Chapter 2
Development of a Chromatographic Method to Estimate the *In Vitro* Percutaneous Absorption of Hydrosoluble UV Filters

2.1 Introduction

In recent years, new sunscreen cosmetic products increasingly more comfortable and easier to apply have been developed, based on an aqueous matrix and the use of hydrosoluble UV filters.

The most used hydrosoluble UV filters approved by the European legislation currently are, using the International Nomenclature of Cosmetic Ingredients, phenylbenzimidazole sulfonic acid (PBS), disodium phenyl tetrasulfonate dibencimidazol (PDT), benzophenone-4 (BZ4) and tereftaliden dicamphor sulfonic acid (TDS). The chemical structures of these compounds are shown in Fig. 2.1.

In the literature, studies that lead to a controversial situation about the side effects of BZ4 and PBS can be found. According to Hughes and Stones, the results obtained by analyzing skin allergy tests show that BZ4 produces significantly more positive reactions in the absence of photo-stimulation that any other UV filter (Hughes and Stone 2007). It was also found that UV filters are the substances that produce positive responses to photo-allergic reactions on the skin more frequently in comparison to the rest of the cosmetic ingredients and, in particular, BZ4 is the responsible of 2 % of the cases that involve patients with clinical diagnosis of photo-allergic contact dermatitis. In the case of PBS, the percentage is 1 % (Rodriguez et al. 2006). However, according to another study, BZ4 did not cause any allergic or photo-allergic reaction (Schauder and Ippen 1997). In this review, a case of allergic reaction and seven cases of photo-allergic reactions were related to the use of PBS, among a population of 402 patients. Accordingly, Darvay and colleagues claim that, despite the marked increase in the use of cosmetic products containing these compounds, contact allergic and photo-allergic reactions to UV filters are considered rare (Darvay et al. 2001).
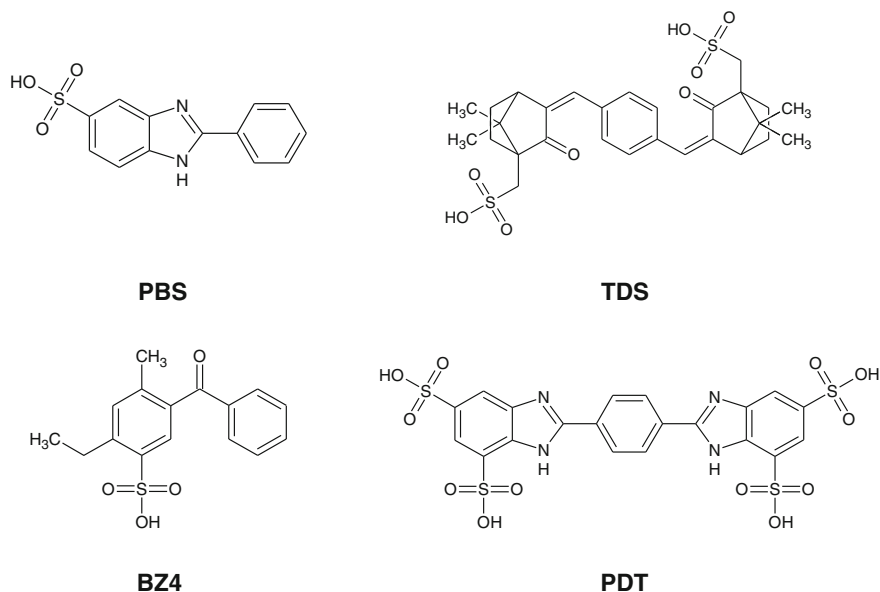

Fig. 2.1 Chemical structures of the most used hydrosoluble UV filters in sunscreen cosmetic products

Other of the side effects of PBS is the formation of free radicals caused by exposure to artificial sunlight (Inbaraj et al. 2002). Its inhibition was achieved through the interaction of the UV filter and derivatives of cyclodextrins, such that the decomposition of PBS induced by UV radiation was reduced (Scalia et al. 2004).

No publications related to the dermatologically harmful effects in the cases of PDT and TDS have been found. On the contrary, several articles show that TDS in contact with skin does not involve any risk to human health (Dean et al. 1992; Seite et al. 1998; Bernerd et al. 2000; Benech-Kieffer et al. 2003; Guenther et al. 2006). Furthermore, the protective properties of TDS regarding cytotoxicity and genotoxicity from solar UV radiation have been proved (Fourtanier et al. 1992; Fourtanier 1996; Marrot et al. 1998; Stege et al. 2000; Seite et al. 2000; Moyal 2004).

