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Study of polyphenol penetration from organic-aqueous cocoa extracts – antioxidant activity

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Summary

Active ingredients of plant origin such as polyphenols and phenolic acids are enjoying increasing acceptance in both the food and cosmetics industries. The Swiss chocolate industry currently benefits from an increased consumption and export. Main driver is of course the exquisite taste, however, health originating from cacao polyphenols play an increasing role. Their antioxidant properties are typically determined using different in vitro assays. However, the high reactivity of antioxidants impedes the development of effective skin care formulations. Besides penetration, the bioavailability of the antioxidants has to be investigated as well. Both depend on molecular size, and also on the molecules' polarity relative to the polarity of the skin. In this paper, the antioxidant activity of an organic-aqueous cocoa extract and its main polyphenols (such as epicatechin) are compared using HPLC-based in vitro antioxidant assays. Interesting is also to compare the intensity of the anti- xidant potential for fraction. This points out how many antioxidant potential the often ignored e.g. condensed polyphenol would have. Following main components of a cocoa extract (beside the alkaloid caffeine, theobromine) will at least from a logP value be appropriate for skin penetration: epicatechin/catechin (EC/Ca), epigallocatechin (EGC), procyanidin B2 (B2) and could as monomer, dimers and trimer strongly contribute to the antioxidant capacity. (Abbe Makeyki et al., 2008)

Solid Lipid Nanoparticles (SLN) are biocompatible, stable during storage and manufacturing, and suitable for scale up. They claim to improve the stability of many actives and can enhance skin penetration. For this reason the application of SLN for the aforementioned cocoa extract and epicatechin as the lead polyphenol has been tested for their application and production feasibility. SLNs particles prepared with and without polyphenol load were prepared using microemulsion technology and were suitably purified.

The penetration behaviour of different aqueous solutions with polyphenolic substances (epicatechin (EC)), theobromine (TH), caffeine (CA) and solid lipid nanoparticles SNL on skin penetration is described in studies using a continuous and sustainable on-line Franz Cells assay (polymeric skin mimicking membranes and excised pig ear skin). These in vitro results about 8 hours were compared to a short in vivo penetration assay (30 min) by confocal Raman spectroscopy. After 8 hours of application EC remained mostly in the donor compartment and a very small amount close to the detection was observed in the skin layer viable epidermis. The recovery for the aqueous 0.05% EC was 83% +/-0.72% after triplicate. While the main barrier function of the skin is located in the stratum corneum, no or little permeation was expected. The residual amount of EC is probably lost due to linkage to proteins of the skin.
Interestingly neither the encapsulated actives by SLN nor main substances of the cocoa extract (TH, EC, CA) in the applied dose could be detected; neither in the donor nor in another skin layer. The

recovery of the encapsulated actives in SLN after triplicate couldn't be assessed. More investigations are needed for an optimized sample preparation and to better understand potential interaction of surface active agents and solid lipids of the SLN ingredients with the stationary phase and the pre-column of the HPLC.
The results obtained by in vivo Raman spectroscopy measuring 30 min after application indicated that the substance remained on the skin surface
The influence of SNL on the perceived sensory properties on the skin assessed through descriptive sensory analysis showed a pleasant skin feeling during the afterfeel phase. The trained sensory young expert panel certified a remarkable increase of the attributes smoothness silky and velvety when comparing a watery gel with a watery dispersion of 5% SLN (Type B). Although of this pleasant sensory characteristic, the chosen SLN techniques and/or the combination of polyphenolic actives (deep load) didn't support in this setting the stability or the penetration behaviour of EC or other cocoa main actives. This actives still remains interesting for an antioxidant efficacy for skin surface application.

Introduction

Polyphenols belong to one of the most interesting classes of compounds in nature with high antioxidant potential. They occur in many plant materials consumed by humans, such as tea, cocoa, coffee and other herbs (Richelle M. et al.,2001). Polyphenols have been claimed to have health-promoting effects like anti-oxidant, anti-microbial, anti-viral, anti-aging, anti-thrombotic and anti-allergic, also govern cellular processes and have metal chelating functions. (Kuhnert N., 2013). Polyphenols used in skin treatment include tea polyphenols (like epicatechine), silymarin, quercetin, resveratrol and tannins (hydrolysable and condensed substances such as proanthocyanidins) (McMullen R. L, 2013). Therefore, these bioactive compounds have to be successfully formulated and incorporated into a vehicle, either as a simple solution, an emulsion or in some other suitable carrier.

