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Article

RP-HPLC simultaneous quantification of rutin, avobenzone, and octyl methoxycinnamate in the presence of hydroxypropyl β-cyclodextrin (HPβCD) and sulfobutyl ether β-cyclodextrin (SBEβCD)

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Development and validation of a simple and fast method of high-performance liquid chromatography with diode array detection (HPLC-DAD) for the simultaneously analysis of rutin, avobenzone, and octyl *p*-methoxycinnamate is presented. These substances were separated using a Kromasil C18 (250×4.6 mm, 5 µm) column, methanol: water (88:12 v/v) as the mobile phase, and a flow rate of 0.8 mL min^{-1}. The experiment was performed at room temperature and elution was under isocratic conditions. Quantification was performed by external calibration at the wavelength of 325 nm. The validated parameters included linearity, selectivity, precision (repeatability), intermediate precision, accuracy, limit of detection, limit of quantification and robustness. The results of validation were statistically treated using the Action Stat version 3.5.152.34. The selectivity was also evaluated in the presence of two cyclodextrins (2-hydroxypropyl-β-cyclodextrin and β-cyclodextrin sulfobutyl ether sodium). The absence of parallelism between the curves of octyl *p*-methoxycinnamate in the absence and presence of the β-cyclodextrin sulfobutyl ether sodium in the mobile phase revealed interference from this matrix, thereby indicating the necessity of validating the method in the presence of this, and other matrices. The proposed method was selective, linear, precise, accurate, and robust for the simultaneous determination of rutin, avobenzone, and octyl *p*-methoxycinnamate.

Keywords: Rutin. Avobenzone. Octyl *p*-methoxicynnamate. HPLC-DAD.

INTRODUCTION

The growing knowledge of the harmful effects of light in the wavelength range of 290 to 400 nm, including erythema, skin photoaging, immunosuppression, and skin cancer, has led to the extensive use of topical formulations containing ultraviolet (UV) filters. The common active ingredients in these sunscreens are organic substances that attenuate the interaction of solar energy with the skin by absorbing UV radiation. An indispensable feature for the effectiveness of UV filters is their satisfactory photostability, since the light-induced decomposition of such compounds not only decreases their UV retention capacity, but may also generate toxic by-degradation substances (Simeoni, Scalia, Benson, 2004).

For effective protection against UV light, at least two types of UV filters that absorb the radiation in different regions of the spectrum (UVA, 190-340 nm; UVB, 340-

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400 nm) must be present in a sunscreen. Avobenzone (BMDBM) and octyl-*p*-methoxycinnamate (EHMC) are among the organic filters most commonly used as UV filters in cosmetic preparations (Scalia *et al.*, 2002; Kim *et al.*, 2015). Research shows that BMDBM exists in 71% of sunscreen products, retaining UVA (λ_{max} = 358 nm) (Kerr, 2011), while EHMC, an UVB filter (λ_{max} = 310 nm) is present in 77% of the products (Hayden, Roberts, Benson, 1998). Sayre and Dowdy (1999) have described the degradation of BMDBM and EHMC in cosmetic preparations exposed to light.

The photodegradation of sunscreeens in cosmetic formulations not only reduces their efficacy but can also lead to the formation of degradation products capable of inducing allergic reactions (Scalia *et al.*, 2002). Thus, in order to guarantee the effectiveness and safety of sunscreens, the addition of antioxidant substances, or the inclusion of these substances in new systems capable of reducing photodegradation, has been described by several authors (Sayre, Dowdy, 1999; Scalia *et al.*, 2002; Simeoni, Scalia, Benson, 2004; Mturi, Martincigh, 2008; Nečasová *et al.*,2017).

Some natural substances can protect UV filters against UVA and UVB radiations. Rutin is a flavonoid traditionally used as an antioxidant and owing to the similarity of absorption spectra in the UV region, can potentially exert photoprotective activity which can reduce the concentration of UV filters in sun care formulations (Savic *et al.*, 2016). According to Oliveira and co-workers (2015), addition of 0.1% rutin to a combination of benzophenone-3 and EHMC led to significant increase in the sun protection factor (SPF) of these samples. Moraes, Arêas, and Velasco (2017) showed that 0.4% rutin was able to reduce the photodegradation of EHMC and benzophenone-3. In parallel, rutin has demonstrated sunscreen effect comparable to that of homosalate (Choquenet *et al.*, 2008).

Several techniques have been used to quantify BMDBM (Mturi, Martincigh, 2008; Yang *et al.,* 2008; Kim *et al.*, 2015), EHMC (Scalia *et al.*, 2002; Gaspar, Maia Campos, 2006) and rutin (Zu *et al.*, 2006; Kuntić *et al.*, 2007; Yan *et al.*, 2011; Araújo *et al.*, 2013). The aim of validation of an analytical method is to provide scientific evidence that the analytical experiment is reliable and consistent before it can be used in the routine analysis of the product and to determine the criteria to ensure its validity. Statistical tools allow us to address all these points (Belouafa *et al.*, 2017).

The objective of this research was to develop and validate a method for the simultaneous quantification of rutin, avobenzone, and octyl *p*-methoxycinnamate using high-performance liquid chromatography with diode array detection (HPLC-DAD). Statistical tools were used to evaluate the reliability of the method.

