separations

MDPI

Article

Quantitation of Copper Tripeptide in Cosmetics via Fabric Phase Sorptive Extraction Combined with Zwitterionic Hydrophilic Interaction Liquid Chromatography and U*V*/*V*is Detection

Pantelitsa Pingou [1], Anthi Parla [1], Abuzar Kabir [2], Kenneth G. Furton [2], Victoria Samanidou [3], Spyridon Papageorgiou [4], Efthimios Tsirivas [4], Athanasia Varvaresou [4] and Irene Panderi [1,*]

[1] Laboratory of Pharmaceutical Analysis, Division of Pharmaceutical Chemistry, School of Pharmacy, National and Kapodistrian University of Athens, Panepistimiopolis Zografou, 15771 Athens, Greece; pantelitsa45@gmail.com (P.P.); aparla@pharm.uoa.gr (A.P.)
[2] Department of Chemistry and Biochemistry, Florida International University, Miami, FL 33199, USA; akabir@fiu.edu (A.K.); furtonk@fiu.edu (K.G.F.)
[3] Laboratory of Analytical Chemistry, School of Chemistry, Aristotle University of Thessaloniki, 54124 Thessaloniki, Greece; samanidu@chem.auth.gr
[4] Laboratory of Chemistry-Biochemistry-Cosmetic Science, Department of Biomedical Sciences, Faculty of Health and Welfare Sciences, Campus 1, University of West Attica, Agiou Spyridonos, 12243 Egaleo, Greece; spypapage@gmail.com (S.P.); tsirivas@cellco.gr (E.T.); avarvares@uniwa.gr (A.V.)
* Correspondence: irenepanderi@gmail.com; Tel.: +30-210-72-74-820

Abstract: The increasing demand for effective cosmetics has driven the development of innovative analytical techniques to ensure product quality. This work presents the development and validation of a zwitterionic hydrophilic interaction liquid chromatography method, coupled with ultraviolet detection, for the quantification of copper tripeptide in cosmetics. A novel protocol for sample preparation was developed using fabric phase sorptive extraction to extract the targeted analyte from the complex cosmetic cream matrix, followed by chromatographic separation on a ZIC®-pHILIC analytical column. A thorough investigation of the chromatographic behavior of the copper tripeptide on the HILIC column was performed during method development. The mobile phase consisted of 133 mM ammonium formate (pH 9, adjusted with ammonium hydroxide) and acetonitrile at a 40:60 (*v*/*v*) ratio, with a flow rate of 0.2 mL/min. A design of experiments (DOE) approach allowed precise adjustments to various factors influencing the extraction process, leading to the optimization of the fabric phase sorptive extraction protocol for copper tripeptide analysis. The method demonstrated excellent linearity over a concentration range of 0.002 to 0.005% *w*/*w* for copper tripeptide, with a correlation coefficient exceeding 0.998. The limits of detection and quantitation were 5.3×10^{-4}% *w*/*w* and 2.0×10^{-3}% *w*/*w*, respectively. The selectivity of the method was verified through successful separation of copper tripeptide from other cream components within 10 min, establishing its suitability for high-throughput quality control of cosmetic formulations.

Keywords: copper tripeptide; biomimetic peptides; cosmetics; analytical method development; DOE; FPSE; HILIC; green analytical chemistry

check for updates

Citation: Pingou, P.; Parla, A.; Kabir, A.; Furton, K.G.; Samanidou, V.; Papageorgiou, S.; Tsirivas, E.; Varvaresou, A.; Panderi, I. Quantitation of Copper Tripeptide in Cosmetics via Fabric Phase Sorptive Extraction Combined with Zwitterionic Hydrophilic Interaction Liquid Chromatography and U*V*/*V*is Detection. *Separations* **2024**, *11*, 293. https://doi.org/10.3390/separations11100293

Academic Editor: Gavino Sanna

Received: 22 September 2024
Revised: 9 October 2024
Accepted: 10 October 2024
Published: 12 October 2024

Copyright: © 2024 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1. Introduction

The pursuit of healthy skin has long been a top priority for individuals. Multiple factors, such as aging, exposure to sunlight, pollution, and adverse weather conditions, increase the likelihood of developing skin imperfections [1]. Over the past twenty years, the incorporation of peptides in cosmetics has gained increasing popularity due to their effectiveness in addressing signs of aging [2,3]. These peptide-based cosmetic products are rooted in early wound healing research originating in the 1930s, where yeast extracts

were used in formulations for wound healing [4]. As the scientific understanding of skin physiology has progressed, the innovation of cosmetics incorporating peptides has also evolved, providing novel methods for preventing skin aging [5]. By interacting with various receptors, peptides play a crucial role in regulating several biological functions, including angiogenesis, cell growth, migration, melanogenesis, inflammatory response, and protein synthesis, offering various therapeutic and cosmetic benefits. Biomimetic peptides, known for their biocompatibility and effectiveness, consist of short amino acid sequences [6]. These peptides can either be synthesized, naturally occurring, or sourced from poisonous organisms. They are classified into categories based on their mechanism of action, including signal peptides, carrier peptides, and those that modulate neurotransmitter activity [7].

Copper tripeptide (GHK-Cu), with the amino acid sequence glycyl-L-histidyl-L-lysine and the IUPAC name copper(2S)-6-amino-2-[[(2S)-2-(2-azanidylacetyl)azanidyl-3-(1H-imidazol-4-yl)propanoyl]amino]hexanoate, is one of the most extensively studied regenerative peptide. Although GHK naturally occurs in human blood, its concentration declines with age [8]. GHK-Cu is a biomimetic peptide that serves as a carrier, stabilizing and transporting copper ions (Cu^{2+}) to the epithelial cells of the skin. Cu^{2+} ions play a crucial role in the human body as a co-factor for enzymes such as superoxide dismutase, which eliminates free radicals, as well as lysyl oxidase, which promotes cross-linking in nascent collagen and elastin fibrils [9]. GHK-Cu resembles the alpha chain of collagen, so it is one of the first peptides used in skincare formulations [10]. Currently, GHK-Cu is extensively used in cosmetics, either alone or in combination with other peptides, for its ability to diminish wrinkles, reverse photodamage, reduce hyperpigmentation, strengthen the skin barrier, and promote keratinocyte proliferation [11,12]. Additionally, it promotes the polarization of M2 macrophages contributing to anti-inflammatory effects [13]. GHK-Cu accelerates skin healing by regulating matrix metalloproteinase expression and supports fibroblast activity by stimulating the production of collagen, glycosaminoglycan, and elastin [14,15]. Liposomes are often used as delivery systems for biomimetic peptides like GHK-Cu in cosmetics [16]. Furthermore, when combined with hyaluronic acid, it may increase collagen type IV synthesis [17]. Research by Pickard et al. in 2018 demonstrated the potential of GHK-Cu as an anticancer agent, particularly for promoting DNA replication in cancer patients undergoing radiation therapy [18]. Moreover, it has shown potential in managing neurodegenerative conditions such as Alzheimer's and Prion diseases by blocking amyloid-β aggregation through copper chelation, thereby reducing neurotoxicity [19,20].

According to the literature, only a few methods have been published for the quantitation of GHK-Cu. Xu et al. (2020) employed pulsed glow discharge mass spectrometry (GDMS) for the quantitative determination of GHK-Cu, focusing specifically on the copper ion signal from the GHK-Cu complex [21]. There is currently only one other HPLC method for GHK-Cu analysis, published by Badenhorst et al. in 2016 [22]. This method utilized reversed-phase HPLC-UV with a highly aqueous mobile phase to study the stability of GHK-Cu in various solutions. While it provided valuable insights into the degradation kinetics of GHK-Cu under different conditions, it was not applied to the analysis of complex cosmetic formulations.

As far as we are aware, there are no established methods in the literature that quantify GHK-Cu in cosmetic products using fabric phase sorptive extraction (FPSE) paired with hydrophilic interaction liquid chromatography (HILIC). Given their hydrophilic nature, peptides generally exhibit minimal retention on standard reversed-phase HPLC columns, frequently requiring additional steps, such as derivatization or the inclusion of ion-pairing agents [23]. HILIC, on the other hand, provides a viable alternative, employing polar packing materials and mobile phases with low aqueous content and high organic composition [24,25]. Thus, HILIC is particularly suitable for the analysis of polar substances [26–28], including peptides [29–31]. Despite numerous studies focused on peptide separation with HILIC columns, limited attention has been given to the quantification of peptides specifically within complex cosmetic matrices [32–35]. To bridge this gap, a fast, eco-friendly, and

sensitive FPSE-HILIC–UV method was designed and validated for the accurate quantification of GHK-Cu in cosmetic creams. Chromatographic separation was achieved using a polymeric zwitterionic ZIC®-pHILIC analytical column under isocratic elution with UV detection. The chromatographic profile of the analyte was carefully assessed, and GHK-Cu was quantified following sample preparation with a newly developed fabric phase sorptive extraction (FPSE) method. FPSE is a recent microextraction method, gaining traction for its sustainability and minimal equipment requirements [36]. During method development, design of experiments (DoE) was applied to systematically explore the impact of various experimental parameters on extraction performance. DoE also helped to enhance GHK-Cu % recovery by identifying significant interactions between various factors [37]. The FPSE technique, which is in alignment with the principles of green analytical chemistry (GAC), effectively isolated GHK-Cu from cosmetic creams with improved sensitivity before zwitterionic HILIC-UV analysis [38].

