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Short communication

Development and validation of an ion-pair RP-HPLC method for the determination of oligopeptide-20 in cosmeceuticals

Panagiota Papagianni [a], Athanasia Varvaresou [b], Spyros Papageorgiou [b], Irene Panderi [a,*]

[a] University of Athens, School of Pharmacy, Division of Pharmaceutical Chemistry, Panepistimiopolis, Zografou 157 71, Athens, Greece
[b] The School of Health and Caring Professions, Technological Education Institution of Athens, Department of Aesthetics and Cosmetology, Ag. Spyridonos str., 122 10 Athens, Greece

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ABSTRACT

Oligopeptide-20 is a growth-factor mimicking peptide used in cosmeceuticals. This article describes the development and validation of an ion-pair reversed-phase liquid chromatography method that allows, after liquid–liquid extraction, the quantification of oligopeptide-20 in cosmetic creams. Chromatographic separation was achieved on a cyanopropyl Hypersil analytical column (100 mm × 2.1 mm i.d., 5 μm particle size), using a mobile phase of acetonitrile–heptafluorobutyric acid (pH = 2.5, 9.0 mM) (70:30, v/v) containing 0.045% diethylamine at a flow rate of 0.50 mL min^{-1}. Ultraviolet (UV) spectrophotometric detection at 225 nm was used. The method had linear calibration curve over the range 1.35–4.95 μg mL^{-1} for oligopeptide-20. The intra- and inter-day RSD values were less than 3.3%, while the relative percentage error, $\%E_r$, was less than 1.9. The developed method was applied successfully to the quality control of a cosmetic cream containing 0.003% (w/w) oligopeptide-20.

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1. Introduction

Over the past ten years there has been a significant increase in the incorporation of bioactive ingredients in the preparation of cosmetics (referred to as "cosmeceuticals") for the improvement of the appearance of photoaged skin and the reduction of face wrinkles [1,2]. Cosmeceuticals were made possible by the increased understanding of skin physiology. Peptide cosmeceuticals are one of the new, popular options to treat aging skin [3]. Since the majority of all natural processes within the body are stimulated or modulated exclusively through the interaction of peptides or proteins with their target partner, peptides with specific amino acid sequences hold future promise for a wide range of therapeutic and cosmeceutical applications. Recent studies have revealed an increasing use of oligopeptides, growth factors and cytokines for wound healing, burns and the reduction of photoaging effects [4,5]. Among the issues that apply to any cosmeceutical ingredient is the quality control of these products and has to be addressed under a more systematic investigation [6,7].

Oligopeptide-20 is a synthetic growth factor mimicking peptide that activates the mechanisms of wound healing [8]. In vitro studies revealed that oligopeptide-20 increases the proliferation rhythm of keratinocyte and fibroblasts, stimulates the collagen and hyalouronic acid synthesis by the fibroblasts and keratinocytes, decreases the apoptosis rhythm, increases the cells adhesion ability and improves the texture of cuticle [9]. Cosmetic creams containing oligopeptide-20 are suitable for wrinkle, rough, and dry skin.

Recently, a LC–MS/MS method has been applied to the determination of a mixture of palmitoyl peptides in cosmetic creams [10]. The objective of this study was to develop an HPLC method for the determination of oligopeptide-20 in cosmeceuticals. Although, RP-HPLC [11–13] and anionic IP-RPLC [14,15] has been extensively applied in the analysis of peptides in many fields of research and development, there is no chromatographic procedure or methodology available for the determination of oligopeptide-20. The chromatographic behavior of oligopeptide-20 on a cyanopropyl analytical column using heptafluorobutyric acid as anionic ion-pair agent under isocratic conditions was thoroughly investigated. The method is the first reported application for the analysis of oligopeptide-20 and could be used for routine analysis of cosmetics containing oligopeptide-20 as it complies well with the validation requirements in the cosmetic industries [16].

2. Experimental

2.1. Materials and reagents

All solvents were of HPLC grade and were purchased from Merck (Darmstadt, Germany). Heptafluorobutyric acid was obtained from Acros Organics (New Jersey, USA). Diethylamine of analytical reagent grade was obtained from Sigma–Aldrich. Tert-butyl methyl ether of analytical reagent grade was

* Corresponding author. Tel.: +30 210 7274820; fax: +30 210 7274747.
E-mail address: ipanderi@pharm.uoa.gr (I. Panderi).