MATERIAL AND METHODS

Standards and Reagents

BMDBM (butyl methoxydibenzoylmethane; trade names: Parsol 1789, Eusolex 90020, Uvinol, Neo Heliopan 357) (98.0% purity) and EHMC (ethylhexyl methoxycinnamate; trade names: Parsol MCX, Eusolex 2292, Uvinol MC80, Escalol 557, NeoHeliopan AV, Tinosorb OMC) (98.8% purity) were obtained from Weihai Sunji Trading Co, Ltd. (Weihai, Sandong, China). Rutin was obtained from Fisher Bioblock (Illkirch, Germany). 2-hydroxypropyl-β-cyclodextrin (HPβCD) (Mw ≈ 1.460, molar substitution degree ≅ 0.6) was purchased from Sigma-Aldrich and β-cyclodextrin sulfobutyl ether sodium (SBEβCD) (Captisol, Mw ≈ 2.163, degree of molar substitution ≅ 6–7.1) was kindly provided by Captisol A Ligand Technology, Inc. UHPLC-Supergradient grade methanol and acetone (AppliChem Panreac ITW Companies, Germany) and ultrapure water Milli-Q Integral (Merck Millipore, Germany) were used as solvents. All other analytical reagents were of analytical grade.

Chromatography

Chromatography was performed using a Shimadzu® Prominence Modular HPLC Liquid Chromatograph with a Shimadzu SPD-M20A photo diode array detector, Shimadzu LC-20AB Pump, Shimadzu DGU-20A Degassing System, Shimadzu SIL-20A/C, Shimadzu CTO-20AC heating system, and Shimadzu CBM-20 controller. The data were treated using the Shimadzu LC-Solution software. Chromatographic separation was achieved using a Kromasil

RP-C18 (AkzoNobel) or Kinetex RP-C18 (Phenomenex) column (100 A, 5 µm, 250 × 4.6 mm), equilibrated at room temperature and eluted under isocratic conditions with methanol:water (88:12, v/v) at pH 6.8–7.0 as mobile phase at a flow rate of 0.8 mL min^{-1}. An injection volume of 20 µL and the wavelength of detection of 325 nm were used. The mobile phase was pre-degassed by vacuum filtration (vacuum pump KNF® mod. N810.FT.18, 100 mbar) through a semi-permeable cellulose acetate membrane (45 µm) followed by sonication (Altsonic Clean mod. 9L) for 15 min. The separation efficiency was evaluated by the chromatographic parameters.

Standard Solutions

Standard solutions of rutin, BMDBM, and EHMC (4 mg mL^{-1}) were prepared in methanol, acetone, and ethanol 96%, respectively. Then, aliquots of 2.5 mL of each of standard solution (rutin, BMDBM, and EHMC) were transferred to 25-mL volumetric flask and the volume was made up with the mobile phase (400 µg mL^{-1}). Standard solutions of cyclodextrins (HPβCD or SBEβCD) (100 µg mL^{-1}) were prepared separately in ultrapure water.

Validation of the Analytical Method and Statistical Treatment

After the development and optimization, validation tests were performed according to the recommendations of ICH Q2 (R1) (ICH, 2005) and National Agency of Sanitary Surveillance (Brasil, 2017a). The parameters used in the analytical validation were selectivity, linearity, precision, accuracy, limit of detection (LOD), limit of quantification (LOQ), and robustness. The results of the validation were treated according to the criteria established by the "Guide for statistical treatment of analytical validation"(Brasil, 2017b) using the Action Stat software version 3.5.152.34 (software R 3.4).

Linearity

Analytical curves were constructed in triplicates from dilutions of rutin, BMDBM, and EHMC standard solutions (400 µg mL^{-1}) in the range of 2.0 to 64.0 µg mL^{-1} using 10 concentrations (2, 4, 6, 8, 12, 16, 20, 32, 40, and 64 µg mL^{-1}). The regression parameters were obtained by the method of ordinary least squares. After regression analysis, the values of each concentration level were evaluated for the homoscedasticity of the variance (Cochran test) and normality of the residues (Shapiro-Wilk test). Observations with standardized and/or studentized residues greater than three were considered extreme values.

Selectivity

The selectivity of the analytical method was assessed by comparing the slope of analytical curves of rutin, BMDBM, and EHMC constructed in the absence and presence of the complex matrix (HPβCD and SBEβCD), using linear regression with Dummy variables. The regression parameters of the curves (equality of the intercept, parallelism, and coincidence) were evaluated by the hypothesis test at the level of significance (α) of 0.05. Complex matrix (100 µg mL^{-1}) of cyclodextrins (HPβCD or SBEβCD) was fortified with standard solutions of rutin, BMDBM, and EHMC (400 µg mL^{-1}) in the range of 2.0 to 64.0 µg mL^{-1}, employing 10 concentrations (2, 4, 6, 8, 12, 16, 20, 32, 40, and 64 µg mL^{-1}).

Precision (repeatability) and Intemediate Precision

Repeatability was determined in the linear range of the method for rutin, BMDBM, and EHMC at three concentrations (16, 20, and 22 µg mL^{-1}), corresponding to the theoretical levels of 80, 100, and 110%, respectively. Three replicates were analyzed for each concentration, individually prepared by the same analyst in a single day, and the results were expressed as mean, standard deviation, and coefficient of variation. The repeatability criterion was defined according to the working concentration, with a coefficient of variation of 2% being considered adequate (AOAC International, 2016; Brasil, 2017a).

