Isothiazolinones Quantification in Shampoo Matrices: A Matter of Method Optimization or Stability Driven by Interactions?

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Abstract: Methylisothiazolinone (MI) is one of the most used preservatives in shampoos and also one of the most effective. A preservative mixture known as Kathon™ CG is commercially available. It contains 5-chloro-2-methyl-4-isothiazolin-3-one (CMI) and 2-methyl-4-isothiazolin-3-one (MI) (3:1) and stabilizers. The aim of this study is to evaluate the influence of formulation factors in the quantification and stability of isothiazolinones in shampoos. Two shampoo bases containing Kathon™ CG as a preservative were prepared. Some ingredients that are at risk of interfering with the preservative stability were added to these formulations. The preservative was quantified by HPLC-DAD (High-performance liquid chromatography with a diode-array detector) after preparation of the formulation and after storage at room temperature and at 40 °C. The addition of magnesium silicate proved to be essential for the breakdown of the interaction between the matrix and the analytes in the extraction procedure. The content of CMI/MI decreased right after preparation indicating that immediate interactions between CMI/MI and the ingredients may have occurred after preparation resulting in a decrease in the preservative concentration. Detrimental interactions between the ingredients, regarding the stability of the isothiazolinones were detected immediately after preparation and over time resulting in the reduction of CMI/MI concentration in these cosmetic shampoos.

Keywords: cosmetics; shampoo; cosmetic ingredients; preservatives; methylisothiazolinone; chloromethylisothiazolinone; dermatitis

1. Introduction

Hair cosmetics, currently perceived as substances or mixtures that aim to clean, perfume, protect or keep the hair in good condition [1], represent a wide variety of products that are used with high frequency. In one study conducted by Ficheux et al. among the French population, it was reported that 98% of adult women use shampoo (frequency of use 0.44–0.67 per day) and 57% use conditioner [2]. In fact, in the middle of the 20th century there was an exponential growth in the market of these products, as a consequence of changes in socioeconomic status, greater image appreciation and facilitated access to products [3].

Hair cleaning is the main purpose of shampoo as a cosmetic product, so surfactants are core ingredients of such products [3]. Surfactants are molecules presenting a lipophilic end that binds to small tallow droplets and a hydrophilic end, which allows the formation of micelles and have the ability to lower surface tension, destroy clusters and emulsify fats, in order to facilitate their

elimination [3–6]. Based on their hydrophilic group, surfactants are classified as anionic (deep cleaning ability and high foaming capacity, e.g. sulfates), cationic (less cleaning capacity, but confer combing and smoothness properties to conditioners), amphoteric and nonionic (both mild surfactants mostly used in the formulation of baby shampoos) [3,4,6].

In addition to surfactants, shampoos contain other components that make them more appealing regarding the preferences of consumers, such as viscosifiers, opacifiers, perfumes, complexing agents, colorants, conditioners, antioxidants, pH stabilizers, humectants, moisturizers and preservatives dispersed or dissolved in water [3,4,6–10].

Sulfate-free shampoos are a trend in hair cosmetics, particularly for those consumers with dry hair and a sensitive scalp. In fact, despite the excellent cleansing and sebum removal properties associated with sulfate surfactants, they have the disadvantage of being very aggressive to the scalp, which can cause irritation and dry hair by removing the lipids from the cuticle [3,7].

Cosmetic ingredients are frequently mixtures of substances, which, according to the European regulation, shall all be mentioned in the label of the final product in descending order of their concentration, using the International Nomenclature of Cosmetic Ingredients (INCI). As a result of this complex mixture, cosmetic labels are often long lists of ingredients that can be difficult to understand [1,11].

The presence of microorganisms in cosmetics can be a threat to the health of consumers so it is mandatory that, although these are generally non-sterile products, the level of microorganisms is within the defined limits throughout the storage and use period. The use of preservatives inhibits the growth of microorganisms and extends the period of safe use of the product [12,13]. This is particularly important for cosmetics, since, unlike other products, they may be labeled only with indication of the "period after opening" (instead of the expiration date) provided that the product has been shown to be stable for more than 30 months.

