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# Penetration studies of vitamin E acetate applied from cosmetic formulations to the stratum corneum of an *in vitro* model using quantification by tape stripping, UV spectroscopy, and HPLC

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Synopsis

The skin activation and penetration capability of vitamin E acetate as an ingredient in a basic o/w cream (lamellar type), in liposomes (Rovisome®) and microparticles (Roviparts®), was investigated under *in vitro* conditions (BUS model) by the adhesive stripping method. The aim of the study was to compare the analytical results obtained by UV spectroscopy (transmission) and the conventional HPLC method. For the quantitative spectrometric assay, a classical least-squares evaluation of the spectra between 265 and 350 nm, based on the constituent spectra, was used. UV spectroscopy is an economic analytical method for evaluating a large population of samples of the horny layer taken by the adhesive tape stripping method, which is an established tool for depth profiling of substances within the stratum corneum.

With regard to the irritation test, no cytotoxicity was recorded for all formulations tested. However, the Roviparts® and Rovisome® cream formulations induced a considerable activation of the epidermal cells that may contribute to the penetration efficiency of Rovisome®-formulated vitamin E acetate. The Rovisome®-formulated cream delivered a maximum amount of vitamin E acetate into the horny layer compared to the other formulations tested. The difference can be explained by an alteration of the plasticity of the horny layer inducing a strong reservoir capacity and an activation of upper epidermal cells. Moreover, the opening of the potential pathway for a follicular penetration may be part of the increased reservoir capacity.

## INTRODUCTION

Today's cosmetic emulsions usually contain several ingredients whose function is to ameliorate the condition of the skin with respect, for example, to skin hydration or

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barrier properties of the stratum corneum. Well known is the importance of vitamins, such as vitamin E or D-panthenol, as bioactive substances in cosmetic formulations. However, the release of vitamins from cosmetics into the stratum corneum requires certain application conditions and exposure times (1,2). After application, the formulations undergo a dramatic change due to evaporation processes, which may influence the penetration of ingredients into the horny layer, to be demonstrated by analytical methods. The use of liposome- and microparticle-based formulations as transport vehicles may also increase the bioavailability of the compounds within the surface skin layer as well as diminish the exposure period for penetrating the skin surface (3–5). Depth profiling for substances penetrated can be carried out by repeated adhesive tape stripping and subsequent tape analysis (3).

Experimental dermatological *in vivo* studies for cosmetics on animals are prohibited nowadays due to ethical and legal restrictions. Therefore, the lateral follicular skin of the isolated perfused bovine udder skin (BUS model) was used here as a viable *in vitro* substitute for the penetration efficiency studies carried out (6).

UV spectroscopy is well suited for fast quantitative determination of trace amounts of vitamin E acetate due to the strongly absorbing chromophore. Multivariate calibration models have come into wide use when implementing quantitative spectroscopic assays based on the Beer-Lambert law in the case of overlapping spectral bands from complex samples. Classical least-squares (CLS) modeling has been used for the quantitative analysis of spectra under the premises that the linear additive model is valid and all component spectra contributing to the sample spectrum are known (7).

The applicability of such a model for vitamin E acetate-loaded adhesive tape strips was tested in this study. The results spectrometrically obtained are compared with those from a more complex and time-consuming HPLC method, which is mainly used for such dermatological studies.

## MATERIALS AND METHODS

### LIPOSOMES/MICROPARTICLES

Liposomes and microparticles are vesicles composed of phospholipids, phophatidylcholine in particular, surrounding an inner fluid compartment. The liposomes consist of an aqueous inner compartment, and therefore the lipophilic vitamin is found in the lipid bilayer, in contrast to the microparticles that have an oily compartment surrounded by only one layer of lipids. For this study, the tocopheryl acetate was dissolved in the natural oil phase. Liposomes and microparticles were prepared by dissolving all lipophilic components in ethanol and than carefully adding the buffer while stirring. These crude carrier suspensions were then homogenized and extruded several times through a microporous filter of 0.2 μm diameter. The particles described in this study have a size of 150 nm, measured by laser light scattering. Both types of vesicles are used for cosmetics due to their ability to transport active ingredients into the skin and to stabilize sensitive active components (4).