The UV/VIS absorption spectra of the target hydrosoluble UV filters shown in Fig. 2.2 indicate their protective ability to electromagnetic radiation on UV-A (320–340 nm) and UV-B (290–320 nm) areas.
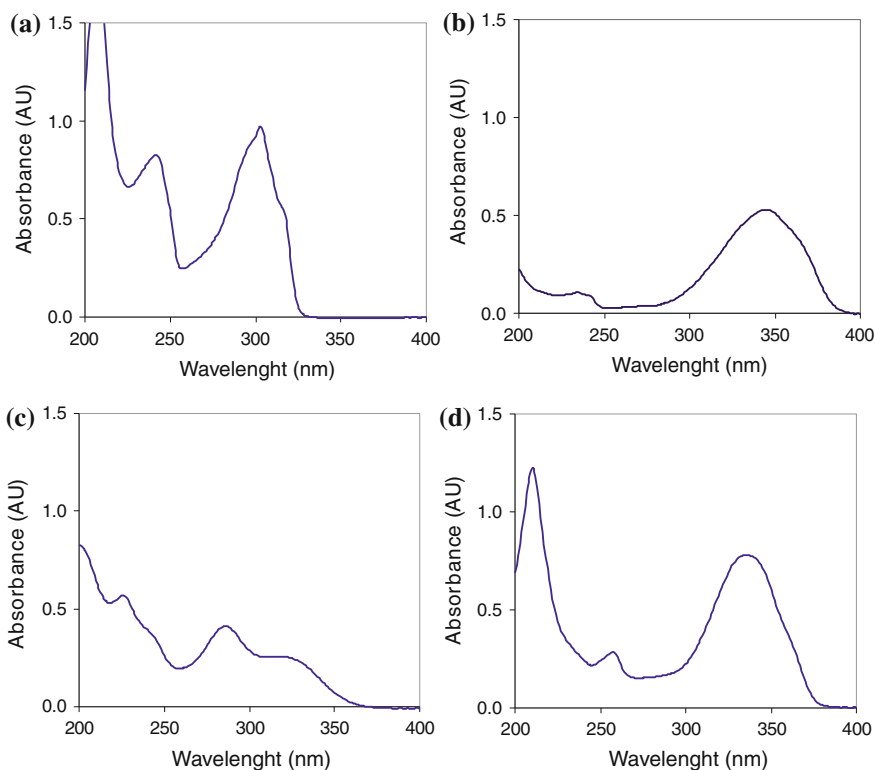
Fig. 2.2 UV/VIS absorption spectra obtained from 5 μg mL^{-1} aqueous solutions of PBS (**a**), TDS (**b**), BZ4 (**c**), and PDT (**d**)

2.1.1 Aim of the Study

As noted in Chap. 1, the fact that some of the organic UV filters penetrate through the skin is real. Considering this phenomenon, the need to perform skin permeation studies of these compounds becomes necessary. Hence, the development of analytical methods to determine UV filters with appropriate parameters of sensitivity, selectivity and accuracy and using preferably *in vitro* or non-invasive *in vivo* methodologies is required.

In this sense, the aim of this work is the development of an analytical method based on liquid chromatography-ultraviolet/visible spectrometry (LC-UV/VIS) to estimate simultaneously the *in vitro* percutaneous absorption of the most used hydrosoluble UV filters in sunscreen cosmetic products. The methodology is based on the application of a cosmetic product containing the UV filters of interest on human epidermis placed in a diffusion cell and the determination of the UV filters in the solution used as receptor fluid. The addition of an ion-pairing reagent to the mobile phase provides a good signal/noise ratio, which allows the determination of

the low content of the analytes in the receptor fluid from the first hours after the application of the cosmetic product.

Given that the *in vitro* percutaneous absorption procedures are complex and require a large number of experiments, the use of analytical methods to simultaneously study several components and evaluate the possibility of synergy effects can be very useful.

2.1.2 Background and Current Status of the Issue

In connection with the hydrosoluble UV filters considered in this study, analytical studies regarding percutaneous absorption by using different types of *in vitro* methodologies have been found in the literature (Part II, Fig. 2.2). Thus, diffusion cells have been used in the cases of BZ4 (Brinon et al. 1999; Potard et al. 1999; Benech-Kieffer et al. 2000) and TDS (Benech-Kieffer et al. 2003), as well as PDT, conducted by our research group (Balaguer et al. 2006b). Furthermore, *in vivo* tape-stripping techniques have also been used with BZ4 (Couteau et al. 2001; Potard et al. 1999).

On the other hand, studies to determine the target UV filters in blood and excretion products (urine or feces) from volunteers who had previously been applied cosmetics containing these sunscreen compounds have been conducted, as in the cases of TDS (Benech-Kieffer et al. 2003), PDT (Balaguer et al. 2006a) and PBS (Vidal et al. 2003) being the last two works from our research group. All these studies were carried out using analytical techniques such as LC-UV/VIS, molecular fluorescence spectrometry and radioactive techniques.

In previous articles published by our research group, the chromatographic separation and simultaneous determination of PBS, PDT, BZ4 and TDS in sunscreen cosmetics was achieved by using a LC-UV/VIS method (Salvador and Chisvert 2005b). However, the limits of detection of this method are not appropriate to determine the target UV filters in the receptor fluid from the *in vitro* diffusion cells, because UV filters are at low levels in the first hours after topical application of the cosmetic product.

It should be noted that the ion interaction chromatography is a technique that allows the separation of ions using conventional LC-UV/VIS instrumentation, unlike ion chromatography. The mobile phase is usually a hydro-organic solution containing a suitable ion-pairing reagent. In comparison to ion chromatography, this type of ion interaction methods provides advantages as lower equipment and columns cost, and the possibility of use in laboratories which only have conventional LC-UV/VIS systems. Furthermore, resolution and sensitivity of both techniques are comparable, given the appropriate choice of ion- pairing reagent.