Intermediate precision (inter-day precision) was determined by the analysis of six replicates at the theoretical level of 100% (20 µg mL^{-1}) for each substance individually prepared by two analysts, on different days. The results were evaluated through Variance Analysis (ANOVA).

Accuracy

The accuracy of the method was established by the recovery test at three levels of concentration (16, 20, and 22 μg mL^{-1}) for each test substance (rutin, BMDBM, and EHMC), corresponding to theoretical levels of 80, 100, and 110%, respectively. Three replicates that were individually prepared were analyzed for each concentration. Recovery values were assessed using the Student's t-test.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

The LOD and LOQ values were calculated using the mean of the regression parameters of the analytical curves obtained for each substance (rutin, BMDBM, and EHMC) in triplicate, according to equations 3.3 (σ/S) and 10 (σ/S), respectively, where σ is the standard deviation of the residuals (linear coefficient) and S is the slope of the regression line (slope).

Robustness

The robustness of the chromatographic method was evaluated using fractional factorial design, using four factors at two levels, resulting in eight experiments. The following factors were evaluated: (P1) mobile phase [(-): methanol: water 88:12, (+): methanol: water 80:20], (P2) (+): 1.0 mL min^{-1}], (P3) mobile phase flow [(-): 0,8 mL min^{1}, (+): 1,0 mL min^{1}], and (P4) column [(-): Kromasil 5- 100-C18, (+): Kinetex 5-100-C18]. The significance of the effects was assessed by Student's t-test and Lenth's method.

Photostability assay

Photostability tests involve forced degradation tests and confirmation tests. The objective of the forced degradation test is to evaluate the photosensitivity of the substance in its solid state and/or in solutions/suspensions, for the development of analytical methods and/or elucidation of the degradation path. It is appropriate to limit exposure and avoid extensive decomposition. For photostable materials, studies should be discontinued after an used suitable exposure level. Under forced conditions, decomposition substances can be observed that would be hardly formed under the circumstances used for confirmatory investigations, however, this information is useful for the development and validation of analytical methods. For the studies, the samples must be packed in chemically inert and transparent containers (ICH, 1996).

Forced degradation tests of rutin, BMDBM and EHMC, and their inclusion complexes with the CDs (HPβCD and SBEβCD), was evaluated in the solid state and, in solution, at different time intervals (0, 24, 48, 72, 168 hours) at 25°C. The tests were performed in Ethik Technology Mod. 424/CF (Ethik Technology, São Paulo, Brazil), with exposure area of 1400 cm^2, equipped with white fluorescent lamps associated with ultraviolet lamp, with spectral distribution between 320 and 400 nm, and maximum emission of energy between 350 and 370 nm (ICH, 1996). The distance between the irradiation sources and the exposure surface where the samples are located was 0.10m. The exposure time of the samples was obtained through the calculation of the luminosity efficiency using the Quinine Chemical Actinometry (quinine monohydrochloride dihydrate) option 2 (Sager, Baum, Wolters, 1998).

For the samples in the solid state, 100 mg of rutin, BMDBM and EHMC were uniformly accommodated in clear glass petri dishes; rutin, BMDBM and EHMC 1 mg mL^{1} solutions were prepared in 96°GL ethanol, and transferred to clear glass ampoules type 1 (10 mL) hermetically sealed. After exposure to irradiation at the predefined time intervals, aliquots were removed, diluted in mobile phase (500 μg mL^{1}), and the remaining content of the substances was quantified by HPLC-DAD using analytical curves fortified with cyclodextrins.

RESULTS AND DISCUSSION

Considering the hydrophilic nature of rutin (log $k_{o/w}$ 0.15) and the lipophilic natures of BMDBM (log $k_{o/w}$ 4.98) and EHMC (log $k_{o/w}$ 6.1), a C18 reverse phase column was selected. Preliminary tests were performed using methanol:water in different proportions as the mobile phases; however, when the water content was increased, BMDBM and EHMC were retained in the column, resulting in a high analysis time. Thus, 88:12 (v/v) methanol:water was established as the mobile phase for the elution of the substances.

Rutin, BMDBM, and EHMC showed symmetrical peaks, with retention times of 3.282, 20.216, and 21.973 min, respectively (Figure 1). The mean values of the chromatographic parameters obtained from the average of three determinations for each of the three substances are listed in Table I.

