II Antioxidant *activity* of hydroxy substituted phenols

Chemical name	Molecular structures	AP (AU)	t_r (min)	w_c (mg)
Catechol (1,2-dihydroxybenzene)		2 534 870	0.12	0.00108
Resorcinol (1,3-dihydroxybenzene)		1179	2.2	0.127
Hydroquinone (1,4-dihydroxybenzene)		343 752	0.98	0.000981
Hydroxyacetaphenone		3	>3	>18

antioxidant will undergo a free radical reaction. The lower t_r, (higher reactivity), for the catechol (1,2-dihydroxybenzene) compound compared to resorcinol (1,3-dihydroxybenze), and hydroquinone (1,4-dihydroxybenzene) is attributed to its lower BDE. Catechol has a lower BDE compared to 1,3- and 1,4-dihydroxybenzene due to stabilization of the phenoxy radical (ArO·) by H-bonding interaction with ortho OH groups [13].

In addition, HAT reactions may be hindered by the presence of electron withdrawing groups in the 3- and 5-positions (*meta*) via deactivation of the aromatic ring [14]. The capacity factor (w_c), which is the concentration of phenol required to reduce a fixed concentration of DPPH radical, was dependent on the number and position of OH groups. Hydroxy groups in the ortho or para positions led to a higher capacity factor (or lower w_c) compared to the

Table III Antioxidant activity of methoxylated phenols

Chemical name	Molecular structures	AP (AU)	t_r (min)	w_c (mg)
Guaiacol (2-methoxyphenol)		50 979	2.22	0.00292
3-methoxyphenol		130	2.06	1.23
4-methoxyphenol		108 150	1.91	0.00160

meta position. When the hydroxy groups were in ortho or para positions, the phenoxy radical formed by donation of a hydrogen atom to the DPPH radical was stabilized by resonance delocalization and could form quinones. Hence, two hydroxy groups in either the ortho or para positions are important for a higher capacity. A single hydroxyl group does not allow for resonance stabilization and results in a very low antioxidative capacity and low reactivity as illustrated by the hydroxyacetaphenone molecule.

The effect of methoxylation on activity was also assessed. It was seen that if hydroxyl groups were blocked by methoxylation, both the antioxidative capacity and reactivity were reduced (Table III). Both meta and para methoxylated phenols showed a large drop in capacity, but a small change in reactivity. These results can be explained by electronic and H-bonding effects on BDE for ortho disubstituted compounds. H-bonding with ortho OH in dihydroxy compounds stabilizes the phenoxy radical (ArO·) by 8 Kcal mole^{-1}, which is lost on methoxylation [13]. As the BDE of a methoxylated compound is higher, the reactivity is lower. Capacity reduction is attributed to the presence of one less hydroxy group in methoxylated compounds compared to the corresponding dihydroxy compounds.

To analyse the effects of the side chains on the catechol moiety, suitable molecules were chosen as shown in Table IV. Hydroxytyrosol (HT) and hydroxytyrosol acetate (HTA) showed similar capacity to catechol, but with a slightly slower reactivity. The alkyl side chain in HT and HTA which lack the potential to stabilize radical intermediates seems to have a minor impact. However, as seen with the caffeic acid, the presence of a vinylic side chain improved reactivity and capacity tremendously compared to that of HT and HTA. The higher activity of caffeic acid is attributed to the lower BDE of OH due to additional resonance and conjugation effects afforded by the vinylic group [15].
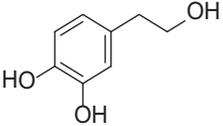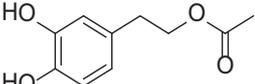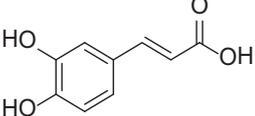
Finally, the impact of methoxylation of 2-OH groups in 1,2,4 trisubstituted phenols was characterized and showed decline in reactivity and capacity when compared to the corresponding non-methoxylated compounds (e.g. HT and caffeic acid in Table IV vs. homovanillyl alcohol and coniferyl alcohol in Table V). A similar trend was observed for di-substituted phenols upon methoxylation (Table II vs. Table III).