Since the compounds of interest are acids that can undergo ionization acquiring anion form, a salt formed by a bulky cation (tetrabutylammonium, TBA) has been proposed as ion-pairing reagent. Thereby, ion pairs with the analytes can be formed, thus increasing the retention because of the greater affinity to the C18

stationary phase. However, this hypothesis of the mechanism of retention is not unique, and various alternatives have been proposed to explain how retention occurs (Gennaro and Angelino 1997).

The mobile phase composition should be optimized considering the different variables that control retention, such as content of organic modifier, concentration and nature of the ion-pairing reagent and pH. However, the choice of the experimental conditions to develop a method by ion interaction does not follow a general rule. In this sense, the dependence of the separation of analytes on so many different experimental conditions provides versatility to solve technical problems regarding retention and resolution.

The ability to obtain a good signal/noise ratio and therefore, a lower limit of detection, is another important consequence of the ion-pairing effect.

According to literature sources consulted to conduct this study, there are no published articles related to the estimation of *in vitro* percutaneous absorption of the target UV filters simultaneously and, moreover, published methods to estimate simultaneously the penetration through the skin of other UV filters are very limited (Potard et al. 1999).

2.2 Experimental

2.2.1 Reagents and Samples

Phenylbenzimidazole sulfonic acid (PBS), also called 2-phenylbenzimidazole-5-sulfonic acid, 99 % from Guinama (Valencia), disodium phenyl dibencimidazol tetrasulfonate (PDT) or acid 2-2′-bis-(1, 4-phenylene)-1H-benzimidazole-4,6-disulfonic, >99 % from Haarmann and Reimer (Parets del Valles), benzophenone-4 (BZ4) or 2-hydroxy-4-methoxybenzophenone-5-sulfonic acid, 99.9 % from Roig Farma (Terrassa) and tereftaliden dicamphor sulfonic acid (TDS) or triethanola-mine salt of 3,3′-(1,4-phenylendimethyliden-bis-(7,7-dimethyl-2-oxo-bicy-clo[2.2.1]heptane-1-methanesulfonic, 99 % supplied by L'Oreal (Madrid) were used as standards.

The solvents used for the preparation of the mobile phase were HPLC grade ethanol (EtOH) from Scharlab (Barcelona) and deionized water obtained from a NANOpure II water purification system ($R \geq 18$ mΩ cm) from Barnstead (Boston).

To adjust the pH of the mobile phase, glacial acetic acid for analysis (AcOH) from Panreac (Barcelona) and an 25 % ammonium hydroxide solution ($d = 0.91$ g mL^{-1}) from Scharlab (Barcelona) were used. The ion-pairing reagent was tetra-n-butylammonium fluoride (TBA) >99 % from Acros Organics (Geel).

To prepare the solution used as receptor fluid, sodium monohydrogen mono-hydrate, anhydrous potassium dihydrogenphosphate, sodium hydroxide and sodium chloride, all from Scharlab (Barcelona), were employed.

To check the integrity of the skin, the phenol red (PR) marker >99 % from Scharlab (Barcelona) was used.

2.2.2 Equipment and Material

The LC-UV/VIS system consisted of a PU-2089 Plus® chromatograph connected to a MD-2010 Plus® UV/VIS detector, both from Jasco (Madrid). The chromatographic separation was performed using a Kromasil® 100 C18 analytical column (5 µm particle size, 125 mm long, 4 mm internal diameter) from Scharlab (Barcelona). A injection loop (20 µL) from Rheodyne (Wertheim-Mondfeld) was used.

Franz-type diffusion cells designed by Prof. Herráez and Prof. O. Díez, from the Department of Pharmaceutical Technology of the University of Valencia (Diez-Sales et al. 1991) were used.

A MicropH 2001pHmeter from Crison (Alella) and a Precisterm® thermostated water bath from JP Selecta (Barcelona) were also used.

2.2.3 Analytical Method for the Simultaneous Determination of Hydrosoluble UV Filters in the Receptor Fluid from Diffusion Cells

2.2.3.1 Preparation of Epidermis

All skin permeation studies were performed using abdominal skin samples obtained from plastic surgery of Caucasian women aged between 30 and 40 held at the University Hospital (Valencia). Previously, the patients signed an informed consent.

Abdominal skin samples were stored in a freezer at −40 °C for a maximum period of one month after conducting the removal of connective tissue and fat excess. After thawing, the skin was entered in a thermostated bath at 60 °C for 1 min and then, after holding the skin on a silicon support with pins, the epidermis was separated from the rest of the skin with tweezers. This separation process was carried out with extreme caution so as not to break the thin layer of epidermis. Finally, the epidermis was immersed in the receptor fluid for a few minutes to obtain a more effective conditioning.

In such assemblies, it is usual to place the epidermis on absorbent paper to prepare the diffusion cell in order to obtain greater rigidity. However, in this case, absorbent paper was not used because of the possible retention of UV filters on the paper, since it was observed in a previous study that the sulfonic groups present in the structure of these compounds enhance the retention over this support (Balaguer

et al. 2006b). Thus, the only barrier between the sunscreen containing the UV filters and the receptor fluid was the epidermis.