2. Equipment

2.1. Chemical and Reagents

High-purity HPLC-grade water was obtained using a combination of the Direct-Pure Water System, RO 10 (Rephile Bioscience Inc., Europe), which produces reverse osmosis (RO) water from tap water, and the Millipore Synergy® UV water purification unit (MerckMillipore, Darmstadt, Germany), which generates ultra-pure (Type 1) water from a pure-water feed. The latter system is equipped with a 185 nm photooxidation UV lamp specifically designed to target organic traces. All other solvents used in this study were of high-purity HPLC grade and obtained from Merck (Darmstadt, Germany). Ammonium formate and ammonium acetate were supplied by Acros Organics (Geel, Belgium). Prior to use, the mobile phases were passed through 0.45 μm pore size nylon membrane filters, supplied by Gelman Sciences (Northampton, UK) and vacuum degassed. Copper tripeptide, specifically copper glycyl-histidyl-lysine with 98.8% purity, was provided by Cellco Chemicals Ltd. (Athens, Greece) which is the Greek distributor of the Caregen Co., Ltd. (Anyang, Republic of Korea).

Two different batches of a commercial anti-wrinkle cream containing 0.004% (*w*/*w*) copper tripeptide were purchased from the local market. For validation purposes, a placebo cream (without copper tripeptide) was prepared by the Department of Aesthetics and Cosmetology at the Laboratory of Chemistry, Biochemistry, and Cosmetic Science, University of West Attica (Athens, Greece). The placebo cream (without GHK-Cu) consisted of various excipients, including aqua, xalifin-15, propylene glycol, sabowax FX-65, squalene, butylated hydroxyl toluene (BHT), and germall 115.

2.2. Equipment

A Waters 2695 Star Alliance HPLC System (Waters Co, Milford, MA, USA) was used for this work, equipped with a temperature-controlled oven, an autosampler, and a Waters 2487 dual wavelength detector, which is designed to provide high performance in UV/Vis detection. Separation was achieved with a ZIC®-pHILIC polymeric zwitterionic analytical column (dimensions: 150 mm × 2.1 mm, particle size: 3.5 μm, pore size: 200 Å) sourced from Merck Millipore (Darmstadt, Germany). The mobile phase comprised 40% of a 133 mM ammonium formate aqueous solution (pH adjusted to 9 with ammonium hydroxide) and 60% acetonitrile, pumped at a flow rate of 0.2 mL/min. Chromatographic analysis was performed using a 20 μL injection volume, the column oven was set at 25 ± 2 °C, detection wavelength was set at 224 nm, and the total run time was 10 min. Data acquisition and subsequent analysis were accomplished with Empower 3 software (Waters, Milford, MA, USA).

Hydrophobic polytetrafluorethylene microfilters (PTFE phobic 0.22 μm pore size, 13 mm diameter) were sourced from Rephile Bioscience Ltd., Europe, through Novalab (Athens, Greece). Sonication was carried out using a transonic water bath (Model 460/H, Elma, Germany), while vortex mixing was conducted with a vortex mixer from FALC

Instruments (Treviglio, Italy). Mild stirring (up to 110 g) was achieved with a Hei-Connect magnetic stirrer (Heidolph Instruments, Schwabach, Germany), which also featured precise temperature control through its integrated heating function. The fractional factorial design for the experiment, along with data visualization, was managed using the software Design Expert® (version 22.0.1, Stat-Ease, Minneapolis, MN, USA). Statistical analysis of data was managed using SPSS® ver. 29.0.2.0 (IBM SPSS Statistics, IBM Corp., Armonk, NY, USA).

The FPSE membranes utilized in this study, composed of methyl-trimethoxy silane (MTMS), phenyl-triethoxy silane (PheTES), and Carbowax 20 M (CW20M), were fabricated by Kabir and Furton using a sol-gel coating process at the International Forensic Research Institute Laboratory, Department of Chemistry and Biochemistry, Florida International University (Miami, FL, USA). These fabric phase sorptive extractors were patented by Kabir and Furton in 2014 [36,39].

2.3. Stock and Working Standard Solutions

A stock solution of GHK-Cu was initially prepared at a concentration of 500 µg/mL using a 20:80 (*v*/*v*) mixture of water and acetonitrile. The stock solutions were subsequently diluted with acetonitrile to produce working standards at concentrations ranging from 5.0 to 25.0 µg/mL. To maintain stability, amber bottles containing these solutions were kept at 4 °C for several weeks.

Matrix-matched calibration standards were prepared daily by spiking placebo cream with adequate aliquots of working standards to obtain containing GHK-Cu concentrations ranging from 0.002% to 0.005% (*w*/*w*). Similarly, quality control (QC) samples were prepared fresh at concentrations of 0.002%, 0.004%, and 0.005% (*w*/*w*) using an alternative stock solution.

2.4. Sample Preparation

Due to the complexity of the cosmetic cream matrix, the FPSE technique was selected for sample pretreatment, employing a sol-gel membrane composed of MTMS/PheTES/CW20M. Circular disks of the membrane were cut, each having a diameter of 1.5 cm and a surface area of 1.77 cm^2. Initially, the membrane was immersed for 5 min in a 2 mL mixture of acetonitrile and methanol (50:50 *v*/*v*), followed by a 5 min immersion in 2 mL of deionized water. Afterward, the membrane was placed into a 2 mL glass vial with a screw cap, and 0.04 mL of 40 mM aqueous ammonium acetate (AMA), 1.86 mL of acetonitrile, and 100 mg of cosmetic cream were added. The extraction was conducted with magnetic stirring at 50 rpm for 30 min. Subsequently, the membrane was placed in a 0.55 mL mixture of acetonitrile and water (40:60 *v*/*v*) for desorption and heated to 35 °C for 30 min without stirring. Before chromatographic analysis, the resulting extract was passed through a 13 mm hydrophobic PTFE syringe filter with a 0.22 µm pore size. A glass syringe was used during the process to avoid peptide loss, as GHK-Cu can adhere to plastic materials. Figure 1 presents a schematic workflow of the FPSE pretreatment method.

2.5. Method Validation

The method validation was performed in terms of specificity, linearity, accuracy and precision, limits of detection, and quantification following the guidelines outlined in ICH Guideline Q2(R2) [40]. Calibration curves were constructed by analyzing matrix-matched calibration standards at five different GHK-Cu concentration levels. The preparation and analysis of the samples were conducted in duplicate over three separate analytical runs. To assess precision and accuracy, five replicates of QC samples were assessed at three different GHK-Cu concentration levels during three independent runs. The % recovery of the FPSE method was assessed by comparing the peak area of GHK-Cu in the QC samples, prepared using the proposed sample preparation procedure, to the peak area of samples prepared in a water/acetonitrile mixture (40:60, *v*/*v*) at the same concentration.