### FORMULATIONS

Several formulations (Table I) that include liposomes (Rovisome®, RS) or microparticles (Roviparts®, RP) (ROVI GmbH & Co., Kosmetische Rohstoffe KG, Schluechtern,

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**Table I**
Chemical Composition (INCI) of Three Cosmetic Formulations (LC, RP, and RS) Containing Vitamin E Acetate

| Product | INCI |
|---|---|
| Lamellar cream, LC (vitamin E acetate) | Aqua, hexanediaol, cetearyl glucoside, *Oenothera biennis,* isopropyl isostearate, behenyl alcohol, cetearyl isononanoate. glycerin, tocopheryl acetate, sorbitol, dimethicone, talc, sodium carbomer, tocopherol, hydrogenated palm glycerides citrate |
| Lamellar cream, RP (20% Roviparts®) | Aqua, (Roviparts® E acetate), hexanediaol, cetearyl glucoside, *Oenothera biennis,* isopropyl isostearate, behenyl alcohol, cetearyl isononanoate, glycerin, sorbitol, dimethicone, talc, sodium carbomer, tocopherol, hydrogenated palm glycerides citrate |
| Lamellar cream, RS (20% Rovisome®) | Aqua, (Rovisome® E acetate), hexanediaol, cetearyl glucoside, *Oenothera biennis,* isopropyl isostearate, behenyl alcohol, cetearyl isononanoate, glycerin, sorbitol, dimethicone, talc, sodium carbomer, tocopherol, hydrogenated palm glycerides citrate |

Germany) and that contain vitamin E acetate (2.0%, in wt % active substance, Roche, Basel, Switzerland), along with controls, were tested for their pharmacokinetic properties within the horny layer.

The galenic formulations selected for this study exhibit physicochemical similarities. The basic o/w cream (lamellar type, LC) contains 2% vitamin E acetate without any form of liposomes/microparticles. For the other two creams (RP, RS) the vitamin E acetate (10%) was packed either in Roviparts® (20%) or in Rovisome® (20%). The analytically determined content of vitamin E actetate in the different creams was slightly lower, as shown in Table II.

### IRRITATION ASSAY IN THE *IN VITRO* SKIN MODEL (BUS)

The isolated perfused bovine udder skin (BUS) model was introduced as an *in vitro* model to study skin penetration and irritation. Due to continuous perfusion, the horny layer demonstrates barrier and reservoir functions similar to those in the *in vivo* human situation. A comparison of the BUS and human skin stratum corneum by infrared spectroscopy was recently carried out (8). The natural skin model is widely used in dermatological and cosmetic research as well, and provides information concerning the penetration kinetics of active ingredients, their interaction in the skin, and skin compatibility.

Depending on the barrier function and the strength of the activant/irritant, increased arachidonic acid metabolism and a diminished cell viability can be analyzed biochemi-

**Table II**
Amount of Vitamin E Acetate (in wt%) in Different Lamellar Creams Determined by HPLC

| Products | Analyzed in the product | Analyzed on the strip |
|---|---|---|
| Lamellar cream, LC (vitamin E acetate) | 1.8 | 1.8 |
| Lamellar cream, RP (20 % Roviparts®) | 1.4 | 1.4 |
| Lamellar cream, RS (20 % Rovisome®) | 1.8 | 1.8 |

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cally in whole skin biopsies. In determining the relevant part of the latter compound metabolism, the concentration of eicosanoids (ng $PGE_2$/μg DNA) was measured in the skin ($PGE_2$ prostaglandin $E_2$). The cell viability was assayed by the MTT assay (μg Formazan/μg DNA). (MTT methyl-thiazol-tetrazolium is a dye transformed by active mitochondria into a water non-soluble compound.) Details of the method concerning skin penetration and irritation are described elsewhere (9,10).

### APPLICATIONS

For this investigation three independent udder studies were performed. The udders were perfused by an oxygenized and warmed-up Tyrode's solution. The skin surface temperature was maintained at approximately 30°C.

After maintaining the perfusion of the udder for a certain period in order to switch in an aerobic metabolism, the emulsions were applied topically with a surface density of 3–4 g/100 $cm^2$. The high dosage was intended to prevent any depletion of the vitamin concentration in the liposomal vesicles during the exposure period. Thirty minutes after starting the application, a dry paper towel carefully removed the residual cream. Whole skin punch biopsies with a diameter of D = 6 mm were taken 30 minutes after the end of the exposure to study the irritation potential of the creams applied.