2.2.3.2 Diffusion Cell and Sampling

The receptor fluid consisted of 20 mL of an anhydrous potassium dihydrogen phosphate (10.4 g L^{-1}) solution and 80 mL of a sodium monohydrogen phosphate monohydrate (11.9 g L^{-1}) solution. Additionally, to get experimental conditions in the receptor fluid closer to those present in body fluids, pH and saline content were adjusted to 7.4 with sodium hydroxide and to 0.44 % with sodium chloride, respectively.

The diffusion cell consisted of both donor and receptor compartments. The 6 mL receptor compartment contained inside the receptor fluid and a magnetic stirrer. On this compartment, a portion of epidermis obtained as indicated above was placed completely stretched over the meniscus formed by the receptor fluid. Next, the donor compartment was carefully placed on the receptor, and the cell assembly was sealed with clamps. Figure 2.3 shows three diffusion cells completely assembled and ready to perform studies of *in vitro* percutaneous absorption.

Next, the skin permeability was studied by adding 100 µL of a cosmetic product containing the target UV filters on the available epidermis portion which had an area of 0.79 cm^2. To finish the installation, a cap was placed in the hole on the receptor compartment that allows sampling and donor compartment was covered with a piece of Parafilm® to prevent the sunscreen cosmetic product from evaporation. The diffusion cell was placed in a thermostatted water bath at 37 °C with magnetic stirring (see Fig. 2.4) and preventing from sunlight.

The assembly was maintained for 48 h. Two hours after the sunscreen cosmetic addition, 200 µL were obtained from the receptor compartment using a syringe. This volume was immediately replaced in the diffusion cell with fresh receptor

Fig. 2.3 Diffusion cells prepared in our laboratory to carry out *in vitro* percutaneous absorption studies
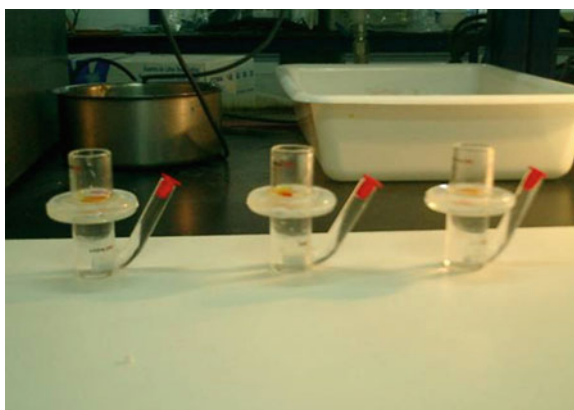
Fig. 2.4 *In vitro* diffusion
cells immersed in a
thermostated bath with
magnetic stirring

fluid so that the volume of receptor fluid in the receptor compartment was kept
constant throughout the experiment. The sampling process was repeated at 7 and
48 h. The obtained receptor fluid samples were stored at 4 °C.

2.2.3.3 Checking the Integrity of the Skin

To evaluate the integrity of the epidermis during the experiment and ensure that
the passage of the analytes through the skin was essentially due to percutaneous
absorption processes, thus avoiding the consideration of damaged epidermis, a PR
marker test was performed. Hence, after the last sampling, 1 mL of receptor fluid
solution containing 0.05 % PR was added to the donor compartment. The
assembly was maintained for 1 h under the same experimental conditions used to
study the *in vitro* percutaneous absorption and then, 200 µL from the receptor fluid
was obtained. Using an analytical method based on a solid-phase extraction (SPE)
on line coupled to a UV/VIS spectrophotometric detector using a sequential
injection that was previously developed by our research group (Balaguer et al.
2006b), PR was determined in the receptor fluid.

2.2.3.4 LC-UV/VIS Analysis

To construct the calibrate, BZ4, TDS, PDT and PBS standard solutions were
prepared in a concentration range between 0.1 and 10 µg mL^{-1}, using as solvent
the same solution that was used as a receptor fluid, and stored at 4 °C. 20 µL of
these solutions and the obtained sample solutions (in triplicate) were injected in
the chromatographic system.

   The chromatographic separation was carried out using a mobile phase con-
sisting of an ammonium acetate [pH 4, containing TBA (50 mM)] solution and
EtOH. An isocratic elution 65:35 (*v/v*) using a flow rate of 1 mL min^{-1} at 50 °C
was selected. The detection was performed at the wavelength corresponding to the

absorption maximum of each analyte, i.e. 304, 344, 288 and 344 nm for PBS, PDT, BZ4 and TDS, respectively.

According to the obtained volume of receptor fluid in each sample, data were adequately corrected and interpolated into the calibrate representing the area of each chromatographic peak versus the standard concentration.

2.3 Results and Discussion

2.3.1 Study of the Chromatographic Variables

To obtain the best resolution of the chromatographic peaks using the shortest analysis time possible, a series of experiments were conducted to select the experimental variables associated with the mobile phase (organic modifier content, pH, ion-pairing reagent concentration) and to evaluate the temperature effect over the analytical separation.

To select the chromatographic variables, a standard solution containing all four analytes (50 μg mL^{-1}) was used and the analytical signals obtained by measuring at the wavelength corresponding to the absorption maximum of each analyte were evaluated.

2.3.1.1 Mobile Phase

In preliminary studies, the percentage of organic modifier in the mobile phase consisting of a mixture of aqueous AcOH (1 %) containing 20 mM TBA and EtOH was studied. The best result concerning resolution and analysis time was obtained by using a mobile phase with a percentage of 35 % EtOH.