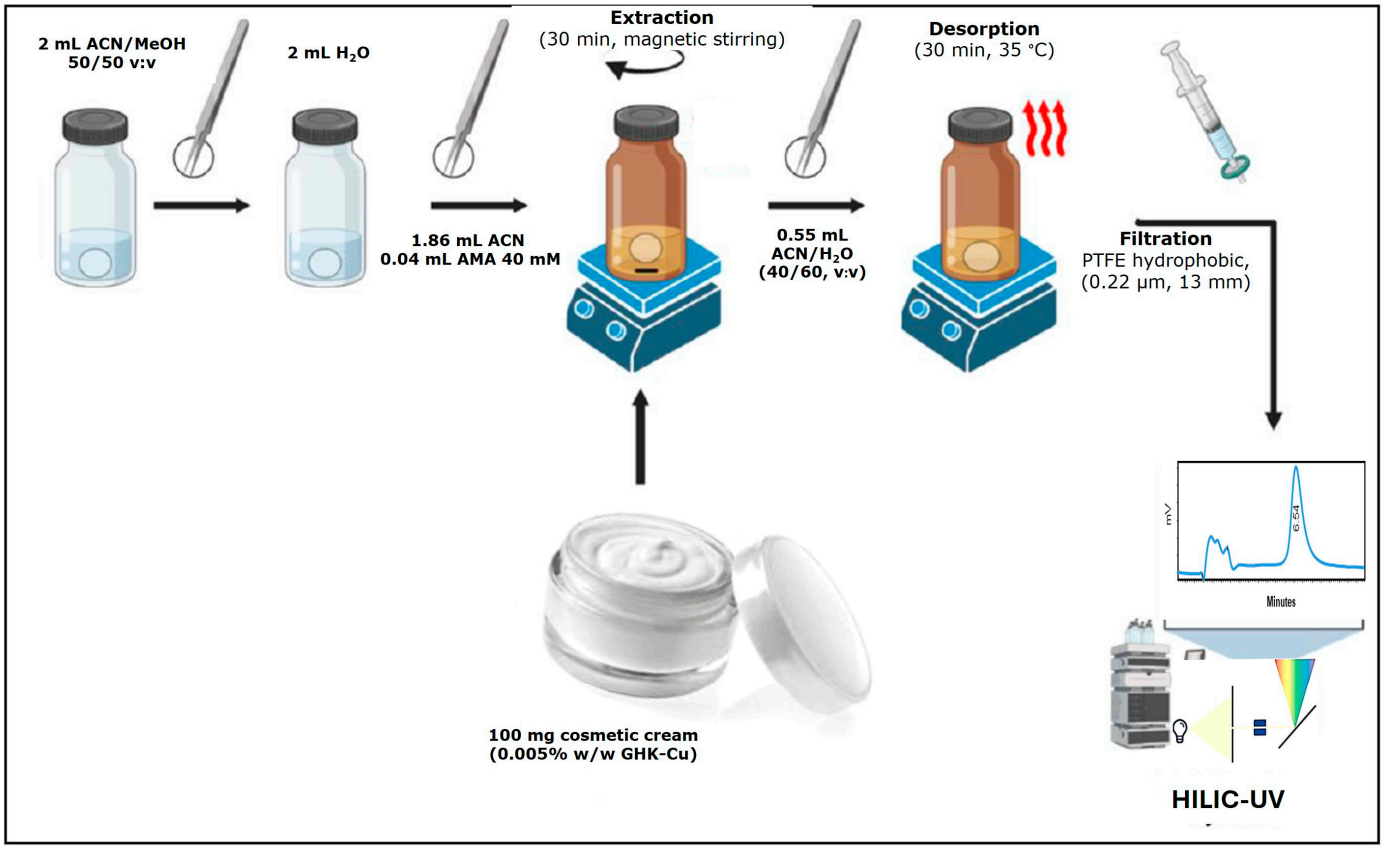


Figure 1. Schematic workflow diagram of the fabric phase sorptive extraction method. Arrows with a depiction of the membrane being moved with forceps indicate membrane relocation between different vials.

3. Results and Discussion

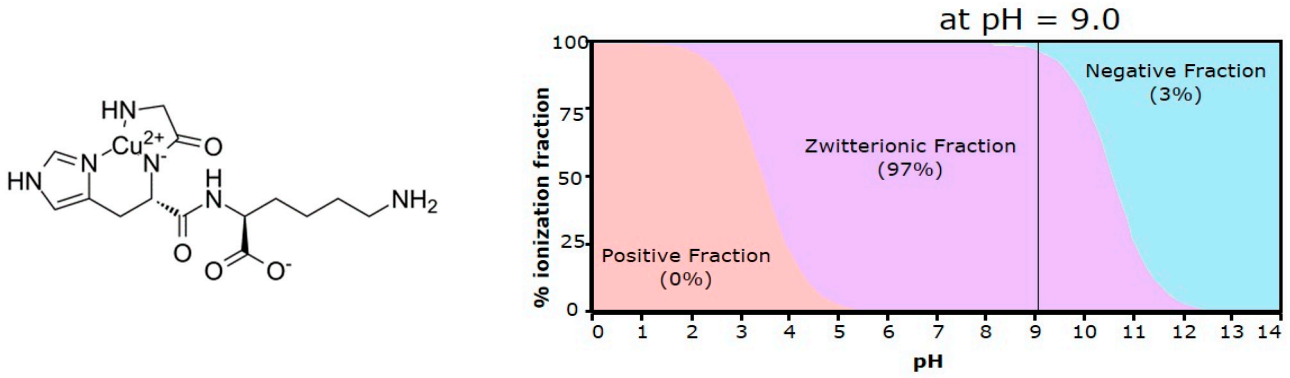
3.1. Optimization of the Chromatographic Conditions

To develop a chromatographic method for the efficient separation and retention of GHK-Cu within a short run time, we first evaluated its physicochemical properties. GHK-Cu is a high-affinity complex formed when the tripeptide glycyl-histidyl-lysine freely binds with Cu^{2+} (Figure 2a). According to the literature, the LogD values of GHK-Cu at pH 4.5 and 7.4 are −2.38 and −2.49, respectively [22]. These negative LogD values indicate the high lipophilicity of the targeted analyte and are consistent with its reported water solubility of 325.09 mg/mL. A diagram showing the ionization fraction of GHK-Cu as a function of pH is presented in Figure 2b, calculated using ADME Boxes software (version 3.0.3) from Pharma Algorithms Ltd., Toronto, ON, Canada. As illustrated in the ionization-fraction-versus-pH diagram, GHK-Cu exists predominantly in its zwitterionic form (97%) at pH 9, with only 3% present in its negatively charged form.

Based on the physicochemical data of GHK-Cu, HILIC is an excellent choice for this work, as it is well suited for the retention of polar hydrophilic compounds. This method operates through a mixed retention mechanism involving partitioning, adsorption, ionic interactions, and hydrophobic interactions. Typically, HILIC mobile phases consist of 40 to 97% organic solvent, such as acetonitrile, mixed with an aqueous buffer solution.

For this study, a zwitterionic ZIC®-pHILIC column was used, featuring a polymer-bonded packing material functionalized with sulfoalkylbetaine groups. These groups contain strongly acidic sulfonic acid groups and strongly basic quaternary ammonium groups, connected by short alkyl spacers. The electrostatic forces between the opposite charges are partially neutralized due to their close proximity. Although the accessibility of the positively charged quaternary ammonium groups is limited, the negatively charged sulfonic acid groups may still contribute to weak but meaningful electrostatic interactions

with analytes [41]. Due to the polymeric bonding, this column operates effectively across a wide pH range, from 2 to 10.



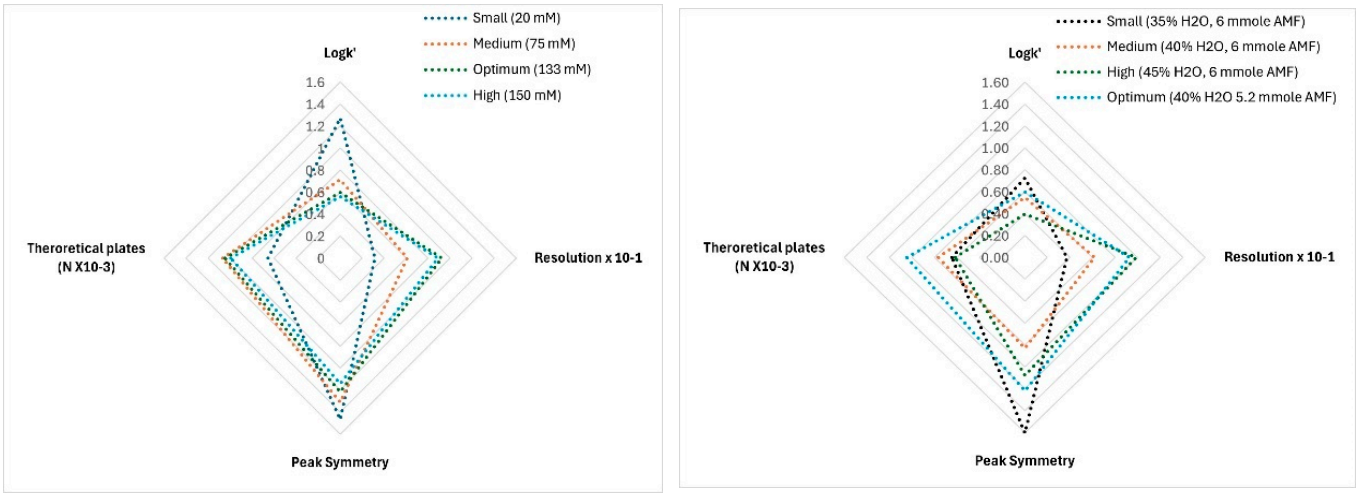
(**a**) (**b**)

Figure 2. (**a**) Chemical structure of GHK-Cu and (**b**) ionization profile of GHK-Cu computed by ADME Boxes software as a function of pH with highlighted fraction at pH 9.

