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Quantitative HPLC analysis of sunscreens and caffeine during in vitro percutaneous penetration studies

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Abstract

This report describes rapid analytical HPLC for the quantification of five UV filters (octyl methoxycinnamate, benzophenone-3, benzophenone-4, octyl triazone and octocrylene) and of caffeine in various skin layers (stratum corneum, dermis, epidermis and receptor fluid) and in cosmetic preparations. The predominant purpose of the study was to establish standard operating procedures for rapid analysis of the compounds in various skin samples. Particular attention was paid to the preparation of biological samples whose natural constitution could interfere with the quantitative analysis. Our methods used the isocratic chromatographic mode in an RP-HPLC with UV detection and did not involve centrifugation or evaporation. Our results were validated in terms of specificity, linearity, precision, accuracy and limits of detection and quantification. The first results, obtained after in vitro experiments, are presented in this report.

Keywords: HPLC quantification; Human skin; Percutaneous absorption; Sunscreens–caffeine

1. Introduction

Analytical methods, employed for the quantitative determination of drugs in biological samples, can influence the evaluation and interpretation of bioavailability, bioequivalence and pharmacokinetic data. It is therefore essential to employ well-characterized and fully validated analytical methods to give reliable results and which can be interpreted with satisfaction (Shah et al., 1992)

UV filters play a significant role in our lives, they protect us against UV radiation, thus prevent skin cancers and they decrease cutaneous aging. However for efficiency and to avoid toxicity, sunscreens should stay on the skin surface and penetrate as little as possible the skin. That is why in vitro studies, to monitor the distribution of the compounds in the skin, are very important.

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The aim of this study was to develop simple, rapid and reliable operating procedures for the quantification of UV filters and caffeine in numerous skin layer samples (stratum corneum (SC), epidermis, dermis, and receptor fluid) and in cosmetic products. The challenge was to quantify many different samples containing different chemical products, in the shortest time, for a further investigation of in vitro percutaneous penetration. One task was to try to homogenize the different sample preparations. The methodology needed to respect the following requirements:

- find a common means of preparation for each category of biological samples named above, with similar conditions of extraction or solubilization, considering the stability of each product;
- define the composition of the mobile phase in order to obtain satisfactory specificity for each compound using the same stationary phase;
- carry out experiments using an isocratic mode since all compounds are assayed separately;
- achieve the elution with a capacity factor less than 5 in order to quantify a lot of samples quickly; and
- validate the method in order to assess the specificity, linearity, repeatability, intermediate precision, accuracy and limit of detection.[1]

Very few methods were found in the literature for assaying some of the compounds or derivatives but they did not correspond exactly to our objectives: octyl methoxycinnamate was assayed by HPLC and gas chromatography (Margineanlazar et al., 1996), benzophenone metabolites in plasma by HPLC with elution gradient (De Vos et al., 1995), octyl triazone by HPLC with gradient elution (Gagliardi et al., 1989) and caffeine in plasma or its derivatives in urine by capillary-performance liquid chromatography–ftir-FAB mass spectrometry (Hieda et al., 1995) or HPLC (Klebovich et al., 1993; Mellini et al., 1993).

The filters studied, whose formulae and characteristics are given in Table 1, were contained separately in a simple lipophilic/hydrophilic emulsion: octyl methoxycinnamate (OMC), benzophenone-3 (BP3) benzophenone-4 (BP4), octyl triazone (OT) and octocrylene at (OC). Another emulsion with caffeine (CAF), an amphiphilic substance often used as an 'anti-cellulite' substance, was used as a control of the skin integrity. Different UV filter concentrations were especially defined in order to compare the distribution of UV filters in formulations having the same SPF (sun protection factor). The purpose of the in vitro work was to determine the quantity of UV filters in different human skin layers, after a definite period of contact on the skin surface.

2. Materials and method

2.1. Instruments

For in vitro experiments, specific instruments were used, such as a dermatome Brown® (Zimmer, Vitry 94401 France), static diffusion cells, 3 ml, 1.86 cm^2 (Lara-Spiral, Courtenon France), a Tewameter T120 (Courage-Khasaka, Monaderm, Monaco) in order to measure the transepidermal water loss (TEWL), D-Squam® Tapes (CuDerm, Dallas, TX, USA), and a constant pressure device with a calibrated spring (80 g/cm^2) (Monaderm, Monaco) was used. The cutting machine was especially conceived in our labs to grind skin using a reduced quantity of solvent.