In the European Union, the Annex V of Regulation (CE) 1223/2009 defines which substances are allowed to be used as preservatives in cosmetics, in what type of products (for example leave-on or rinse-off products) and their maximum concentration allowed. Over the years several preservatives have been limited in use or even withdrawn from Annex V as a result of risk evidence mainly related with their sensitization or endocrine disruption potential [14]. Updates on Annex V are mainly driven by the opinions of Scientific Committee on Consumer Safety (SCCS) of the European Commission, which, after analyzing the available scientific information on the ingredient, including reported adverse effects (as a result of cosmetovigilance), may recommend to maintain, restrict or even exclude the substance from Annex V [15]. Limitations on the availability of allowed preservatives for cosmetics raise concerns and difficulties among formulators and cosmetic companies.

Methylisothiazolinone (MI) is one of the most used preservatives in shampoos and one of the most effective [16–18].

A preservative mixture known as Kathon™ CG is commercially available. It contains 5-chloro-2-methyl-4-isothiazolin-3-one (CMI) and 2-methyl-4-isothiazolin-3-one (MI; 3:1), magnesium nitrate and magnesium chloride as stabilizers [18].

Isothiazolinones exhibit excellent broad-spectrum antimicrobial activity against gram positive, gram negative bacteria and fungus at low concentrations and over a wide range of pH [12,17–20].

Isothiazolinones are electrophilic compounds due to the sulfur heterocycle and consequently react with nucleophilic molecules. Thus, they bind to the thiol groups of proteins and consequently inhibit the activity of enzymes that are essential for growth and metabolism, which leads to microbial cell death after a few hours of contact [17,19,21].

The quantification of preservatives in cosmetic products is essential to ensure that the product is protected from microbial growth while complying with the legal concentration limits, thus ensuring the safety of the product.

In the case of MI and CMI the main associated adverse effect is contact dermatitis, as they are skin irritants and sensitizers. The binding of an activated N–S bond to a nucleophilic center present on the

skin proteins explains the potential cutaneous toxicity. Additionally, the presence of contaminants or impurities resulting from the preservative manufacturing process, even in small amounts, may contribute to this toxicity [18,22–28].

Some studies suggest that repeated exposure to MI even in rinse-off products, although very short, allows for the penetration of the preservative into the stratum corneum, binding to skin proteins and accumulation, reaching an epidermal concentration that initiates immune responses responsible for contact dermatitis. This may be particularly significant in products containing surfactants as they increase the penetration of MI through the skin [29].

In a study carried out in Portugal, the number of positive CMI/MI patch tests increased from 1.5% in 2006 to 2.9% in 2011 and 3.6% in 2012 [24,30]. These data are in accordance with other European countries: in Germany the positive patch test results increased from 2.3% (2009) to 3.9% (2012), and in the UK from 0.9% to 4.9% [24,31,32].

Due to the potential induction of contact dermatitis, maximum limits of use have been defined. The maximum concentration of the CMI/MI mixture in the ratio 3:1 was set by Regulation (EC) 1003/2014 as 0.0015% in rinse-off products, while MI alone was still allowed for leave-on products. [33]. Later on, Regulation (CE) 2016/1198 limited the use of MI to rinse-off products, keeping the maximum authorized concentration (0.01%) [25]. The latter was recently reduced to 0.0015% by Regulation 2017/1224 [34]

Stability of the preservative system throughout the production, storage and use processes are essential for microbiological quality of the products [13].

For CMI/MI it is known that the temperature is one of the factors that influence their stability and so it should not exceed 50 °C. Another factor to consider is pH, because the CMI/MI mixture is stable only up to pH 8, which is not problematic for most shampoos [18].

Additionally, there is an inherent instability of the isothiazolinone molecule driven by the opening of the isothiazolinone ring by a nucleophilic attack on the sulfur ring, which results in the loss of the electrophilic reactivity and, therefore, of the antimicrobial activity. In the CMI molecule, there is an additional electrophilic center, and the chlorine atom is responsible for the increased instability of CMI compared to the MI molecule [24,35].

The high levels of impurities resulting from the manufacturing processes also contribute to make the mixture unstable, so it becomes necessary to add stabilizers (such as magnesium salts). Various attempts have been made to manufacture pure isothiazolinones so that they are not so dependent on stabilizers, allowing for the reduction of the magnesium salts concentration.

