Development of an innovative peat lipstick based on the UV-B protective effect of humic substances

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SUMMARY

Humic acids (HA) are known for their antiviral and UV-B protecting effects, and are considered promising as ingredients for a UV-protective lipstick which is being developed to minimise or even prevent recurrences of UV- induced herpes. In this study, the UV/Vis spectra of three natural HA and three synthetic HA-like substances are analysed to determine the appropriateness of their UV-absorbing characteristics for the product under development. The contribution of a matrix component (castor oil) to the total UV absorption of the lipstick is also assessed. The results confirm the expected high UV-B absorption of the individual test substances, but reveal considerable differences in the UV-A wavelength range. Castor oil absorbs only UV-B radiation; and when mixed with HA it enhances total absorption in the UV-B range, but reduces it in the UV-A range. This is probably due to molecular interactions between castor oil and HA. Preliminary results from cultures of human U937 cells assayed for survival 24 hours after exposure to UV-B radiation show that both HA and castor oil exert a significant concentration-dependent UV-B protective filter effect similar to that of the UV-B absorbing reference substance p-aminobenzoic acid (PABA).

KEY WORDS: castor oil; natural and synthetic humic substances; UV/Vis spectra

INTRODUCTION

The sunlight that reaches the earth's surface has a wide spectrum consisting of 52 % visible light, 42 % infrared radiation, 5.6 % UV-A and 0.4 % UV-B radiation (Umbach 2004). UV-B has both positive and negative effects on human skin. On the one hand, it is necessary for the activation of melanin formation and for the development of delayed pigmentation which, like a natural sunshade, protects the skin against UV light. On the other hand, prolonged exposure to UV-B radiation can cause skin erythema, sunburn and the formation of mutagenic DNA lesions capable of inducing skin cancer as a long-term effect. UV-B radiation is also a major provocative factor for the re-activation of herpes labialis, a recurring viral infection caused by Herpes Simplex Virus (HSV) Type 1 (Schöfer 2010). The use of sunscreen products for preventing UV-light induced herpes labialis (Rooney *et al.* 1991), and of antivirals for its treatment (Spruance *et al.* 1991) has been a subject of basic and clinical research since the early 1990s (for review see Harmenberg *et al.* 2010).

Humic substances, and in particular their alkali-soluble humic acids (HA) fraction, are known for UV-absorbing properties and show some structural similarities to human eumelanin (Cataldo 1998). They are effective in protecting bacterial (Bitton *et al.* 1972, Muela *et al.* 2000) and human cells from UV-induced damage (Hübner 2004, Klöcking *et al.* 2004, Kühn 2005, Klöcking 2010). Moreover, humic substances are antivirally active against various DNA and RNA viruses (Klöcking *et al.* 2006) and exert anti-inflammatory activity *in vivo* (Klöcking *et al.* 1968).

Current research aims to utilise the photo-protective properties of HA in a peat lipstick that could be used to prevent re-activation of the herpes simplex virus by UV light. This article reports the UV/Vis spectra of three natural HA and three synthetic HA-like substances, which were analysed to identify appropriate ingredients. Castor oil, as an important matrix component of the lipstick, was also studied; and the UV-protective effects of HA, castor oil and some combinations of both were examined in human U937 cells.

METHODS

Test substances

Humic acids from Altteich peat
Both sodium and potassium humate were prepared from a black peat layer occurring at the Altteicher Moor (north-east Saxony, Germany) according to the method described by Meyer & Klöcking (2011). The freeze-dried peat sample was extracted with a mixture of methyl acetate and cyclohexane in a Soxhlet extractor to remove organic impurities, then dried under vacuum. The peat was then suspended in water and extracted with an aqueous solution of sodium or potassium hydroxide for two hours under mild conditions (pH 9.0; 30 °C). The sludge was separated by centrifugation (ten minutes at 4,000 r.p.m.) and floating particles were removed by filtration. Humic acids were precipitated with an aqueous solution of oxalic acid at pH 1.5. The precipitate was collected by centrifuging at 11,000 r.p.m. for three hours, then washing with water until it was free of oxalic acid. The residue was suspended in water and dissolved by adjusting to pH 7.0 using NaOH or KOH as appropriate. The resulting solution was centrifuged to remove insoluble components, then freeze-dried (Kleiner *et al*. 2010).

Synthetic HA-like substances
Synthetic HA-like substances were prepared according to Helbig *et al*. (1997) by periodate oxidation of (2E)-3-(3,4-dihydroxyphenyl) propenoic acid (*caffeic acid*), 3-(3,4-dihydroxyphenyl)-propanoic acid (*hydrocaffeic acid*) and 3-(3,4-dihydroxyphenyl)alanine (*DOPA*) to the corresponding oxidation products (OP), namely: caffeic acid OP (KOP 466), hydrocaffeic acid OP (HYKOP 441), and DOPA-OP, respectively. Peak molecular weights determined by HPSEC (Kloecking *et al*. 2008) were found to be 2.64 kDa with a shoulder at about 25 kDa (KOP); 1.95 kDa (HYKOP); and two separate peaks at 1.07 and 33.17 kDa (DOPA-OP).