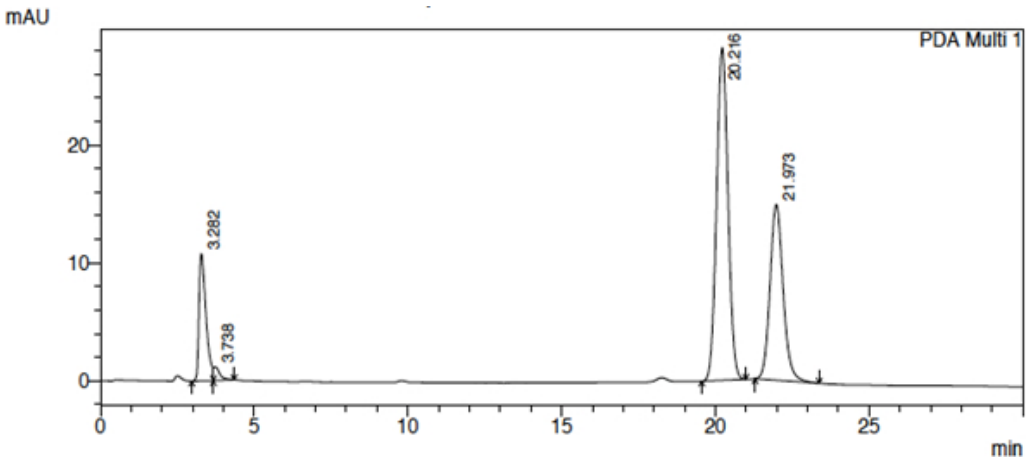

FIGURE 1 - Chromatogram of rutin (3.282 seconds), BMDBM (20.216 seconds), and EHMC (21.973 seconds), all at a concentration of 64 μg mL^{-1}, obtained using a Kromasil RP C18 chromatographic column (250 × 4.6 mm, 5 μm) at a flow rate of 0.8 mL min^{-1}.

TABLE I - Mean values of the chromatographic parameters (t_m, t_r, W, k', α, and R)

	t_r	W	N	k'				
RUT	3.28	0.60	478.73	3.21				
BMDBM	20.22	0.90	8072.82	24.92	7.77		22.58	
EHMC	21.97	1.10	6384.30	27.17	8.47	1.09	21.99	1.76

t_r (retention time, min), W (peak width, cm), N (number of theoretical plates), k' (retention factor), α (secectivity factor) e R (resolution)

The resolution (R) of a column indicates the ability to separate two solutes and depends on the selectivity (α), the retention factor (k'), and the efficacy of the column (N). R values equal to 1 indicate almost complete separation of the substances and, R values greater than 1.5 indicate complete separation of the substances (Brito *et al.*, 2003). The selectivity (α) is represented by the measurement of the separation between two solutes (α> 1). The retention factor (k') describes the rate at which a compound migrates along the column and usually varies between 1 and 10; values of k' close to 1 suggest that the analyte emerges at a time close to the eluent and, values of k' greater than 20 or 30 indicate that the retention time of the analyte in the column is very long. The efficiency of a separation is related, among other factors, to the quality of the chromatographic column. The greater the efficiency of the column (N), the better the separation and the narrower the peak (Brito *et al.*, 2003).

The selectivity values (α) of BMDBM and EHMC in relation to rutin and EHMC in relation to BMDBM were higher than 1, that meant adequate separation of the substances. The k' values observed for BMDBM and EHMC showed lower migration rates of these substances along the column than that of rutin, which can be explained by the difference in the solubility between the substances and consequent affinity for the chromatographic column. The R values for BMDBM and EHMC, calculated in relation to rutin, and the R value for EHMC, calculated in relation to BMDBM, were higher than 1.5, indicating complete separation of the substances.

Validation of the Analytical Method and Statistical Treatments

Linearity

Table II presents the regression parameters, residue diagnosis, and homoscedasticity of the models for the substances analyzed, obtained at the level of significance (α) of 0.05. Linear relationships were obtained in the concentration range between 2.0 to 64.0 μg mL^{-1} for BMDBM, EHMC, and rutin (R>0.99) (Table II, Figure 2) (Brazil, 2017b). As the P-values for rutin, BMDBM, and EHMC for residue diagnosis and homoscedasticity were found to be greater than 0.05, at 5% level of significance, we did not reject the hypothesis of residue normality and homoscedasticity of the variances. No extreme values were detected.

TABLE II - P-values obtained for the tests of normality of residues (Shapiro-Wilk Test) and homoscedasticity (Cochran Test) (α = 0.05) and regression parameters obtained from the analytical curves of rutin, BMDBM, and EHMC

Substances	P-value		Regression Parameters		
	Shapiro-Wilk Test	Cochran Test	Pearson Correlation coefficient	Slope	Linear Coefficient
RUT	0.5686	0.3257	0.9999	17093.5144	1027.7068
BMDBM	0.9834	0.2745	0.9999	38222.69	-25458.32
EHMC	0.7508	0.2642	0.9999	72809.8524	22568.9443