Furthermore, the presence of amines, especially secondary amines, and amine derivatives are detrimental to the stability of the isothiazolinones. This drawback can be overcome with a pH reduction below 7, which allows the amine to be converted to the corresponding acid salt. Another interference that has been referred in the literature occurs with thiols (present in cysteine and zinc pyrithione, for example). Shampoos with repairing and fortifying claims often contain keratin hydrolysates (rich in thiol groups), which are available to react with the isothiazolinone ring. Reducing agents contained in some sulfated and sulphonated surfactants as the residual sulphite or bisulfite groups may impair the stability of the preservative [18].

The complexity of cosmetic products matrices may represent a source of interactions and, therefore, a challenge for ingredient stability, particularly for CMI/MI stability. Liquid chromatography has been one of the most widely used methods to quantify preservatives in cosmetics, namely isothiazolinones [36–39]. In previous studies, Baranowska and Wojciechowska described the quantification of CMI and MI in shower gel and shampoo through HPLC. Furthermore, the recovery percentages were determined in two different cosmetic matrices (gel and cream with a composition similar to the composition of the tested samples, but not containing the analytes). In the cream matrix, the average recovery for MI was 97.3% and for CMI was 79.5%. In the gel matrix, the average recovery for MI was 92.8% and for CMI was 84.3%. These data suggest that the quantification of isothiazolinones is affected by the type of cosmetic matrix [38].

In another research, Alvarez-Rivera et al. developed a methodology to quantify isothiazolinones in cosmetic products by matrix solid-phase dispersion followed by high-performance liquid chromatography–tandem mass spectrometry. The average recoveries of preservatives from the shampoos were 45.6% for MI and 92.0% for CMI (shampoo 1) and 60.6% for MI and 86.4% for CMI (shampoo 2). These data suggest the influence of cosmetic ingredients on the quantification of isothiazolinones [39].

The aim of this research is to study the influence of formulation factors on the quantification and stability of isothiazolinones in shampoos.

2. Materials and Methods

2.1. Material

For the shampoos' preparation the following cosmetic ingredients were used: Amisoft®ECS-22SB—disodium cocoyl glutamate (Ajinomoto, Tokyo, Japan), Dehyton®AB30—coco-betaine (BASF, Madrid, Spain), Eur-amid®V—cocamide DEA (Sapeq Quimica, Setúbal, Portugal), Kathon^TM CG—methylchloroisothiazolinone and methylisothiazolinone (Dow Inc., Midland, MI, USA), Oramix^TM NS10—decyl glucoside (Seppic, Marinha Grande, Portugal), zinc pyrithione (Acofarma, Madrid, Spain), Plantapon®LGC Sorb—sodium lauryl glucose carboxylate (BASF, Madrid, Spain), Sepimax Zen^TM—polyacrylate crosspolymer-6 (Seppic, Marinha Grande, Portugal), Tensagex®EOC670—sodium laureth sulfate (DSProd. Quimicos, Portugal), Varisoft®100—hydrolyzed keratin (Evonik Nutrition & Care GmbH, Essen, Germany) and Varisoft®BT85—behentrimonium chloride (Evonik Nutrition & Care GmbH, Essen, Germany).

Citric acid (Guinama, Valencia, Spain), sodium hydroxide (VWR, Amadora, Portugal) were used for adjustment of pH and ultra pure water was obtained in-house (MilliQ, Millipore, Burlington, MA, USA).

Other reagents used in the experimental procedures were: acetic acid 0.4% v/v (VWR, Amadora, Portugal), Florisil®—magnesium silicate (VWR, Amadora, Portugal), methanol (VWR, Amadora, Portugal) and ProClin™ 150—methylisothiazolinone and chloromethylisothiazolinone (Sigma–Aldrich, Lisbon, Portugal).

2.2. Formulations' Preparation

The base formulation 1 (BF1) was prepared as follows: 40% Tensagex®EOC670 (sodium laureth sulfate) was heated in a water bath (VWR B26®) to 30–40 °C. 30% Dehyton®AB30 (coco-betaine) and 30% ultrapure water were weighed into a second beaker and 100 μL of Kathon™ CG (methylisothiazolinone and chloromethylisothiazolinone) per 100 g of formulation were added. The second beaker was placed in the bath and shaken occasionally. When the contents of both beakers reached 30–40 °C, the contents of the second beaker were added to the first and then manually stirred. The mixture was cooled to room temperature and then homogenized with a helix stirrer (Heidolph RZR 2041®), at 130 rpm for 2 min. pH was adjusted to 5.5–6.5 (pH meter Mettler Toledo SevenCompact pH/ion S220)).

