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Short communication

Permeation profiles of resveratrol cream delivered through porcine vaginal mucosa: Evaluation of different HPLC stationary phases

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ABSTRACT

Trans-resveratrol affects biological systems in a multitude of ways, but its oral bioavailability is remarkably poor due to in vivo metabolization. This drawback has fomented the development of new strategies for systemic delivery, such as transmucosal delivery via the vaginal route, which is our main focus here. In this sense, our pioneering study purposed to evaluate the *trans*-resveratrol permeation efficacy through this route. For that, we used a previously validated method and tested it with three different stationary phases: a commercial C18 column and two laboratory-made chromatographic columns containing poly(methyloctadecylsiloxane) (PMODS) thermally immobilized onto zirconized silica (Zr-PMODS) or titanized silica (Ti-PMODS). The permeation experiments showed that resveratrol, in the formulation used, was not successfully delivered to the bloodstream – it was actually retained within the vaginal mucosa, which suggests a local use rather a systemic one.

1. Introduction

In this work, we focused on resveratrol, which is a nonflavonoid phenolic compound produced by certain spermatophytes such as grapes [1] found in grape wines [2]. The *trans*-resveratrol (Fig. 1) isomer is a more biologically active compound [3]. Although *trans*-resveratrol has numerous positive effects on biological systems, including antioxidant, neuroprotective, antiphotoaging, and antiviral benefits [4], its poor oral bioavailability due to in vivo metabolization [5] limits *trans*-resveratrol's potency as a prophylactic agent [6]. This is why new oral delivery systems, such as micellar solutions, cyclodextrins, solid lipid nanoparticles, liposomes, multiparticulate calcium-pectinate carriers, acoustically active lipospheres, and chitosan microspheres, are currently being studied. Nevertheless, these methods have limited use due to their inefficient systemic delivery [7], which encouraged new strategies such as transdermal delivery [8,9] and transmucosal delivery via the vaginal route, which is our main focus here, as it has not been studied yet, to the best of the authors' knowledge.

Although the commercial columns packed with octadecyl-bonded silica (C18) has traditionally been the most employed stationary phase (SP) for resveratrol analysis, our group has been utilizing silica-oxide materials (SiO_2-Zr or SiO_2-Ti) as chromatographic supports for SP in HPLC with considerable success [10–12]. Using this process, the primary objective of this work was to evaluate the possibility of vaginal creams to successfully deliver *trans*-resveratrol to the blood stream via an *ex vivo* permeation experiment. For that, we quantified the permeated resveratrol using three HPLC columns: a commercial C18 column and two laboratory-made chromatographic columns containing poly(methyloctadecylsiloxane) (PMODS) thermally immobilized onto zirconized silica (Zr-PMODS) or titanized silica (Ti-PMODS).

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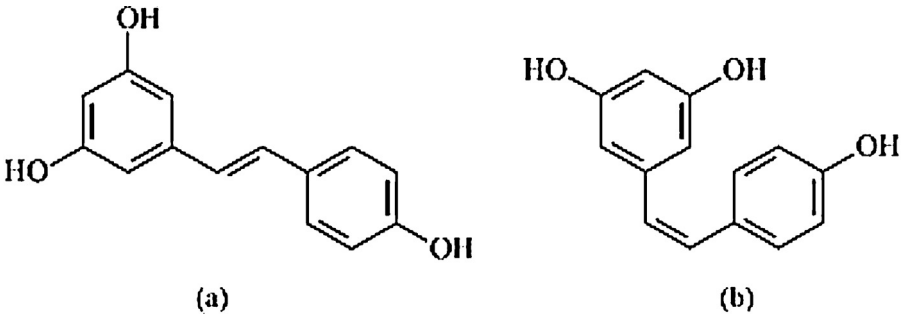

Fig. 1. Chemical structures of *trans*- (a) and *cis*-resveratrol (b).