### TAPE STRIPPING

Adhesive tape stripping (Tesa R, 4204, BDF, Hamburg) was used to remove the outermost layers of the stratum corneum (SC) in sequence. Thirty and 90 minutes after the end of application, the adhesive tape strips for corneocyte layer removal were peeled off. Two parallel series of 15 successive strips (1.9 × 10 cm = 19 $cm^2$) were taken to analyze the vitamin E acetate content, either by the conventional HPLC method or UV spectroscopy. The chemical analysis for vitamin E acetate in either treated or untreated strips was performed in the Corporate Analytical Center of Henkel KGaA (Düsseldorf, Germany).

### DETERMINATION OF VITAMIN E ACETATE BY HPLC

For the determination of vitamin E acetate the adhesive tapes were extracted by means of acetonitrile. After solvent removal, the residue was dissolved in a well-defined volume of acetonitrile. This solution was injected in an HPLC system. The chromatographic investigation was performed using a reversed-phase HPLC column. Since vitamin E acetate shows fluorescence, this can be used for selective and highly sensitive detection. Application of an excitation wavelength of 276 nm and detection at 320 nm allows the quantification of vitamin E acetate as well as of underivatized vitamin E. The wavelengths selected for excitation and detection do not induce fluorescence either of other ingredients of the cosmetic products or of skin constituents. The acquisition of a blank chromatogram is mandatory to ensure that any fluorescent components of the adhesive tapes are extracted. The limit of determination for standard injected samples is approximately 5 ng.

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UV SPECTROSCOPY

For the UV spectroscopic investigation, spectra of adhesive tapes loaded with corneocytes (second set of strips) were measured in transmission. In Figure 1 the experimental setup for the transmission measurements of the adhesive tapes is sketched. The UV light of a deuterium lamp is projected via a mirror through the adhesive tape. Above the adhesive tape a fiber-optic probe was used to take the transmitted and collected light to a diode array minispectrometer. The outer diameter of the optic fiber was 200 μm; its numerical aperture of 0.22 provides a half angle of about 18° for the radiation collection cone.

Spectroscopic data were recorded with a dispersive photodiode array fiber-coupled minispectrometer (Ocean Optics, Inc., Dunedin, FL) with a microcomputer-based data acquisition system within the spectral range of 260 to 525 nm (the visible part of the spectrum was not used for the spectrometric assay). A Cary 5G scanning dispersive spectrometer with double monochromator (Varian, Darmstadt, Germany) was also used for comparison and for recording reference spectra of different cream components. Examples of the spectra are shown in Figure 2.

Five visually controlled spots on each tape (tapes 1–15) and several unloaded tapes (controls) were included in the investigation. Visually controlled spots were identical to highly packed locations covered by corneocytes. Tape areas with a low content of flat cells from the skin surface were strictly avoided.

Before analysis, the resulting spectra from single adhesive tape measurements were averaged. The evaluation of the loaded tape spectra by least-squares fitting was carried out after spectral subtraction of the spectral adhesive tape component. As fitting components, a reference spectrum of vitamin E acetate, the corneocytes' spectrum, and a linear baseline were sufficient for modeling the loaded tape spectra. For selecting appropriate spectral intervals, measures of matrix orthogonality such as the condition number have been used in the past. Such a criterion was also applied so that the optimal spectral interval between 265 nm and 350 nm was exploited for quantitative analysis. An example with the measured spectrum and the fitting results for corneocytes and vitamin E acetate is given in Figure 3.

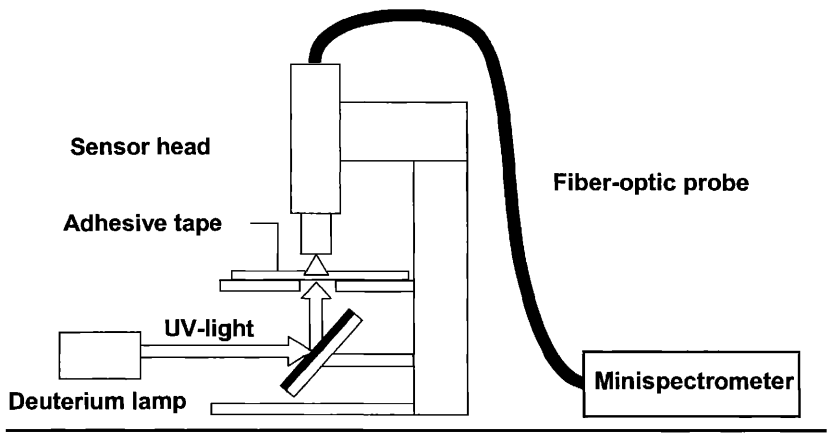


Figure 1. Experimental setup used for UV spectroscopy of adhesive tapes in transmission mode.