HuminFeed® was obtained from Humintech GmbH, Düsseldorf, Germany. It is produced by alkaline extraction from highly oxidised German lignites (Leonardite). The water-soluble product contains 70–80 % HA (as sodium salts) and has a pH value between 9 and 10.

Waskish peat humic acid (IR107H) derived from a *Sphagnum* peat that formed at Pine Island Bog in Koochiching, Minnesota (USA) was purchased from the International Humic Substances Society (IHSS) and used as reference HA.

Castor oil (Rizinusöl native Ph. Eur. 5.0, Charge K00034) purchased from D. Köhler, Sonsbeck, Germany was also studied, both alone and in combination with HuminFeed.

PABA = p-aminobenzoic acid, (Merck KGaA, Darmstadt, Germany) was used as UV-B absorbing reference substance.

UV/Vis spectra

The UV-Vis spectra were measured with the spectrophotometer Synergy HT Multi-Mode Microplate Reader (BioTek) using 96-well microplates UV-Star® (Greiner Bio-One GmbH). The samples (except for castor oil) were dissolved in water and measured at three substance concentrations (10, 100 and 1000 µg/ml) and a layer thickness of 2.2 mm. The spectra between 280 and 800 nm were displayed as the percentage reduction of light transmission.

Cell culture

The human cell line U937 (CRL 1593) was cultivated in RMPI 1640 medium containing 10 % foetal bovine serum, and incubated at 37 °C in a humid atmosphere containing 5 % CO_2. To determine the cell count and the viability of cells, a Neubauer counting chamber was used. The cells were stained with a solution of 0.1 % trypan blue. The live/dead staining shows living cells colourless and dead cells stained blue. For the experiments, the cell concentration was adjusted to 5×10^5 cells/ml by adding RPMI medium.

UV-B irradiation, determination of UV-induced cell damage and UV-protective effect

Cells were irradiated using the microprocessor-controlled device Bio-Sun (Vilber Lourmat). For this, 100 µl of cell suspension was transferred into each well of a 96-well flat-bottom microtitre plate. Before irradiation, a UV-transparent 96-well plate (UV-Star®) was placed on top of the primary (lower) plate and each well was filled with 100 µl of the humic substance dilution or distilled water (for positive controls). The "double plate" was exposed to UV-B radiation at a wavelength maximum of 312 nm and an irradiation dose of 80 mJ/cm². After that, the cell cultures were incubated at 37 °C / 5 % CO_2

for 24 hours. This was followed by determining cell toxicity employing the XTT tetrazolium reduction assay (Roche Diagnostics) according to manufacturer's specifications. The optical density (OD) of formazan produced by cellular dehydrogenases was measured after incubating for three hours at 37 °C, at a wavelength of 450 nm (reference wavelength 620 nm). The UVB-protecting effect (PE) was calculated (as a percentage) from the measured values of OD according to the equation:

$$PE_{UV-B} = \frac{x_i - x_0^*}{x_0 - x_0^*} \times 100 \quad [1]$$

where x_0 represents the OD for non-irradiated controls, x_0^* the OD after irradiation with distilled water in the upper plate, and x_i the OD after irradiation with the humic substance dilution under test in the upper plate.

RESULTS

UV/Vis spectra

The UV-Vis spectra of the three potential lipstick ingredients natural HA, synthetic HA and castor oil are presented in Figures 1–3. The spectrum of the UV-absorbing reference substance p-aminobenzoic acid (PABA) is shown for comparison in Figure 3.

The UV/Vis spectra shown in Figures 1 and 2 are typical for substances of the HA type. They are characterised by high UV absorption (low transmittance) in the higher-energy range (short-wave light) and by a continuously decreasing UV absorption (increasing transmittance) in the lower-energy range (long-wave light).

The spectra of sodium and potassium humate from Altteich peat (AP) are nearly identical irrespective of the solvent (NaOH, KOH) used for alkaline extraction of the peat material. At concentrations of 100 µg/ml HA and a layer thickness of 2.2 mm they reduce the UV_{280} transmittance to 77 % and 80 %, respectively. HuminFeed reaches 76 % under the same conditions (Figure 3). Na-humate from Waskish peat reduces the UV_{280} transmittance to only 52 %. The corresponding values for UV_{340} are 59 % (Na-humate AP), 55 % (K-humate AP), 56 % (HuminFeed) and 31 % (Na-humate WP).