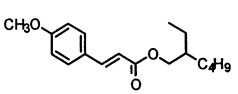
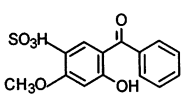
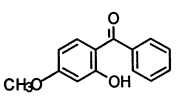
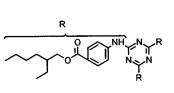
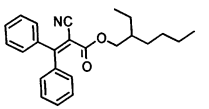
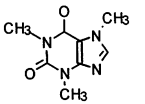
Chromatographic measurements were made with Hitachi–Merck equipment: a 6000/6200 pump, an L4000/4200 UV visible detector, an AS-2000 autosampler and a multi HSM manager integrator. A Novapack C18 (4 μm, 150×3.9 mm) and a guard column (Novapack C18, 4 μm, 10×3.9 mm) from Waters (Milford, MA, USA) were used to quantify the five UV filters, another RP column Spherisorb (C18, 5 μm, 250×4 mm, Prolabo, France) was used for caffeine.

2.2. Chemicals

Chemical products used for in vitro experimentation were: sodium lauryl ether sulfate (Texapon N40, Sidobre Sinnova, France), distilled water, bovine serum albumin (SL-S Grade Miles,

[1] All definitions and terminology come from the European Agency for the Evaluation of Medicinal Product (Bass et al., 1995).

Table 1
Formulae and characteristics of all compounds

INCI Name	Octyl methoxycinnamate (OMC)	Benzophenone-4 (BP4)	Benzophenone-3 (BP3)	Octyl triazone (OT)	Octocrylene (OC)	Caffeine (CAF)
Formulae						
Molecular weight	290	308	228	823	361	194
Solubility coefficients log $P_{oct/water}$	6	1.9	3.78	–	–	−0.07
Supplier characteristics	UVB filter	UVB+UVA filter	UVB+UVA filter	High UVB filter	High UVB filter	Present in tea, coffee, in guarana paste and cola nuts
	Concentration recommended 2–7%	Concentration recommended 5%	Concentration recommended 10%	Concentration recommended 5% for high SPF	Concentration recommended 10%	Soluble in water, alcohol, acetone chloroform, ether
	Used for the formulation of effective water-resistant or water-proof sunscreen preparations	Soluble in water. Its acid group must be neutralised with, e.g. triethanolamine + NaOH (giving a pH of 6). Used in highest sun protection factor when it is combined with an oil soluble UV filter.	Soluble in oil.Miscible with polar oil. Stable toward light. Used in creams to prevent premature skin ageing and in lipsticks.	Polar nature: good affinity with the skin keratin. Completely insoluble in water. Stable toward light. Used in day creams because of its low penetration into the skin.	Hydrophobic, oil soluble. Stable toward light. Preferred for water-resistant and water repellent formulations.	Used in cosmetics as an anticellulite

France), gentamicin sulfate (G-1264 Sigma, France) and phosphate-buffered saline (Sigma, France) containing NaCl 0.120 M, KCl 0.0027 M and phosphate buffer 0.01 M (pH 7.4).

All reagents used for chromatography were of analytical-reagent grade. Methanol (Fisher Scientific, Elancourt, France) was of chromatography grade, ultra-high quality water was obtained from an Alpha-Q system (Millipore, St.-Quentin, France). Isopropanol and sodium hydroxide 1 N were obtained from Fisher Scientific, acetic acid (Ac.A.) and orthophosphoric acid (H_3PO_4) from Prolabo (Nogent/Marne, France), Pic A reagent from Waters (Milford, MA, USA), 1-heptane sulfonic acid (H.S.Ac.) from Sigma (Saint-Quentin Fallavier, France). The five filters were obtained from BASF (Ludwigshafen, Germany) and introduced in formulations made by l'Oreal (Aulnay-sous-Bois, France) OMC at 5%, BP3 at 4.9%, BP4 at 6.9%, OT at 4%, OC at 8% and caffeine at 3%.