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obtained from Fisher Scientific (Leicestershire, UK). Water was deionised and further purified by means of a Milli-Q Plus Water Purification System, Millipore Ltd. Oligopeptide-20, Arg–Arg–Leu–Glu–Met–Tyr–Cys–Ala–Pro–Leu–Lys–Pro, was donated from Cellco Chemicals Ltd. (Athens, Greece), distributor in Greece of Caregen Co. Ltd. (South Korea).

Cosmetic cream containing 0.003% (w/w) oligopeptide-20 and placebo cream (cream matrix without oligopeptide-20) were produced in the Department of Aesthetics and Cosmetology of the Technological Education Institution of Athens, Greece. The excipients present in cream are: aqua, C12-20 acid PEG ester, glyceryl stearate, PEG-100 stearate, cetyl alcohol, dimethicone, octyl palmitate, xanthan gum, propylene glycol, propylparaben, methylparaben, imidazolidinyl urea, BHT.

2.2. Instrumentation

A SpectraSeries P100 isocratic pump (SP ThermoSeparation, UK), and a Rheodyne Model 7725i injector, (Rheodyne California, CA, USA) with a 10 μL loop along with a Waters 486 UV–vis detector, (Waters, Milford, MA, USA) operated at 225 nm were used to perform the experiments. Data acquisition and analysis were performed using Empower software (Waters, Milford, MA, USA). Chromatography was performed at ambient temperature on a cyanopropyl CPS Hypersil analytical column 100.0 mm × 2.1 mm i.d., particle size 5.0 μm from Thermo (UK) with a mobile phase of acetonitrile–heptafluorobutyric acid (pH = 2.5, 9.0 mM) (70:30, v/v) containing 0.045% diethylamine at a flow rate of 0.50 mL min^{-1}. Chromatographic run time was less than 6.0 min.

2.3. Stock standard solution and spiked cream samples

Oligopeptide-20 was dissolved in acetonitrile–water (50:50, v/v) to obtain a 90.0 μg mL^{-1} stock standard solution that was stored in amber bottles at −20 °C and was found to be stable for at least one month.

Calibration spiked cream samples of oligopeptide-20 were freshly prepared every working day at the concentration levels of 1.35, 2.25, 3.15, 4.05, 4.95 and 5.85 μg mL^{-1} for oligopeptide-20 by addition of the appropriate aliquot of the stock standard solution of the analyte to 100 mg of placebo cream. QC samples at three concentration levels (1.35, 3.15 and 4.95 μg mL^{-1}) were prepared in an analogous manner.

2.4. Assay procedure of cosmetic creams

Due to the high complexity of the cosmetic cream a liquid–liquid extraction was adopted for the sample pretreatment. Exactly 850 μL of HCl 0.01 M and 50 μL of acetonitrile are added to 100 mg of cream sample. The mixture is shaken for a few seconds and centrifuged at 18,000 × *g* for 30 min. In 700 μL of the aqueous phase, exactly 350 μL of tert-butyl methyl ether is added and the mixture is vortexed at 150 × *g* for 2 min and centrifuged at 16,000 × *g* for 10 min. The organic layer is discarded while aliquots of the aqueous layer are injected into the HPLC system.

2.5. Validation procedures

The method was validated according to ICH guidelines [16]. External standard calibration curves with five calibration points were obtained by analysing the calibration spiked cream samples that were prepared and analyzed in duplicate on three different analytical runs. Quantitation was performed using the peak area of oligopeptide-20. QC samples were processed in five replicates at each concentration level for five different analytical runs in order to evaluate the intra- and inter-assay accuracy and precision. The standard addition method was used to evaluate the effect of the excipients on the determination of oligopeptide-20.