Bioavailability depends on molecular size, but also on the difference between the molecule's and the skin's polarity. This difference can be estimated from the partition coefficient n-octanol-qater (logP). In theory, a moderate lipophilic octanol-water partitioning coefficient (*P*ow between 10 and 100, which corresponds to a log*P* between 1 and 2) and a molecular mass lower than 500 g/mol represent promising chances for a skin penetration of these substances through the stratum corneum to the skin epidermis. Unfortunately materials with a logP between 1 and 2 are mostly sparingly soluble either in water or in oil. Therefore application and skin penetration must be supported and enhanced by the topical formulation (Daniels R. et al., 2007)

In general, the penetration of polyphenols is improved with smaller molecular size and moderate hydrophilicity (negative logP) according to Zillich et al. (2013). Following main components of a cocoa extract (beside the alkaloids caffeine, theobromine) will at least from a logP value be appropriate for skin penetration: epicatechine/catechine (EC/Ca), epigallocatechine (EGC), procyanidin B2 (B2).

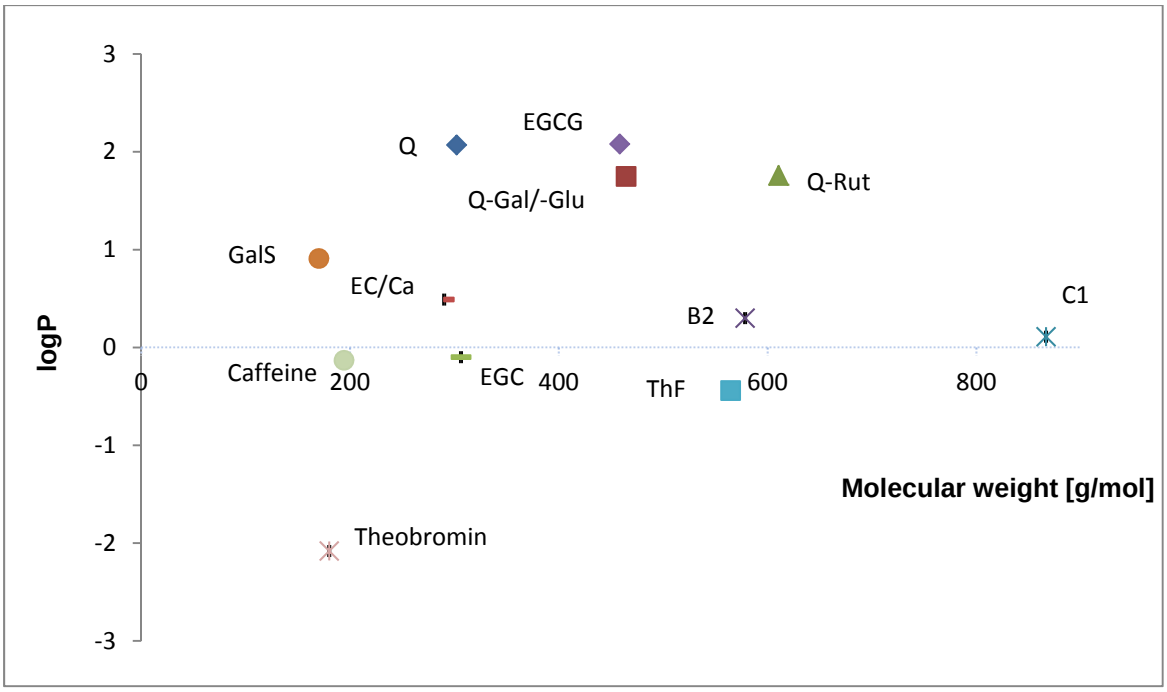

Fig. 1: Polyphenols and alkaloids assigned according to molecular weight and logP (logP + = Lipophilic, - = Hydrophilic) with the calculated logP (ACD/ChemSketch V. 12.01) (Q = Quercetin; Q-Glu = Quercetin-3-O-glucosid; Q-Gal = Quercetin-3-O-galactosid; Q-Rut = Quercetin-3-O-rutinosid; B2 = Procyanidin B2; C1 = Procyanidin C1; Ca = (+)- Catechin; GalS = Gallic acid; EC = (-)-Epicatechin; EGC = (-)-Epigallocatechin; EGCG = Epigallocatechingallat; ThF = Theaflavin) (Largey, O., 2014).