2.3. Method for in vitro study

The in vitro study was conducted with fresh dermatomed (344 ± 61 μm) human Caucasian skin from abdominal or breast surgery (females aged from 17 to 65) and put on static diffusion cells. The 3-ml receptor fluid (pH 7.4) was maintained at 32°C and consisted of 1% bovine serum albumin, 0.9% NaCl, 0.02% KCl and 0.04% gentamicin sulfate in distilled water. TEWL (transepidermal water loss) was recorded on each site with a Tewameter®, and 3 mg/cm^2 of cream were applied. After an exposure time of 16 h, a specific washing procedure of the treated area was carried out: once with 0.5 ml of sodium lauryl ether sulfate solution at 1% (w:w) and twice (2×0.5 ml) with water (cotton swabs were used to dry the skin). Then the receptor fluid was taken out of the cell and 16 strippings were carried out on the skin surface with D-Squam™ tapes by applying constant pressure (80 g/cm^2) for 5 s. The epidermis was separated from the dermis with a hair dryer and dermis was ground by the cutting machine. For the HPLC an isocratic phase, degassed in ultrasonic bath for 30 min was used and the flow-rate was set at 1 ml/min. An amount of 20 μl of each sample was injected after the treatments summarized in Table 2.

3. Results and discussion

3.1. Initial HPLC conditions

The study of bibliographic references enabled us to choose some bases for the chromatographic conditions. It was possible to quantify UV filters and caffeine by RP-HPLC (grafted silica C18) and by UV detection set on the λ_{max} of each product. The use of an isocratic methanol:water mobile phase was kept since each product was quantified separately. The percentages of methanol:water were adjusted in order to obtain a capacity factor between 2 and 5 for each compound. The mobile phase was acidified by the addition of either acetic acid or orthophosphoric acid. According to the structures laid out in Table 1, caffeine and BP4 were ionized at the pH compatible with silice, and therefore counterions were added to the mobile phase: 1-heptane sulfonic acid 5×10^{-3} M (anionic) and tetrabutylammonium phosphate 1×10^{-3} M (Pic A reagent, cationic) for caffeine and BP4, respectively.

3.2. Preparation of samples and stability

The samples studied were of three types: solutions (receptor fluids/washing solutions), solids (stratum corneum on tapes, epidermis/dermis) and emulsions (cosmetic preparations). Consequently they necessitated different treatments to obtain the best analytical solution to be injected. Adequate concentration range, satisfactory recovery and good stability had to be achieved, in a standardized general operating procedure.

The solutions did not need any particular treatment. The receptor fluids were injected directly and the washing solutions were only diluted in order to fit the concentration range. The treatment of cosmetic preparations generally consisted of a dilution to break the emulsion. In contrast, the solid samples were subjected to more thorough preparation in order to separate the analyte from the sample matrix. Alkaline digestion, before neutralization by HCl was generally used to treat the skin samples. Because of the caffeine degradation in a strong alkaline medium, this

Table 2
Sample preparations

	Stratum corneum (tape)	Epidermis	Dermis	Receptor fluid	Washing solution	Cream
Operation No. 1	–	–	Cut in little pieces	–	–	Weigh 10 mg in a flask
Volume of appropriate solvent (ml)	2	1	6	–	30	100
Operation No. 2	–	–	Grind for 2 min with cutting machine	–	–	–
Contact time			One night at room temperature			Until complete solubilization
Ultrasound			3 min			15 min
Filtration			0.45 μm			

technique was abandoned. The extraction or dissolution of products in these samples, with an appropriate solvent, was directly considered for the epidermis or dermis. Stratum corneum samples had to be removed from the tapes (3.8 cm^2 polymeric support and polymeric glue). The eight first tapes were pooled in pairs, tapes 9–16 were pooled together. Two methods for removing the stratum corneum (SC) from the tape were considered: first a disorganization of the polymeric configuration of glue to favor an extraction of the chemical product, second the dissolution of the overall tape in order to obtain total recovery. Two solvents were tested: isopropanol and acetone, according to the solubilities of the studied compounds. After one night of contact with the tape, the first solvent (isopropanol) destructured the polymeric glue, whereas the second one (acetone) dissolved the polymeric glue as well as the hard polymeric support of the tape. Due to numerous interferences encountered on the chromatogram of the corresponding acetonic solution, the use of acetone was abandoned. Isopropanol led to a more selective extraction. However, from a chromatographic point of view, isopropanol generated a system peak when the mobile phase contained a counter-ion (i.e. for the analysis of the most hydrophilic compounds: BP4 and caffeine). Thus, different mixtures of methanol:water as extraction solvent, were tested for both of them to finally set at a 50:50 mixture for caffeine and at a 25:75 mixture for BP4.