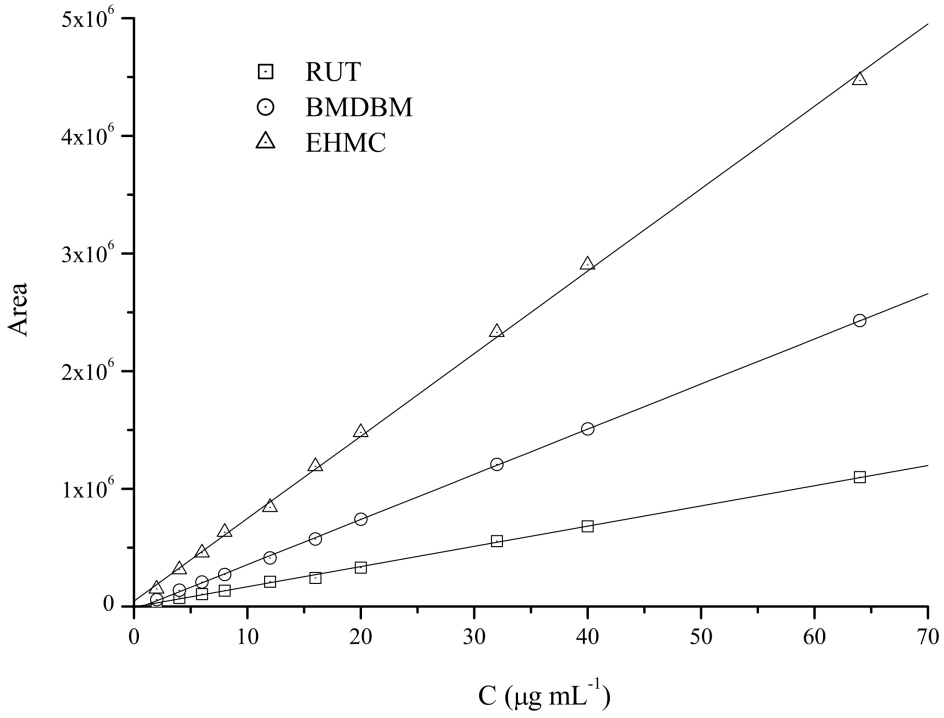

FIGURE 2 - Values adjusted for rutin, BMDBM and EHMC obtained in the range of 2 to 64 μg mL^{-1}.

Selectivity

Cyclodextrins (CDs) are cyclic oligosaccharides that can accommodate molecules in their hydrophobic cavity. The inclusion of molecules inside the CDs has been widely used in the development of new products with the aim of increasing the aqueous solubility of poorly soluble molecules and/or increasing the light-stability of photosensitive substances (Loftsson, Brewster, 1996).

The photodegradation of BMDBM (Scalia *et al.*, 1998, Simeoni, Scalia, Benson, 2004), octyldimethyl-*p*-aminobenzoic acid (Scalia, Villani, Cosalari, 2010), and EHMC (Scalia *et al.*, 2002) has been demonstrated to reduce in the presence of CDs.

The inclusion of molecules in CDs for increased solubility and stability has shown to be a promising strategy; however, high solubility of these oligosaccharides can alter the chromatographic parameters and the reliability of the analytical method. Therefore, two CDs (HPβCD and SBEβCD) were used as matrices of the samples to evaluate the method selectivity. In the selectivity study, the effect of the matrix allows us to investigate possible interferences caused by the substances that form the sample matrix, resulting in decrease or amplification of the signal or response from the instrument. The parallelism between the curves is an indicative of the absence of the interference of the matrix constituents (Brasil, 2017b).

Table III presents the P-values for the tests of intercept equality, parallelism, and coincidence obtained by comparison of the analytical curves of rutin, BMDBM, and EHMC in the absence and presence of a matrix (HPβCD or SBEβCD).

TABLE III - P-values for the tests of equality of intercept, parallelism, and coincidences of the lines ($\alpha = 0.05$)

	P-value		
	Equality of Intercept	Parallelism	Coincidence
RUT/RUT-HPβCD	0.4855	0.6610	0.2925
RUT/RUT-SBEβCD	0.2741	0.3827	0.5445
BMDBM/BMDBM-HPβCD	0.0048	0.4369	0.0031
BMDBM/BMDBM-SBEβCD	0.0557	0.1130	0.0000
EHMC/EHMC-HPβCD	0.2757	0.2257	0.0087
EHMC/EHMC-SBEβCD	0.8953	0.0000	0.0000

For rutin, as the P-value of the intercept equality, parallelism, and coincidence tests for both CDs evaluated (HPβCD or SBEβCD) were greater than 0.05, we did not reject the hypothesis that the parameters were equal at a significance level of 5%. BMDBM showed parallelism between the curves for both CDs; however, the intercept equality was only observed for the curves in the presence of SBEβCD, while the curves were not coincident for both studied CDs. EHMC, in the presence of HPβCD, presented intercept equality and parallelism between curves, but they were not coincident; the curves obtained in the presence of SBEβCD presented intercept equality, but they were not parallel and coincident. The absence of parallelism between the curves of EHMC in the mobile phase in the absence and presence of the SBEβCD suggested interference from this matrix in the quantification of the analyzed substance, thereby indicating the analytical method validation necessity in the presence of SBEβCD (Brasil, 2017b).

Recent studies have shown that CDs can alter the chromatographic parameters of the substances (Gazpio *et al.*, 2005; González-Ruiz *et al.*, 2011, González-

Luiz, Olives, Martín, 2011; Rodríguez-Bonilla *et al.*, 2011; Zeng *et al.*, 2012). The observed retention times for rutin, BMDBM, and EHMC (64 μg mL^{-1}) in the presence of HPβCD (100 μg mL^{-1}) were 3.01, 16.82, and 18.24, respectively, whereas in the presence of SBEβCD (100 μg mL^{-1}), they were 2.98, 16.00, and 17.77, respectively. Reduction in the retention time of the evaluated substances was observed for both CDs (SBEβCD and HPβCD), indicating affinity reduction of these substances for the apolar stationary phase (Feng *et al.*, 2012). A small solubility difference was observed between HPβCD (~600 mg mL^{-1} at 25°C) and SBEβCD (~700 mg mL^{-1} at 25°C), which accounted for the small difference among the retention times obtained for each substance (Jain *et al.*, 2011; Xu *et al.*, 2017).