Vanillyl alcohol and homovanillyl alcohol showed small differences with each other in the antioxidative activity due to different reactivities. On the other hand, the coniferyl alcohol, which has an electron donating side chain, showed an eight- to nine-fold higher reactivity. Also, ferulic acid and 2-methoxy-4-vinylphenol, which have double bonds in their side chains, show slightly higher antioxidant reactivity compared to vanillyl alcohol.
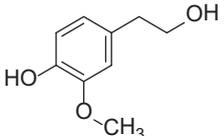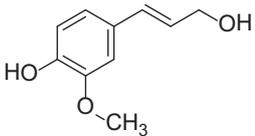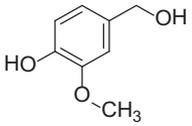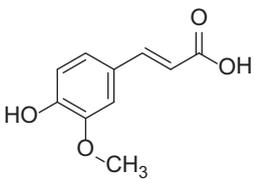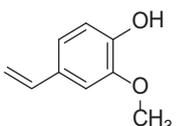
HAT reactions increase with the presence of *t*-butyl groups at the 2- and 6-positions (*ortho*), and methoxy substituents in the 4-position (*para*) by inductive donation of electron density to help in the resonance stabilization of the generated phenoxy radical [16, 17].

Although the results of HAT and SET mechanisms are the same, the kinetics are quite different. In the SET mechanism, a cationic antioxidant radical intermediate is created, with a more or less extended lifetime due to the possibility of a delocalized electron

Table IV Antioxidant *activity* of catechol(s) substituted with side chain

Chemical name	Molecular structures	AP (AU)	t_r (min)	w_c (mg)
Hydroxytyrosol 3,4-dihydroxyphenylethanol		915 919	0.17	0.00212
Hydroxytyrosol acetate 2-(3,4-dihydroxy)phenyl ethyl acetate		781 483	0.17	0.00249
Caffeic acid 3-(3,4-dihydroxyphenyl)-2-propenoic acid		2 032 910	0.16	0.00102

Table V Antioxidant *activity* of methoxylated trisubstituted phenols

Chemical name	Molecular structures	AP (AU)	t_r (min)	w_c (mg)
Homovanillyl alcohol		135 946	0.96	0.00247
Coniferyl alcohol 4-(3-hydroxy-1-propenyl)-2-methoxyphenol		538 199	0.15	0.00409
Vanillyl alcohol		88 287	1.14	0.00267
Ferulic acid		86 564	0.99	0.00384
2-methoxy-4-vinylphenol		408 459	0.25	0.00326

distributed throughout the aromatic ring and substituents. The SET mechanism involves the generation of ionic species. Therefore, the ionization potential (IP) can be used to predict the antioxidant activity *via* SET. The lower the ionization energy required, the more readily an antioxidant will donate an electron [18, 19]. As the IP increases with lower pH, so the SET reactions are favoured in alkaline solvents.