Figure 2 shows the UV/Vis spectra of the three synthetic HA: KOP 466, HYKOP 441, and DOPA-OP. The shapes of the UV/Vis spectra and the capacity to reduce UV transmittance are very similar to those of natural HA. The reduction of UV_{280} transmittance through KOP, HYKOP and DOPA-OP is 77 %, 72 % and 76 %, and the reduction of UV_{340} transmittance amounts to 45 %, 32 % and 51 %. Unlike the UV/Vis spectra of KOP and HYKOP, the spectrum of DOPA-OP exhibits a shoulder at about 320 nm. At the highest test concentration (1000 µg/ml) all HA and HA-like test substances exceeded the measuring range in the UV-B range and reached at least 94 % reduction of transmission in the UV-A range.

Figure 3 presents the UV/Vis spectra of HuminFeed and castor oil, and of two combinations. Castor oil is one of the basic matrix components of lipsticks, which usually contain 50–70 % of this oil. It is characterised by a relatively high UV-B absorption with a maximum at 270 nm, but absorbs very little UV-A and visible light. The question is whether castor oil, due to its high concentration in the lipstick, influences the UV absorption of the lipstick matrix as a whole. In contrast to humic substances, the oil is measured as pure, undiluted fluid. In Figure 3, the absorption curves of HuminFeed correspond largely with those of the Altteich peat humic acid in Figure 1. However, the data from the combination of HuminFeed with castor oil show a surprising picture. The UV-A absorption of 100 and 1000 µg/ml HuminFeed is clearly diminished while the absorption at wavelengths >560 nm is enhanced. This probably indicates the formation of previously unknown new compounds as a result of interactions between castor oil and HA.

Like castor oil, the reference substance PABA has a significant UV absorption in the UV-B range with a maximum at 280 nm, but hardly absorbs any UV-A radiation.

UV-B protective effect

In the next step, we examine the UV-B protective effect of Altteich Peat HA, Waskish Peat HA, KOP 466/489, HYKOP 441, DOPA-OP and PABA for human U937 cells. According to Kühn (2005) we use a test arrangement where cells and test substances are placed in two separate plates, which enables us to evaluate the UV filtering effect independently of possible interactions of the test substances with the cells. The results are shown in Figures 4 and 5.