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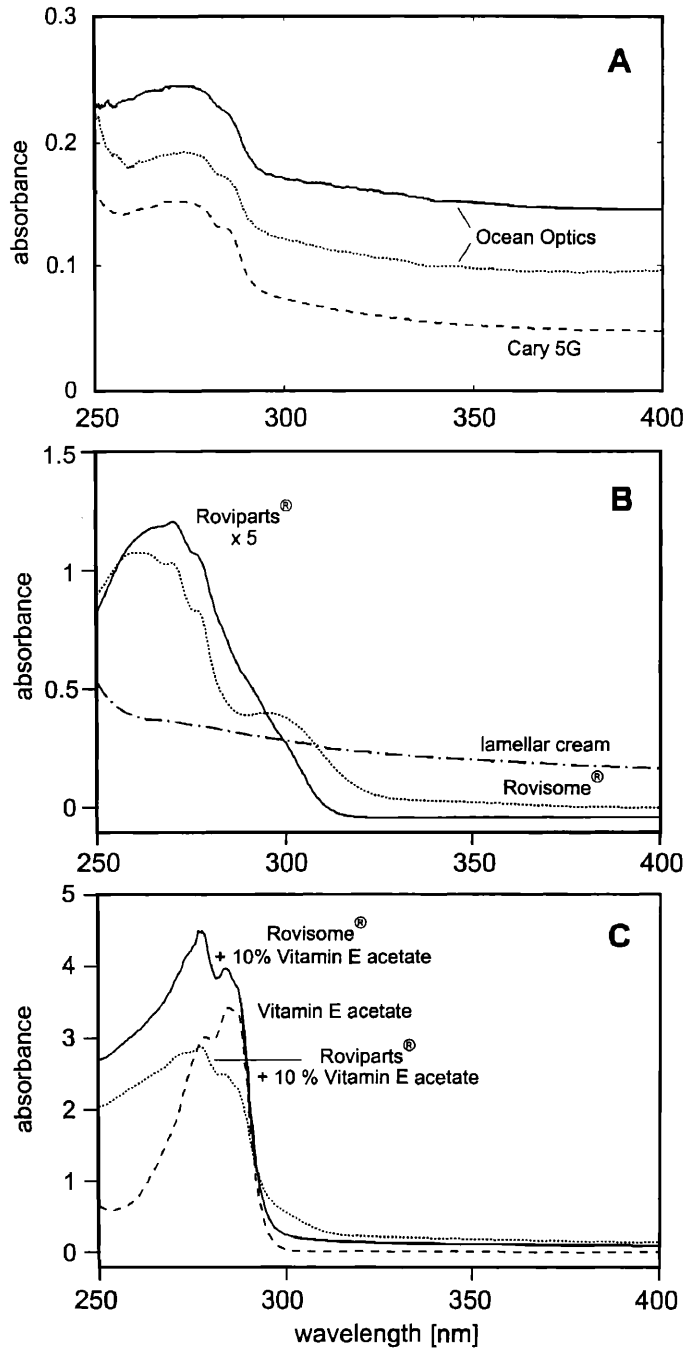


**Figure 2.** (A) Spectra of adhesive tapes measured by a minispectrometer (Ocean Optics) and a Cary 5G double monochromator spectrometer (the upper two spectra are offset for clarity). (B) Formulation components. (C) Rovisome®- and Roviparts®-containing vitamin E acetate and pure vitamin E acetate.

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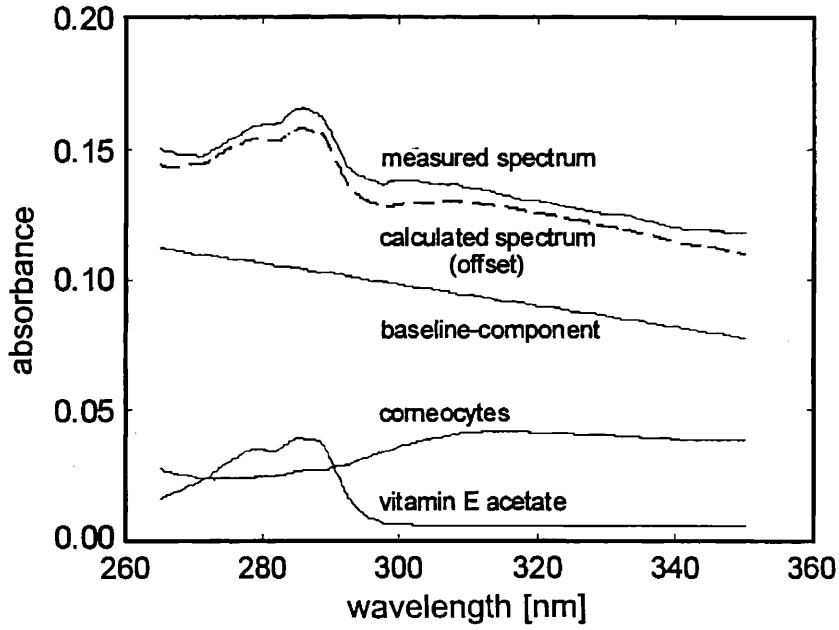