From this first study, the appropriate solvents for the preparation of each kind of sample were isopropanol for lipophilic UV filters (OMC, BP3, OT and OC) and methanol:water mixture for BP4 and caffeine.

The volume of the extraction solvent has then to be defined, taking into account concentrations and treatment conditions. The concentration of the washing solutions was high, corresponding to the excess of cream which had not penetrated within the skin. So each washing solution (1.5 ml) was diluted with 30 ml of the appropriate solvent for HPLC measurement. In contrast, the concentrations of the chemical compounds studied in skin compartments and in receptor fluid were low. Thus, for these latter samples, the volume of solvent had to be as low as possible and compatible with the detectability of the chromatographic measurement. Tapes pooled in pairs were put in 2 ml of solvent, whereas the next tapes were pooled in 5 ml of solvent for a total immersion. The viable epidermis samples were very thin and small (2 cm^2) and had a good contact surface with 1 ml of solvent. The previously defined treatment was successfully applied. The dermis samples were thicker and larger than the epidermis samples (4 cm^2). Their elastic structure made it impossible to grind them with commercial equipment (Potter, Ultraturax®). Thus, a special reduced cutting machine was created in order to obtain, in a minimum of 6 ml of solvent, the ground skin, after cutting the dermis biopsies in to small pieces. Due to the complexity of the receptor fluid mixture and the very low level of products expected, the samples were injected directly without any treatment.

The specific operating conditions for each product are summarized in Table 2. After one night at room temperature in the solvent, each sample was sonicated for 3 min for biological samples or 15 min for the creams, and then filtered (0.45 μm) before injection.

3.2.1. Stability

Considering the possible phenomena of degradation or isomerisation in daylight (Jiang et al., 1996), the penetration experiments were performed with the skin, shielded from light and all samples were stored at -20°C in a freezer until their quantification. Indeed, it was shown (Marginean-Lazar et al., 1997) that octyl methoxycinnamate (OMC), contained in hydro-alcoholic solution, is altered after artificial irradiation. Their conclusion is that each sunscreen preparation has a particular photochemical behavior with relation to filter mixture and emulsion type. The stability of each product in its respective solvent was investigated during the night at room temperature and at -20°C during a period of 2 weeks in a dark environment. No degradation was observed by the HPLC method. Nevertheless, for quality monitoring, the formulations were regularly quantified and the mass balance of each biopsy was calculated.

3.3. Qualitative criteria and adjustment of the HPLC conditions

The specificity of the method was assured by the injection of the emulsion solution without an active component (blank) and also by the injection of a solution containing a tape or a piece of skin (stratum corneum, viable epidermis and dermis), by the injection of washing solution and receptor fluid. The specificity of the different samples was investigated for all components at each analytical wavelength and each mobile phase composition (Table 3).

There was no interference with receptor fluids or with washing solutions, regardless of the chemical component active ingredient. There was no interference with the other skin samples for OMC, BP3, OT and OC. However, interferences were detected for BP4 and caffeine with skin samples. The specificity was obtained by modifying the methanol:water proportions of the mobile phase for BP4 compound. To assess the caffeine specificity, it was also necessary to increase the system efficiency by increasing the column length from 150 to 250 mm. The resulting resolution was satisfactory: 1.4 for caffeine/tape interference and 2.98 for the most critical pair of caffeine/skin components (Fig. 1). Thus, the injection of all