The characterization of the antioxidant activity of an organic-aqueous cocoa extract and its main polyphenols (such as epicatechin) was performed using in vitro assays DPPH. The antioxidant activity of the main substances was screened for potential candidate with ideal molecular weight and logP . A promising method is an on-line based screening NP-HPLC-DPPH antioxidant assay. This offers an interesting alternative to screen the antioxidant capacity, especially of condensed polyphenols directly from e.g. unroasted cocoa beans extract. The polyphenols are detected by 275nm. After incubation time nearly one of 2 min with the DPPH-reactant reagent the newly formed reaction products are detected by 515 nm. The Normal Phase (NP) allows the fractionation separation of condensed proanthocyanidins based on their degree of polymerization and therefor on their molecular size e.g. of a homologous series. (Pedan V. et al., 2015)

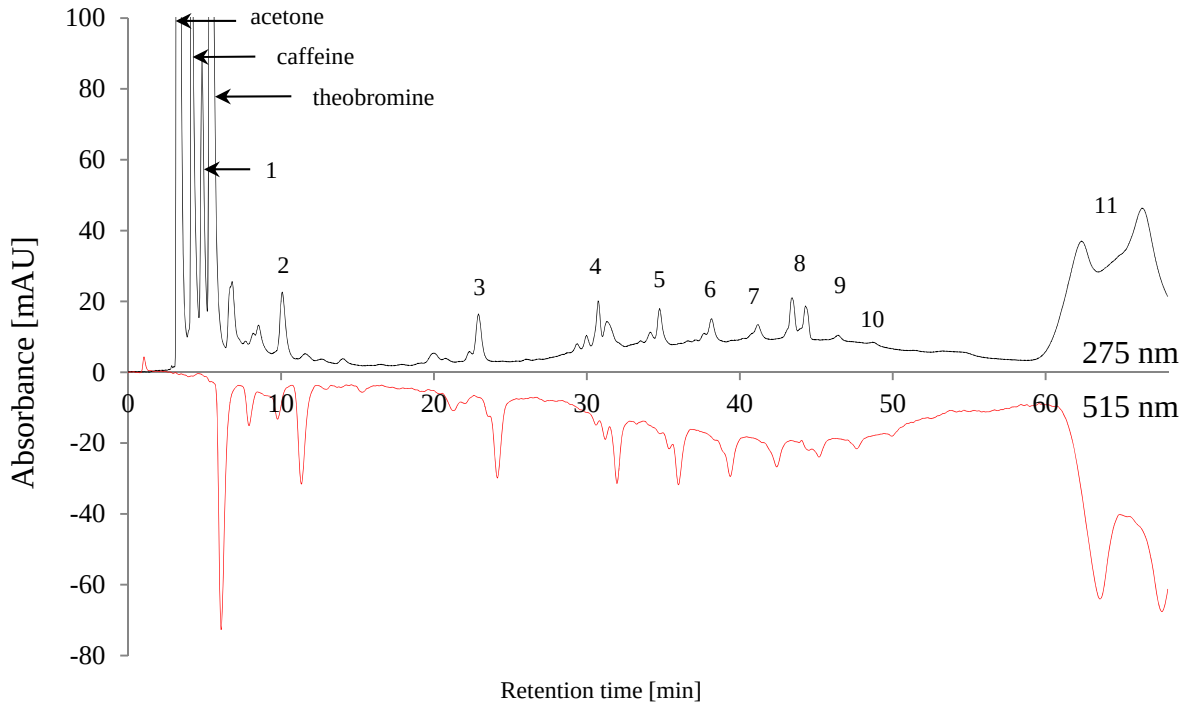

Fig. 2: Bioactive screening of condensed polyphenols from unroasted cocoa beans extract using online screening NP-HPLC-DPPH (injection volume: 1µ; UV wavelength: positive 275 nm, negative 515 nm) (**b**) Identified PA's: epicatechin monomer (1), dimers (2), trimers (3), tetramers (4), pentamers (5), hexamers (6), heptamers (7), hexamers (8), nonamers (9), decamers (10) and unresolved condensed polyphenols (11). (Pedan V. et al., 2015)

Depending on the polarity and the stability of an active substance, the right formulation or even the application in a carrier system can improve the penetration. For this reason we investigated vesicular systems like solid lipid nanoparticles (SLN). SLN are colloidal lipid particles present in the solid state at body temperature at the point of their application. They offer alternative nanoparticulate systems to polymeric encapsulations and liposomes, and are developed for lipophilic and hydrophilic drugs.