Since this modification in the retention characteristic of the molecule is related to the formation of water-soluble complexes, the extent of the interaction of the solute with the CDs can be estimated by chromatographic parameters (Scalia *et al.*, 2002; Feng *et al.*, 2012). Figure 3 shows the influence of various concentrations of the CDs (HPβCD and SBEβCD) in the methanol-water (88:12, v/v) mobile phase on the retention factor (k'). No significant differences in the k' values were observed with the increase in the CD concentration in the mobile phase for rutin. In contrast, the addition of HPβCD or SBEβCD in the eluent produced a significant decrease in the BMDBM and EHMC retention factor (k'). The results indicated that BMDBM and EHMC reacted more strongly with the CD (HPβCD or SBEβCD) than rutin.

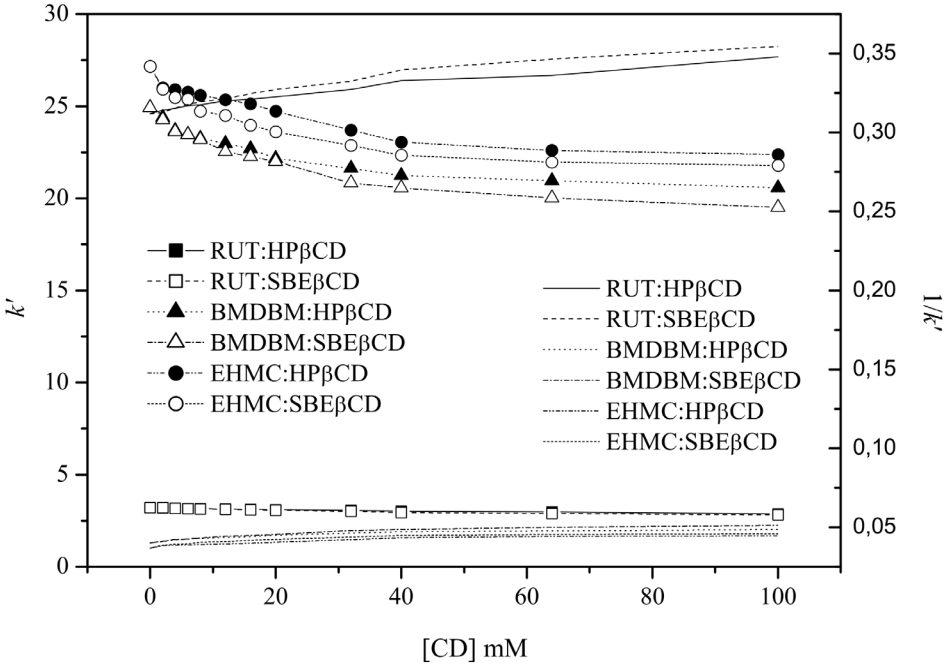

FIGURE 3 - Dependence of the retention factor (k') as a function of CD concentration (HPβCD or SBEβCD) in the mobile phase, methanol-water (88:12, v/v) at 25°C.

Several factors influence the complexation of a substance with CD, such as the chemical composition, size, geometry of the host molecule, its water solubility, ionization state, molecular weight, and melting point, in addition to the conditions of the medium, such as the temperature, pH, and solvents used. In general, complexation occurs more favorably when the host molecule has a molecular mass between 100 and 400 Da, water solubility less than 10 mg mL^{-1} and melting point below 250°C. Larger molecules can also be complexed,

since they have appropriate side chains for partial inclusion, which will also lead to modifications in the original molecule solubility and stability (Rama *et al.*, 2005).

Rutin has a high molecular weight (610.5175 Da) and is hydrophilic in nature (125 mg mL^{-1} at 25°C), which makes it difficult to fit into the hydrophobic cavity of the CDs. In contrast, due to their lower molecular weights and lipophilicity, BMDBM (310.39 Da, 10 μg mL^{-1} at 25°C) and EHMC (290.397 Da, 1 mg mL^{-1} at 25°C) readily formed inclusion complexes with CDs. Wang *et al.,* (2011) evaluated the influence of natural CDs (αCD, βCD, and γCD) on the separation of oleanolic and ursolic acids by HPLC. Lower retention times were observed for these acids in the presence of γCD, indicating the formation of more soluble and stable complexes (Del Valle, 2004). Feng *et al.* (2012) evaluated the retention times of isoflavone in the presence of CDs (βCD, HPβCD, and RMβCD) using the ratio, k_1/k_0. The values of k_1/k_0 followed the order, RMβCD> HPβCD> βCD, suggesting that CD derivatives decreased the retention times of a solute more strongly than βCD did. This phenomenon can be attributed to the higher hydrophilicity of βCD derivatives (RMβCD and HPβCD), leading to the formation of more soluble complexes, and consequently, lower affinity for the stationary phase.

Precision (repeatability) and Intermediate Precision

The values of the coefficient of variation for theoretical levels of 80, 100, and 110% for rutin, BMDBM, and EHMC were lower than 2%, therefore the precision (repeatability) met the criteria recommended by the regulatory agencies (USP, 2015; AOAC International 2016; Brasil, 2017a, 2017b) (Table IV). Intermediate precision was assessed using ANOVA two factors (day and analyst) (Table V). As P-values for day and analyst factors were greater than 0.05, we did not reject the hypothesis that the effects were zero at the significance level of 5%. There were no significant differences between day and analyst for rutin, BMDBM, and EHMC, but significant interaction between day and analyst was observed for rutin at the significance level of 5%. As the coefficient of variation observed was less than 2%, we did not find significant differences between the factors, day and analyst, and we can conclude that the methodology of evaluation is precise.