Another of the conclusions drawn from these preliminary studies was that the use of aqueous AcOH (1 %) buffered with ammonium hydroxide to pH 7.7 reduces the analysis time considerably in comparison to the use of aqueous AcOH (1 %) at pH 2.6. Thus, before performing a more comprehensive study of pH, an aqueous ammonium acetate (1 %) phase adjusted to pH 7.7 was used to perform the study of the other chromatographic variables.

2.3.1.2 Ion-Pairing Reagent Concentration and pH of the Mobile Phase

To select the concentration of TBA in the mobile phase, the analytes were analysed using different mobile phase solutions consisting of aqueous TBA (20–100 mM) in ammonium acetate (1 %) pH 7.7:EtOH 65:35 (*v/v*). The best results were obtained when using TBA concentrations between 40 and 60 mM as shown in Fig. 2.5.

Fig. 2.5 Results obtained by
LC-UV/VIS analysis to select
the concentration of TBA.
Mobile phases composed of
aqueous solutions of TBA
(20–100 mM) in ammonium
acetate (1 %) pH 7.7:EtOH,
65:35 (*v/v*) were used.
Experiments were performed
at room temperature. PBS,
(*blacklozenge*); PDT,
(*blacksquare*); BZ4,
(*blacktriangle*); TDS, (✕)
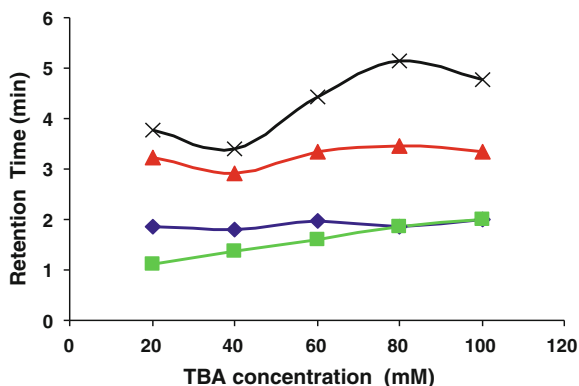
While concentrations of TBA lower than 40 mM caused the elution of PDT
next to the elution front, values higher than 60 mM caused the overlap of PDT and
PBS. For the other two analytes, the increase in the concentration of TBA did not
involve any significant variations in the case of BZ4, but increased the elution time
for TDS.

In general, it can be conclude that, on the one hand, a high amount of TBA in
the mobile phase did not allow the obtaining of a proper separation of analytes and,
on the other hand, a low concentration of TBA did not allow the separation of the
first eluted chromatographic peak from the elution front, although the analysis time
was reduced considerably. Therefore, a concentration of 50 mM TBA was
selected. Under these conditions, it was possible to achieve full resolution of all
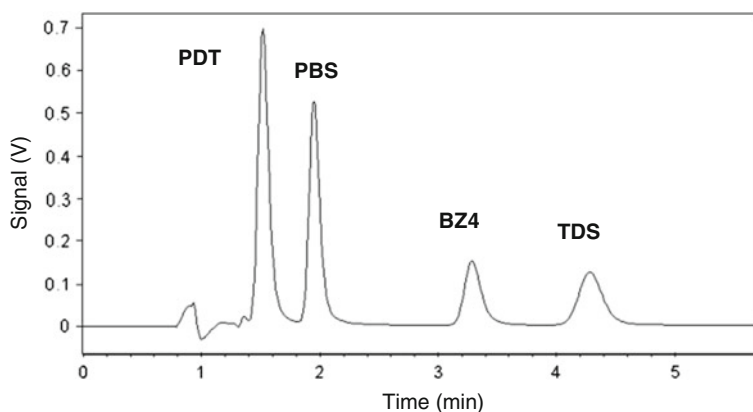the peaks in less than 5 min, as shown in Fig. 2.6.

Fig. 2.6 Chromatogram obtained by LC-UV/VIS analysis of a PDT, PBS, BZ4 and TDS
(50 µg mL^{-1}) solution using a mobile phase consisting of TBA aqueous solutions (50 mM) in
ammonium acetate (1 %) pH 7.7:EtOH, 65:35 (*v/v*). Experiments performed at room temperature.
Detection carried out at 316 nm

Fig. 2.7 Results obtained by LC-UV/VIS analysis to select the pH of the mobile phase. Mobile phases composed of TBA (50 mM) aqueous solutions in ammonium acetate (1 % and different pH values):EtOH, 65:35 (*v/v*). Experiments were performed at room temperature. PBS, (*blacklozenge*); PDT, (*blacksquare*); BZ4, (*blacktriangle*); TDS, (✕)
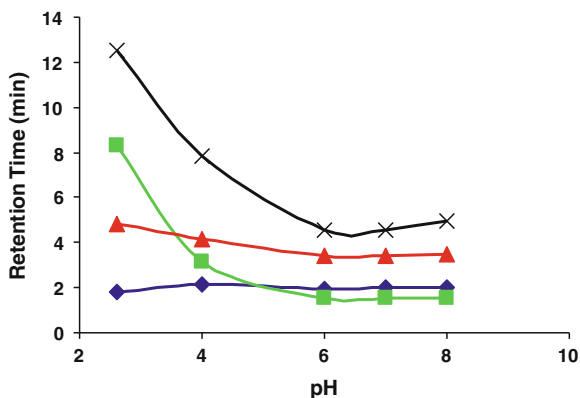
However, as the chromatographic peak corresponding to PDT still eluted close to the front of elution where other polar ingredients from the matrix could co-elute, other variables were explored to achieve a better separation from the front.