2. Materials and methods

2.1. Materials

All reagents for the mobile phase and receptor medium were obtained from Sigma–Aldrich Corp. (St. Louis, Missouri). Ultrapure water (H_2O) obtained through the AquaMax-*Ultra* 370 Series (Young Lin Instrument Co. Ltd., Seoul, South Korea) was used throughout our analyses. *Trans*-resveratrol 98% and its standard, ethoxydiglycol and Pentravan (vehicle for emulsion compounding) were kindly donated by Fagron (São Paulo, Brazil).

2.2. Vaginal cream

We compounded an emulsion consisting of resveratrol, 2%; ethoxidiglycol, 0.5%; and Pentravan in quantity sufficient for 1 g. The resveratrol was accurately weighed, transferred to an agate mortar, ground stepwise with the ethoxydiglycol, and then homogenized with the vehicle. The product was passed through a roll mill (Fagron, USA), collected, and packed into white airless plunger packing (Emphasys, São Paulo, Brazil).

2.3. Percutaneous absorption

2.3.1. Tissue preparation

Porcine vaginal mucosal tissues were obtained from freshly killed pigs from a local slaughterhouse. After removal with a bistoury, the subcutaneous fat and connective tissue were cleaned with water and saline, wrapped in Parafilm, and stored at −80 °C prior to use (<1 month). They were withdrawn from the freezer 10 min before use.

This protocol was approved by the Ethics Committee of Universidade Federal de Juiz de Fora, (approbation no. 021/2014).

2.3.2. Permeation

A protocol previously developed and validated by our group was used [8]. In brief, experiments were conducted using 7 mL static vertical diffusion cells (commonly known as Franz cells) with automatic sampling (Microette Plus, Hanson Research, Chatsworth, California). The donor compartment contained the vaginal cream ($n = 6$) and the receptor compartment was filled with the receptor medium (artificial human sweat with 10% ethanol to ensure high solubility for the resveratrol). Diffusion testing was performed at 600 rpm and 37 ± 2 °C. The full thickness porcine vaginal mucosa discs were positioned between both cell compartments, and doses of the formulation (7.44 mg of cream containing 0.15 mg of resveratrol per dose, or 0.08 mg of resveratrol cm^{-2}) were applied to the mucosal surface under occlusion. The available diffusion area was 1.86 cm^2; a clamp was used to hold the compartments together. Aliquots (1 mL) were withdrawn at regular time intervals (0.5, 1, 2, 4, 8, and 12 h), collected in HPLC vials, and immediately replaced with receptor medium at equal temperatures. The resveratrol concentrations were correspondingly corrected at each replenishment.

The permeated amount of the drug ($Q_{real,t}$), in the time t, was calculated using Eq. (1):

$$Q_{real,t} = C_{measured,t} \times V_r \times V_a \times \Sigma^{n-1} C_a, \quad (1)$$

where $C_{measured,t}$ is the concentration measured at sampling time t, V_r is the volume of the diffusion cell, V_a is the aliquot volume and C_a is the concentration of the aliquot. Mathematical models were applied to determine the kinetics of diffusion. Cumulative amounts of drug diffusion per unit area (μg cm^{-2}) were plotted against time (h) for zero-order kinetics; and cumulative amounts of drug diffusion per unit area (μg cm^{-2}) were plotted against the square-root of time (μ g cm^{-2}) for the Higuchi model; and the log of the cumulative amounts of drug diffusion per unit area (log μg cm^{-2}) were plotted against time (h) for the first-order kinetics. Coefficients of determination (R^2) higher than 0.99 were considered linear. For those, the steady-state flux (J_s) was determined from the linear slope of the cumulative amount of resveratrol *versus* the time curve. The lag time (T_L) represented the time required to achieve the steady-state flux.

2.3.3. Drug retention in the mucosa

After the permeation experiments, the mucosa tissues were cut into small pieces using a surgical scissor and then placed in 10 mL conical polypropylene tubes (Eppendorf AG, Hamburg, Germany) containing 10 mL of methanol. All tubes were shaken mechanically, sonicated for 1 h, filtered using 0.45-μm filters, and then transferred to HPLC vials for quantification.