A one-factor-at-a-time approach was used to study the effects of three factors—namely, salt type, concentration, and the percentage of water—on four parameters: retention (Logk′), resolution (R), peak symmetry (T), and the number of theoretical plates (N) for the GHK-Cu peak. In these experiments, a working standard solution containing GHK-Cu at a concentration of 10.0 µg/mL was prepared using a 40/60 (*v*/*v*) ACN/H_2O mixture.

During preliminary experiments considering the hydrophilicity of GHK-Cu and the zwitterionic nature of the ZIC®-pHILIC column, we examined the retention of the analyte in three different mobile phases, each containing a high salt concentration of 150 mM ammonium acetate, ammonium formate, or ammonium bicarbonate, all adjusted to pH 9 and 60% acetonitrile. Among these, ammonium acetate resulted in non-symmetric peak shapes with a peak symmetry (T) value below 0.82, while ammonium bicarbonate caused peak splitting and distortion of the chromatogram. In contrast, ammonium formate provided the best results, achieving a peak symmetry value of 1.28.

Following this, further optimization was performed by varying either the ammonium formate concentration or the percentage of water in the mobile phase, while keeping all other conditions constant. Experiments on ammonium formate concentrations, ranging from 20 to 150 mM, revealed that higher concentrations of AMF led to faster elution of GHK-Cu, thereby shortening its retention time. Figure 3a presents a radar chart illustrating the effect of ammonium formate concentration, adjusted to pH 9 and 60% acetonitrile, on the retention, resolution, peak symmetry, and theoretical plates of GHK-Cu. The radar chart in Figure 3a shows that the retention factor (Log k′) decreases with increasing salt concentration, indicating reduced retention at higher concentrations. This behavior is attributed to the crucial role of salt concentration in modulating electrostatic interactions between the analyte and the stationary phase packing material [42]. Resolution is maximized at the 133 mM concentration, making it the optimal salt level for achieving the best separation with the solvent front and lipophilic cream excipients. Column efficiency, as measured by theoretical plates, is highest at 75 mM (medium) and 133 mM salt concentrations, suggesting that these conditions offer the most efficient separations. In conclusion, the 133 mM salt concentration adjusted to pH 9 with ammonium hydroxide is identified as the optimum salt concentration, providing the best balance between resolution, peak symmetry, and column efficiency.



(**a**) (**b**)

Figure 3. Combined radar charts depicting the impact of (**a**) ammonium formate concentration and (**b**) water content percentage on the retention (Logk′), resolution ($R \times 10^{-1}$), peak symmetry (T), and theoretical plates ($N \times 10^{-3}$) of GHK-Cu. Resolution and theoretical plate values have been normalized to ensure all variables are presented in a comparable scale.

To evaluate the effect of water content (Φ_{water}) on GHK-Cu chromatography, experiments were conducted using a mobile phase with constant ammonium formate content at 6.0 mmoles in 100 mL mobile phase, while adjusting the pH of the aqueous phase to 9.0. The Φ_{water} was varied at 35, 40, and 45%, each with 6.0 mmoles ammonium formate. Additionally, an experiment with 40% acetonitrile and 5.2 mmoles ammonium formate in 100 mL mobile phase was performed. Figure 3b illustrates the impact of Φ_{water} on the retention, resolution, peak symmetry, and theoretical plates of GHK-Cu. The results show that the retention factor (Logk′) decreases as water content increases, yet the optimum condition (40% water, 5.2 mmol ammonium formate) achieves a good balance between retention and separation efficiency. In addition, the Logk′ values for GHK-Cu decrease linearly as Φ_{water} increases, pointing out to a partition mechanism for the retention for GHK-Cu with reduced electrostatic interactions. Resolution improves with increasing water content, peaking at 45% Φ_{water} with 6.0 mmoles ammonium formate, though the optimum condition (40% Φ_{water}, 5.2 mmoles ammonium formate) also provides effective separation. Peak symmetry is most favorable at medium water content (40% Φ_{water}, 6.0 mmoles ammonium formate) and under the optimum condition, ensuring ideal peak shapes. The number of theoretical plates (efficiency) is highest at the optimum condition, indicating the most efficient separation. In conclusion, the optimum condition (40% Φ_{water}, 5.2 mmoles ammonium formate) reaches the best balance between peak symmetry, efficiency, and resolution, making it the ideal setting for GHK-Cu chromatography on the ZIC-pHILIC column. The final composition of the mobile phase included 40% 133 mM ammonium formate (adjusted to pH 9 with ammonium hydroxide) and 60% acetonitrile. This mobile phase composition facilitated the isocratic elution of GHK-Cu in less than 10 min, with the peptide eluting at 6.41 min, as shown in Figure 4.

3.1.1. Fabric Phase Sorptive Extraction

FPSE is a modern microextraction method that uses fabric substrates, typically made from materials like synthetic fibers (polyesters) or natural cellulose layered with various polymers through sol-gel technology. The sol-gel process deposits a thin, porous, and durable layer onto the substrate, enhancing its ability to capture a variety of analytes [42]. This technique allows for the effective extraction of analytes without requiring extensive pretreatment steps. The open-bed design of the fabric substrate facilitates both equilibrium-based and exhaustive extraction modes, offering flexibility across different

analytical applications. Unlike traditional microextraction methods, FPSE relies on the inherent chemical characteristics of the fabric substrate, which significantly influences the effectiveness and specificity of extraction. The combination of the substrate's surface chemistry and the sol-gel coating is essential in the performance of the FPSE membrane. Different substrates, even when coated with the same polymer, can yield varying extraction results due to their hydrophilic or hydrophobic nature. Therefore, selecting the appropriate substrate is essential for optimizing the efficiency of the FPSE process, though this adds complexity to method development.

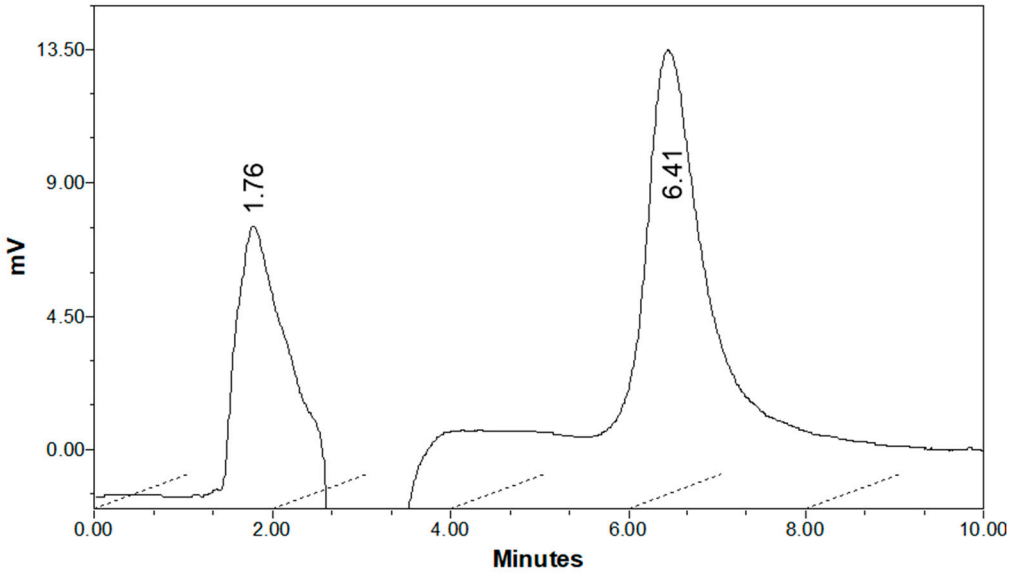


Figure 4. HILIC-UV chromatogram of a 10.0 µg/mL GHK-Cu standard solution prepared in a 60:40 (*v*/*v*) water/acetonitrile mixture, using a *z*-axis in Empower® Software. Chromatographic conditions: ZIC®-pHILIC column; mobile phase: 133 mM ammonium formate water solution (pH 9.0) in acetonitrile at a ratio of 40:60, *v*/*v*; flow rate: 0.2 mL/min; λ = 224 nm.

For this study, a sol-gel membrane consisting of methyl-trimethoxy silane (MTMS), phenyl-triethoxy silane (PheTES), and Carbowax 20 M (CW20M) was used for extracting the hydrophilic GHK-Cu peptide from a cosmetic cream formulation (Figure 5). The geometric properties of the membrane were modified by using a handheld punch to produce circular discs with a diameter of 1.5 cm, ensuring consistency across the extractions.

In preliminary experiments, we observed that magnetic stirring at 50 rpm was important during the extraction step, and heating at 35 °C without magnetic stirring played a crucial role in desorption. As a result, these factors were kept at their observed levels without further optimization. A design of experiments (DoE) approach was employed to thoroughly assess the influence of selected experimental conditions on extraction effectiveness and to enhance the % recovery of GHK-Cu. Specifically, the impact of both extraction and desorption parameters on % recovery was analyzed using a 2^{6-1} fractional factorial design, which allowed for a more systematic exploration of these variables. The categorized experimental factors and their corresponding levels are shown in Table 1. A matrix-matched calibration standard, consisting of a 100 mg cream sample spiked at 0.005% *w*/*w* with GHK-Cu, was employed for the DoE experiments.

