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Coffee based polyphenols with potential in skin care

Antioxidant activity and skin penetration assessed by *in vivo* Raman spectroscopy

KEYWORDS: polyphenols, coffee fraction, confocal Raman spectroscopy, formulation, enhancer

Abstract Active ingredients of plant origin such as polyphenols and phenolic acids are enjoying increased acceptance in both the food and cosmetics industries. Their often excellent antioxidant properties are determined by **in vitro** assays. To develop an effective skin care formulation, their penetration ability and bioavailability has to be investigated as well. Both depend on molecular size, but also on the molecules' balance of lipo- and hydrophilicity. In this paper, the antioxidant activity of individual coffee fractions (roasted and unroasted) is compared by means of *in vitro* antioxidant assays, and the influence of the formulation on skin penetration of the antioxidant active is examined by in vivo Raman spectroscopy.

INTRODUCTION

Polyphenols and in-situ (roasting) reaction products

Polyphenols belong to one of the most interesting classes of compounds in nature with antioxidative potential. While they occur in plant materials such as tea, cocoa, coffee, herbs and phytochemicals, coffee as beverage has been shown by an LDL oxidation assay to have among the highest contents (1). 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. Although the specific mechanisms responsible for the effective and cumulative health-promoting effects are not yet clear, there is an observed correlation between an intake of anti-oxidants and health benefits (2). Polyphenols used in skin treatment include tea polyphenols (like epicatechin), silymarin, quercetin, resveratrol and tannins (hydrolysable and condensed substances such as proanthocyanidins). However, smaller molecules such as phenolic acids like caffeic acid and ferulic acid are also used in anti-aging skin care (3).

Epidemiological studies have increasingly revealed the positive benefits of coffee consumption on our health and wellbeing (4). Antioxidant molecules, such as chlorogenic acid (CGA) in coffee, represent a group of compounds that has been frequently associated with beneficial health effects (5). Other than the main CGA, several other isomers make up this family of water-soluble phenolic acids, which are esters of (-)-quinic acid and one or more hydroxycinnamic acids (6). CGA occurs in large quantities in unroasted and to a lesser degree in roasted coffee (7). Furthermore, during roasting high molecular weight Maillard reaction end-products, the melanoidins, are formed and these exhibit antioxidant activity as well (8). For measurements of the antioxidant capacity in coffee, a wide variety of assays is available (9). The aim of this study was to examine how coffee (green and roasted coffee as well as CGA) can be used in topically applied skin care formulations.

Therefore, these bioactive compounds had to be successfully formulated and incorporated into a vehicle, either as a simple solution, an emulsion or in some other carrier. Bioavailability of the antioxidant actives after topical application was then studied by in vivo Raman spectroscopy.

Controlling skin penetration by the topical formulation

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 up to 500 g/mol (corresponds to Daltons) indicate an ideal penetration of the substances through the stratum corneum of the skin epidermis. Yet even with these ideal characteristics, penetration must be supported and enhanced by the topical formulation (10).

Phenolic acids like ferulic acid with a molar mass of 194 g/mol and a moderate lipophilicity (log*P* of 1.64±0.36) (11) resp. ($\log P_{pH\ 7.4}$ of -1.19) (12) showed a better penetration than CGA (354 g/mol and log*P* -0.37 ±0.05 (13) resp. $\log P_{pH\ 7.4}$ of -3.05) (12). During pre-tests, an improved penetration of CGA with a lipophilic enhancer was observed, while the penetration of ferulic acid was negatively affected.

Chemical penetration enhancers affect mostly the intercellular lipid bilayer in the stratum corneum (14). Various esters and fatty alcohols such as isopropyl myristate and isopropyl alcohol can be used as enhancers, while fatty acids like oleic acid change the structure and thermodynamic state of the skin's lipid barrier (15). In addition, solvents like various glycols and ethanol interacted with the intercellular lipids and enhance skin penetration when used in cosmetic emulsions or gels.
Depending on the polarity and the stability of an active substance, the application in a carrier system like conventional liposomes (phospholipids as main component) improves the penetration. The size of the liposomes and the choice of building components determine whether they create a skin reservoir or increase percutaneous permeation (16). Other vesicular systems functioning as dermal delivery systems are microemulsions or carriers with a solid lipid core matrix, so-called solid lipid nanoparticles (SLN) or nano lipid carriers (NLC) (17).