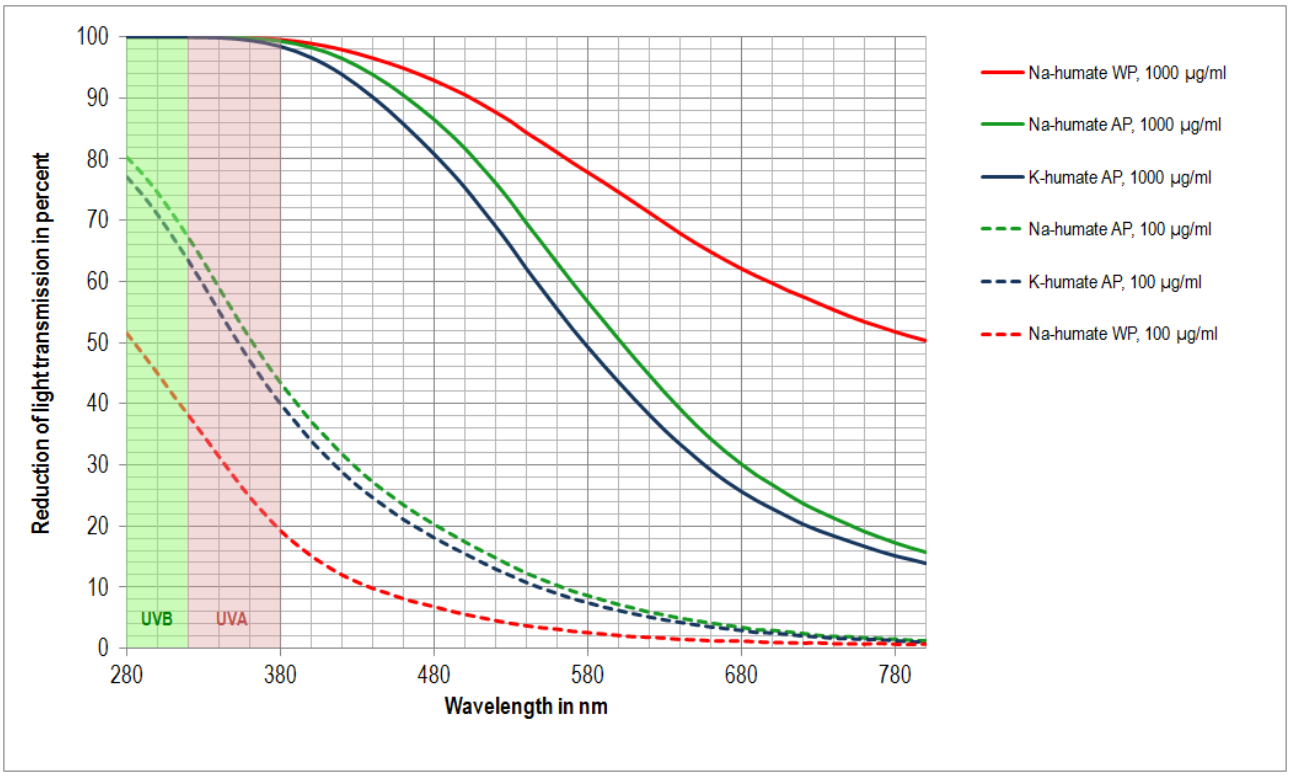

Figure 1. UV/Vis spectra (280–800 nm) of naturally occurring HA from Altteich peat (AP) as Na- and K-humate and from Waskish Peat (WP) as Na-humate. Concentrations of test substances: 100 and 1000 µg/ml; layer thickness: 2.2 mm.

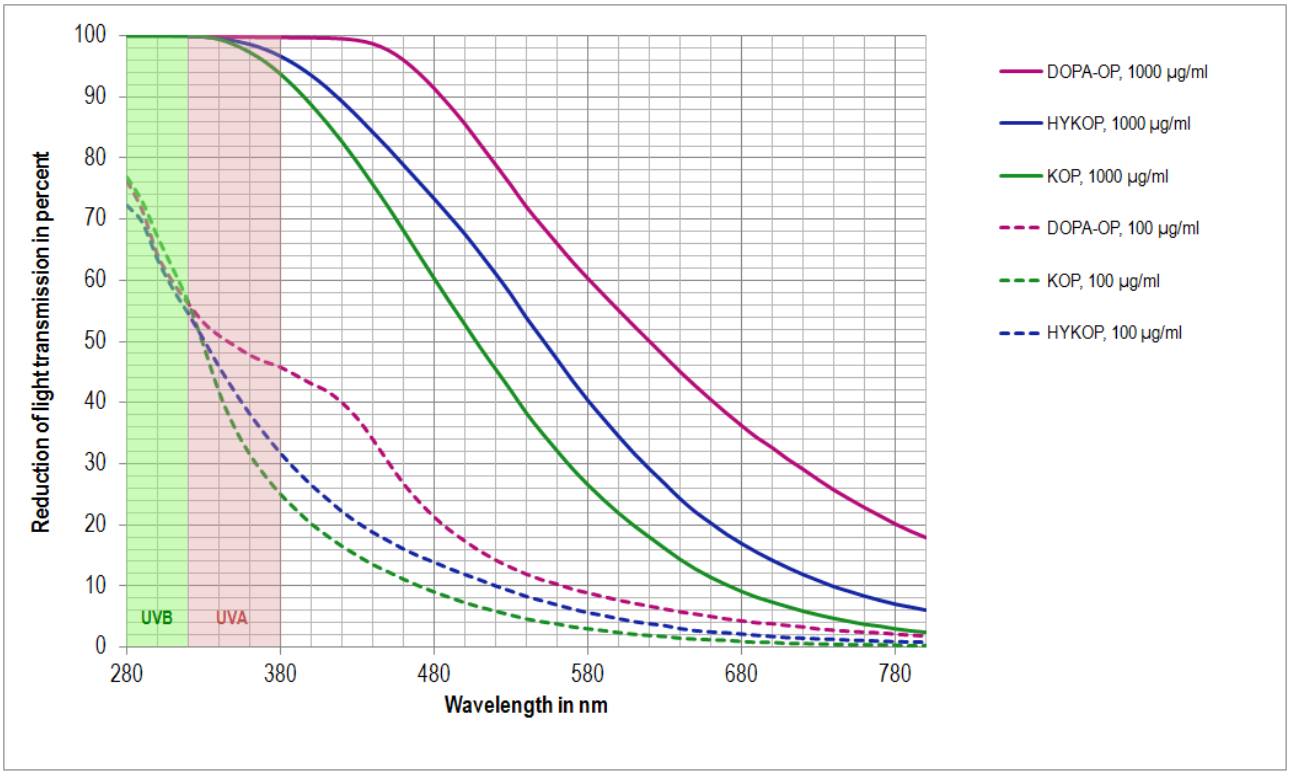

Figure 2. UV/Vis spectra (280–800 nm) of HYKOP 441, DOPA-OP and KOP 466 (as sodium salts). Concentrations of test substances: 100 and 1000 µg/ml; layer thickness: 2.2 mm.