**Figure 3.** Component spectra, measured spectrum of a tape loaded with corneocytes and vitamin E acetate, and a calculated spectrum as a result from a spectral least-squares fit.

## RESULTS

### ACTIVATION/IRRITATION POTENTIAL

The irritation assay using whole skin biopsies differentiates biochemically between the cytotoxic and irritant potential of substances applied to the skin surface. Using both parameters, the reversible (activation/irritancy) and the non-reversible (cytotoxicity) parts of the irritation potential of test material, depending on the skin barrier function, can be characterized (11).

With lapses of 60 minutes after starting the application and 30 minutes after finishing the application, different results were observed with regard to the cytotoxic and irritant potential of the three types of emulsion in comparison to the untreated area, as shown in Figure 4. No major differences between the three emulsions (LC, RP, and RS) were manifested by the cytotoxicity assay (MTT). Generally, a negative deviation between 6% and 7% from the untreated control sites was found, which was within the biological variation. The results for the untreated site are characterized by normalized mean and standard deviation (100% ± 5%). Compared to the untreated controls, the increase of the $PGE_2$ tissue concentration (activation/irritancy) induced by the LC using Rovisome® (RS) as liposomal carrier was higher (119% ± 8%) than the concentration induced by the other emulsions (RP: 110% ± 3%; LC: 105% ± 2%).

### ADHESIVE TAPE STRIP ANALYSIS: RESULTS OF THE HPLC METHOD

The first set of 15 successively taken adhesive tape strips was analyzed by a conventional HPLC method, and the analytical results were calculated as $\mu g/cm^2$ per tape strip. This

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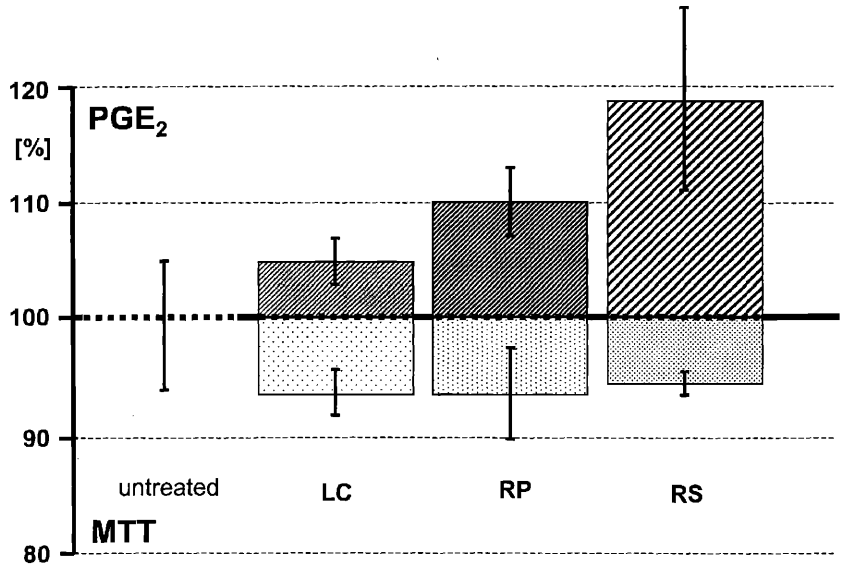


**Figure 4.** Results for LC, RP, and RS formulations from the cytotoxicity MTT assay (below) and the activation/irritancy test using the tissue concentration of $PGE_2$ (above) after an exposure period of 0.5 h, compared to untreated skin areas (100%).

methodology prefers a high dosage of 30–40 mg/cm$^2$, which is roughly tenfold the amount applied under normal use conditions, in order to prevent any depletion of vitamins encapsulated in liposomes. In general, increasing an application to more than 10 µl/cm$^2$ is not expected to increase percutaneous absorption significantly (3).