3. Results and discussion

3.1. Chromatographic characteristics

Oligopeptide-20 is a synthetic 12 amino acids peptide that consists of hydrophobic (Ala, Cys, Leu, Met, Pro, Tyr) as well as hydrophilic (Arg, Lys, Glu) amino acids. Chromatography was performed on a cyanopropyl analytical column using aqueous acetonitrile mobile phases with different percentages of organic modifier ranged from 45 to 80% (v/v). In all cases problems were experienced due to strong retention of oligopeptide-20, retention times were greater than 80 min, and excessive peak broadening, asymmetry factors greater than 3.4. These results can probably be explained by the fact that the peptide adsorbs strongly to the stationary phase and elute only when the solvent strength is sufficient to compete with the hydrophobic forces keeping it there. The situation can be rectified by adding a strong acid in the mobile phase that will have the dual function of salting out the silanol groups and, by lowering the pH, tends to protonate arginine, lysine and glutamine residues. The addition of 0.02 M formic acid (pH = 3.0, adjusted with concentrated ammonia solution) to the mobile phase using acetonitrile as organic modifier in the percentages 45–80% (v/v) did not improve the chromatography as retention times were greater than 35 min with asymmetry factors greater than 3. When 0.1% trifluoroacetic acid (pH = 2.5, adjusted with concentrated ammonia solution) was used as ion-pair agent in the mobile phase, oligopeptide-20 was co-eluted with matrix excipients. Thus, we finally used heptafluorobutyric acid (HFBA) as ion-pair agent to the mobile phase, with acetonitrile as organic modifier. In all cases the mobile phase was adjusted to pH 2.5 with concentrated ammonia solution. The addition of HFBA reduced the retention time of oligopeptide-20 and improved peak shape (asymmetry factor 1.75). This effect can be attributed to the fact that, under these conditions, the peptide is positively charged in the basic residues and it forms an ion-pair with the anionic ion-pair agent and changing selectivity. Moreover, HFBA when used in the mobile phase tends to suppress the absorption of the peptide to the surface silanols of the reversed phase column support. In an attempt to further improve peak shape, diethylamine was also added to the mobile phase.

To optimize the chromatographic conditions percentages of acetonitrile, concentration of the ion-pair agent (HFBA), concentration of diethylamine and pH of the mobile phase were selected for examination. The method development was conducted in detail using a univariate procedure (change one-variable-at-a-time approach). A linear decrease in the logarithm of retention coefficient ($\log k$) of oligopeptide-20 was observed with increasing the percentage of acetonitrile (φ_{ACN}) in the mobile phase over the ranges 50–80% (v/v) (Fig. 1a). A reduction in the retention time of oligopeptide-20 that is observed with increasing HFBA concentration from 5 to 14 mM (Fig. 1b) can most probably be attributed to the low hydrophobicity of the cyanopropyl reversed-phase column used in this investigation. Diethylamine was also added to the mobile phase in an attempt to further improve peak shape. As the concentration of diethylamine increases from 0 to 0.05% (v/v), retention of oligopeptide-20 decreases and peak shape sharpens (Fig. 1c). The effect of pH changes on the retention of oligopeptide-20 is illustrated in Fig. 1d. The retention of the analyte increases at pH values below 4.0 due to protonation of the N-terminal and the basic residues of the peptide. Oligopeptide-20 is adequately retained and well resolved from matrix excipients using a mobile phase of acetonitrile–heptafluorobutyric acid