Typically, penetration or permeation data are acquired *in vitro* using diffusion cells and excised human or animal skin. In recent years, Raman spectroscopy has been established as an alternative non-invasive method to directly determine penetrated actives in human skin in vivo or ex vivo by focusing a laser into the top layers of skin and recording depth dependent scattered Raman signals of the compound of interest (Adlhart C. et al., 2011).

Materials and Methods

Preparation of aqueous cocoa extract and solutions

Cocoa extract (defatted) was dissolved in phosphate buffer (0,02M) and mixed with solvent 1:10 at 80 °Celsius and stirred at a rate of 600 rpm. After centrifugation, the extract was lyophilised.

For the donor solution in the in vitro penetration studies 0,5 g/l EP, 0,5 g/l TH or 1 g/l CA of phosphate buffer solution were used as infinite dose. The application amount for the penetration tests were 4 ml for CA and 8 ml for EC and TH corresponding to 4 mg of substance on a surface of 4.91 m2 (synthetic membrane or pig ear skin). For the in vitro penetration studies with SLN the applied average dose of SLN/EC was 1,025 g corresponding to 29.72 mg EC, respectively SLN/Cacao extract 1.026 g corresponding to 109.33 mg TH, 23.02 mg CA, EC was under the limit of detection.

The used concentration for the Raman assay were a 1% aqueous-solvent solution of EC and ever a 10% aqueous solution mix with SLN Type B (m/m); with and without load of main actives of the Cocoa extract corresponding to a final concentration of 1.06 % TH, 0.22 % CA and 0.026 % EC.

SLNs unloaded and with cocoa extract were prepared by hot melted modified homogenization (Perugini et al., 2012) technique and purified. In particular, the melted lipid phase (myristyl myristate and cetearyl glucoside) was added to the hot aqueous surfactant solution under homogenization by Silverson SL 2T at 11,000 rpm for 2 minutes. The obtained emulsion O/W emulsion has been cooled in an ice bath to recrystallize the lipid phase to the solid state in the form of a SLN aqueous suspension. Drug loaded SLN have been prepared by adding cocoa extract in the aqueous phase. For the investigations two different types were tested: SLN loaded purified (Type A) and lyophilized SLN loaded purified (Type B). Type A SLN were added with preservatives solution (0.02% sodium azide) and were used for the penetration studies with excised pig ear skin. The loadings of the SLN were quantifed with solvent extraction (dichlormethan/ethanol/water) followed by HPLC analysis. The final loading with epicatechin was 28.97 mg/g SLN corresponding to 2.9 % and the main actives of the Cocoa extract corresponds to 10.6 % TH, 2.2 % CA and 0.26 % EC.

Type B SLN were lyophilized using as cryoprotector a 5% sucrose solution. Lyophilized sample should guarantee better storage properties and no preservative has to be added. The loadings of the SLN with EC was 0.0162 mg/g SLN (0.0016 %) and also for the SLN loaded with the Cocoa extract were very low (TH 1.56 %, CA 0.0045 %, EC under the limit of detection). These SLNs were used for sensorial testing und for the Raman investigation due to signal interference issues by the preservative.

Quantification of the antioxidant activity

The characterization of the antioxidant activity of an aqueous-organic cocoa extract (50% water/acetone) and its main polyphenols (such as epicatechin or proanthocyanidin dimer B2) was performed according the DPPH assay (Thaipong et al. , 2006) This screening helps to identify which of the main actives have a high antioxidant activity calculated as EC/Troloxequivalents (EE/ET).