Assessing skin penetration by in vivo Raman microscopy
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 by focusing a laser into the top layers of skin and recording the scattered Raman signals (18-22). With this technique, concentration profiles of penetrated actives and skin constituents can be directly extracted within the first 200 µm of the skin. This covers the full stratum corneum, the most important lipophilic barrier layer in terms of penetration. Since tests can be performed in vivo, permeation characteristics can be studied with skin that is in a natural equilibrium between the living body and the surrounding atmosphere with ambient humidity. Raman-based skin concentration data in human tissue have been recorded for a large variety of substances, including the antioxidant carotenoid in human tissue (23, 24, 25) but so far, no data are available for the important antioxidant CGA, present in coffee.

MATERIAL AND METHODS

CGA was purchased from Acros Organics. Cayoma Green Coffee (INCI: Aqua, *Coffea arabica* seed Extract, Ethanol) is an alcoholic extract made of green coffee beans with CGA content in the range 6 percent to 10 percent CGA and was provided by Qenax AG, Switzerland.

Arabica coffee (*Coffea arabica* from Costa Rica) was roasted at high temperature (fast roasting) to a light roast degree to avoid extensive degradation of CGA (26). After grinding the roasted beans and brewing the coffee with a French Press (27), the filtered coffee was freeze dried and the lipophilic part of the coffee removed by Soxhlet extraction with dichloromethane. After dissolving the defatted soluble coffee in water, size exclusion chromatography was performed on a Sephadex G-25 (GE Healthcare) column to obtain several fractions of the coffee after the elution with Millipore water (28). The eluent was monitored by a DAD spectrometer with a flow-through cuvette and several wavelengths were recorded (280 nm for caffeine, 325 nm for CGAs and 405 nm for melanoidins) (Figure 1).

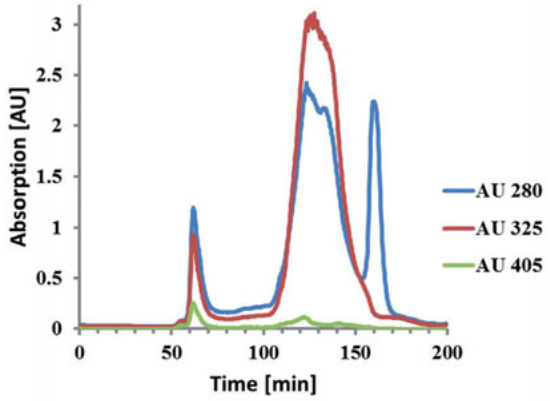


Figure 1. Size exclusion chromatography was performed on coffee brew to obtain fractions to be used in penetration studies. The effluent from the column was recorded with a DAD spectrometer and three wavelength were recorded (280 nm for caffeine, 325 nm for chlorogenic acids and 405 nm for melanoidins). Fraction A (50 to 70 min) and Fraction C (105 to 150 min) were collected, while Fraction B (70 to 105 min) and Fraction D containing caffeine (150 min to 170 min) were discarded.

Antioxidant assays
The fresh coffee brew, the freeze dried and extracted coffee as well as fraction A and fraction C were diluted so that its components fell within the dynamic ranges of the individual assays and analysed by flow injection analysis (FIA) or sequential injection analysis (SIA) with a FIAlab-3200 (FIAlab Instruments Inc., U.S.A.) (28). All results were related to the antioxidant activity of gallic acid and presented as gallic acid equivalent (GAE, see table 2).