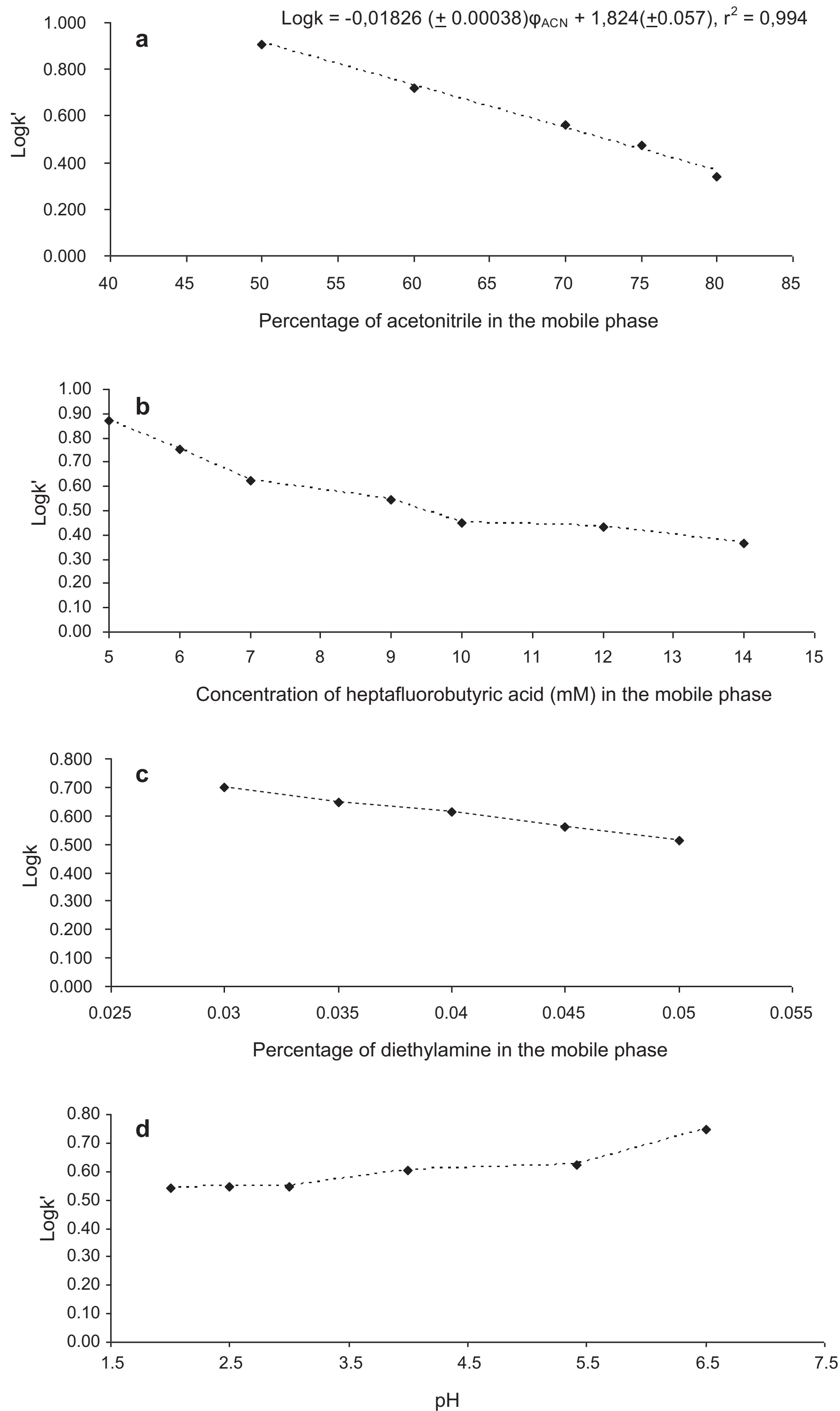

Fig. 1. Plots of the logarithm of capacity factor, log *k*, versus (a) the percentage of acetonitrile in the mobile phase, (b) the heptafluorobutyric acid concentration (in mM) in the mobile phase, (c) the percentage of diethylamine in the mobile phase and (d) the pH of the mobile phase.

(pH = 2.5, 9.0 mM) (70:30, v/v) containing 0.045% diethylamine. The selectivity of the proposed chromatographic procedure is illustrated in Fig. 2 with representative IP-RPLC chromatograms obtained from the analysis of (a) a blank cream matrix sample, (b) a cream sample containing 3.00 μg mL^{-1} oligopeptide-20 and (c) a calibration spiked cream sample at the LOQ level (1.35 μg mL^{-1}).

3.2. *Linearity and reproducibility*

Linear relationships were observed between the peak area signals of oligopeptide-20 and the corresponding concentrations (Table 1); the correlation coefficient was greater than 0.998. The LOD was calculated based on the concentration exhibiting a peak-to-noise ratio of 3 and it was found to be 0.45 μg mL^{-1}. The LOQ