Franz cell diffusion studies with tape stripping

The in vitro experiments were carried out in Franz-type diffusion cells by means of excised pig ear skin and polymeric membranes (Strat-M®, (Merck Millipore). The procedure followed OECD No 428 protocol and Lademann J. et al. 2009. Pig skin was collected from the ears of seven month old Swiss landrace pigs (lat. Sus scrofa domestica). For the assays a split-thickness skin of 300 μm was prepared using a dermatome. The stratum corneum fraction were collected using a tape stripping technique, the remaining skin (viable epidermis/dermis) was cut and squashed. The procedure was performed in triplicate. The endpoints and the concentration of the acceptor solution is measured by HPLC (Agilent Technologies 1260 Infinity - LC-ESI-MS) or with UV-on line detection (Agilent Technologies Cary 60 UV-Vis/ Fiberoptic Dip Probe micro). The Franz cells from Permegear (SES GmbH –Analysesysteme, Germany), type flat ground joint, jacketed, had a diameter of 25 mm (corresponding 4.91 cm2 surface), with a acceptor fluid volume of 20 ml. As acceptor fluid PBS (0.02M) buffer at pH 6.85 was used. The temperature was controlled at 32 ± 0.5° C.

Fig.3: Franz cells from Permegear (SES GmbH –Analysesysteme, Germany)

In vivo skin penetration profiles by confocal Raman microscopy

Three tests samples (refer to section “preparation”) were applied to the volar forearm of one volunteers. 80 µl were applied to a test area of 4 cm^2. After 30 min. the skin was gently wiped with a soft cellulose tissue, other than the 1% aqueous-solvent solution of EC, which could evaporate on the skin. Then the test area was placed on the measurement window of the Raman spectrometer (Model 3510 SCA, *River Diagnostics)*

Raman spectra were acquired using an inverse confocal Raman microscope. The Raman microscope was equipped with a 60x oil-immersion objective and a 785 nm excitation laser. The axial resolution was determined to 6 µm. Raman spectra (400 – 1800 cm^{-1}, integration time 10 s) were acquired starting from the skin’s surface down to a depth of 30 µm (step size 2 µm). Ten penetration profiles were recorded for each test area at random positions.

Sensory assessment

The sensory young expert panel consisted of 8 female volunteers and were trained following the protocol of an in-house manual. The application amount of 0,04ml was applied by the project leader directly on the marked test zones on the internal side of the forearm. According to the defined procedure, as described in the ZHAW standard manual, the attribute smoothness velvety was assessed as follows in comparison to their specific reference substances low (0) and intense value (10). (Huber P. , 2008, 2015)

Results

The characterization of the antioxidant activity of an aqueous-organic cocoa extract (50% water/acetone) and its main polyphenols (such as epicatechin or proanthocyanidin dimer B2) was performed using DPPH assay. This screening helps to identify which of the main actives have a high antioxidant activity calculated as EC/Troloxequivalents (EE/ET). Gallic acid has (according to the DPPH assay, fig. 3) very high potential followed by procyanidin C1 and epigallocatechingallate EGCG.

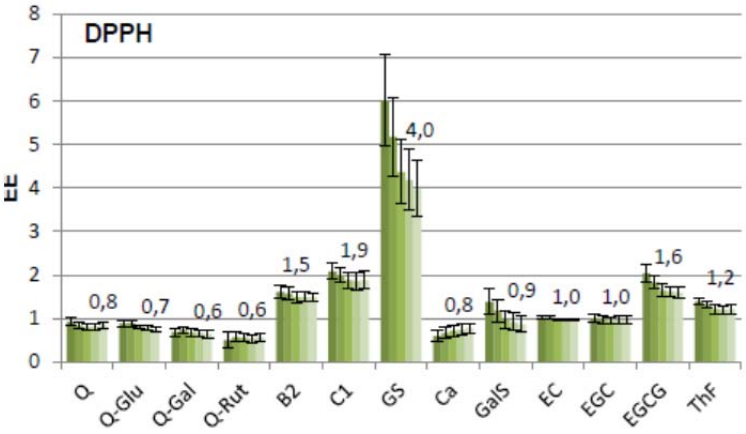

Fig. 4: Screening of polyphenols by a DPPH antiox assay to identify the antioxidant activity calculated as EC/Troloxequivalents (EE/ET) of the main actives. (Q = Quercetin; Q-Glu = Quercetin-3-O-glucoside; Q-Gal = Quercetin-3-O-galactoside; Q-Rut = Quercetin-3-O-rutinoside; B2 = Procyanidin B2; C1 = Procyanidin C1; Ca = (+)- Catechin; GalS = Gallic acid; EC = (-)-Epicatechin; EGC = (-)-Epigallocatechin; EGCG = Epigallocatechingallate; ThF = Theaflavine) (Largey, O., 2014).