(i) In the ABTS assay, sometimes also known as Trolox equivalent AO assay (TEAC), the reduction of the 2,2-azinobis-(3-ethylbenzothiazoline-6-sulphonate) stable radical ($ABTS^+$) by the sample is monitored (29). The ABTS reagent is oxidized with potassium persulfate for a miniumum of 16 h. Applying a SIA method, the sample or gallic acid standard (injection volume 40 µl), the $ABTS^+$ solution (injection volume 20 µl) and carrier solution (0.1 M sodium phosphate, pH = 7.4) are aspirated sequentially in a heated holding coil, which is kept at a steady temperature of 35°C. By aspirating and dispensing the components through the holding coil, an even dispersion is achieved, and by stopping the instrument, a reaction delay of 30 sec is applied to extend reaction times. After dispensing the mixture to the detector cell (flow rate 30 µl/sec), the absorbance of the $ABTS^+$ solution is measured photometrically (λ = 734 nm).
(ii) In the Folin-Ciocalteu (FC) reagent assay, which is often described as a total phenol assay, the total antioxidant capacity (TAC) (30) is determined measuring the reduction of "molybdenum blue" at high pH, which is formed from a mixture of phosphomolybdate and phosphotungstate (FC reagent). With an FIA method, the sample or gallic acid standard is injected (injection loop 100 µl) into the flow stream (flow rate 30 µl/sec) of the FC reagent (0.2 M concentration). After mixing with NaOH (0.25 M concentration, flow rate 30 µl/sec) to raise the solution pH for higher reactivity, dispersion in the reaction coil (1m tubing length) mixes the components, and the reaction product (blue coloured metal complexes) is measured photometrically (λ = 765 nm).
(iii) In the oxygen radical absorbance assay (ORAC), the ability of the sample to inhibit the reaction between peroxyl radicals and fluorescein is assessed (31). With an SIA setup,

Test Substance (formulation)	Actives	Mass Fraction CGA [percent]	AUC [mmol CGA / g Keratin]
1	Pure CGA (Acros Organics)	0.50	0.37
2	Cayoma green coffee (bean) extract, containing mainly CGA	0.66	0.86
3	Coffee fraction C (lyophilised water soluble extract of roasted coffee) containing mainly CGA	0.42	0.30
4	Liposomes loaded with CGA from Coffee fraction C (lyophilised water soluble extract of roasted coffee)	0.25	0.05

Table 1. Summary of applied test substances (formulations), all actives were incorporated to the base formulation. See results section for the area under the curve, AUC.

the sample or gallic acid standard, fluorescein solution, and the AAPH solution (injection volumes of 25, 25 and 50 µl) are aspirated sequentially into the holding coil. Introducing the components and the carrier into the holding coil produces an even dispersion and subsequently the reagents-sample mixture is dispensed to the detector cell. At the peak of the fluorescein signal, the flow is stopped and the degradation of fluorescein is monitored for 1h. The fluorescence excitation peak wavelength is at λ = 494 nm and the emission wavelength peak at λ = 514 nm. Broadband filters were used for both excitation and emission, with a colour blue filter as an exsiccation source and an interference green filter for emission.

The antioxidant capacity of the treated coffee, compared to the analysis of fresh coffee brew, was reduced between 12 to 28 percent after freeze drying and Soxhlet extraction, depending on the assay. The relation of the antioxidant activity of the fractions and the treated coffee resulted in recovery of 72 to 82 percent (N=3) of the antioxidant activity in the fraction C, while only 8 to 12 percent was allotted to fraction A. The remaining 8 to 16 percent seem to have been lost in fraction B or D, which were not sampled as fractions. While caffeine as the main constituent of fraction D gave no response in the antioxidant assays, fraction B showed no peak and it was therefore decided to omit fractions B and D.