2.4. Quantification of resveratrol by HPLC

Resveratrol quantification was achieved through standard curves (prepared immediately before sample analysis) using our previously optimized method [8]. The HPLC runs were performed via a Young Lin (Corea) chromatography system. Separation was achieved at 25 °C using a ZORBAX Eclipse XDB-C18, 250 × 4.6 mm, 5 μm column (Phenomenex Inc., Torrance, California); a 250 × 4.6 mm, 5 μm column containing Zr-PMODS as described by da Silva and Collins [10,11]; or a 250 × 4.6 mm, 5 μm column containing PMODS thermally immobilized onto the surface of porous titanized silica particles as described by da Silva and Collins [12]. The mobile phase entailed a mixture of acetonitrile and water (50:50, v/v) at a flow rate of 1.2 mL min^{-1}. The samples and standards were injected in a volume of 20 μL and monitored using UV detection at 307 nm.

and Table 1

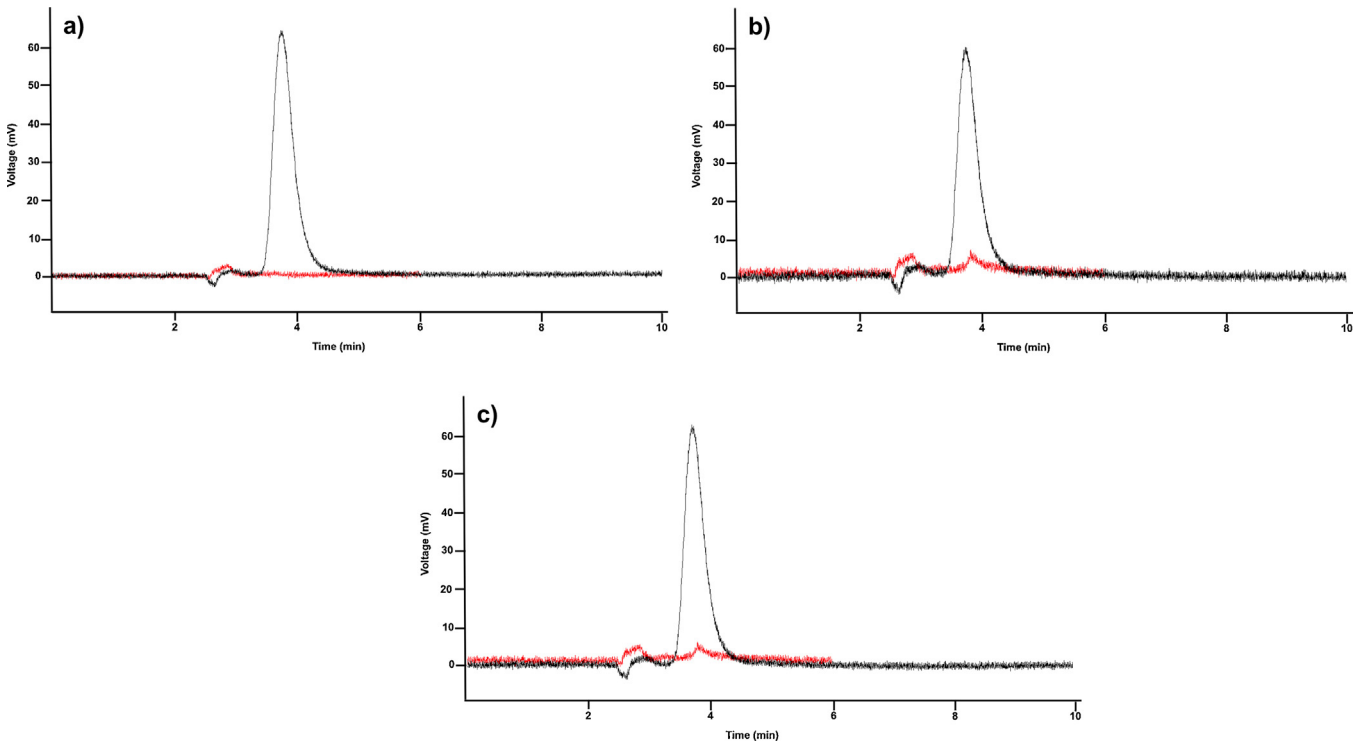

Fig. 2. Chromatograms of resveratrol vaginal cream obtained using: a) C18 commercial column, b) Zr-PMODS column, and c) Ti-PMODS column. In red: placebo/matrix; in black: sample. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3. Results and discussion

The *ex vivo* determination of skin permeability using static vertical diffusion cells, also known as Franz-type diffusion cells, is a widespread method used extensively for the design and development of new formulations [13–15]. However, there are a number of factors that directly affect the results of these types of experiments. These factors include stirring variables (speed, time, homogeneity); membrane type (material, brand, time of hydration); donor solution (volume, composition, purity); sampling (sampling times, rate); and experimental issues, setup, quantification method, and operator training. A study conducted by Shiow-Fern et al. [16] demonstrated the degree to which these factors impacted permeation. They found that when the abovementioned factors were validated and optimized, the coefficient of variation of the results could be diminished from 25.7% to 5.3%.