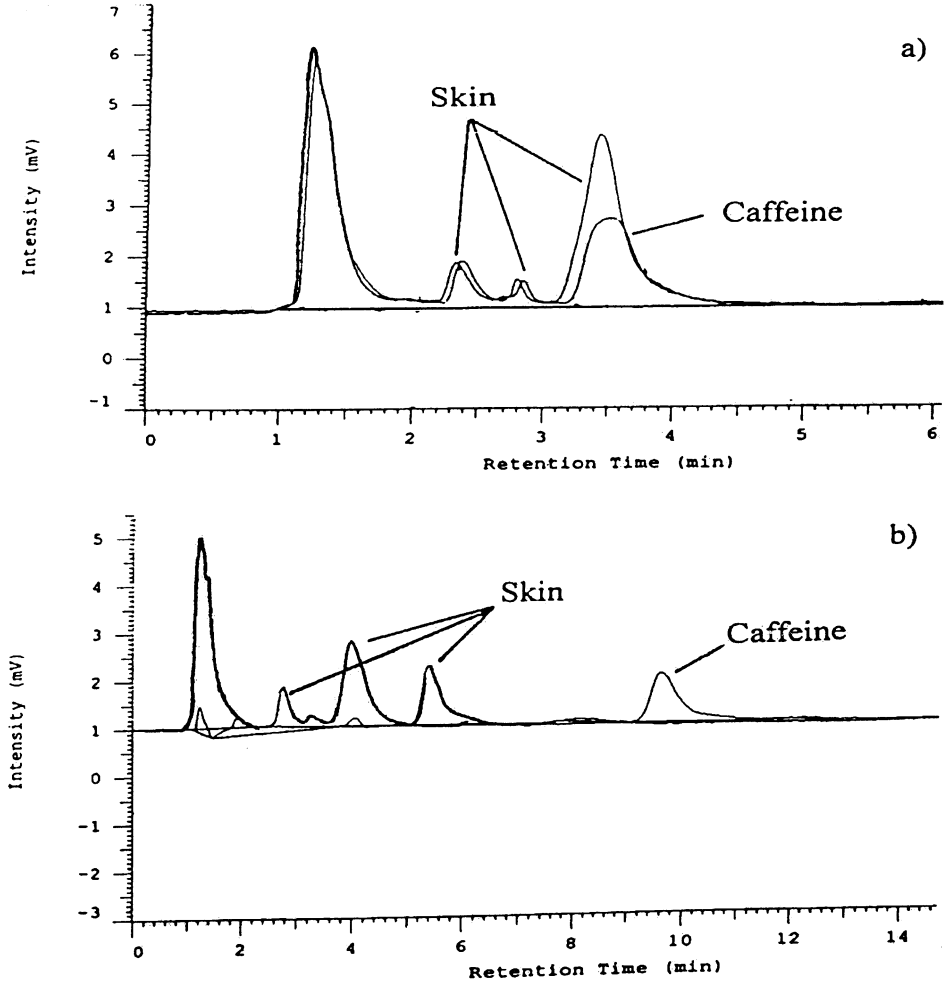


Fig. 1. Chromatogram of skin sample and caffeine with (a) a column C18 of 150 m and with (b) a column C18 of 250 mm.

Table 3
Schedule of operating conditions for chromatographic analyses[a]

INCI name	Octyl methoxycinnamate (OMC)	Benzophenone-4 (BP4)	Benzophenone-3 (BP3)	Octyl triazone (OT)	Octocrylene (OC)	Caffeine (CAF)
Wavelength	311 nm	285 nm	291 nm	310 nm	307 nm	272 nm
Mobile phase	MeOH 88%; H_2O 12%; (H3PO4 0.5g/l)	MeOH 40%; H_2O 60%; (0.3% PicA (v:v))	MeOH 69%; H_2O 31%; (1%Ac.A. (v:v))	MeOH 98%; H_2O 2%; (1% Ac.A.(v:v))	MeOH 79%; H_2O 21%; (1% Ac.A.(v:v))	MeOH 25%; H_2O 75%; (1%Ac.A. (v:v) + HS.Ac. 5.10^{-3}M)
Dissolution solvent	Isopropanol	MeOH 25%; H_2O 75%	Isopropanol	Isopropanol	Isopropanol	MeOH 50%; H_2O 50%

[a] Ac.A, acetic acid, HS.Ac., heptane sulfonic acid-All percentages are given in (v:v).