To study the pH effect of the aqueous phase in the chromatographic separation (see Fig. 2.7), the analytes were analysed using different mobile phases composed of TBA (50 mM) aqueous solutions in ammonium acetate (1 %) pH (from 2.5 to 8.5):EtOH, 65:35 (*v/v*).

Values of pH above 4 caused the elution of PBS at 2 min steadily and the strong alteration of the PDT elution. Thus, at pH values of 6 and above, there was a reversal in the elution of PDT and PBS. Under these conditions, PDT showed a lower affinity to the stationary phase and increasingly approached to the front of elution. Increasing pH values allowed the suddenly elution of BZ4 and TDS, although their retention times remained constant from pH values higher than 5. For both analytes, no overlap occurred at any time under these conditions.

Furthermore, the analysys time was substantially lengthened at pH 2.6 and the definition of the peaks was worse, especially for PDT. Under these conditions, the retention time of PDT was even higher than that of BZ4.

Figure 2.8 shows a chromatogram obtained using a mobile phase composed by an aqueous solution containing 50 mM TBA in ammonium acetate at pH 4

As can be seen, the four chromatographic peaks eluted with good separation and resolution and additionally, the separation of the first eluted peak from the front of elution was achieved.

2.3.1.3 Effect of Temperature

The temperature can reduce the retention time of the analyte and therefore the analysis time, but also can affect the chromatographic resolution. Using a mobile phase consisting of an aqueous solution of TBA (50 mM) in ammonium acetate (1 %) pH 4:EtOH, 65:35 (*v/v*) at 50 °C or lower, the first chromatographic peak
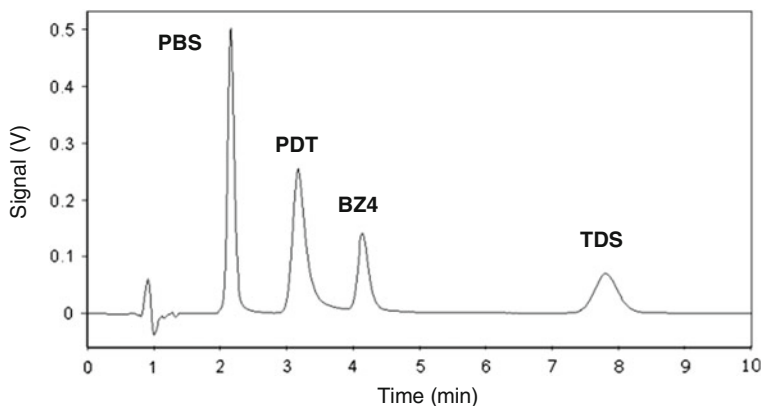
Fig. 2.8 Chromatogram obtained by LC-UV/VIS analysis of a PDT, PBS, BZ4 and TDS (50 μg mL^{-1}) solution using a mobile phase consisting of TBA aqueous solutions (50 mM) in ammonium acetate (1 %) pH 4:EtOH, 65:35 (*v/v*). Experiments performed at room temperature. Detection carried out at 316 nm

Fig. 2.9 Results obtained by LC-UV/VIS analysis to select the temperature. Mobile phase composed of TBA (50 mM) aqueous solutions in ammonium acetate (1 %) pH 4:EtOH, 65:35 (*v/v*) at different temperature values. PBS, (*black lozenge*); PDT, (*black square*); BZ4, (*black triangle*); TDS, (✕)
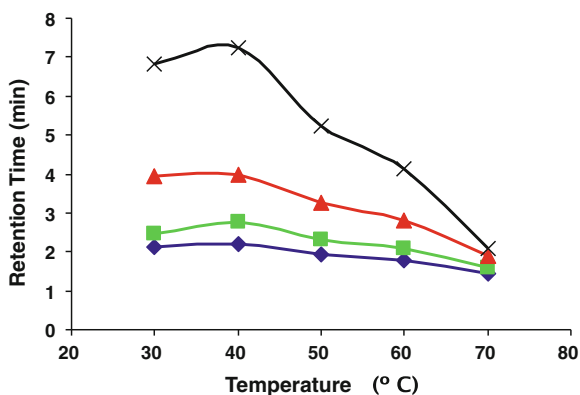
(PBS) eluted at 2 min. When increasing temperature, its retention time decreased (see Fig. 2.9).

The chromatographic profile showed some variations, especially with TDS. Basically, the retention time was reduced from 8 min to less than 6 min, when the temperature reached 50 °C. In contrast to that, values higher than 50 °C caused the elution of the first chromatographic peak closer to the elution front and a very noticeable loss of resolution, resulting in an overlap of the peaks.