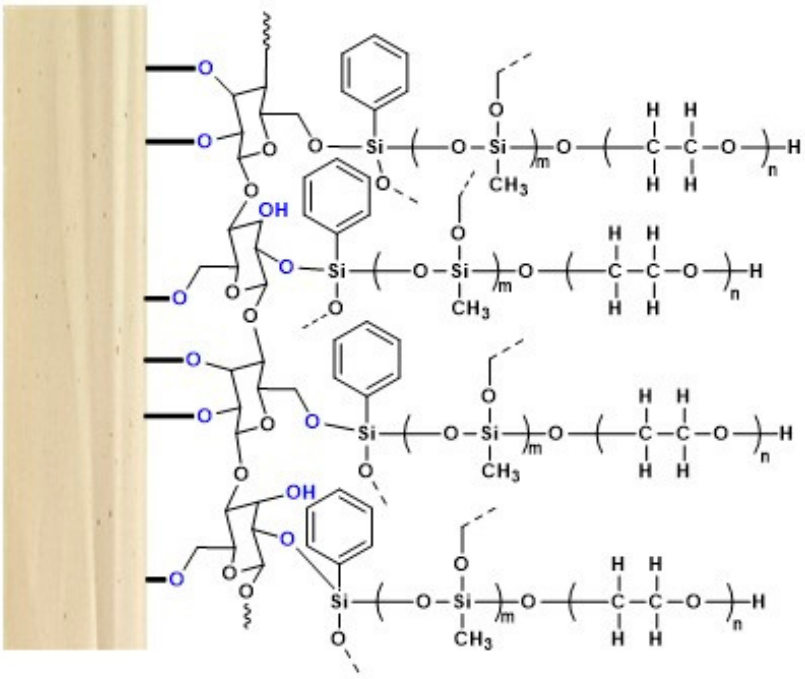


Figure 5. Scheme demonstrating sol-gel MTMS/PheTES/CW20M membrane.

Table 1. Experimental factors and their levels.

Factor Codes	Name (Unit)	Levels	
		Low (−1)	High (+1)
A	Extraction volume (mL)	0.5	2
B	Extraction time (min)	5	30
C	40 mM ammonium acetate aqueous solution (40 µL)—Extraction	No	Yes
D	Desorption volume (mL)	0.4	0.8
E	Acetonitrile (% *v*/*v*)—Desorption	0	40
F	Desorption time (min)	5	30

Following data collection and analysis, a linear first-order model was derived to describe the recovery of GHK-Cu, as shown in Figure 6. This model, expressed in coded values, captures the relationship between the experimental factors and GHK-Cu recovery, as articulated in Equation (1).

$$Y_{\text{GHK-Cu}} = -0.1381A - 0.0526B - 2.20E - 0.0845F + 0.8243AB + 0.05216AC + 0.00521AC - 0.7678AE - 0.1917AF - 0.3894BE + 0.5216BF - 0.1906CF - 0.5867ABE + 0.7024ACF + 3.86 \quad (1)$$

The developed model was assessed through analysis of variance (ANOVA) with a 95% confidence level ($p < 0.05$), and the results were highly significant ($p < 0.0001$). The model's F-value of 14.23 indicates its statistical significance, with *p*-values below 0.0500 confirming the importance of individual model terms. Additionally, the predicted coefficient of determination (predicted R^2) of 0.7187 was in close alignment with the adjusted R^2 of 0.8606. The precision value is 13.50 and suggests that the signal is strong enough to navigate the design space effectively.

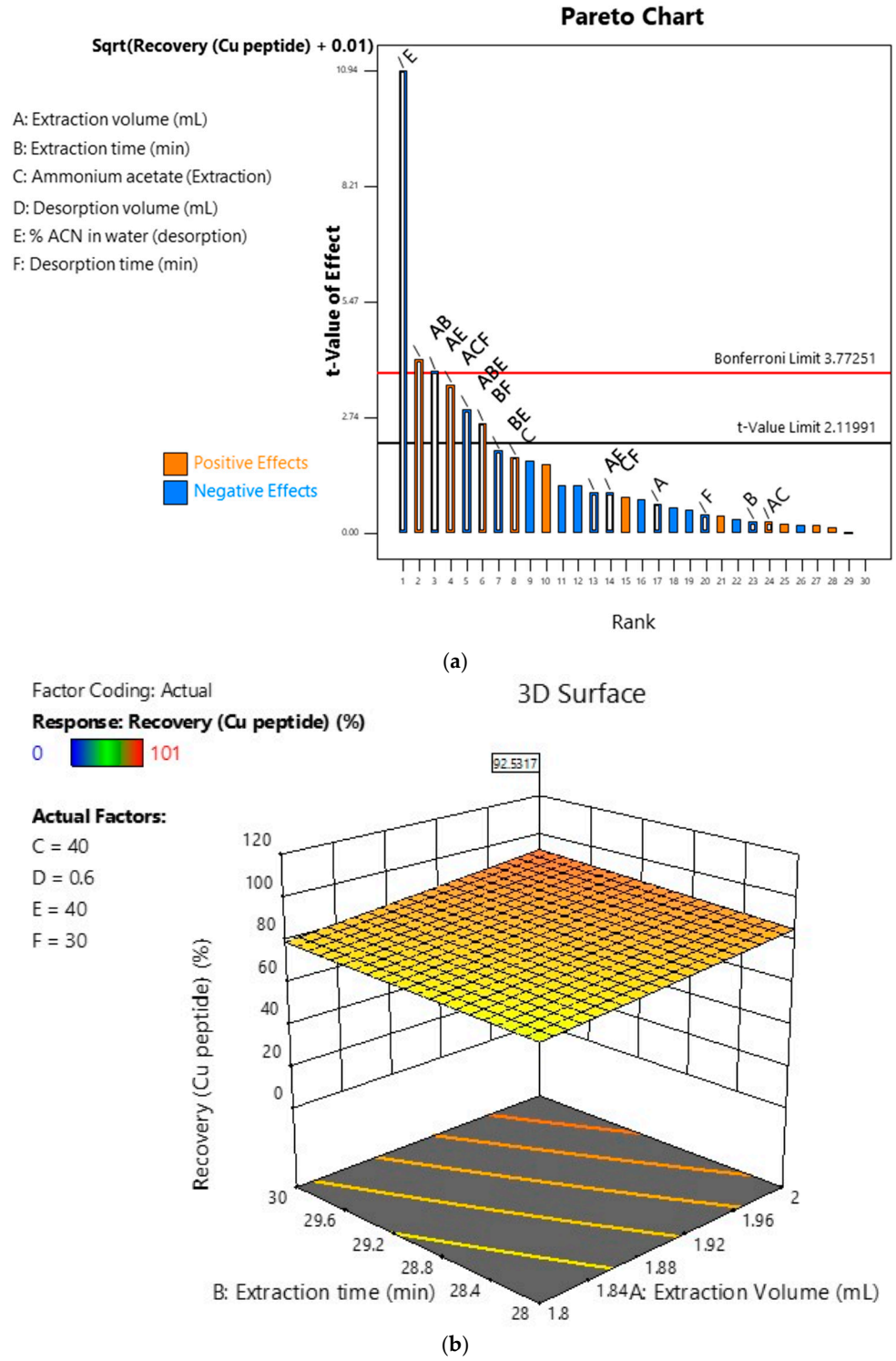


Figure 6. (**a**) Pareto chart displaying the ranked influence of key factors on GHK-Cu recovery (%), in order of decreasing statistical significance, and (**b**) 3D surface plot illustrating the effects of extraction time (min) and extraction volume (mL) on GHK-Cu recovery (%).

This DoE approach offered valuable insights into the influence of various experimental conditions on the % recovery of GHK-Cu from the complex cosmetic cream matrix. As illustrated in the Pareto chart (Figure 6a), the % acetonitrile (ACN) content in the solvent used for desorption had a significant negative impact on the % recovery of GHK-Cu, emerging as the most influential factor with the lowest *p*-value ($p < 0.0001$). This negative effect is likely due to the hydrophilic nature of GHK-Cu, which requires a higher water

content for effective desorption. Several statistically significant interactions between factors were identified, highlighting the value of the DoE approach in investigating the FPSE procedure. Notably, the interaction between extraction volume and extraction time (AB) positively influenced GHK-Cu % recovery, even though neither factor was individually significant. This is demonstrated in Figure 6b, which presents a 3D surface plot showing the effects of extraction time (min) and extraction volume (mL) on GHK-Cu recovery (%). Additionally, the interaction between extraction volume and the % ACN content in the solvent used for desorption (AE) had a negative impact on % recovery. Another significant interaction was observed among extraction volume, the presence of ammonium acetate (40 µL 40 mM ammonium acetate aqueous solution in the extraction solvent mixture) in the extraction, and desorption time, which collectively had a positive effect on % recovery. These inter-parameter interactions underscore the limitations of the one-factor-at-a-time approach, which would likely have led to suboptimal outcomes in optimizing % recovery.