Table 4
Data obtained by the analytical validation of all compounds

Linearity	OMC $r^2 = 0.998$	BP4 $r^2 = 0.999$	BP3 $r^2 = 0.999$	OT $r^2 = 0.998$	OC $r^2 = 0.999$	CAF $r^2 = 0.998$
Repeatability (%)	1	2.5	2	2.5	1.6	2
Intermediate repeatability (%)	3.9	3.9	3.4	6.6	1.7	3.0
Accuracy (%)	95	97	101	101	102	101
Limit of detection (μg/l)	10	90	20	10	40	80
μg/cm² for						
Tape (SC)	0.01	0.10	0.02	0.01	0.04	0.08
Epidermis	0.01	0.05	0.01	0.01	0.02	0.04
Dermis	0.03	0.29	0.06	0.03	0.12	0.24
Receptor fluid	0.02	0.15	0.03	0.02	0.06	0.12
Washing solution	0.16	1.50	0.30	0.16	0.60	1.20
Limit of quantification (μg/l)	370	930	50	50	150	400

'blank samples' showed no interference between UV filters or caffeine and the different matrices.

3.4. Validation of the analytical procedure

The analytical methods were validated in terms of linearity, precision, accuracy and limits of detection and quantification.

As chemical compounds were found in very small quantity in some skin layers, a large range of concentrations had to be considered in order to detect from 0 to 100% of the dose applied on the skin. For each of the six components, stock solutions were prepared by dissolving about 100 mg of pure product (exact weight) in 100 ml of dissolution solvent. Then diluting aliquots of the stock solution with the same solvent to give concentrations ranging from 1 to 50 mg/l (1–2–5–10–25–50 mg/l) produced six standard solutions. Precision was defined as the repeatability and the intermediate precision. The repeatability was established by the relative standard (CV%) calculated from the ten injections of low (1 mg/l) and high (50 mg/l) concentrations. The intermediate precision was evaluated with the relative standard deviation of response factors obtained from the data of three calibration curves performed on three different days. The accuracy was calculated by the recovery yield between the value found with a calibration curve and the true value incorporated in the cosmetic cream. The detection limit was calculated as the concentration that led to a signal three times the noise level, the quantification limit as ten times the noise level. Characteristics concerning the validation terms for all products are related in Table 4.

The coefficients of linearity (r^2) were 0.999 or 0.998. The CV% of repeatability was lower or equal to 2.5%, the coefficient of intermediate precision was lower than 4%, except for OT (6.6%). Those results were acceptable for the quantification of human skin samples. The accuracy was between 95 and 102%. The limits of detection or quantification were quite different according to components, but enough low to appreciate the quantity of product contained in each sample.

3.5. In vitro application: results and discussion

The first results obtained after an exposure time of 16 h on human fresh skin are summarized in Table 5 and Fig. 2. It is shown, under our conditions, that UV filters have different distribution profiles in human skin. The mean mass balance obtained for all experiments was 95%. Their mean amount found in the receptor fluid is zero or very low, except for BP3 with 1.0 ± 0.4 μg/cm^2. This penetration rate can be compared with that of caffeine (1.1 ± 0.8 μg/cm^2), caffeine known as a good penetrating compound. The mean quantities found in the epidermis and dermis are very low: not more than 0.4 μg/cm^2 for both compartments

Table 5
In vitro quantification of products in human skin, after an exposure time of 16 h (mean values ± S.D.)[a]

	OMC ($n=9$)	BP4 ($n=9$)	BP3 ($n=6$)	OT ($n=7$)	OC ($n=7$)	CAF ($n=9$)
SC (μg/cm^2)	9.5 ± 3.2	4.0 ± 1.8	8.5 ± 3.3	4.7 ± 1.4	10.3 ± 6.0	2.3 ± 2.0
Epidermis (μg/cm^2)	0.2 ± 0.2	0.1 ± 0.1	0.3 ± 0.2	0.1 ± 0.1	0.2 ± 0.4	0.1 ± 0.1
Dermis (μg/cm^2)	0.1 ± 0.2	0.2 ± 0.4	0.4 ± 0.1	0.03 ± 0.05	0.01 ± 0.02	0.1 ± 0.2
Receptor fluid (μg/cm^2)	0.03 ± 0.07	BLD	1.0 ± 0.4	BLD	BLD	1.1 ± 0.8
Washing solution (%)	87.1 ± 3.6	93.6 ± 4.0	85.7 ± 4.5	92.0 ± 5.4	90.1 ± 6.0	90.4 ± 3.4
Recovery (%)	94.4 ± 3.1	96.0 ± 4.2	93.4 ± 3.1	96.5 ± 4.4	95.7 ± 4.7	94.7 ± 3.0