Taken into consideration all of this, 50 °C was finally selected. Thus, the analysis time was reduced to less than 6 min, which led to a further improvement to the above advantages achieved of high resolution and good chromatographic separation from the front of elution. Figure 2.10 shows the separation of the target
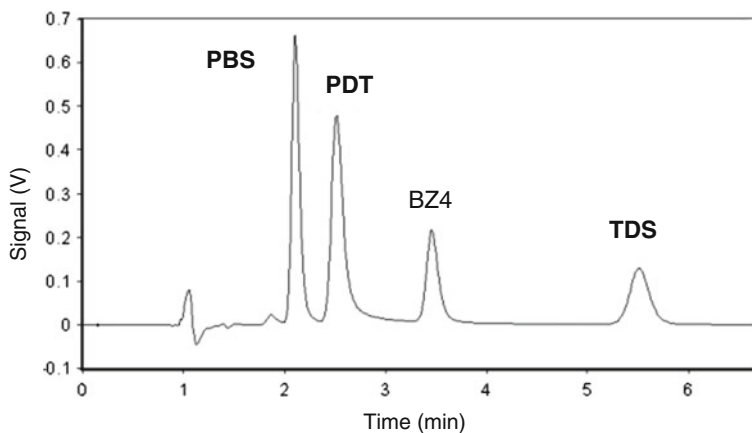
Fig. 2.10 Chromatogram obtained by LC-UV/VIS analysis of a PDT, PBS, BZ4 and TDS (50 μg mL^{-1}) solution using a mobile phase consisting of TBA aqueous solutions (50 mM) in ammonium acetate (1 %) pH 4:EtOH, 65:35 (*v/v*). Experiments performed at 50 °C. Detection was carried out at 316 nm

UV filters conducted under the selected chromatographic conditions. The resulting retention times (expressed in min) were: 1.9 to PBS, 2.3 to PDT, and 3.2 to 5.1 for TDS BZ4.

2.3.2 Validation of the Proposed Analytical Method: Study of the Interferences and Accuracy

Firstly, a study on the matrix effects caused by skin and/or the cosmetic product in the determination of the analytes was conducted. For this, a series of standard solutions of the four analytes (0.1–2 μg mL^{-1}) were prepared using, on the one hand, a solution of anhydrous potassium dihydrogen phosphate and sodium monohydrogen phosphate monohydrate used as receptor fluid and, on the other hand, receptor fluid obtained at 24 and 48 h from a skin permeation experiment based on the application of a cosmetic lotion containing no analyte.

The receptor fluid analysis obtained at both times showed the absence of peaks after the front of elution (1.8 min). Furthermore, when comparing by Student's *t*-tests (Annex III.4) the intercepts and slopes between the two calibrates, they were found statistically comparable for all analytes (see Table 2.1). In this way, the absence of proportional and/or constant interferences from the skin or the cosmetic sunscreen was showed.

Table 2.1 Statistical comparison of the linearity parameters obtained by conventional calibration and standard addition calibration

Parameter	t_{cal} [c]			
	PBS	PDT	BZ4	TDS
a (μV s)[a]	1.86	2.10	1.63	0.55
b (mL μg^{-1})[b]	0.33	1.15	2.17	0.79

[a] Intercept

[b] Slope

[c] Statistical $t_{tab(0.05,(N1-2)+(N2-2)=6)} = 2.45$

2.3.2.1 Analytical Parameters

Calibrates exhibited excellent linearity in the different concentration ranges for PBS, PDT, BZ4 and TDS with regression coefficients greater than 0.995 in all cases, establishing the working range between 0.1 and 10 μg mL^{-1} for all analytes. Table 2.2 shows the values corresponding to the limits of detection (LOD) and the slopes of the calibrates, which is the parameter used to estimate the sensitivity of the method, for each target UV filters.

The instrumental repeatability was studied by fivefold injecting a solution containing the analytes (10 μg mL^{-1}) prepared with receptor fluid. The relative standard deviations (RSD) in area values (N = 5) obtained for each analyte are also shown in Table 2.2.

2.3.3 Application of the Analytical Method

After preparing three diffusion cells according to the protocol described in Sect. 2.2.3, a volume of 100 μL of a sunscreen cosmetic lotion containing 3 % of the target hydrosoluble UV filters (Annex II.1) was added to the available epidermis. The cosmetic lotion was prepared in the laboratory using a protocol adapted from the manufacture of cosmetic products (Jordán and Jordán 1991).

As indicated above, the integrity of the epidermis was evaluated by an assay with PR marker, accordingly to a previous work (Balaguer et al. 2006b). As no PR values greater than 0.1 % of the total amount of PR applied to the donor compartment to none of the cells were detected in the receptor fluid, it was concluded that the skin remained intact during the experiment and then, the percutaneous absorption study was considered valid. The proposed chromatographic method was carried out to determine PBS, PDT, BZ4 and TDS in the receptor fluid.