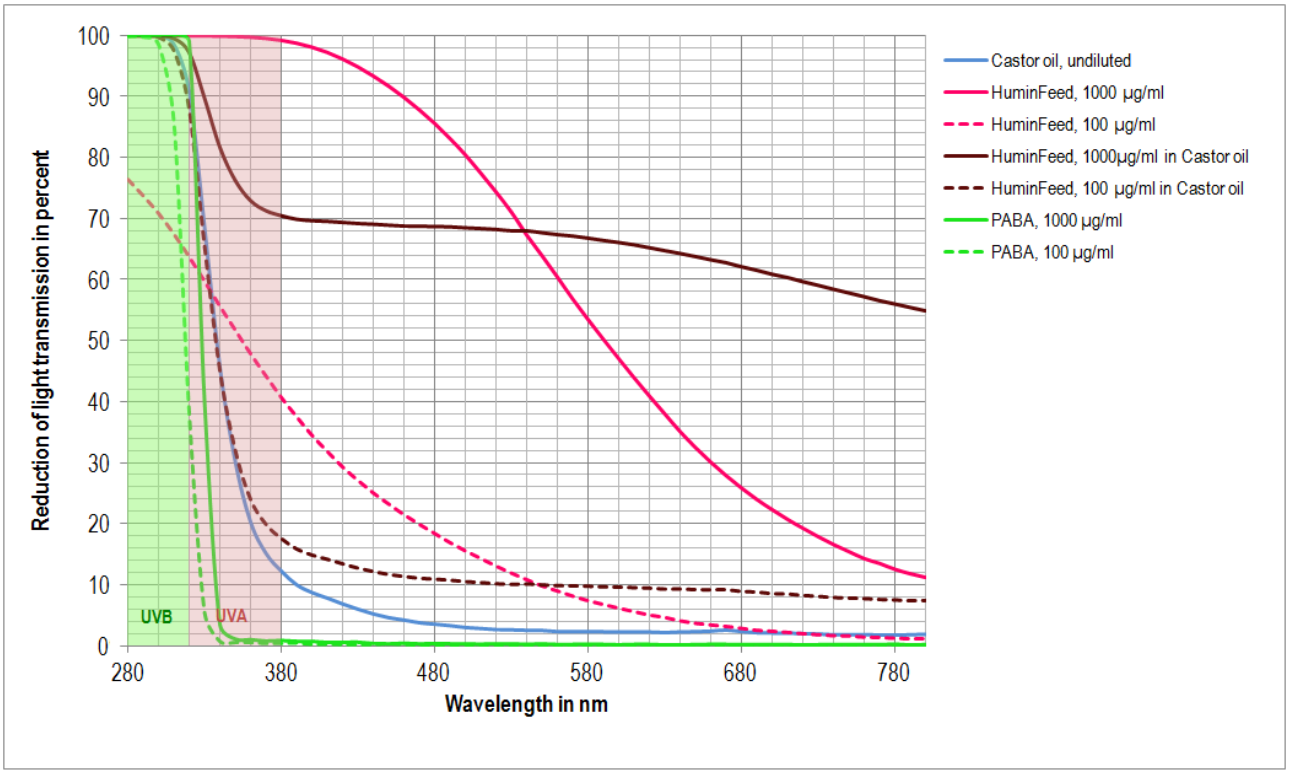

Figure 3. UV/Vis spectra (280–800 nm) of HuminFeed® (100 and 1000 µg/ml), undiluted castor oil, and two mixtures. The UV/Vis spectrum of PABA (100 and 1000 µg/ml) is also shown. Layer thickness: 2.2 mm.

Figure 4 shows the UV-B protective effect of natural HA from Waskish peat and of sodium and potassium humate from Altteich peat for U937 cells. It is clear that humic substances applied as a UV filter are able to protect U937 cells from UV-induced damage. While HA concentrations of 10 µg/ml are too low to shield effectively against UV rays, clear positive effects are detectable at 100 and 1000 µg/ml. Na-humate and K-humate AP at a concentration of 100 µg/ml prevent cell damage almost completely, as does HA from Waskish peat at the highest concentration tested (1000 µg/ml).

The same pattern is obtained with the synthetic HA-like substances HYKOP, DOPA-OP and KOP (Figure 5). So, at the lowest concentration (10 µg/ml) tested, these substances do not exert any protective effect on the cells; but at higher substance concentrations (100 and 1000 µg/ml), they prevent UV-induced cell damage by more than 60 %. Almost complete UV protection is provided by HYKOP, KOP, and DOPA-OP at the highest substance concentration (1000 µg/ml).

Finally, the two experimentally used lipstick ingredients HuminFeed and castor oil are examined for their UV-protective capacity in the same way. The Leonardite HA HuminFeed behaves very similarly to the other HA shown in Figures 4 and 5. While the UV-protecting effect is negligible at a concentration of 10 µg/ml, it amounts to about 75 % at 100 µg/ml and reaches almost 100 % at the highest test substance concentration (1000 µg/ml).