Although such factors are already known to play a role in permeation studies, to the best of the authors' knowledge, the influence of the HPLC stationary phase used for quantification has not yet been studied, notwithstanding that HPLC is the technique most frequently used in these studies. Yet, quantification may be quite challenging when biological tissues such as human skin or mucosa are involved. Problems can arise because of compounds that can co-elute and that are not always predictable due to expected variations in the composition of different biological tissues. In this study, the quantification of the drug permeation across a vaginal mucosa was assessed using three different SP.

Fig. 2 show chromatograms and the chromatographical parameters for each column. All columns presented the same retention times and effective separations, although slight differences in retention factor, efficiency, and symmetry could be observed: Zr-PMODS and Ti-PMODS presented equally good chromatographic parameters, both being slightly (yet not significantly) better than the commercial C18 phase.

As for the permeation using our particular SP, the permeation profiles were nearly equivalent in the C18 and Zr-PMODS columns,

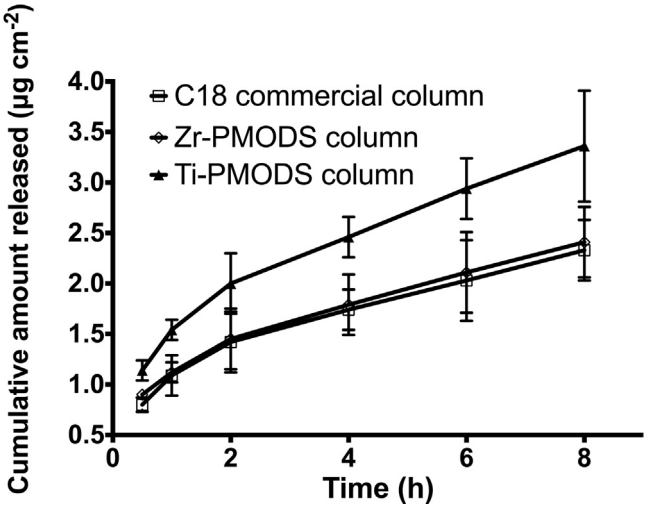

Fig. 3. Permeation profiles of resveratrol vaginal cream using different stationary phases for separation/quantification. Values represent mean ± standard deviation ($n = 6$).

but greatly differed from the one obtained using the Ti-PMODS SP (Figs. 3 and 4). Even considering average point variations, the standard deviations from the final column did not intersect the other columns at all sampling points. In this study, permeation profiles were conducted only once (with six replicates); therefore, all three quantifications were performed using the same aliquots from the same HPLC vials, thus reducing the influences that an entire permeation experiment could have on quantification.