The base formulation 2 represents the "sulphate free" trend for shampoo. For the preparation of this base, 1% of Sepimax Zen™ (polyacrylate crosspolymer-6) was added to 60% ultrapure water, at room temperature and manually shaken. The mixture was then heated at 70–80 °C, in a water bath, and stirred occasionally. Fifteen percent Plantapon®LGC Sorb (sodium lauryl glucose carboxylate), 15% Oramix ™ NS10 (decyl glucoside) and 10% Dehyton®AB30 (coco-betaine) were weighed and added to the above mixture. The homogeneous mixture was cooled to room temperature. Then 100 μL of Kathon™ CG (methylisothiazolinone and chloromethylisothiazolinone) per 100 g of formulation was added. The mixture was homogenized through mechanical stirrer at 130 rpm for 2 min and the pH was adjusted to 5.5–6.5. The following ingredients were added to the base formulations: 1% zinc

pyrithione, 0.1% Varisoft BT85 (behentrimonium chloride, 1% Vari®ker 100 (hydrolyzed keratin), 4% Amisoft ECS-22SB (disodium cocoyl glutamate) and 2.5% Eur-amid V (cocamide DEA).

Two lots of formulations were prepared: one lot was analyzed after preparation and another lot was analyzed after a storage period. Samples were stored for 5 months at room temperature and for 2 months at room temperature followed by 3 months at 40 °C. All formulations were packed in opaque plastic containers of similar characteristics, with an identical air exposure area (when open, this is when the cap is removed), all opaque.

2.3. Extraction and Quantification Procedure

The extraction and quantification procedure were optimized.

For the sample extraction, 1 g of shampoo, 1 g of Florisil® (magnesium silicate) and 10 mL of methanol were added. Vortexing was then performed for 30 s (the time required for visually identified homogenization) and centrifugation at 4500 rpm for 5 min (centrifuge Heraeus Megafuge 8R®).

The quantification of the CMI/MI mixture in the formulations was performed by high performance liquid chromatography (HPLC)using an equipment composed by a diode array detector (DAD): CM 5430; oven: CM 5310; automatic sampler: CM 5260; pump: CM 5160; ACE Equivalence ™ C18 reverse phase column with 3.5 μm porosity, 25 cm in length and 4.6 mm in diameter.

In the assay, the gradient mode was followed. The percentage of acetic acid 0.4% decreased from 80% to 20% and the percentage of methanol increased from 20% to 80% over the course of 20 min.

The flow was 1 mL minute^{-1} and the wavelength 275 nm. The running time was 20 min and the balance time between injections was 5 min. The injection volume was 5 μL. The temperature in the oven was 30 °C and in the autosampler was 4 °C.

For the preparation of the calibration curves the ProClin™ 150 standard (a solution containing 1.14% of CMI and 0.38% of MI) was used [40].

Table 1 presents the preparation of standards for these curves.

Table 1. ProClin^{TM} 150 standards preparation for the calibration curves.

	Concentration MI μg mL^{-1}	Concentration CMI μg mL^{-1}
P1	0.1187	0.3562
P2	0.2375	0.7125
P3	0.4750	1.4250
P4	0.9500	2.8500
P5	2.3750	7.1250

The quantification limit (LQ) corresponds to the first calibration curve standard (P1). For this extraction volume the MI LQ was 0.1187 μg mL^{-1} (equivalent to 0.0001187% w/w) and for CMI was 0.3562 μg mL^{-1} (equivalent to 0.0003562% w/w).

Recovery assays were performed to evidence the preservative extraction. For this purpose, the formulations were fortified with a known concentration of preservative: Proclin™ 150 standard (0.000855% (w/w) MI and 0.002565% (w/w) CMI) was added to the samples.

The fortification of the base formulations with Proclin™ 150 standard was performed on the day the formulations were prepared, representing the addition of 0.0034% (w/w) CMI/MI. Furthermore, fortification of the formulations with Kathon^{TM} CG 3 days after the preparation was also performed, which represents the addition of 0.0015% (w/w) CMI/MI.

The recovery percentage was calculated according to the following equation [39]:

$$Recovery\ \% = \frac{(Concentration\ obtained\ after\ fortification\ - Concentration\ obtained\ before\ fortification)}{theoric\ concentration\ added} \times 100.$$

2.4. Preservative Quantification after Preparation and Recovery Tests

Recovery tests with Proclin[TM] were initially performed on the base formulations with and without Florisil®in the extraction procedure.