The penetration ability of a substance is described as permeability coefficient (K_p). A comparison of the diffusion rates on a synthetic membrane (Strat-M®, Merck Millipore) showed, that the selected pure compounds EC, TH and CA, applied at “infinite dose” (amount of active on the skin surface remains constant) had very different penetration abilities. With this efficient pre-screening on-line method, we could observe a small permeation amount for an aqueous solution of EC (0.05%) in comparison to CA (see Fig.5).

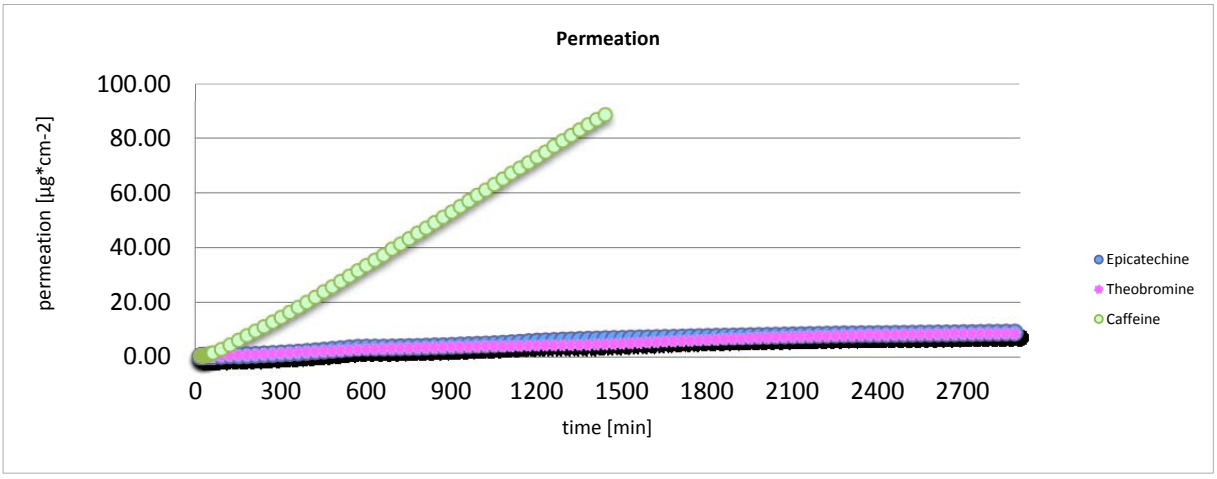

Fig. 5: Permeation amount obtained after application of 4ml of aqueous solution of EC, TH and CA applied on Strat-M®, (Merck Millipore) membranes by Franz cells. (Kp for CA 9.07 *10^{-7} cm/s)

At the end of the ex vivo penetration experiment, the total amounts of catechins were determined for each individual compartment of the Franz diffusion cell (applied dose/donor/tape stripping for stratum corneum/epidermis/dermis/acceptor). After 8hours of application EC remained mostly in the donor compartment and a very small amount was detected in the skin layer viable epidermis, but was at the limit of detection. The recovery for the aqueous 0.05 % EC was 83 % +/-0.72 % after triplicate. While the main barrier function of the skin is located in the stratum corneum, no or little permeation was expected. The missing amount of EC could be permanently bound to skin proteins or eventually been oxidized and destroyed that way.