A visual analysis of the permeation profiles in Fig. 3 shows that the quantification limit was reduced in the Ti-PMODS SP, thereby enabling a higher quantification of the resveratrol within the receptor media. To ensure the main peak was solely due to resveratrol, all columns were previously run with blanks composed of recep-

Table 1
Chromatographic parameters of the different stationary phases using an optimized HPLC method.

Column	t_R (min)	Retention factor (k)	Symmetry/Ttailing	Efficiency (N)
C18 commercial	3.7	3.33	1.57	3958
Zr-PMODS	3.7	3.34	1.53	4039
Ti-PMODS	3.7	3.36	1.58	4015

t_R: retention time. N: number of theoretical plates.

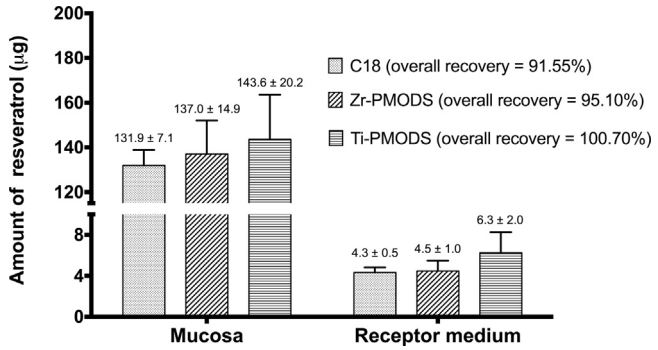

Fig. 4. Mass balance of resveratrol permeation study from vaginal cream. Values represent mean ± standard deviation ($n = 6$). Overall recovery is the total drug that was quantified in the mucosa and in the receptor medium divided by the total amount applied (148.8 µg). Results given as percentage.

tor media and mucosa that were not included in the permeation studies. Even then, an unexpected variation in the results was found, which can be hypothesized to have occurred due to: (i) some degree of co-elution of a particular component of each mucosa disc—although blanks were run, we have in mind that each part of a biological tissue may have an unique biochemical composition; (ii) the occurrence of *cis*/*trans* isomerization, possibly at a different rate on the different columns if some SP act as catalysts for this reaction (the UV spectra of *cis* and *trans* species are slightly different, with different molar absorptivities and a small spectral shift of the band that we were observing at 307 nm); and (iii) both phenomena abovementioned, of even other one diverse from the hypothesized here.

To determine permeation kinetics, the results were analyzed by the linear regression method (Table 2), with the ultimate aim of discovering the mechanism for drug release. These data can predict the in vivo drug permeation rate, which fosters an understanding of the physics regarding resveratrol permeation phenomena [17]. The graphical evidence shows that the release profiles were not linear, as the product best fitted the pseudo-first-order model. Also known as the Higuchi model, our schema was in agreement with the linear correlation coefficient (R^2) that scored higher than 0.99 for the relationship between the amount of permeated resveratrol (µg cm^{-2}) and the square root of time ($\sqrt{}$h). The release mechanism of these drugs involves a multilayered process based on Fick's law of diffusion, which depends on the square root of time, as is typical in matrix-type products. Thus, even when there were differences in quantification within the receptor medium, the kinetics study was unaffected in terms of process modeling.

These data were complemented by determining drug flux (J_S, µg cm^{-2} h^{-1}) and lag time (L_T, h) parameters, also presented in Table 2. Once again, fluxes were similar between the C18 and Zr-PMODS columns and different between those SPs and the Ti-PMODS, a direct reflection of the permeation profiles. The release rate, or the steady-state flux, is formulation-specific and can be used to monitor product quality. As for lag times, the differences were more scattered among the three SPs. A lower lag time was also found when using the Ti-PMODS SP (0.23 h), suggesting that the Ti-PMODS was more efficient in quantifying the smaller amounts that permeated during the first samplings.