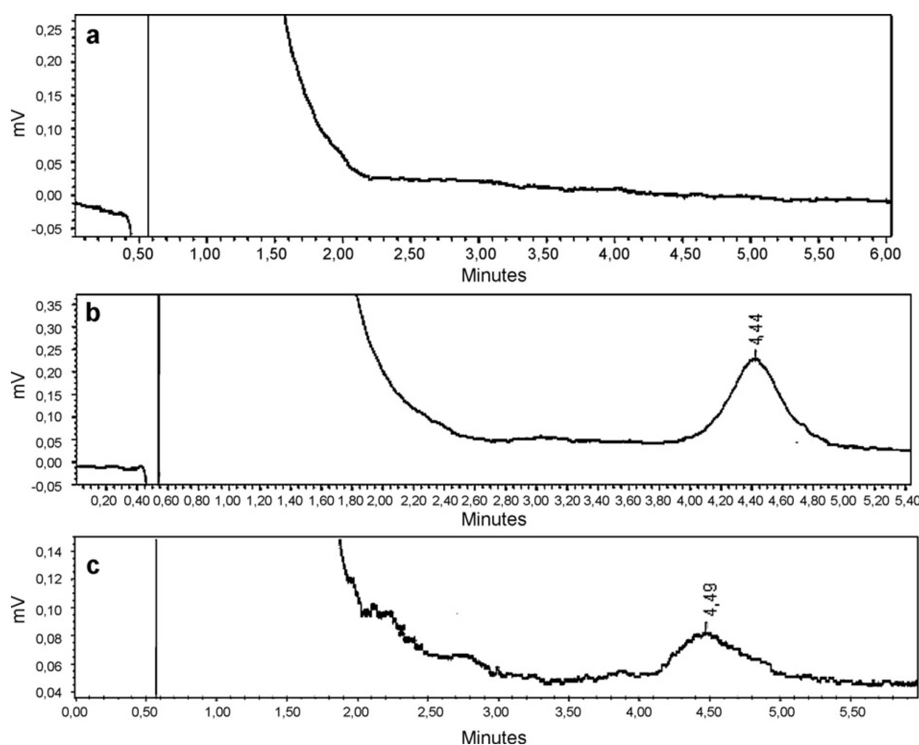

Fig. 2. Representative IP-RPLC chromatograms obtained from the analysis of (a) a blank cream matrix sample, (b) a cream sample containing 3.00 μg mL^{-1} oligopeptide-20 and (c) a calibration spiked cream sample at the LOQ level (1.35 μg mL^{-1}). Chromatographic conditions: IP-RPLC on a CPS Hypersil column; mobile phase: acetonitrile–heptafluorobutyric acid (pH = 2.5, 9.0 mM) (70:30, v/v) containing 0.045% diethylamine; flow rate 0.50 mL min−1 and a UV detector at 225 nm.

Table 1
Analytical concentration parameters of the calibration equations for the determination of oligopeptide-20, by IP-RPLC method.

Medium	Concentration range (μg mL^{-1})	Regression equation[a]	r[b]	Standard deviation		S_r[c]
Water samples				Slope	Intercept	
Run1	1.35–4.95	$S_{olig\text{-}20} = 3.78 \times C_{olig\text{-}20} - 1.71$	0.998	0.12	0.52	0.27
Spikes cream samples						
Run 1	1.35–4.95	$S_{olig\text{-}20} = 2.985 \times C_{olig\text{-}20} - 2.97$	0.9991	0.077	0.34	0.22
Run2	1.35–4.95	$S_{olig\text{-}20} = 2.828 \times C_{olig\text{-}20} - 2.21$	0.998	0.074	0.32	0.23
Run3	1.35–4.95	$S_{olig\text{-}20} = 2.796 \times C_{olig\text{-}20} - 2.19$	0.9995	0.080	0.35	0.20
Mean of three calibration curves over a period of 1 month						
Spikes cream samples	1.35–4.95	$S_{olig\text{-}20} = 2.87 \times C_{olig\text{-}20} - 2.16$	≥0.998	0.10	0.56	≤0.23

[a] Signal × 10^{-3}of oligopeptide-20, $S_{olig\text{-}20}$ versus the corresponding concentration, $C_{olig\text{-}20}$.
[b] Correlation coefficient.
[c] Standard error of the estimate.

was calculated as a peak-to-noise ratio of 10. LOQ was found to be 1.35 μg mL^{-1} and resulted in accuracies within ±20% of the nominal concentration and precisions ≤20%. One-way analysis of variance (ANOVA) was used to evaluate the intra- and inter-assay precision (Table 2). Precision was assessed by intra-assay and inter-assay %RSD values and were lower than 3.3 and 2.3%, respectively, while the overall accuracy was assessed by the relative percentage error, %Er, was ranged from −0.3 to 1.9% for oligopeptide-20.

Tert-butyl methyl ether was chosen as the optimum solvent for the liquid–liquid extraction procedure. It was found that the addition of a small amount of acetonitrile along with 0.1 M HCl improves the recovery of the analyte that was quantified in the aqueous layer. The recovery of the extraction procedure was evaluated by comparing the slope of the regression equation obtained from the analysis of calibration spiked cream samples over the slope of the regression equation obtained from the analysis of calibration samples prepared in water solution and analyzed immediately without sample preparation procedure (Table 1). The data, under the optimum extraction conditions indicate a recovery of 75.9% for oligopeptide-20.