Pure undiluted castor oil improves the viability of UV-B irradiated U937 cells by approximately 73 % (Figure 6). The protective effect increases to about 80 % by adding 100 µg/ml HuminFeed to this oil. Almost complete UV protection is provided by 1000 µg/ml HuminFeed with and without castor oil. It follows that the degree of UV protection may benefit from the presence of UV-B absorbing oils, especially at low and mean humic substance concentrations. The UV-B absorbing reference substance PABA already provides almost complete protection from UV-induced cell damage at a concentration of 100 µg/ml PABA.

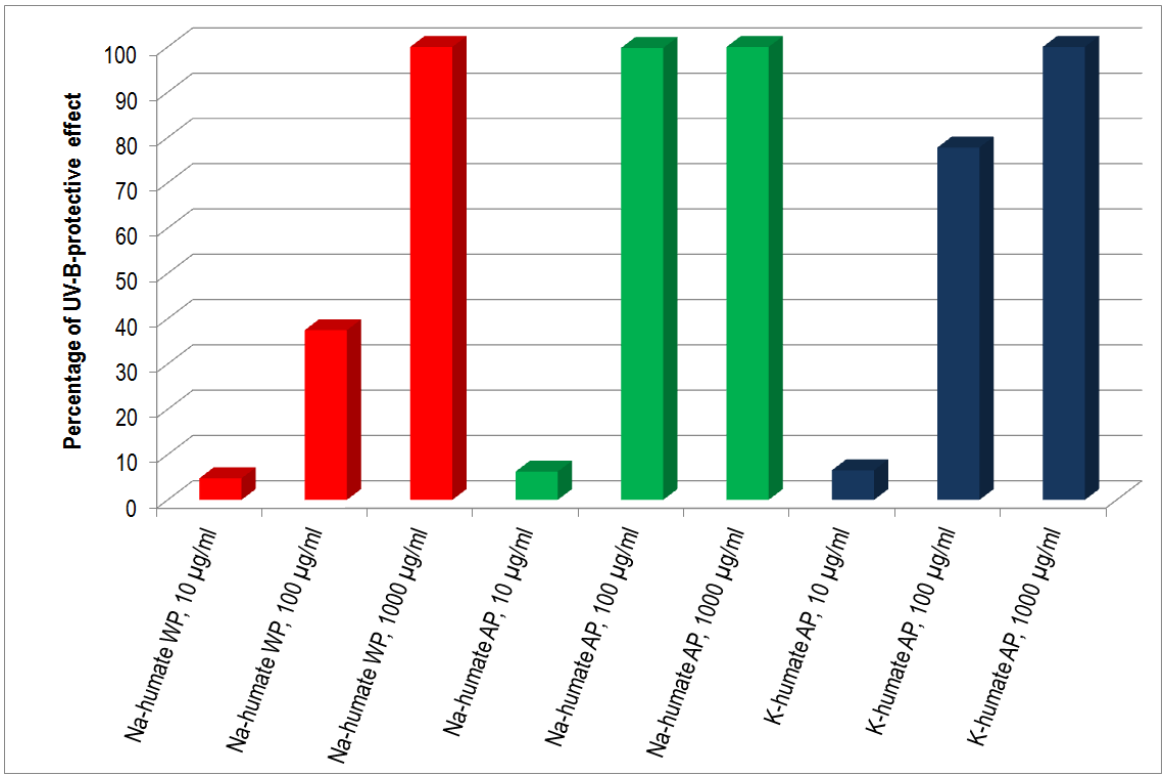

Figure 4. UV-B protective filter effect of HA from Waskish peat (WP) and of sodium and potassium humate from Altteich peat (AP) for U937 cells. Length of the optical path through the HA-containing filter medium of the upper plate: 2.2 mm. XTT test 24 h after irradiation; the data are means of three single measurements.