In many cases, [15] both SET and HAT mechanisms occur simultaneously or in mixed forms, depending on the stoichiometry, the structure, molecular size, free radical, pH, solvent, ionic strength and presence of metal ions.
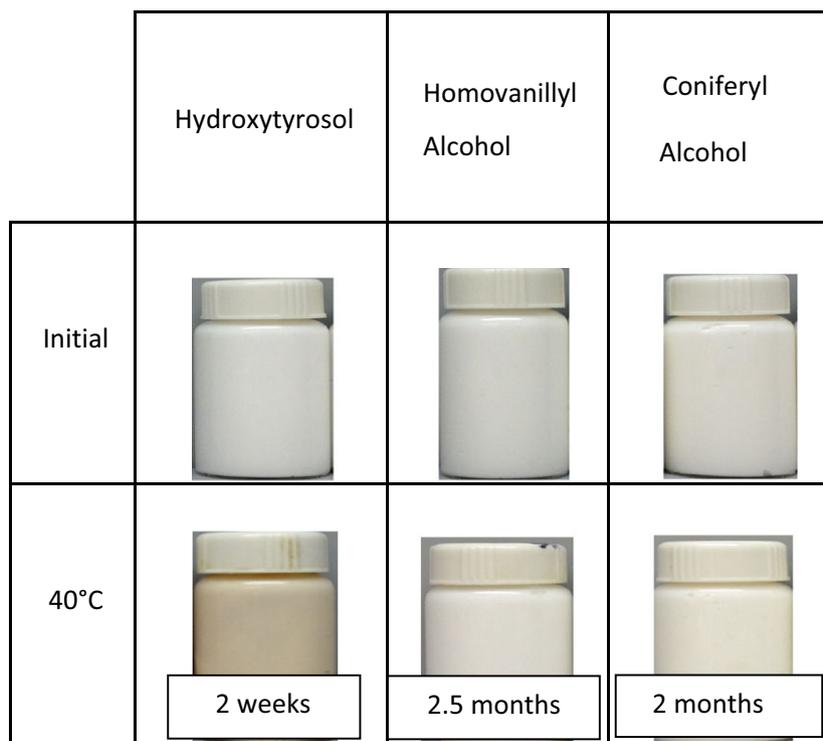
It might be expected that phenols with short reaction times would readily undergo oxidative degradation due to their high reactivity. An example of the reactivity of the substituted phenol, hydroxytyrosol is presented in two different systems (Table VI). Hydroxytyrosol (0.5%w//w) was formulated into two different proprietary cosmetic vehicles. These systems were similar and contained triglycerides (capric/caprylic triglycerides, shea butter), glycols (such as glycerine), polymers (carbomers, xanthan gum and cellulose derivatives), emulsifier (such as phosphatidylcholine) and water; however, their relative levels varied. The AP values were determined immediately post manufacture and then 40 days later after storage at 40°C. The results indicate that there are strong differences in the reactivity between the two media

(t_r = 0.12 min in Formula B versus 0.2 min in Formula A) and illustrate how formulation changes are sometimes necessary to optimize antioxidant performance. After storage for 40 days at 40°C, the main difference observed is a lower reactivity in Formula A, meanwhile the decay in antioxidant capacity is similar for both formulations.

Table VI Antioxidant activity of 0.5%w/w hydroxytyrosol in two different cosmetic formulations

	Antioxidative power					
	$t = 0$			$t = 40$ days*		
	AP (AU)	t_r (min)	w_c (mg)	AP (AU)	t_r (min)	w_c (mg)
Formula A	998	0.20	0.166	262	0.42	0.301
Formula B	1987	0.12	0.139	733	0.18	0.251

*Storage at 40°C.

	Hydroxytyrosol	Homovanillyl Alcohol	Coniferyl Alcohol
Initial			
40°C	2 weeks	2.5 months	2 months

Figure 2 Comparison of colour changes observed in antioxidant systems formulated into the same formulation vehicle. Significant differences in colour stability are apparent that can be related to chemical structure and AP values. Hydroxytyrosol shows significant darkening after 2 weeks storage at 40°C whereas homovanillyl alcohol and coniferyl alcohol show relatively little change. Furthermore, oxidative polymerization occurs easily in non-methylated catechols [20].

The images in Fig. 2 reveal how the AP values can be used for formulation development purposes. Hydroxytyrosol, homovanillyl alcohol and coniferyl alcohol were formulated at 0.5%w/w levels into the same formulation vehicle. A rapid colour change is observed in the hydroxytyrosol (AP = 915 919) containing formulation after 14 days. It is observed that homovanillyl alcohol (AP = 135 946) and coniferyl alcohol (AP = 538 199) show less evidence of colour change after 2.5 months and 2 months at 40°C, respectively. These data appear to suggest that AP values may have the potential to be used predictively in formulation design to identify colour stable systems.

Conclusions

A series of phenolic molecules were studied and insights gained into how their chemical structure related to their antioxidant potential using a DPPH electron spin resonance method. The structure activity analysis revealed the best antioxidants to be those compounds that contain electron donor groups directly attached to the aromatic ring, especially if the group is in ortho position to ArOH and has the ability to form H-bond with ArO· radical, for example catechol.

The formulation data revealed that a high reactivity of the antioxidant molecule may possibly translate into poor stability within the cosmetic product. Hence, careful attention needs to be paid to both capacity and reactivity of the molecule when selecting molecules that would be effective as well as colour stable.

The described DPPH electron spin resonance approach is a potentially useful tool for formulation design and has application in identifying agents that can be formulated into cosmetic products.

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