TABLE IV - Mean, standard deviation, and coefficient of variation obtained for rutin, BMDBM, and EHMC in the precision study (repeatability)

	Theoretical Levels, %	Concentration (μg mL^1)	Mean±Standart Deviation (μg mL^1)	Coefficient of variation, %
RUT	80	16	16.137±0.1733	1.0738
	100	20	19.7927±0.1208	0.6105
	110	22	22.0974±0.1969	0.8911
BMDBM	80	16	15.8441±0.2196	1.3857
	100	20	19.8702±0.1622	0.8162
	110	22	21.9700±0.1053	0.4792
EHMC	80	16	15.7043±0.0482	0.307
	100	20	19.8729±0.0052	0.0259
	110	22	22.0842±0.0313	0.1418

TABLE V - P-values obtained from the Analysis of Variance (ANOVA) and values of coefficient of variation for the day, analyst, and interaction factors ($\alpha = 0.05$)

	RUT		BMDBM		EHMC	
	P-value	**CV, %**	**P-value**	**CV, %**	**P-value**	**CV, %**
Repeatability		1.2169		0.4870		0.3393
Day	0.5208	0.0000	0.2153	0.1752	0.9191	
Analyst	0.6222	0.0000	0.1888	0.2009	0.2745	
Interaction	0.0374	1.6034				
Intermediate precision		2.0130		0.5552		0.3491

Coeficiente of variation (CV)

Accuracy

The maximum concentration recommended for BMDBM and EHMC in sunscreens is 5.0 and 10.0%, respectively (Cabral, Pereira, Partata, 2011), while 10.0% rutin is usually found in topical preparations. Based on the working concentration of the substances, the expected lower (LIE) and higher (LSE) specification limits are 98 to 102%, respectively (Brasil, 2017b).

The recovery values (%) were within the established criteria, and therefore, we did not reject the equivalence hypothesis at the significance level of 5% (Table VI).

TABLE VI - Accuracy values obtained from Student's t-test ($\alpha = 0.05$)

	RUT	**BMDBM**	**EHMC**
Recovery±Standard deviation. %	99.4749±0.8742	100.1366±0.9413	99.5798±0.3239
Degrees of freedom	9	9	9
Inferior limit	98.9682	99.5909	99.392
Upper limit	99.9817	100.6823	99.7676

Limit of Detection (LOD) and Limit of Quantification (LOQ)

The LOD and LOQ obtained for rutin, BMDBM, and EHMC are presented in Table VII and theymet the criteria for quantification of the substances in the samples of interest.

TABLE VII - LOD and LOQ values obtained for rutin, BMDBM, and EHMC

	RUT	BMDBM	EHMC
Standard Deviation of Waste	3519.0739	6881.3716	12562.3526
Slope	17093.5144	38222.6871	72809.8524
Limit of Detection Limit (μg mL^{1})	0.6794	0.5941	0.5694
Limit of Quantification (μg mL^{1})	2.0587	1.8003	1.7254

Robustness

The use of factorial planning is recommended in the robustness tests, since it allows determining if a combination of influences can cause significant differences in the results.

The Lenth's method is considered an efficient method to evaluate if the active effects are significant. According to the Lenth's method, the effects are considered as active (non-zero) when they reach the simultaneous error margin (SME), and inactive when they do not reach the margin of error (ME); the region between ME and SME is considered a region of uncertainty, requiring greater caution in the decision. The values of the effects obtained by the Lenth's method, Students t-test, estimated values, and limit values for the effects are presented in Tables VIIIA, B and C.

TABLE VIIIA - Values of effects: estimated, limit, and Student's t-test for rutin

	Effects values	Estimated values	Inferior Limit	Upper Limit	t-value	P-value
Intercept		100.1998				
P1	-0.2318	-0.1159	-1.7382	1.2745	0.5793	0.6134
P2	0.2716	0.1358	-1.2348	1.778	0.6787	0.5584
P3	0.2216	0.1108	-1.2848	1.7279	0.5536	0.6284
P4	-0.2789	-0.1394	-1.7853	1.2275	0.6969	0.5488
P1:P2	0.2668	0.1334	-1.2396	1.7732	0.6667	0.5649
P1:P3	-0.3804	-0.1902	-1.8867	1.126	0.9504	0.4296
P2:P3	-0.0347	-0.0173	-1.5411	1.4717	0.0867	0.9378

α = 0.05, ME = 1.5064, SME = 3.6051 e tcrit. = 3.7641

TABLE VIIIB – Values of effects: estimated, limit, and Student's t-test for BMDBM

	Effects values	Estimated values	Inferior Limit	Upper Limit	t-value	P-value
Intercept		101.2709				
P1	0.1458	0.0729	-1.397	1.6885	0.3557	0.7517
P2	0.2732	0.1366	-1.2695	1.816	0.6667	0.5649
P3	-0.0533	-0.0266	-1.596	1.4895	0.13	0.907
P4	0.0266	0.0134	-1.516	1.5696	0.0653	0.9531
P1:P2	0.4272	0.2136	-1.1156	1.9699	1.0422	0.3931
P1:P3	0.6375	0.3188	-0.9053	2.1803	1.5554	0.2425
P2:P3	-0.8896	-0.4448	-2.4324	0.6532	2.1705	0.1437