Formulations

The base formulation is an oil-in-water emulsion with a water content of 69 percent. The emulsion was optimized in requirements related to the incorporation of actives and/or carriers without interfering with either the Raman spectra of the skin nor the explored actives. For the main investigation, the ingredients of the base formulation were optimized to assist the penetration of CGA by different enhancer (like oleic acid). The liposomes with phosphatidylcholines were loaded with fraction C. They were prepared by the ethanol injection method following high-pressure homogenization at 1500 bar and stepwise extrusion with a final filter mesh size of 100 nm in accordance with Xia et al. (32).
Care was taken that in samples 1 to 4, the active compounds were dissolved and the final formulas with respect to their relationship to the hydrophilic and lipophilic phase were as comparable as possible. Due to reasons concerning formulation (load limit of the liposomes and subsequent incorporation into the base formulation), the maximum final concentration for sample 4 was 0.25percent CGA.

Application of test substances

Upon written consent, 2 mg cm^{-2} of the test substances were applied onto the volar forearm of 7 healthy volunteers (4 male and 3 female, Caucasian skin, aging from 25 to 33 years) under standardized conditions. 30 min after application, Raman penetration profiles were recorded once the test area had been cleaned of any remaining test substance using a soft cotton cloth. The application tests were repeated four times for each test substance randomized through the 7 volunteers.

Confocal Raman microscopy

Raman spectra were acquired using an inverse confocal Raman microscope (Model 3510 SCA, *River Diagnostics)*. The Raman microscope was equipped with a 60x oil-immersion object lens and a 785 nm excitation laser. The axial resolution was determined to 6 µm. Raman spectra (400 – 1800 cm^{-1}, integration time 5 s) were acquired starting from the skin's surface down to a depth of 24 µm (step size 2 µm). Ten penetration profiles were recorded for each test area at random positions. Averaged concentration profiles of CGA were extracted from the Raman profiles following the method described by Caspars *et al.*. (18,19) Spectra were fitted with those of the major skin constituents, CGA and the base formulation. They were corrected for the variation in absolute Raman intensity by normalizing to the Raman signal of keratin. The local content of CGA relative to keratin was determined by calibrating the response factors against CGA and BSA solutions, assuming similar Raman cross sections for keratin and BSA. The boundary between the SC and the viable epidermis was determined based on changes in keratin signal. The area under the curve AUC values were calculated from the concentration profiles for the first 20 µm of the skin.

Raman distribution maps

Distribution maps were extracted from Raman maps (250 x 200, points, 200 nm spacing) acquired on a confocal Witec Raman microscope (532 nm excitation, integration time 50 ms).