To optimize GHK-Cu recovery, Derringer's desirability function was applied, generating multiple potential solutions. The one with the highest desirability score was chosen for further use. The optimized extraction protocol included an extraction volume of 1.9 mL, a 30 min extraction time with magnetic stirring, and 40 µL 40 mM ammonium acetate aqueous solution in the extraction solvent mixture. For desorption, a solvent mixture of 0.55 mL acetonitrile/water (40:60, v/v) was used, and the process was carried out at 35 °C without magnetic stirring.

3.1.2. Eco-Friendly Aspects of Fabric Phase Sorptive Extraction

FPSE is recognized as a modern and environmentally sustainable sample preparation technique. As outlined in the 12 principles of green analytical chemistry (GAC) [38], FPSE aligns with 10 of these guidelines. A key environmental benefit of FPSE involves simplifying the sample preparation workflow, which reduces the number of steps involved. This streamlining significantly lowers the consumption of organic solvents, removes the need for both pretreatment and post-treatment stages, and enhances its applicability for fieldwork. What sets FPSE apart is the ability to customize membrane sizes depending on the sample volume being analyzed. In the case of biological samples, FPSE enables direct analysis of whole blood without requiring the separation of plasma or serum. By eliminating pretreatment, FPSE minimizes the loss of analytes and enhances the precision and reliability of analytical results.

3.2. Statistical Analysis of Data

3.2.1. Selectivity

The selectivity of the HILIC-UV method for analyzing GHK-Cu in cosmetic creams was thoroughly evaluated. Figure 7 presents a HILIC-UV chromatogram, comparing a cosmetic cream sample (black line) with a blank cream sample (blue line). Both were analyzed using a ZIC®-pHILIC analytical column, with a mobile phase comprising a 133 mM ammonium formate and acetonitrile in a 40:60 (v/v) ratio. The flow rate was set at 0.2 mL/min, and detection was performed at 224 nm.

The chromatograms in Figure 7 show no peaks corresponding to GHK-Cu in the blank cream sample, confirming the high selectivity of the proposed method in distinguishing the targeted analyte from the placebo cream matrix.

Table 2 summarizes the column performance parameters for the analysis of GHK-Cu. The resolution between the GHK-Cu peak and the solvent front is 3.51, indicating adequate separation between adjacent peaks (solvent front and matrix excipients). The symmetry factor is 1.31, meeting the acceptance criterion of 0.8 to 1.8 according to Ph. Eur. guidelines [43]. These results confirm that the proposed system is appropriate for the analysis of GHK-Cu by the proposed zwitterionic HILIC-UV method.

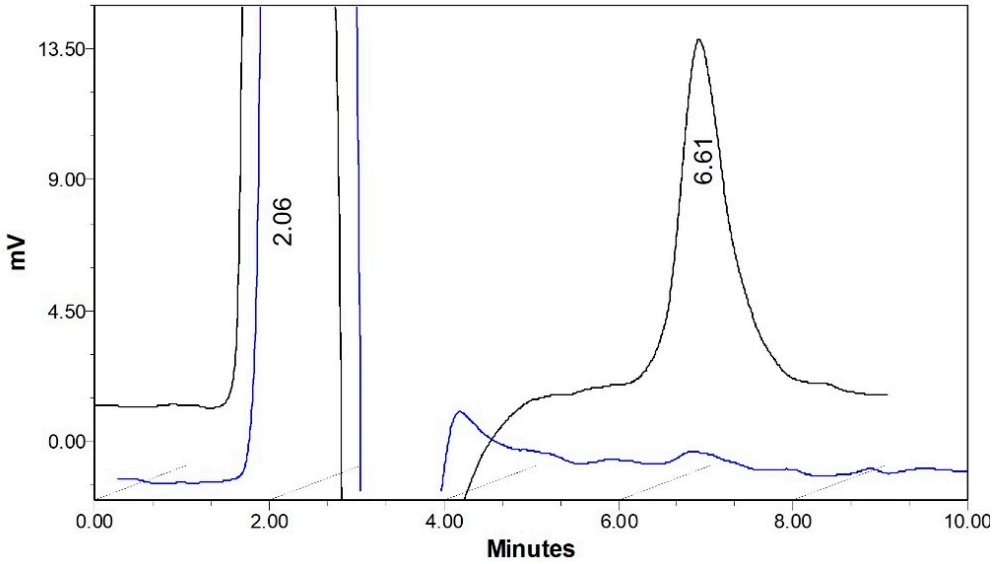


Figure 7. Overlaid chromatograms from FPSE-HILIC-UV analysis of a cosmetic cream containing 0.004% *w*/*w* GHK-Cu (black line) and a placebo cream (blue line), visualized using the *z*-axis in Empower® Software. Chromatographic conditions: ZIC®-pHILIC column; mobile phase: 133 mM ammonium formate (pH 9.0) in acetonitrile (40:60, *v*/*v*); flow rate: 0.2 mL/min; detection at 224 nm.

Table 2. System suitability parameters of the HILIC-UV method upon analysis of a working solution containing 10 µg/mL copper tripeptide (GHK-Cu).

Compound	System Suitability Parameter (CV%) [1]			
	Retention Time (min)	Resolution [2]	Symmetry Factor	N [3]
t0	1.44 (2.0%)			
GHK-Cu	6.44 (0.8%)	3.51 (6.2%)	1.31 (8.9%)	987 (1.9%)

[1] Coefficient of variation was based on ten replicate determinations; [2] resolution is calculated between consecutive peak pairs; [3] EP count number.

3.2.2. Robustness

Robustness was evaluated by measuring system suitability parameters following small, deliberate variations in mobile phase composition. The factors examined included the concentration of ammonium formate in the aqueous phase, the percentage of water in the mobile phase, and the pH of the aqueous buffer at three levels. Replicate injections (n = 3) of a matrix-matched QC sample at 0.004% *v*/*v* GHK-CU were performed under small changes in the parameters. In the data presented in Table 3, it is shown that %CV values ranged from 1.0 to 4.1% for the retention time of GHK-Cu and from 1.9 to 4.1% for the resolution of GHK-Cu peak from the solvent front.

Robustness evaluation data indicate that, under the deliberately varied chromatographic conditions, GHK-Cu was adequately resolved from the solvent front and matrix excipients, with most parameters remaining unchanged. The most prominent effect observed was on the retention time of the analyte, particularly when the pH of the aqueous content in the mobile phase was altered by 0.3. Therefore, precise control of the mobile phase composition in terms of pH is crucial.

Table 3. Robustness evaluation of the HILIC-UV method for the quantification of GHK-Cu.

		Parameters				
Factor Variations	**Level**	**Peak Area**	**Retention Time**	**Peak Symmetry**	**Resolution**	**EP Plate Count**
A: Ammonium formate concentration (mM)						
131	−1	52,353	6.563	1.29	3.496	990
133	0	50,384	6.632	1.31	3.469	991
134	1	47,485	6.502	1.29	3.593	993
% CV		4.9	1.0	1.1	1.9	0.2
B: ΦH_2O (% *v*/*v*)						
39	−1	51,391	6.585	1.40	3.587	981
40	0	50,384	6.632	1.32	3.369	991
41	1	53,841	5.887	1.28	3.348	994
% CV		3.4	2.4	4.6	3.8	6.8
C: pH of the aqueous eluent in the mobile phase						
8.7	−1	48,249	6.743	1.34	3.660	979
9	0	50,384	6.632	1.32	3.470	991
9.3	1	52,553	6.234	1.24	3.380	996
% CV		4.2	4.1	4.0	4.1	1.1

3.2.3. Linearity Data

To quantify GHK-Cu in cosmetic formulations, matrix-matched calibration standards were prepared following the optimized sample preparation procedure, with GHK-Cu concentrations ranging from 0.002% to 0.005% *w*/*w*. Calibration curves were generated and analyzed using linear regression, and data of a typical calibration curve are presented in Table 4. A strong linear correlation ($r^2 > 0.996$) was observed between the normalized peak area ($\times 10^{-4}$; $S_{GHK\text{-}Cu}$) and GHK-Cu concentration ($C_{GHK\text{-}Cu}$). The results of the Student's *t*-test (Table 4) confirmed that the intercept of the regression line was not significantly different from zero, indicating no interference from the cream matrix.

Table 4. Calibration equation parameters for copper tripeptide quantitation.