With a lapse of 30 minutes after the end of exposure, by thorough cleansing of the treated skin, the lowest total amount observed was by using Roviparts® (58%) as carrier compared to LC (100%) without any liposomes (Table III). The largest amount of vitamin E acetate in the horny layer was observed after application of the Rovisome®-containing emulsion (150%).

After the additional 60 minutes of exposure and 90 minutes after starting the application, the largest amount of vitamin E acetate was observed in the horny layer treated with the lamellar cream (100%). The formulations RP and RS induced much less penetration of

**Table III**
Natural Amount and Supplemented Total Amount of Vitamin E Acetate (µg/per cm$^2$) as Determined by HPLC and UV Spectroscopy (given in brackets) and Ratio After an Exposure Duration of 1.5 h Versus That After 0.5 h

| | Exposure period | | Ratio |
|---|---|---|---|
| Strips 1–15 | 0.5 h | 1.5 h | 1.5 h vs 0.5 h |
| Tocopherol (natural amount) | 0.06 | Not determined | — |
| Lamellar cream, LC (vitamin E acetate) | 30.0 (29.9) | 34.6 (31.5) | 1.15 |
| Lamellar cream, RP (20% Roviparts®) | 17.6 (18.5) | 24.0 (18.6) | 1.36 |
| Lamellar cream, RS (20% Rovisome®) | 45.4 (30.6) | 20.2 (19.7) | 0.44 |

The ratio value was calculated using the HPLC results.

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vitamin E acetate into the SC (69% and 58%, respectively). Independently of the formulation type and period of exposure, nearly 40% of the total amount of 15 strips was concentrated on the first strip. The amount of natural vitamin E was nearly 1000-fold lower than the supplemented amount. Again, about 50% were located on the first strip.

The different kinetics for the formulations at the two time points were calculated as the differences between the two values analyzed. The ratio can be expressed as total amount at the exposure period of 1.5 h versus 0.5 h (see also Table III). Regarding the total transferred amount, a steady state was nearly reached after 0.5 h for the LC-treated skin. The creams containing microparticles/liposomes influenced the penetration efficiency for vitamin E acetate differently. In contrast to the effect when applying the RP formulation, a severe decrease in the total amount was observed for the formulation using Rovisome® as liposomes, as the ratio calculation indicates (see Table III).

The Rovisome®-based formulation released a significant amount of vitamin E acetate into the horny layer within the short exposure period. Compared to the other formulations, the Rovisome®-containing cream only enables further penetration of vitamin E acetate into the lower epidermal layers after the additional exposure period.

### ADHESIVE TAPE STRIP ANALYSIS: SPECTROSCOPIC EVALUATION

In Figure 2A, two representative transmission spectra of adhesive tape samples (average of five tape measurements) are shown. Under the recording conditions chosen, the spectra are similar, apart from some slight deviations just above 250 nm wavelength due to low intensities observed for the minispectrometer in the short-wavelength region. In the ensuing diagrams, UV spectra of cream formulation components (Figure 2B), and Rovisome® and Roviparts® (liposomes/microparticles) containing vitamin E acetate, as well as pure vitamin E acetate (Fig. 2C), are shown. UV spectra of adhesive tape samples loaded with stripped skin and pure tape were used to generate a difference spectrum for the corneocyte component, which mainly consists of bovine skin keratin (see Figure 3).

In Figure 5, the comparison of the spectroscopic (mean, 2 × SD) and conventional (mean) results per strip (one udder) is shown for the RS cream after exposure periods of 0.5 h and 1.5 h, respectively. A major deviation between the two different types of evaluation can be observed on the first strip only.

After the exposure period of 30 minutes, the total amount of vitamin E acetate (strips 1–15) analyzed by HPLC and spectroscopy was quite comparable for the samples taken after application of the LC and the RP, respectively. However, for the cream using Rovisome® as liposomal carrier, a significant difference was observed. Considering the total amount, about 33% less vitamin E acetate was determined in the horny layer by the spectroscopic assay than by the HPLC method. The difference between the two methods is reduced almost completely when the total amount is calculated without the values of the first adhesive tape strip taken. The detailed analysis (Figure 6) of the strips (first strip and collected strips) shows the results of both methods in a direct comparison. The averaged value of the first tape strip of the Rovisome® group measured by HPLC was more than twofold the value evaluated by spectroscopy. Except for this result, there was a substantial similarity in the means and the standard deviations evaluated by both analytical methods.