Quantification was also performed on the mucosae that underwent the permeation experiments (Fig. 4). This was done to calculate overall recovery, which must lie within 85–115% [14,15], and also to verify whether the cream possessed a good potential for application in vivo. Our experiments led to very similar results among the three SPs used, probably due to the large quantities of drug found in the tissue. Even with average amounts fluctuating, the standard deviations showed that the differences remained within normal variations for this type of experiment. As for the *in vitro* phenomena, inferences may sometimes be tricky; vascularized tissue in vivo accelerates drug delivery to the bloodstream [18]. However, one must consider that in normal use (in vivo) the cream could also be washed out within 8 h. Thus, limited conclusions can be drawn here, although the majority of the drug was not able to cross the mucosa.

This indicates that the vaginal mucosa forms a significant barrier acting against the transport of resveratrol in this formulation compounded with Pentravan, a transdermal liposomal vehicle often used by compounding pharmacies [19]. This liposomal vehicle was already found to be suitable for delivering resveratrol transdermally [8,9], because the vesicles are intended to increase the solubility of hydrophobic drugs, improving both release kinetics

Table 2
Kinetic parameters for the vaginal permeation of resveratrol.

MathematicalModel	Equation	R^2	J_S(µg cm^{-2} h^{-1})	K_P(cm h^{-1})	T_L (h)	Cumulative drug in receptor media (µg)
C18 commercial						
Zero-order	$y = 0.19x + 0.89$	0.957				
Higuchi	$y = 0.69x + 0.37$	0.994	0.69	4.53	0.29	4.33
First-order	$y = 0.05x - 0.03$	0.877				
Zr-PMODS column						
Zero-order	$y = 0.19x + 0.94$	0.972				
Higuchi	$y = 0.69x + 0.42$	0.998	0.69	4.54	0.36	4.47
First-order	$y = 0.05x + 0.002$	0.911				
Ti-PMODS column						
Zero-order	$y = 0.28x + 1.25$	0.965				
Higuchi	$y = 1.01x + 0.49$	0.996	1.17	7.80	0.23	6.26
First-order	$y = 0.05x + 0.12$	0.890				

J_S: steady-state flux. K_P: permeability coefficient. T_L: lag time. Results expressed as a mean of six replicates.

and bioavailability. Our study hypothesized that the same could happen via the vaginal mucosa, but it seems this was not the case. To achieve confirmation, the next step is to perform in vivo pharmacokinetic/pharmacodynamics studies.

In summary, our permeation experiments showed that resveratrol, in the formulation used, was not successfully delivered to the blood stream, demonstrating the need for new technological strategies to overcome this issue. However, as the drug was mainly retained within the mucosa, the formulation would be suitable for local vaginal treatments that could benefit from the diverse biological effects of resveratrol.