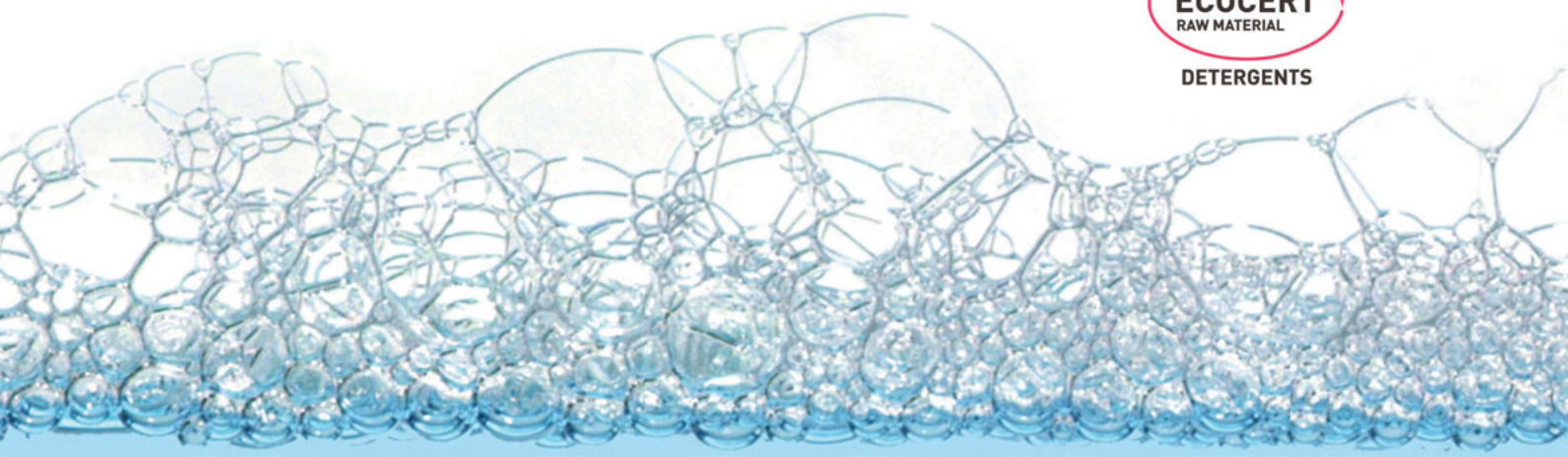
RESULTS AND DISCUSSION

The antioxidative potential of selected coffee fractions (*in vitro* assays)

The high molecule melanoidins were sampled in fraction A. Due to their lower antioxidant capacity (see Table 2) and to their high molecular weight that impedes skin penetration, fraction A was not used in further penetration studies. CGA and its isomers were collected in fraction C, while fraction B was not sampled due to the low fractionation yield, which

Sample	FC assay (GAE) [mg/L]	ABTS assay (GAE) [mg/L]	ORAC assay (GAE) [mg/L]
Fresh coffee brew	2232	654	5875
Dried + extracted coffee brew	1818 ± 25	468 ± 38	5220 ± 160
Fraction A	149 ± 2	57 ± 4	465 ± 43
Fraction C	1387 ± 227	335 ± 37	4304 ± 656

Table 2. Antioxidant values of different coffee samples analysed with FC, ABTS and ORAC assays and presented as gallic acid equivalent (GAE) values in mg/L as mean value (N=3) ± standard deviation. The fresh coffee brew was analysed only once.



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would not have been enough for skin penetration experiments. Hereby other small molecules produced by the Maillard reaction were also collected in fraction C, although great care was taken to exclude caffeine (fraction D). Fraction D was not collected since caffeine gave no response in the antioxidant assays. The quantification of the CGA content in fraction C by HPLC-MS resulted in 0.17 mg total chlorogenic acids per 1 mg of fraction C.

The antioxidant capacity of the treated coffee, and depending on the assay, was reduced by 12 to 28 percent after freeze drying and Soxhlet extraction, as compared to the analysis of fresh coffee brew. The relation of the antioxidant activity of the fractions and the treated coffee resulted in recovery of 72 to 82 percent (N=3) of the antioxidant activity in the fraction C, while only 8 to 12 percent was allotted to fraction A.
The remaining 8 to 16 percent seem to have been lost in fraction B or D. While caffeine as the main constituent of fraction D gave no response in the antioxidant assays, fraction B showed no peak and it was therefore decided to omit the fractions B and D.

The higher antioxidant capacity of fraction C compared to fraction A is probably due to its high content of CGAs.

A direct comparison of the antioxidant capacity of the selected coffee fractions with other natural antioxidants and polyphenols is difficult due to the variety of preparative extraction methods, the difference in antioxidant assays using different parameters and the expression of the results as different equivalents.