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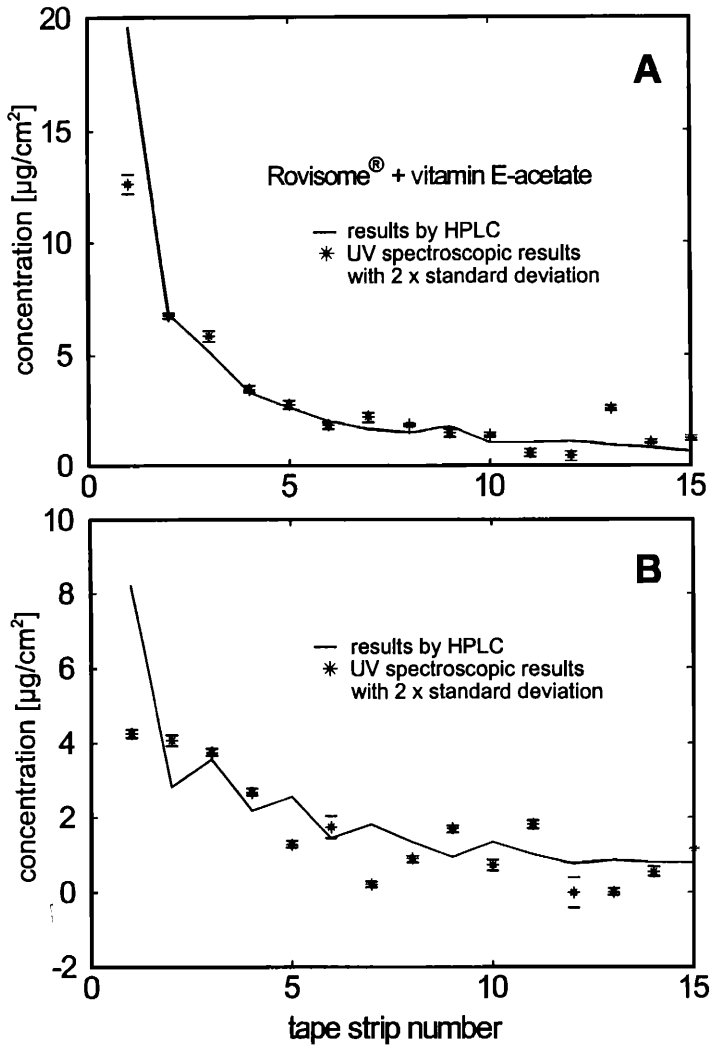


**Figure 5.** Results (one udder study) performed by spectroscopy (mean, 2 × SD) and the HPLC method (mean) at the exposure periods of 0.5 h (A) and 1.5 h (B) for the Rovisome® cream formulation.

## DISCUSSION

The aim of this *in vitro* study was to test the applicability of UV/VIS spectroscopy in order to facilitate the analytical procedure of a large number of horny layer samples, usually analyzed by HPLC. In the case of the BUS model the loaded strips exhibit a large representative area (19 $cm^2$) of the horny layer. Generally, compared to human adhesive tape stripping, the size of the tapes used is significantly increased. Therefore, the logistics and the manipulation of several hundred strips during chemical analysis are labor-intensive. Transmission spectroscopy is much easier and faster and can at least halve the costs.

The results indicate that the choice of five spots loaded with corneocytes and avoiding lower loaded or empty spots are appropriate. Except for the case of the first tape, the

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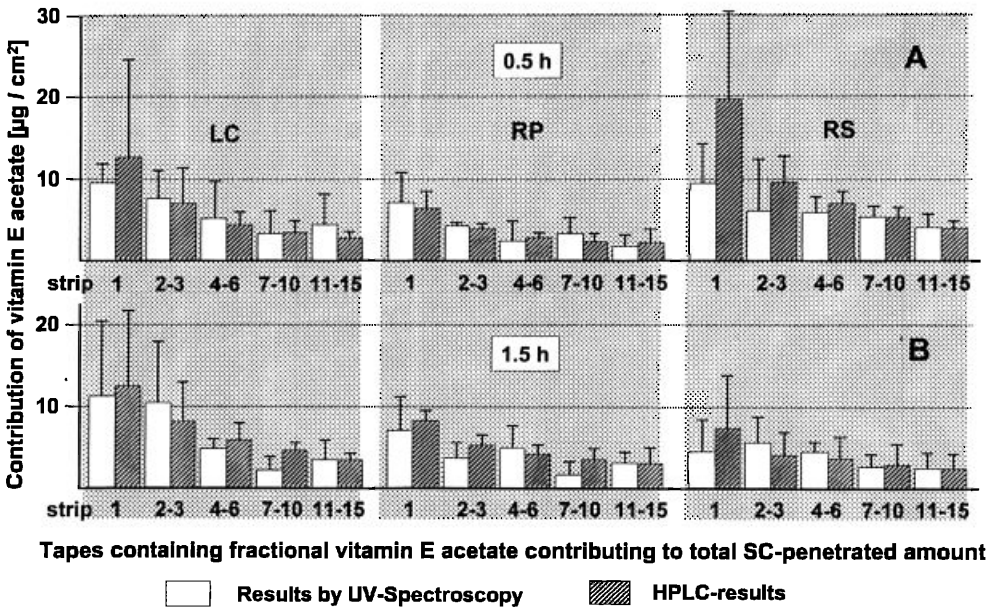