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References

[1] J.J. Docherty, J.S. Smith, M.M. Fu, T. Stoner, T. Booth, Effect of topically applied resveratrol on cutaneous herpes simplex virus infections in hairless mice, Antiviral Res. 61 (2004) 19–26.
[2] M.A. Baxter, Anti-aging properties of resveratrol: a review and report of a potent new antioxidant skin care formulation, J. Cosm. Dermatol. 7 (2008) 2–7.
[3] F. Orallo, Comparative studies of the antioxidant effects of *cis*- and *trans*-resveratrol, Curr. Med. Chem. 13 (2006) 87–98.
[4] J.A. Santos, G.S.G. Carvalho, V. Oliveira, N.R.B. Raposo, A.D. da Silva, Resveratrol and analogues: a review of antioxidant activity and applications to human health, Rec. Pat. Food Nutr. Agric. 5 (2013) 144–153.
[5] A. Amri, J.C. Chaumeil, S. Sfar, C. Charrueau, Administration of resveratrol: what formulation solutions to bioavailability limitations? J. Control. Release 158 (2012) 182–193.
[6] R.D. Sale, D. Verschoyle, D.J.L. Boocock, N. Jones, K.C. Wilsher, G.A. Ruparelia, P.B. Potter, W.P. Farmer, Pharmacokinetics in mice and growth-inhibitory properties of the putative cancer chemopreventive agent resveratrol and the synthetic analogue trans 3,4,5,4′-tetramethoxystilbene, Br. J. Cancer 90 (2004) 736–744.
[7] A.C. Santos, F. Veiga, A.J. Ribeiro, New delivery systems to improve the bioavailability of resveratrol, Exp. Op. Drug Deliv. 8 (2011) 973–990.
[8] H.C. Polonini, C.A. Bastos, M.A.L. Oliveira, C.G. Silva, C.H. Collins, M.A.F. Brandão, N.R. Raposo, In vitro drug release and ex vivo percutaneous absorption of resveratrol cream using HPLC with zirconized silica stationary phase, J. Chromatogr. B 947–948 (2014) 23–31.
[9] P.A. Almeida, M.C. Campos, H.C. Polonini, S.L. Calixto, T.B.B. Gomes, A.A.B. Lataliza, C.D.S. Ramos, N.R.B. Raposo, A.O. Ferreira, M.A.F. Brandão, Studies with emulsion containing trans-resveratrol: in vitro release profile and ex vivo human skin permeation, Curr. Drug Deliv. 12 (2015) 157–165.
[10] C.G.A. da Silva, C.H. Collins, Improved chemical stabilities for end-capped high performance liquid chromatography stationary phases based on poly(methyloctadecylsiloxane) thermally immobilized onto metalized silicas, J. Chrom. A 1260 (2012) 81–87.
[11] C.G.A. da Silva, C.H. Collins, Preparation and characterization of a poly(methyloctadecylsiloxane) thermally immobilized onto zirconized silica stationary phase for high-performance liquid chromatography, J. Chrom. A 1232 (2012) 248–256.
[12] C.G.A. da Silva, C.H. Collins, Stationary phases based on poly(methyloctadecylsiloxane) thermally immobilized onto titanized silica for high-performance liquid chromatography, Microchem. J. 109 (2013) 150–157.
[13] FDA. –Food and Drug Administration, U.S. Department of Health and Human Services, Center for Drug Evaluation and Research (CDER). Guidance for Industry on Nonsterile Semisolid Dosage Forms –Scale-up and Postapproval Changes: chemistry, manufacturing, and controls, In vitro Release Testing and In Vivo Bioequivalence Documentation, 1997.
[14] OECD Environmental Health and Safety Publications, Series on Testing and Assessment no. 28. Guidance document for the conduct of skin absorption studies, Paris, March 2004.
[15] COLIPA. Cosmetics ingredientes: Guidelines for percutaneous absorption/penetration. 1997.
[16] N.G. Shiow-Fern, J.J. Rouse, F.D. Sanderson, V. Meidan, G.M. Eccleston, Validation of a static Franz diffusion cell system for in vitro permeation studies, AAPS PharmSciTech 11 (2010) 1432–1441.
[17] R. Sharma, R.B. Walker, K. Pathak, Evaluation of the kinetics and mechanism of drug release from econazole nitrate nanosponge loaded carbapol hydrogel, Ind. J. Pharm. Ed. Res. 45 (2011) 25–31.
[18] J. Reichling, U. Landvatter, H. Wagner, K.H. Kostka, U.F. Schaefer, In vitro studies on release and human skin permeation of Australian tea tree oil (TTO) from topical formulations, Eur. J. Pharm. Biopharm. 64 (2006) 222–228.
[19] H.C. Polonini, M.A.F. Brandão, A.O. Ferreira, C. Ramos, N.R.B. Raposo, Evaluation of percutaneous absorption performance for human female sexual steroids into Pentravan cream, Int. J. Pharm. Compound. 18 (2013) 332–340.