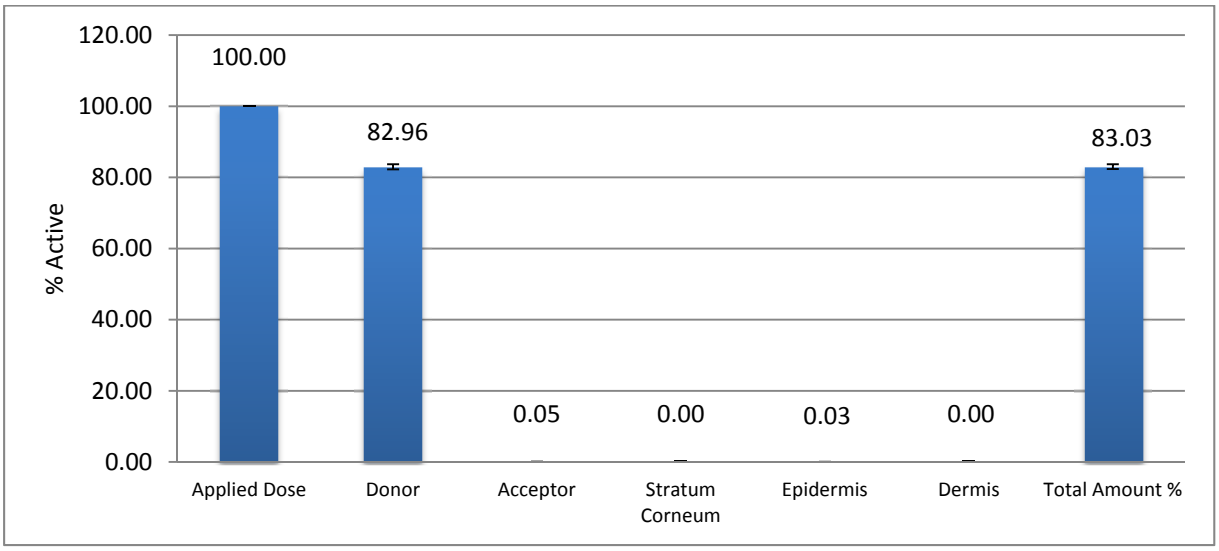

Fig. 6: Delivery and recovery of aqueous 0.05% EC by in vitro permeation study after 8 hours (on pig ear skin by Franz cells). The final recovery for EC was 83% +/-0.72%.

Interestingly none of the encapsulated actives or main substances of the cocoa extract (TH, EC, CA) in the applied dose could have been detected neither in the donor nor in another skin layer. The recovery of the encapsulated actives in SLN after triplicate couldn't be found at all. It is assumed that in the process of lipid crystallization, encapsulated extract may have been expelled. For this reason it should be re-evaluated the lipid composition of the SLN and more investigations are needed for the sample preparation and to better understand potential interaction of surface active agents and solid lipids of the SLN ingredients with the stationary phase and the pre-column of the HPLC.

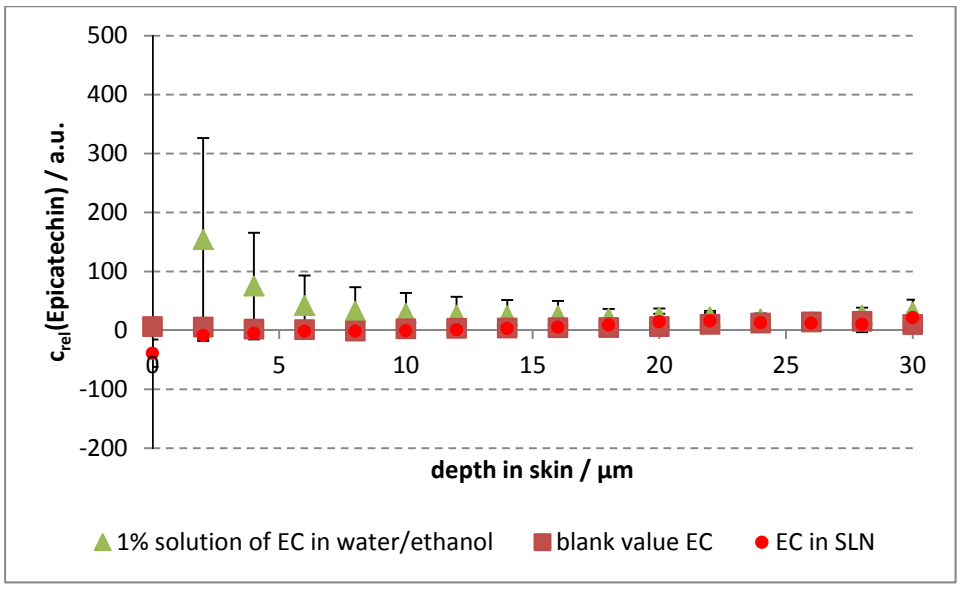

Fig. 7: In vivo skin penetration profile of EC relative to Keratin obtained from confocal Raman spectra. The apparent penetration of epicatechin is caused by interfering Raman signal from remaining epicatechin on the skin's surface.