Table 2
Accuracy and precision evaluation of quality control samples for oligopeptide-20 (n = 5 runs, five replicates per run).

Compound	Concentration (μg mL^{-1})		
Oligopeptide-20			
Added concentration	1.35	3.15	4.95
Run 1 (mean ± SD)	1.382 ± 0.022	3.196 ± 0.012	4.977 ± 0.091
Run 2 (mean ± SD)	1.387 ± 0.016	3.142 ± 0.086	4.993 ± 0.072
Run 3 (mean ± SD)	1.374 ± 0.044	3.135 ± 0.066	5.04 ± 0.13
Run 4 (mean ± SD)	1.349 ± 0.041	3.089 ± 0.062	4.97 ± 0.14
Run 5 (mean ± SD)	1.351 ± 0.028	3.147 ± 0.024	4.995 ± 0.085
n	25	25	25
Overall mean	1.369	3.142	4.995
Intra-assay CV (%)[a]	3.3	1.9	2.2
Inter-assay CV (%)[a]	2.3	1.8	2.1
Overall accuracy % E_r[b]	1.4	−0.3	1.9

[a] Coefficient of variation; intra- and inter-assay CVs were calculated by ANOVA.
[b] Relative percentage error = [(overall mean assayed concentration – added concentration)/(added concentration) × 100].

Table 3
Robustness evaluation of the IP-RPLC method for the determination of oligopeptide-20 in cosmetic creams.

Chromatographic changes	Measured responses			
Parameters[a]	tr[b]	k[c]	T[d]	Concentration (μg mL^{-1})
A: concentration of heptafluorobutyric acid (8.9–9.1 mM)				
Mean (%RSD):	4.45 (0.8)	3.68 (1.0)	1.23 (0.8)	3.12 (0.7)
B: flow rate (0.49–0.51 mL min^{-1})				
Mean (%RSD):	4.41 (0.3)	3.64 (0.4)	1.23 (0.5)	3.13 (0.7)
C: % of acetonitrile in the mobile phase (69–71%, v/v)				
Mean (%RSD):	4.40 (0.7)	3.63 (0.9)	1.23 (0.5)	3.13 (0.8)

[a] Three parameters (*A*, *B* and *C*) were slightly changed at three levels (1, 0, −1); each time a parameter was changed from level (0) the others remained at level (0).
[b] Retention time.
[c] Capacity factor.
[d] Tailing factor.

To verify method robustness, small deliberate variations were introduced around the optimal conditions and the influence of these variations in the retention time, capacity factor, tailing factor and concentration of the analyte was thoroughly investigated and presented in Table 3. The evaluation of the method robustness indicates that there is no significant difference in the measured responses after small variations of the selected parameters.

3.3. Application of the method

The proposed method was evaluated in the assay of cosmetic creams containing 0.003% (w/w) of oligopeptide-20 with ten replicate determinations on accurately weighted amounts of the cream equivalent to 3.0 μg of oligopeptide-20. The percent label claims for the analyte were found to be 98.1 ± 0.7 (n = 10, RSD = 0.7%).

The standard addition method was used to further assess the specificity of the proposed method. Thus, six equal amounts of the cosmetic cream equivalent to 3.0 μg of oligopeptide-20 were spiked with known and different amounts of oligopeptide-20 (0.90, 1.35, 1.80, 2.25, 2.70 and 3.15 μg). The regression line of the instrumental response versus the added concentration of the analyte is plotted and the negative intercept on the concentration axis (x-axis) corresponds to the concentration of the analyte in the cream sample. This value is given by the ratio of the intercept and the slope of the regression line, that were found to be 6.911 ± 0.071 and 2.331 ± 0.032, respectively. The label claim for oligopeptide-20 using the standard addition method was 0.00296% (w/w).

4. Conclusion

There is a real need to set up analytical methods in order to quantitate the active compounds in cosmeceuticals. The proposed IP-RPLC method has been evaluated over the linearity, precision, accuracy and specificity and proved to be convenient and effective for the determination of oligopeptide-20 in cosmetic creams. There is no doubt that the proposed methodology could be extended in the future for the determination of other peptides in complex cosmetic matrices.

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