Table 2.3 shows the concentrations found in the receptor fluid of the three cells for each analyte, considering representative sampling times at 2, 7 and 48 h after starting the experiment. The study was not extended beyond 48 h because the used *in vitro* system does not guarantee the integrity of the epidermis after this period of time. From the obtained concentrations, it is possible to estimate the penetration

Table 2.2 Analytical parameters of the proposed chromatographic method

Parameters	Analyte			
	PBS	PDT	BZ4	TDS
a (μV s)[a]	$(3 \pm 2) \times 10^{-5}$	$(-3 \pm 2) \times 10^{-5}$	$(-2 \pm 2) \times 10^{-5}$	$(-19 \pm 9) \times 10^{-5}$
b (mL μg^{-1})[b]	$(185 \pm 1) \times 10^{-5}$	$(134 \pm 1) \times 10^{-5}$	$(83 \pm 1) \times 10^{-5}$	$(93 \pm 2) \times 10^{-5}$
R^{2c}	0.99997	0.99995	0.99996	0.998
Repeatability (%)[d]	1.4	5.8	3.2	3.7
LOD[e] (ng mL^{-1})	16	44	36	65

[a] Intercept
[b] Slope
[c] Regression coefficient
[d] Expresed as peak area RSD ($N = 5$)
[e] Estimated as $3.s_{y/x}/b$, where b is the slope and $s_{y/x}$ the standard deviation of the residuals of the calibration curve

Table 2.3 Estimation of the *in vitro* percutaneous absorption of the target hydrosoluble UV filters obtained by applying the described method to receptor fluid samples

Diffusion cell	Analyte	Concentration (μg mL^{-1})		
		2 h	7 h	48 h
1	PBS	0.31 ± 0.03	0.34 ± 0.02	4.59 ± 0.03
	PDT	0.29 ± 0.01	0.35 ± 0.03	4.19 ± 0.02
	BZ4	0.34 ± 0.02	0.46 ± 0.07	5.52 ± 0.08
	TDS	0.70 ± 0.03	0.75 ± 0.05	2.74 ± 0.07
2	PBS	0.15 ± 0.01	0.20 ± 0.01	1.75 ± 0.03
	PDT	0.18 ± 0.01	0.21 ± 0.02	1.29 ± 0.03
	BZ4	0.21 ± 0.02	0.31 ± 0.04	2.41 ± 0.01
	TDS	0.54 ± 0.03	0.56 ± 0.03	1.07 ± 0.03
3	PBS	N.D.[a]	0.16 ± 0.01	5.06 ± 0.06
	PDT	N.D.[a]	0.16 ± 0.02	5.8 ± 0.9
	BZ4	N.D.[a]	0.22 ± 0.01	6.21 ± 0.04
	TDS	N.D.[a]	0.13 ± 0.03	1.98 ± 0.02
Average	PBS	0.2 ± 0.1	0.2 ± 0.1	4 ± 2
	PDT	0.2 ± 0.1	0.2 ± 0.1	4 ± 2
	BZ4	0.2 ± 0.1	0.3 ± 0.1	5 ± 2
	TDS	0.4 ± 0.1	0.5 ± 0.3	2 ± 1

[a] ND not detected

through the skin, so that the percentage of percutaneous absorption after 48 h from the application of the lotion on the skin was 0.8 ± 0.4, 0.8 ± 0.5, 0.9 ± 0.4 and 0.4 ± 0.2 % for PBS, PDT, BZ4 and TDS, respectively.

The accuracy of the results can be estimated by considering the standard deviation of each cell to a specific time (see Table 2.3). In this case, the obtained results can be considered accurate, since the average of the RSD values obtained in the determinations reaches 5.8 %. However, it is not so easy to draw the same conclusion when considering the standard deviation of the three cells to a specific time, because the RSD values increase significantly, probably due to the own peculiarities of the skin portion used in each cell.

It should be emphasised that the delicate step of separation of the epidermis from the dermis can cause the obtaining of epidermis portions seemingly similar but having different biomechanical parameters, such as elastic and viscoelastic deformation, porosity, thick, etc., which would explain the variability of the results obtained. Only the application of the described analytical method to the study of a large number of diffusion cells using a wide variety of skin types will enable the average of these inherent differences and provide more specific data.

2.4 Conclusions

In this Chapter, a liquid chromatography analytical method based on ion inter-action with UV/VIS to estimate the *in vitro* percutaneous absorption of the most used hydrosoluble UV filters in sunscreen cosmetic products has been proposed.

The addition of an ion-pairing reagent to the mobile phase allowed the simultaneous determination of the analytes in the fluid receptor from diffusion cells with a low detection limit, which allows the quantification of the target compounds from the first hours after the application of the cosmetic product on the epidermis.

Although the estimation of the obtained percutaneous absorption may not be conclusive, a general idea of how the analyte passes through the skin surface is achieved. No more than 1 % of the total applied amount of each UV filter was determined in the receptor fluid after 48 h in any case. It is not possible to ensure that more percutaneous absorption occurs after this time due to the used *in vitro* system does not guarantee the integrity of the epidermis (possible occurrence of decomposition processes). It would be reasonable to assume that the absorption of the tested compounds occurs over a time less than 48 h, although a more comprehensive biokinetic study would be necessary to confirm this. Moreover, it should be noted that the actual *in vivo* situation would be even more adverse, since users of such cosmetic products rarely stay with the product applied after 24 h, tending to remove the product in the shower, when covering with clothes, etc.

This method may be useful in future studies to develop more exhaustive biokinetic studies regarding percutaneous absorption of these UV filters, thus considering different times and employing a greater number of diffusion cells that minimize variability in the used skin portions. In this context, the study of synergy effects of the UV filters would be also possible by using this analytical method.

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