[a] *n*, number of different biopsies; BLD, below the limit of detection.

for all products. In contrast, the compartment containing the highest quantity of substance is the stratum corneum, which contains a minimum of 2.3 ± 2.0 μg/cm^2 for caffeine and a maximum of 10.3 ± 6.0 μg/cm^2 for OC. We can conclude here with those last results, that the quantity of product found in the stratum corneum does not always reflect the quantity penetrated under the skin (receptor fluid).

The stripping technique enables the determination of the distribution of chemical products in the stratum corneum. In Fig. 3 we observe, as Trebilcock et al. (1994) did, that the quantity of product decreases according to tape strippings and thus according to the depth in the stratum corneum. After the sixth strip, it becomes impossible to compare each product because the amounts are too small and the curves are superposed. Nevertheless, we can see that the first six strips contain more than 70% of product compared to the total amount found in the stratum corneum. In addition, we can also see that the

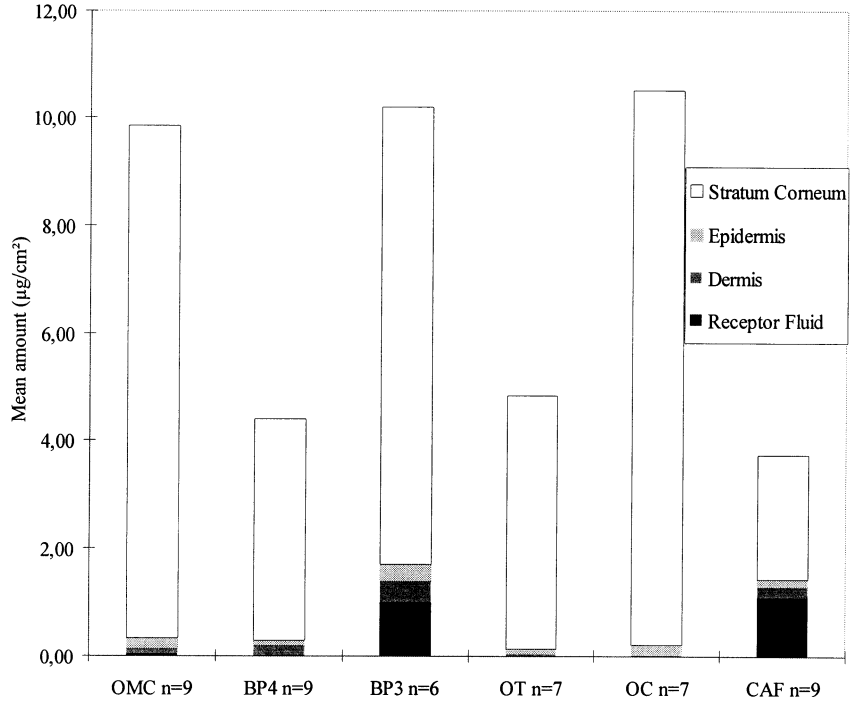


Fig. 2. Distribution of UV filters and caffeine in human skin, after an exposure time of 16 h (mean values, *n* different biopsies).