Parameter	**Value**
Selected linear range in (% *w*/*w*)	0.002–0.005
Regression equation	$S_{GHK\text{-}Cu} = 1818 \times C_{GHK\text{-}Cu} - 0.398$
Correlation coefficient (r)	0.996
Standard error (slope)	88
Standard error (intercept)	0.320
Standard error of the estimate (Sr)	0.1990
LOD % *w*/*w*	5.3×10^{-4}
LOQ % *w*/*w*	0.002
$t_{experimental}$: a/Sa [1]	1.24
t_p, f = 3, *p* = 0.05 [2]	3.182

[1] $t_{experimental}$ = experimental t-value; a = intercept; Sa = standard deviation of the intercept; [2] tp = theoretical t-value; f = degrees of freedom; *p* = *p*-value.

The limit of detection (LOD) and the limit of quantitation (LOQ), for the quantitation of GHK-Cu by the proposed FPSE-HILIC-UV method was calculated based on a signal-to-noise ratio of 3:1 and 10:1, respectively. These values were obtained by analyzing dilute GHK-Cu solutions of known concentration in a placebo cream matrix, prepared

using the optimized sample preparation procedure [44], and the results are presented in Table 2.

3.2.4. Accuracy and Precision

To evaluate the precision of the method within one run and over multiple runs, one-way analysis of variance (ANOVA) was used. Table 5 reports repeatability expressed as intra-day coefficients of variation (CV %) in the range of 1.6% to 2.3%, while total accuracy was expressed as the percent recovery of the analyte, yielding results between 97.9% and 101.3%.

Table 5. Accuracy and precision assessment for quality control samples of Copper Tripeptide (n = 3 runs over 5 replicates).

Concentration Level (% w/w)	0.02	0.03	0.04
Run 1 (mean ± SD)	20.40 (±0.29) × 10^{-4}	29.32 (±0.55) × 10^{-4}	38.96 (±0.62) × 10^{-4}
Run 2 (mean ± SD)	20.46 (±0.68) × 10^{-4}	29.40 (±0.35) × 10^{-4}	40.75 (±1.04) × 10^{-4}
Run 3 (mean ± SD)	19.9 (±0.32) × 10^{-4}	29.43 (±0.45) × 10^{-4}	40.07 (±0.51) × 10^{-4}
Overall mean	20.26 (±0.49) × 10^{-4}	29.38 (±0.43) × 10^{-4}	39.93 (±1.04) × 10^{-4}
Intra-day precision (Repeatability) CV (%) [1]	2.3	1.6	1.9
Total precision, CV (%) [1]	3.9	1.7	5.4
Total accuracy, recovery (%) [2]	101.3	97.9	99.8

[1] Coefficient of variation, calculated by ANOVA; [2] relative percentage recovery.

3.3. Recovery Evaluation

The % recovery of the optimum FPSE procedure was evaluated at two GHK-Cu concentration levels, 0.002% and 0.005% w/w, with recoveries of 80.2 ± 1.6% (n = 3) and 78.5 ± 2.4% (n = 3), respectively.

3.4. Application for the Analysis of Actual Cream Samples

The applicability of the developed FPSE-HILIC-UV method was tested by analyzing three different batches of a cosmetic formulation containing 0.004% w/w GHK-Cu. The results are presented in Table 6. As shown, the method exhibited excellent precision and accuracy, with %CV values ranging from 2.6% to 2.9% and GHK-Cu recoveries between 99.1% and 103.4%.

Table 6. Quantification of GHK-Cu in cosmetic creams.

Batch No	% Label Claim (±SD) [1] (n=5)	% CV [2]	% Recovery (±SD) [1] (n=5)
Batch No GHK-Cu1	0.00413 (±0.00011)	2.6	103.4 (±2.6)
Batch No GHK-Cu2	0.00406 (±0.00012)	2.9	101.4 (±2.9)
Batch No GHK-Cu3	0.0039 (±0.0001)	2.6	99.1 (±2.6)

[1] Standard deviation; [2] % coefficient of variation.

4. Conclusions

The increasing use of peptides in cosmetic formulations underscores the need for precise analytical methods to quantify active ingredients, such as biomimetic peptides, at very low concentrations. In this study, an FPSE-HILIC-UV method was developed and validated for the accurate analysis of copper tripeptide (GHK-Cu) in cosmetic creams.

The chromatographic behavior of GHK-Cu on a Zic-pHILiC column was thoroughly investigated, providing valuable insights into its retention behavior in zwitterionic HILIC columns. Furthermore, a DoE approach was employed to optimize the FPSE sample preparation procedure for maximum % recovery of GHK-Cu. The DoE analysis revealed critical interactions between key factors selected for investigation, such as extraction volume, extraction and desorption time, and the composition of the desorption solvent mixture. This optimization significantly enhanced the % recovery of GHK-Cu. The method proved reliable for the quantitation of the targeted analyte, with effective sample preparation and a chromatographic run time of less than 10 min, making it ideal for high-throughput applications in the quality control of cosmetics containing the analyzed tripeptide. The simplicity of the proposed method and the lack of specialized detection equipment further enhance its suitability for routine analysis of cosmetic creams containing GHK-Cu. Additionally, this method offers significant potential for broader applications in the quantification of other peptides, potentially driving future innovations in the quality control of cosmetics incorporating peptides.

Author Contributions: Conceptualization, I.P., A.K., A.V., E.T., S.P. and V.S.; methodology, A.P., A.K., A.V., S.P., V.S., K.G.F. and I.P.; validation, P.P., A.P. and I.P.; formal analysis, P.P., A.P. and I.P.; investigation, A.P. and P.P.; resources, I.P., A.K., K.G.F., A.V., E.T. and S.P.; writing—original draft preparation, P.P. and I.P.; writing—review and editing, I.P., A.K., K.G.F., V.S., A.V. and S.P.; supervision, I.P. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Data Availability Statement: Data are available upon request to the corresponding author.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Gupta, M.; Gupta, A. Photodamaged skin and quality of life: Reasons for therapy. *J. Dermatol. Treat.* **1996**, *7*, 261–264. [CrossRef]
2. Draelos, Z.D. Cosmeceuticals: Efficacy and Influence on Skin Tone. *Dermatol. Clin.* **2014**, *32*, 137–143. [CrossRef] [PubMed]
3. Ledwoń, P.; Errante, F.; Papini, A.M.; Rovero, P.; Latajka, R. Peptides as Active Ingredients: A Challenge for Cosmeceutical Industry. *Chem. Biodivers.* **2021**, *18*, e2000833. [CrossRef] [PubMed]
4. Algermissen, E. Wound healing: An overview. *BDJ Stud.* **2023**, *30*, 24–26. [CrossRef]
5. Ngoc, L.T.N.; Moon, J.-Y.; Lee, Y.-C. Insights into Bioactive Peptides in Cosmetics. *Cosmetics* **2023**, *10*, 111. [CrossRef]
6. Fields, K.; Falla, T.J.; Rodan, K.; Bush, L. Bioactive peptides: Signaling the future. *J. Cosmet. Dermatol.* **2009**, *8*, 8–13. [CrossRef]
7. Veiga, E. Anti-aging peptides for advanced skincare: Focus on nanodelivery systems. *J. Drug Deliv. Sci. Technol.* **2023**, *89*, 105087. [CrossRef]
8. Pickart, L.; Vasquez-Soltero, J.M.; Margolina, A. GHK and DNA: Resetting the human genome to health. *BioMed Res. Int.* **2014**, *2014*, 151479. [CrossRef]
9. Tapiero, H.; Townsend, D.M.; Tew, K.D. Trace elements in human physiology and pathology. *Copper. Biomed. Pharmacother.* **2003**, *57*, 386–398. [CrossRef]
10. Finkley, M.B.; Appa, Y.; Bhandarkar, S. Copper Peptide and Skin. In *Cosmeceuticals and Active Cosmetics: Drugs vs. Cosmetics*; Elsner, P., Maibach, H.I., Eds.; Marcel Dekker: New York, NY, USA, 2005; pp. 549–563.
11. Schagen, S.K. Topical Peptide Treatments with Effective Anti-Aging Results. *Cosmetics* **2017**, *4*, 16. [CrossRef]
12. Pickart, L.; Margolina, A. Regenerative and Protective Actions of the GHK-Cu Peptide in the Light of the New Gene Data. *Int. J. Mol. Sci.* **2018**, *19*, 1987. [CrossRef] [PubMed]
13. Mazurowska, L.; Mojski, M. Biological Activities of Selected Peptides: Skin Penetration Ability of Copper Complexes with Peptides. *J. Cosmet. Sci.* **2008**, *59*, 59–69. [PubMed]
14. Wegrowski, Y.; Maquart, F.X.; Borel, J.P. Stimulation of sulfated glycosaminoglycan synthesis by the tripeptide-copper complex glycyl-L-histidyl-L-lysine-Cu^{2+}. *Life Sci.* **1992**, *51*, 1049–1056. [CrossRef] [PubMed]
15. Pesáková, V.; Novotná, J.; Adam, M. Effect of the tripeptide glycyl-L-histidyl-L-lysine on the proliferation and synthetic activity of chick embryo chondrocytes. *Biomaterials* **1995**, *16*, 911–915. [CrossRef] [PubMed]
16. Dymek, M.; Olechowska, K.; Hąc-Wydro, K.; Sikora, E. Liposomes as Carriers of GHK-Cu Tripeptide for Cosmetic Application. *Pharmaceutics* **2023**, *15*, 2485. [CrossRef]
17. Jiang, Y.; Wu, Z.; Liu, Z.; Hong, M.; Huang, Y. Synergy of GHK-Cu and hyaluronic acid on collagen IV upregulation via fibroblast and ex-vivo skin tests. *J. Cosmet. Dermatol.* **2023**, *22*, 2598–2604. [CrossRef]