In vivo penetration of CGA by confocal Raman microscopy

The Raman spectrum of CGA is shown in Fig. 2. CGA is a strong Raman scattering molecule that is characterized by its intensive phenyl ring and ethylenic stretch at 1605 and 1627 cm^{-1} respectively (33). These characteristic peaks are also observed in the spectra of formulation with pure CGA that had been applied to the volunteers. Figure 2 shows also a typical Raman spectrum of skin before and after treatment with the test substance at a skin depth of 8 µm. The alterations in the spectrum due to the presence of CGA are clearly observed.
Penetration profiles (Fig. 3) depict the local content of CGA relative to keratin at different depths of the skin. These profiles, which were recorded 30 min after application, are consistent with Fick's diffusion equations and similar to profiles reported for topically applied caffeine (34). Since the amount of CGA applied was different, due to a different content in the test substances (Table 1), penetration profiles were normalized to one for the amount of CGA at the skin surface (i.e. 0 µm depth in the skin). Interestingly, these profiles are identical within experimental error for different sources of CGA (pure CGA, CGA from Cayoma green coffee bean and CGA, contained in fraction C), when they had been formulated into the same base formulation. When CGA contained in fraction C had been loaded into liposomes, penetration was only observed down to 6 µm depth in the skin. Within the limit of detection, no CGA was observed below the stratum corneum (SC) that showed an average thickness of 17.4 µm for the seven volunteers – a value close to the 22.6 µm was observed by Egawa et al. (35). The concentration profiles were linear with respect to the amount of CGA applied and the content of CGA had doubled after 2-fold application. At increasing intervals of 120 min after application, the local CGA had slightly decreased - a situation that has also been reported for carotenoids (23).
The absolute amount of penetrated CGA was calculated as area under the curve (AUC), Table 1.
The more CGA applied due to higher concentration in the test substance, the more CGA was observed in the SC. No positive effect was observed when fraction C had been loaded into liposomes, and even less CGA was observed in the SC (0.05 mmol / g keratin for a test substance containing 0.25 percent CGA compared to 0.30 mmol / g Keratin for 0.42 percent).
To better understand the reduced bioavailability of CGA when fraction C had been loaded into liposomes, distribution maps of the test substances were recorded (Fig. 4). These clearly indicate that the hydrophilic CGA is mainly located in the aqueous phase of the formulation. Phosphatidylcholine, the main constituent of the liposomes' double lipid layer is found in the oil phase. This implies partial disintegration of the liposomes and may explain the missing enhancement in bioavailability.

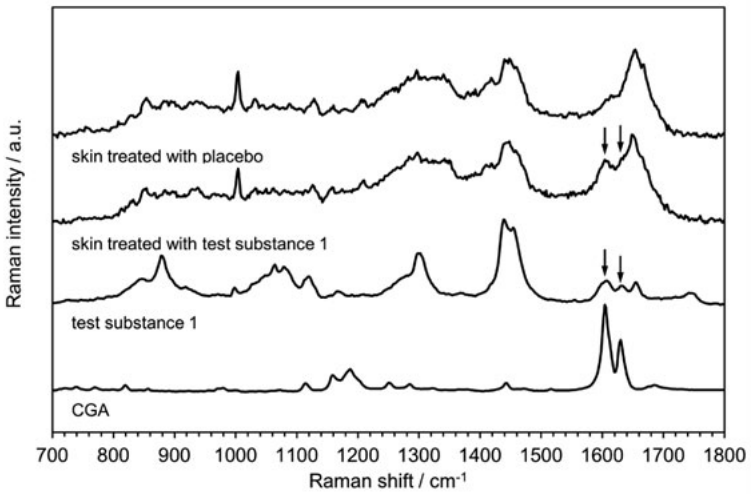

Figure 2. Raman spectra of pure CGA, 0.5 percent in base formulation (test substance 1) and human skin at a depth of 8 µm after 30 min treatment with test substance 1 and placebo. The characteristic phenyl ring and ethylenic stretch of CGA at 1605 and 1627 cm^{-1} are clearly observed in the spectra of test substance 1 and of treated skin.

CONCLUSION AND OUTLOOK

For the development of an effective skin care formulation, actives with excellent antioxidant properties proven by *in vitro* assays are not sufficient. Their penetration capacity and bioavailability have to be investigated as well. Here we have determined the antioxidant capacity of different water soluble coffee extracts from roasted Arabica coffee beans and separated these into different mass fractions. 82 percent of the antioxidant activity was recovered