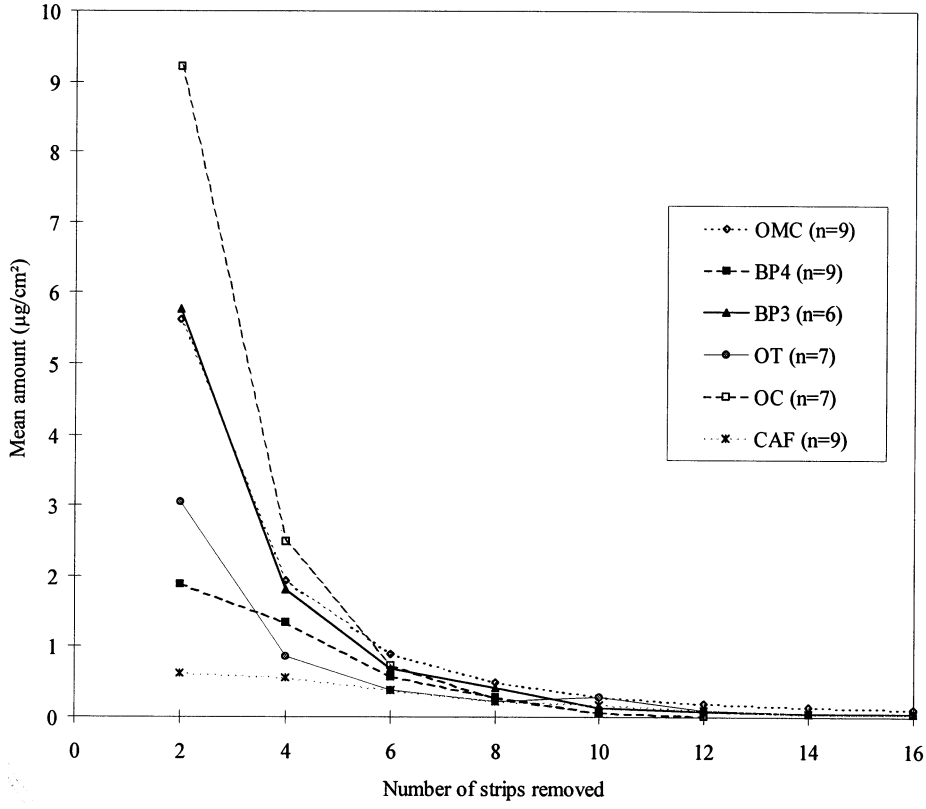


Fig. 3. Distribution of UV filters and caffeine in human stratum corneum according to the strips removed (mean, *n* different biopsies).

amount quantified in the first strip seems to predict which component will have the best substantivity for the stratum corneum (OC) or which one will have the lowest (CAF). Two groups of products can be defined here according to their cumulated amount (µg/cm^2) in the stratum corneum. The first group, composed of the most lipophilic compounds (OC, BP3, OMC and OT), has high substantivity for stratum corneum, the second is composed of hydrophilic compounds (BP4 and CAF) and has the lowest substantivity. In addition, with experiments made for this report, we calculated the intra-individual variability, which was 22%, and the inter-individual variability, which was between 34 and 45%, except for caffeine whose inter-individual variability was 70%. These results confirm the fact that for the same experiment, each human skin reacts in a special way; the skin of each person is unique. Note that this result could be explained by the wide age range of volunteers whose biopsies were used in our experiment. To conclude, this first in vitro experiment confirms that each chemical product is distributed differently in human skin, some are accumulated in the stratum corneum, others penetrate the skin.

4. Conclusion

This study shows the complexity of an analytical technique used when samples are biological and/or contain products introduced by the imperatives of the protocol. In our case, D-Squam® tapes were used to take off the horny layer from the skin in vitro. They often caused, as biological samples do, interferences with the marker to be quantified. Several trials were carried out for each

product taken separately, with different mobile phases, dissolution solvents and eventually by using a column longer in order to obtain perfect results.

The HPLC method of six sunscreens and caffeine, incorporated in cosmetic preparations, is established as the answer to our requirements. We validated methods in terms of specificity, linearity, repeatability, intermediate precision, and accuracy, among other things. The limits of detection and quantification were also defined. We obtained simple and rapid chromatographic techniques that enabled us to obtain the first in vitro results, shielded from light, after applying each cream on the skin for 16 h. Therefore, if experiments have to reflect the real situation, i.e. under strong light for UV filters, their stability has to be studied.

These validated techniques, which respect strict requirements, are absolutely necessary before assaying all new products. Thus they provide a basis for discussing and proving our future in vitro and in vivo experiments.

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