$\alpha = 0.05$, $ME = 1.5428$, $SME = 3.6922$ e $t_{crit.} = 3.7641$

TABLE VIIIC. Values of effects: estimated, limit, and Student's t-test for EHMC

	Effects values	Estimated values	Inferior Limit	Upper Limit	t-value	P-value
Intercept		100.9598				
P1	0.0649	0.0325	-2.5329	2.6627	0.0941	0.9325
P2	0.593	0.2965	-2.0048	3.1908	0.8592	0.4694
P3	-0.0845	-0.0423	-2.6823	2.5132	0.1225	0.9123
P4	-0.555	-0.2775	-3.1528	2.0427	0.8042	0.495
P1:P2	-0.0478	-0.0239	-2.6456	2.55	0.0693	0.9503
P1:P3	0.6972	0.3486	-1.9006	3.295	1.0102	0.4055
P2:P3	-0.4601	-0.23	-3.0579	2.1377	0.6667	0.5649

$\alpha = 0.05$, $ME = 2.5977$, $SME = 6.2170$ e $t_{crit.} = 3.764123072$

As the P-values of the Student's t-test of the Lenth's method were greater than 0.05, we rejected the hypotheses of null effects at a significance level of 5%. However, the effects were considered acceptable since they were within the lower and upper limits, calculated from the 95% confidence interval. The values of effects, in module, did not exceed the ME at 95% level of significance, and therefore, were not considered active.

Photostability studies of inclusion complexes

The efficacy of sunscreens depends on their photostability. The molecules when excited through the absorption of UV radiation return to the fundamental state by distinct mechanisms of radiactive and non-radiative decline. Some of these mechanisms can affect their activity, leading to the formation of new compounds by reactions of photoaddition, substitution, ciceloading, isomerization and photofragmentation. These new compounds can be inactivated (not absorbing UV radiation) or can favor the degradation of the skin components by photosensitization, which can be hazardous for human skin. Thus, the study of photostabilization of molecules used in the formulations of UV filters is of great importance (Vallejo, Mesa, Galhardo, 2011).

BMDBM is an organic UVA filter, highly conjugated in structure, which in contact with solar radiation presents a keto-enolic balance. Photoallergic and cytotoxic reactions have been associated with avobenzone due to its photodegradation products, such as aril glycols and benzyl (Afonso *et al.*, 2014). Lhiaubet-Vallet *et al.* (2010) studied the photodegradation of BMDBM in the presence of different UVB filters. In its fundamental state, avobenzone presents a keto enolic balance, where the enolic form is predominant. After the radiation, the balance is displaced to the formation of the isomer *β*-dicetone. This tautomer absorbs light generating a triplet form, responsible for most of the harmful effects of avobenzone. The triplet form is capable of reacting with molecular oxygen, forming singlet oxygen, a highly reactive species, which with the enolic form, forms radical oxygenated products. The authors suggest the addition to the formulation of a sacrifice filter, which prevents the formation of the isomer *β*-dicetone and thus interrupts the formation of toxic byproducts from its degradation.

EHMC is a filter that protects from UVB radiation. Studies have shown that when exposed to sunlight, this filter changes from octyl p-methoxy-trans-cinnamate to octyl p-methoxy-cis-cinnamate. The combination of BMDBM and EHMC is commonly found in sunscreen formulations (Kockler *et al.*, 2012). In parallel, the addition of antioxidants and cyclodextrins in sunscreens seems to contribute to the increase of their photochemical stability (Scalia *et al.*, 1998, 2002, 2006, 2010).

After 168 hours of irradiation, the remaining amount of rutin, BMDBM or EHMC in the samples followed the order SBEβCD> HPβCD> Standard Substance, suggesting that the presence of the CDs was capable of increasing the photochemical stability of the substances. Inclusion complexes of rutin and cyclodextrins (HPαCD, HPβCD e βCD) confer a moderate degree of protection against heat and ultraviolet radiation, and increase its antioxidant power (Nguyen *et al.*, 2013; Savic *et al.*, 2016). The presence of 30% HPβCD in sunscreens increased the stability of avobenzone, in addition of improving photoprotection effect (Simeoni, Scalia, Benson, 2004; Yang *et al.*, 2008). Inclusion complexes between the EHMC and cyclodextrins were able to reduce the transformation of the *trans*- isomer into its less stable form, the *cis*- isomer, of reduced efficacy (Pattanaargson, Limphong 2001; Scalia *et al.*, 2002; Nečasová *et al.*, 2017).

CONCLUSION

A simple, rapid, and sensitive HPLC-DAD method for the simultaneous determination of rutin, avobenzone, and octyl *p*-methoxycinnamate was developed and validated for selectivity (matrix effect), linearity, precision (repeatability), intermediate precision, accuracy, *limit of detection, limit of quantification* and robustness. In the selectivity studies (matrix effect), the lack of parallelism between the curves of octyl *p*-methoxycinnamate in the mobile phase in the absence and presence of SBEβCD showed interference from the matrix in the EHMC quantification, reinforcing the necessity to validate the analytical method in the presence of this, and other matrices. The statistical treatments of the validation results were fundamental to guarantee the reliability of this analytical method in its routine use.

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