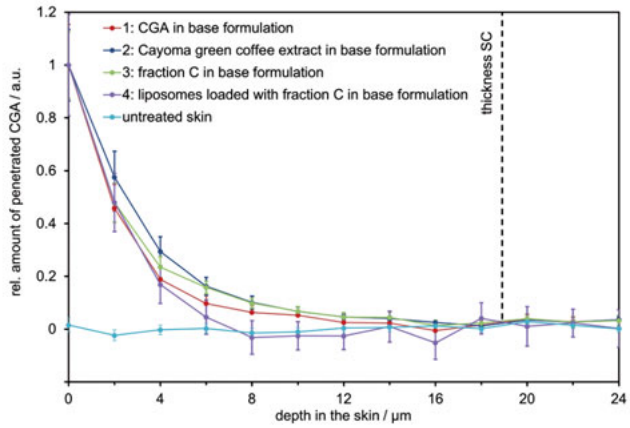

Figure 3. Matrix corrected penetration profiles for CGA 30 min after application normalized equal amount of CGA at the surface of the skin. The dashed vertical line shows the average boundary between the stratum corneum (SC) and the viable epidermis.

in fraction C that contained 17 percent CGA but no caffeine. Chlorogenic acids are the main antioxidant in fraction C, although Maillard reaction products likely contribute to the antioxidative potential as well.
The non-invasive Raman based investigation of topically applied CGA revealed that the effective penetration of CGA was independent from its source (pure CGA, CGA from Cayoma green coffee bean and CGA, contained in fraction C), while bioavailability was slightly reduced when fraction C had been loaded into liposomes.

To optimize the delivery of the active ingredients into the skin, the polarity of the active ingredient with the polarity of skin and the other emollient components must first be

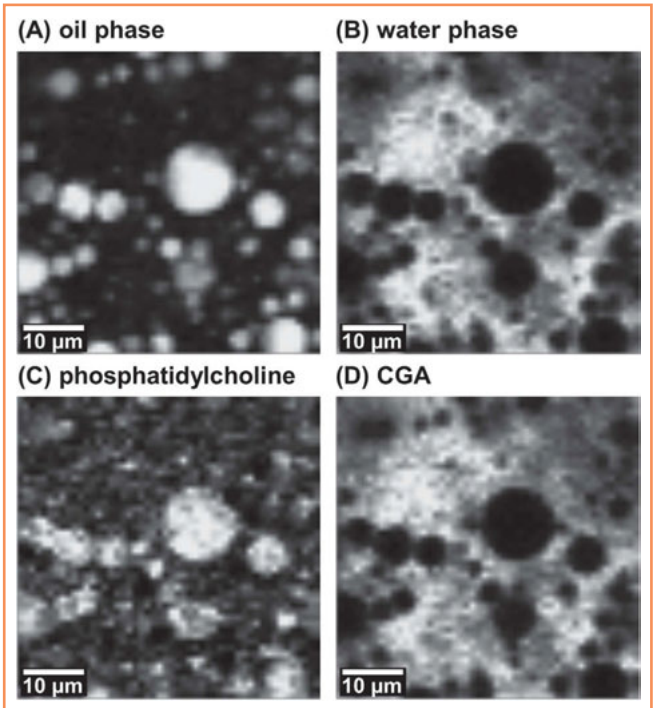

Figure 4. Raman distribution maps of the test substance 4 containing the liposomal loaded fraction C showing the local distribution of (A) the oil phase, (B) the water phase, (C) phosphatidylcholine and (D) CGA.

compared (see concept of the Relative Polarity Index (RPI) by Wiechers (36)). This simple method of formulation should be checked and optimised before further skin application tests are carried out. Handling and benefit of carrier systems such as liposomes are not always suited to supporting the dermal delivery of active ingredients due to loading, compatibility and/or stability problems. These

complex systems have to be experimentally analyzed and optimized for each combination (active ingredient, base formulation and carrier system), while Raman spectroscopy can provide an excellent tool for this.

By means of ESR (Electron Spin Resonance Spectroscopy), the emergence or the inhibition of free radicals in the skin (ex vivo and in vivo) can be determined (37). In this case, it would be interesting to compare the protective effect of the isolated antioxidants of fraction C (coffee brew) with further plant antioxidants; they differ not only by their capacity to neutralize free radicals, but also in their reaction time (38). Gathering indirect evidence of the protective effect of antioxidants by means of proton-transfer-reaction time-of-flight mass spectrometry (PTR-TOFMS) (39) is also conceivable.

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