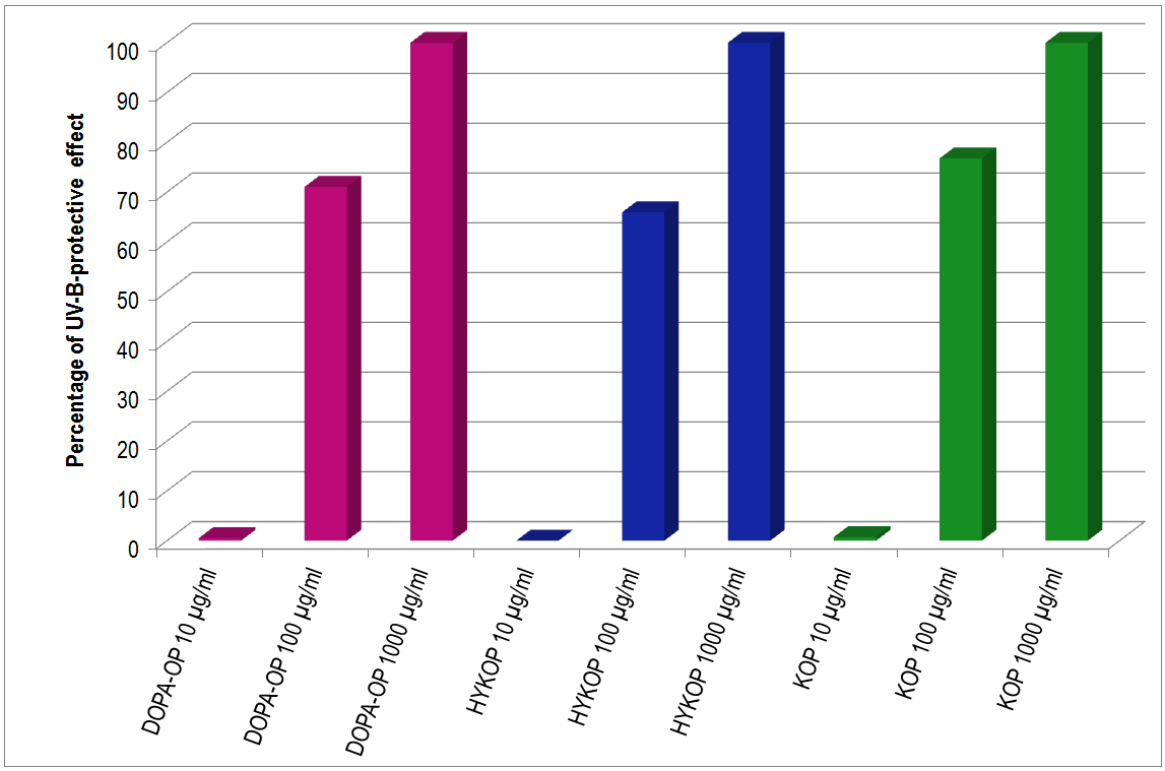

Figure 5. UV-B protective filter effect of HYKOP 441, DOPA-OP and KOP 466/489 for U937 cells. Length of the optical path through the HA-containing filter medium of the upper plate: 2.2 mm. XTT test 24 h after irradiation; the data are means of at least three single measurements.