**Figure 6.** Results (µg/cm$^2$) of vitamin E acetate determination by spectroscopy and the HPLC method after adhesive tape application. Amounts from LC, RP, and RS formulations found in various strips after exposure periods of 0.5 h (A) and 1.5 h (B), respectively.

results of both methods are quite comparable. The result of the first tape may be artefactual due to the entrapped material that was not completely removable. For the HPLC method the whole strip was used, whereas for the spectroscopic assay only strip locations were evaluated. However, for both methods a higher accuracy can be achieved by quantifying the individual number of corneocytes sticking to the tape.

The BUS-irritation assay differentiates biochemically between activation/reversible injury or death of epidermal cells using whole skin biopsies. Therefore, rubbing or stripping the skin induces a reversible activation of epidermal cells, e.g., an increase of the arachidonic acid metabolism, but no cytotoxic action to keratinozytes at all (unpublished results). There are also anatomical differences between irritancy (reversible injury) and cytotoxicity. In reconstructed human skin equivalents (RE-DED cultures) it was shown that the MTT-positive cells could be attributed to the lower cell layers, whereas the terminally differentiated keratinozytes may not be involved (12). In this irritation study with three cream formulations, no difference was observed for the cytotoxic activity, which usually occurs in the basal and suprabasal epidermal cells. Neither type of formulation was able to alter the activity of the basic cream under exposure conditions of 60 minutes from the beginning. The results only indicate biological activity regarding the arachidonic acid metabolism that was more active by using Rovisome® as liposomal carrier than by using microparticles or the liposome-free cream. This indicates activation but no irritation of the upper epidermal cells that may contribute to the high uptake of vitamin E acetate into the horny layer under short-term exposure.

Vitamin E acetate in Rovisome® as liposomal carrier delivered most of the bioactive substance into the skin, compared to other formulations such as the lamellar cream (LC) or a microparticle-based one (Roviparts®). The reasons for the difference can be explained

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by an alteration of the plasticity of the horny layer, inducing a strong reservoir capacity and activation of epidermal cells. Additionally, an opening of the potential pathway for a follicular penetration may be part of an increased reservoir capacity (13).

According to a hypothetical example, Schaefer and Redelmeier (3) illustrated that diffusion through the "shunt" pathway may be relatively more important at an early stage, within a first period of approximately 90 minutes. At a later time, the flux through the stratum corneum is generally more important. Formulations using Rovisome® vesicles may take advantage of both the "shunt" pathway immediately after the application and the conventional pathway during the extended exposure time.

## CONCLUSIONS

For the quantitative spectrometric assay, a classical least-squares evaluation of the spectra between 265 nm and 350 nm based on the constituent spectra was used. It can be concluded that the spectral measurement is extremely fast, and a calibration can be based on a few reference spectra only. Therefore, UV/VIS spectroscopy is an economic analytical method for evaluating large numbers of samples of the horny layer taken by the adhesive tape stripping method. The latter is an established tool for the depth profiling of substances within the stratum corneum.

Regarding the irritation test, no cytotoxicity was recorded for the three formulations. However, Roviparts®- and Rovisome®-formulated creams induced a considerable activation of the upper epidermal cells, possibly contributing to the penetration efficiency of Rovisome®-formulated vitamin E acetate by increasing the reservoir capacity. Rovisome®-formulated creams were most successful in vitamin E acetate delivery into the horny layer, which can be explained by an alteration in the corneal plasticity and by opening the additional pathway for a follicular penetration.

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