The results obtained from in vivo Raman depth profiles recorded 30 min after application indicated that the substance remained on the surface of the skin in case of the EC solution 1%. The apparent penetration in the skin is presumably due to scattering Raman signals from remaining EC at the surface – an artefact caused by the finite depth resolution of the confocal Raman setup (Everall N. J., 2000). The large error bars are due to the heterogeneous surface distribution of EC. For the SLN solution, the Raman based limit of detection was above the potential surface concentration of EC.

The influence of SNL on the perceived sensory properties on the skin assessed through descriptive sensory analysis showed a pleasant skin feeling during the afterfeel phase. The trained sensory young expert panel certified a significant increase of the attribute smoothness velvety when comparing a watery gel with a watery gel-dispersion of 5% SLN (Type B). The rating for the gel-SLN-dispersion was 6.9, whereas the watery gel reached only a rating of 3.3 on 10 point scale.

Conclusions and Discussion

In general, the penetration of polyphenols is according to Zillich et al. (2013) improved with smaller molecular size and moderate hydrophilicity (negative logP). Following main components of a cocoa extract (beside the alkaloid caffeine, theobromine) will at least from a logP value be appropriate for skin penetration: epicatechin/catechin (EC/Ca), epigallocatechin (EGC), procyanidin B2 (B2).

Within the limit of detection penetration of EC could be demonstrated neither by confocal Raman spectroscopy nor by HPLC in a classical Franz-cell setup with excised animal skin. (The limit of detection for HPLC for the main substances based on visual and signal-to-noise was between 0.05 - 3g/l.)
Raman measurements as well as the investigation of skin fractions by in vitro penetration assay with pig ear skin revealed that the main part of substance remained on the skin surface. Raman measurements as well as the investigation of skin fractions by in vitro penetration assay with pig ear skin revealed that the main part of substance remained on the skin's surface. More than 80% of an EC solution remained in the donor phase of the in vitro assay by Franz cells after 8 hours. We presume that the loss of EC could either be through oxidation process and subsequent oligomerisation and polymerisation or by permanent adhesion to skin proteins. In the HPLC chromatogram we observed newly formed peaks beside an EC peak with probably carry over effects. Raman data showed larger standard deviations, which are typically observed when the surface concentration of the test substance is very heterogeneous, e.g. caused by surface crystallisation. Although EC, if properly formulated, is able to permeate the upper layers of human cadaver skin (Wisuitiprot et al. (2011), Suppasrivaseuseth, R. A. et al. (2006)). Following explanation could be possible for the little skin permeability of EC (Wisuitiprot et al. (2011); the hydrophilic nature of catechin and chemical interaction with skin lipid bilayers and the presence of proteins (such as keratin) endows the stratum corneum with both positively and negatively charged groups. This characteristic provides an effective barrier against charged molecules too.

Interesting insights into the synergistic effects of other ingredients within the complex aqueous extract are expected by comparing aqueous solutions of pure active components with solutions containing the extracts' matrix. The synergistic effects could not be shown due to a restricted solubility of the active components and the limit of detection of the chosen methods, but still remain an important topic for further research!

A good formulation is still a challenge and a requirement for substances with poor solubility. The choice of SLN also needs investigation. In this case the polyphenol load was too small to be a sufficient delivery system for EC or Cocoa extract. Although the perceived sensory properties on the skin assessed through descriptive sensory analysis showed a pleasant skin feeling during the afterfeel

phase, the chosen SLN techniques and/or the combination of polyphenolic actives (deep load) didn't support in this setting the stability or the delivery behaviour for EC or other cocoa main actives.

This actives still remains interesting for an antioxidant efficacy for skin surface application. Polyphenols with an affinity to the upper skin layers could be of interest for cosmetic use, e.g. for the treatment and prevention of skin lipid oxidation, e.g. inhibition of squalene hydroperoxide formation, in particular in sebaceous glands for a preventive treatment of acne lesions. To improve the generally skin conditions of dry or sensitive skin the application of polyphenols with an affinity to the stratum corneum could help to protect the lipid barrier of skin.

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