18. Pickart, L.; Margolina, A. Skin Regenerative and Anti-Cancer Actions of Copper Peptides. *Cosmetics* **2018**, *5*, 29. [CrossRef]
19. Pickart, L.; Vasquez-Soltero, J.M.; Margolina, A. The Human Tripeptide GHK-Cu in Prevention of Oxidative Stress and Degenerative Conditions of Aging: Implications for Cognitive Health. *Oxidative Med. Cell. Longev.* **2012**, *2012*, 324832. [CrossRef]
20. Baig, M.H.; Ahmad, K.; Rabbani, G.; Choi, I. Use of Peptides for the Management of Alzheimer's Disease: Diagnosis and Inhibition. *Front. Aging Neurosci.* **2018**, *10*, 21. [CrossRef]
21. Xu, X.; Pan, Y.; King, F. Extending the applicability of pulsed glow discharge mass spectrometry to GHK-Cu determination. *Int. J. Mass Spectrom.* **2020**, *449*, 116274. [CrossRef]
22. Badenhorst, T.; Svirskis, D.; Wu, Z. Physicochemical characterization of native glycyl-l-histidyl-l-lysine tripeptide for wound healing and anti-aging: A preformulation study for dermal delivery. *Pharm. Dev. Technol.* **2016**, *21*, 152–160. [CrossRef] [PubMed]
23. Papagianni, P.; Varvaresou, A.; Papageorgiou, S.; Panderi, I. Development and validation of an ion-pair RP-HPLC method for the determination of oligopeptide-20 in cosmeceuticals. *J. Pharm. Biomed. Anal.* **2011**, *56*, 645–649. [CrossRef] [PubMed]
24. Fountain, K.J.; Xu, J.; Diehl, D.M.; Morrison, D. Influence of stationary phase chemistry and mobile-phase composition on retention, selectivity, and MS response in hydrophilic interaction chromatography. *J. Sep. Sci.* **2010**, *33*, 740–751. [CrossRef] [PubMed]
25. Al Musaimi, O.; Jaradat, D.M.M. Advances in Therapeutic Peptides Separation and Purification. *Separations* **2024**, *11*, 233. [CrossRef]
26. Hemström, P.; Irgum, K. Hydrophilic interaction chromatography. *J. Sep. Sci.* **2006**, *29*, 1784–1821. [CrossRef]
27. D'Atri, V.; Fekete, S.; Clarke, A.; Veuthey, J.-L.; Guillarme, D. Recent Advances in Chromatography for Pharmaceutical Analysis. *Anal. Chem.* **2019**, *91*, 210–239. [CrossRef]
28. Pedrali, A.; Bleve, M.; Capra, P.; Jonsson, T.; Massolini, G.; Perugini, P.; Marrubini, G. Determination of N-acetylglucosamine in cosmetic formulations and skin test samples by hydrophilic interaction liquid chromatography and UV detection. *J. Pharm. Biomed. Anal.* **2015**, *107*, 125–130. [CrossRef]
29. Chirita, R.I.; Chaimbault, P.; Archambault, J.C.; Robert, I.; Elfakir, C. Development of a LC–MS/MS method to monitor palmitoyl peptides content in anti-wrinkle cosmetics. *Anal. Chim. Acta* **2009**, *641*, 95–100. [CrossRef]
30. Doulou, E.; Kalomiraki, M.; Parla, A.; Thermos, K.; Chaniotakis, N.A.; Panderi, I. Hydrophilic Interaction Liquid Chromatography Coupled with Fluorescence Detection (HILIC-FL) for the Quantitation of Octreotide in Injection Forms. *Analytica* **2021**, *2*, 121–129. [CrossRef]
31. Hernandez-Hernandez, O.; Quintanilla-Lopez, J.E.; Lebron-Aguilar, R.; Sanz, M.L.; Moreno, F.J. Characterization of post-translationally modified peptides by hydrophilic interaction and reverse phase liquid chromatography coupled to quadrupole-time-of-flight mass spectrometry. *J. Chromatogr. A* **2016**, *1428*, 202–211. [CrossRef]
32. Raikou, V.; Kalogria, E.; Varvaresou, A.; Tsirivas, E.; Panderi, I. Quantitation of Acetyl Hexapeptide-8 in Cosmetics by Hydrophilic Interaction Liquid Chromatography Coupled to Photo Diode Array Detection. *Separations* **2021**, *8*, 125. [CrossRef]
33. Zhou, W.; Wang, P.G.; Krynitsky, A.J.; Rader, J.I. Rapid and simultaneous determination of hexapeptides (Ac-EEMQRR-amide and H2N-EEMQRR-amide) in anti-wrinkle cosmetics by hydrophilic interaction liquid chromatography-solid phase extraction preparation and hydrophilic interaction liquid chromatography with tandem mass spectrometry. *J. Chromatogr. A* **2011**, *1218*, 7956–7963. [CrossRef] [PubMed]
34. Giannakou, M.; Varvaresou, A.; Kiriazopoulos, E.; Papageorgiou, S.; Kavvalou, E.; Tsirivas, E.; Panderi, I. Quantification of oligopeptide-20 and oligopeptide-24 in cosmetic creams using hydrophilic interaction liquid chromatography with electrospray ionization mass spectrometry. *SCS Plus* **2018**, *1*, 159–167. [CrossRef]
35. Tengattini, S.; Orlandi, G.; Perteghella, S.; Bari, E.; Amadio, M.; Calleri, E.; Massolini, G.; Torre, M.L.; Temporini, C. Chromatographic profiling of silk sericin for biomedical and cosmetic use by complementary hydrophylic, reversed phase and size exclusion chromatographic methods. *J. Pharm. Biomed. Anal.* **2020**, *186*, 113291. [CrossRef]
36. Kabir, A.; Mesa, R.; Jurmain, J.; Furton, K.G. Fabric Phase Sorptive Extraction Explained. *Separations* **2017**, *4*, 21. [CrossRef]
37. Weissman, S.A.; Anderson, N.G. Design of Experiments (DoE) and Process Optimization. A Review of Recent Publications. *Org. Process Res. Dev.* **2015**, *19*, 1605–1633. [CrossRef]
38. López-Lorente, Á.I.; Pena-Pereira, F.; Pedersen-Bjergaard, S.; Zuin, V.G.; Ozkan, S.A.; Psillakis, E. The ten principles of green sample preparation. *TrAC Trends Anal. Chem.* **2022**, *148*, 116530. [CrossRef]
39. Kabir, A.; Furton, K.G. Fabric Phase Sorptive Extractors (FPSE). United States Patent Application Publication. US 2014/0274660 A1, 18 September 2014.
40. ICH Guideline Q2(R2) Validation of Analytical Procedures, Final Version, 1 November 2023. Available online: https://database.ich.org/sites/default/files/ICH_Q2(R2)_Guideline_2023_1130.pdf (accessed on 22 September 2024).
41. Panderi, I.; Malamos, Y.; Machairas, G.; Zaharaki, S. Investigation of the retention mechanism of cephalosporins by zwitterionic hydrophilic interaction liquid chromatography. *Chromatographia* **2016**, *79*, 995–1002. [CrossRef]
42. Kabir, A.; Samanidou, V. Fabric Phase Sorptive Extraction: A Paradigm Shift Approach in Analytical and Bioanalytical Sample Preparation. *Molecules* **2021**, *26*, 865. [CrossRef]

43. European Pharmacopoeia (Ph. Eur.) 11th Edition, 2.2.46. Chromatographic Separation Techniques, Implemented Date 1 January 2023. Available online: https://www.edqm.eu/en/-/general-chapter-2.2.46.-chromatographic-separation-techniques-now-published-in-ph.-eur.-11th-edition (accessed on 10 September 2024).
44. *British Pharmacopoeia*; Her Majesty's Stationery Office: London, UK, 2004; Volume II, pp. 1428–1429.

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.