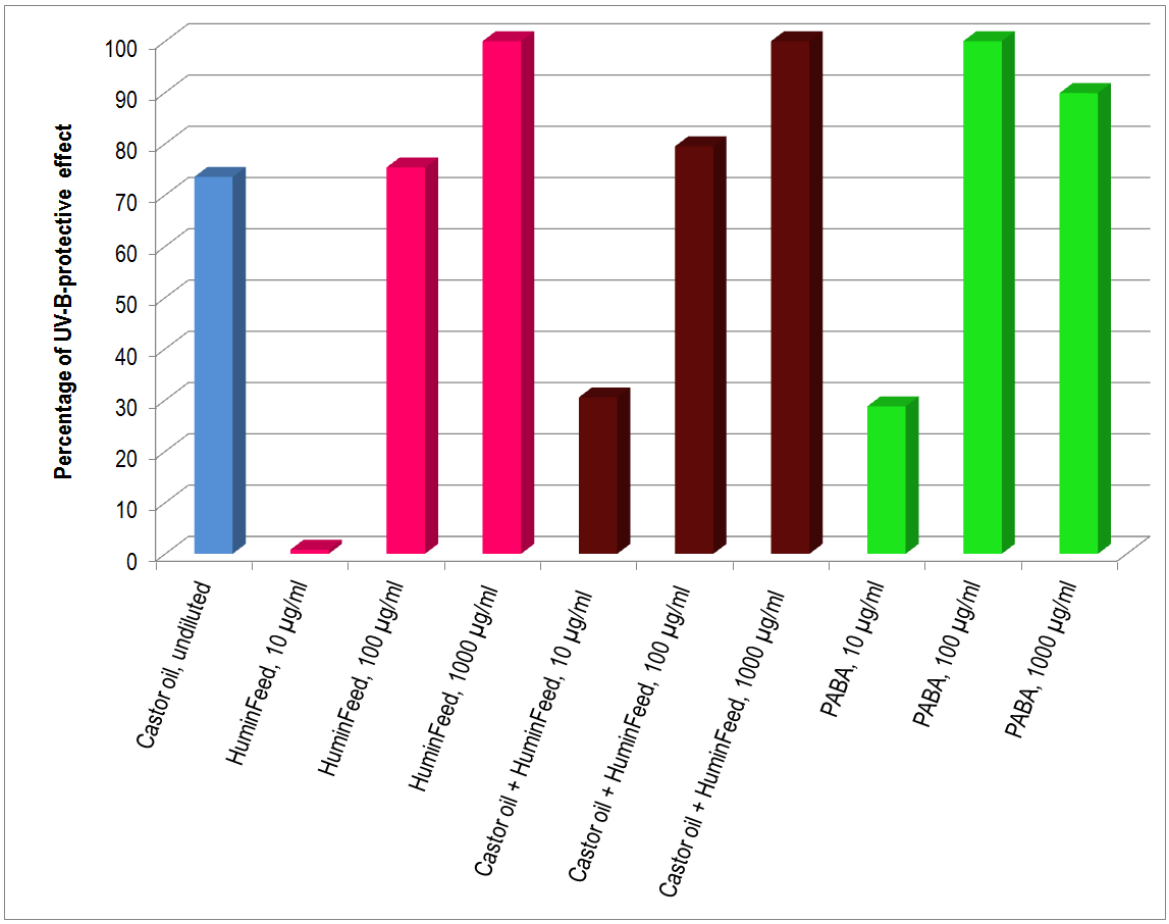

Figure 6: UV-B protective filter effect of castor oil, HuminFeed and three mixtures for U937 cells. Length of the optical path through the HA containing filter medium of the upper plate: 2.2 mm. XTT test 24 h after irradiation; the data represent means of at least three single measurements.

DISCUSSION

With regard to developing a UV-protecting lipstick on the basis of UV absorbing substances, the UV/Vis spectra of the products in question are the primary criterion for assessing the UV-filtering effect. The results obtained from three natural and three synthetic HA show that all of them are fundamentally suitable for UV-B and, to a lesser extent, also for UV-A absorption. Differences (e.g. between HA from Altteich peat and from Waskish peat) may be caused by different preparation methods and/or structural differences, and should be investigated in more detail.

Besides naturally occurring HA, we investigated also the HA-like oxidation products of DOPA, caffeic acid and hydrocaffeic acid. The last two dihydroxylated phenylpropanoids were chosen because of their structural relationship to DOPA, the parent compound of melanin (Mason 1948). DOPA differs from hydrocaffeic acid by just one amino group and from caffeic acid, additionally, by the lack of the ethylenic double bond in the side chain.

The generally high level of UV-B absorption by natural as well as synthetic HA has long been recognised, as has the shape of the typical absorption curve of HA which, in contrast to other UV-filters such as PABA (280 nm), does not show any absorption peaks in the range 280–800 nm (see, for example, the UV absorption spectrum of peat water HA published by Klöcking 2010). Upon closer examination, however, one notices that the absorption curve of DOPA-OP differs from the HA standard curve as well as from the curves of KOP and HYKOP in that it shows a higher absorption level in the UV-A range, which may be due to the N-heterocyclic basic structure shown for DOPA-melanin (Watt *et al*. 2009).

Using a variation of the double-plate test design introduced by Kühn (2005), all of the previously mentioned HA were examined for their UV-protective filter effect on human U937 cells. With

the exception of Waskish peat HA, the protective effect of HA and HA-like substances (100 µg/ml) was found to be between 60 % and 90 %. The results are consistent with earlier findings of Kühn (2005), who reported 50 % UV-B protection at HA concentrations between 30 and 40 µg/ml.

The test design characteristics did not allow possible interactions of HA with the cells, or phototoxicity/photoinstability of the test substances, to be taken into account. In this regard, the assessment of the individual substances is still preliminary and should be complemented by detailed in-vitro toxicological studies.

Oils may contribute significantly to the UV protection of body care products (Teressa *et al*. 2004), especially when they are present in high concentrations. It should be noted that the castor oil content of the lipstick matrix is approximately 1000 times its HA content. This may explain why castor oil and other oils apparently influence the spectral features of other lipstick components. A more detailed analysis of molecular interactions between UV-absorbing substances and matrix ingredients is of high interest and should be included in future studies. This could ensure better utilisation of positive additive and synergistic effects on UV protection, and at the same time prevent possible negative consequences.

CONCLUSIONS

The results of these studies are consistent with the following conclusions.

1. HA from different sources (Altteich peat, Waskish peat, Leonardite), as well as synthetic HA-like substances, are capable of strongly absorbing UV-B and, to some extent, also UV-A radiation.
2. The effective protection of human U937 cells against UV-B induced cell damage is mainly due to the UV-B filter effect of HA.
3. Castor oil, as a usual lipstick component, absorbs UV-B radiation to a significant degree, and also has a UV-B protective effect for human cells. In combination with HuminFeed, castor oil supports the UV-B absorption of HA, but reduces absorption in the range of UV-A.

ACKNOWLEDGMENTS

This work is funded by the German Bundesministerium für Bildung und Forschung (BMBF). We also thank Prof. Dr. rer. nat. habil. Roland Schubert for providing facilities for our studies. This manuscript arises from an oral presentation by Seel *et al*. (2012) at the 14th International Peat Congress, 03–08 June 2012, Stockholm, Sweden.

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Submitted 30 Aug 2012, final revision 22 Apr 2013
Editor: Olivia Bragg

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