Quantification was performed 3 days after the preparation of the formulations as described.

To validate the method, samples were fortified with Kathon[TM] CG. The fortification consisted of adding 1 μL of Kathon[TM] CG to 1 g of sample immediately prior to the extraction procedure.

In order to study if the optimized method allows assaying in complex samples, quantification was performed on commercial shampoos, acquired in a supermarket in Portugal.

2.5. Study of Storage Conditions Influence on the Stability of Isothiazolinones

For the stability testing, the formulations were analyzed regarding organoleptic characteristics and preservative amount.

The color and appearance of the product were observed in the lot of samples analyzed after preparation and in the lot of samples subject to storage. These parameters were visually determined by the same operator. Photographs were taken throughout time to support the identification of changes regarding organoleptic characteristics.

Quantification after storage was performed as described above.

3. Results and Discussion

3.1. Extraction and Quantification Procedure

Table 2 shows the results obtained for the quantification of CMI and MI in the base formulations BF1 and BF2 with and without magnesium silicate addition in extraction procedure. Recovery tests were initially performed with ProclinTM 150 addition on the base formulations (Table 3).

Table 2. Extraction of the CMI/MI mixture in the base formulations with and without magnesium silicate addition in extraction procedure [36].

Extraction Procedure Tested	% w/w MI	% w/w CMI	% w/w CMI/MI
BF1 without Magnesium Silicate	0.000262	0.000862	0.001124
BF1 with Magnesium Silicate	0.000155	0.000888	0.001043
BF2 without Magnesium Silicate	<LQ [1]	<LQ [1]	
BF2 with Magnesium Silicate	0.000239	0.000874	0.001112

[1] LQ (limit of quantification) MI = 0.0001187% w/w and LQ CMI = 0.0003562% w/w.

Magnesium silicate addition was fundamental to MI/CMI quantification in the BF2 (Table 2) since it was not possible to quantify MI/CMI without magnesium silicate addition (the result was inferior to the limit of quantification for both analytes).

Whenever the determination was lower than the limit of quantification (LQ), the concentration of P1 (lower concentration standard of the calibration line) was considered for the purpose of calculations.

The base formulations were fortified with ProclinTM 150 (reference substance) and the extraction was performed with and without magnesium silicate addition. The fortification with ProclinTM 150 represents the addition of 0.000855% (w/w) MI and 0.002565% (w/w) CMI.

This quantification and fortification were made on the day the formulations were prepared.

After fortification with the standard it was found that the highest recovery percentages were achieved in samples where magnesium silicate had been added on extraction (Table 3). Therefore, this was the extraction method chosen for all samples (1 g of shampoo, 1 g of Florisil® and 10 mL of methanol were added. Vortexing was then performed for 30 s and centrifugation at 4500 rpm for 5 min). The recovery percentages were within the limit usually accepted (80% to 120%) [41].

Magnesium silicate acts as a purifying agent because of its adsorbent capacity, since it is a porous substance with a large surface area. So, magnesium silicate can remove interferents present in the matrix, acting as an interference retainer and dispersive clean-up sorbent. Its efficiency in isothiazolinones extraction was also proven by Alvarez-Rivera et al., who used the solid phase matrix dispersion, where Florisil®(magnesium silicate) was the dispersive phase and methanol was the eluting solvent [39].

Table 3. Fortification of formulations with standard Proclin[TM] 150 (addition of 0.00342% CMI/MI) with and without magnesium silicate addition.

Fortification of Formulations	% (w/w) MI	% (w/w) CMI	% (w/w) CMI/MI	% Recovery
BF1 (fort) without Magnesium Silicate	0.001135	0.003487	0.004622	102
BF1 (fort) with Magnesium Silicate	0.001233	0.003746	0.004979	115
BF2 (fort) without Magnesium Silicate	0.000677	0.002851	0.003528	103
BF2 (fort) with Magnesium Silicate	0.001221	0.003632	0.004853	109

3.2. Preservative Quantification 3 Days after Preparation and Recovery Tests

The concentration of CMI/MI obtained in all formulations was lower than the theoretically added concentration (15 mg/Kg), which may indicate an immediate interaction between ingredients, since the quantification was performed 3 days after preparation.

The addition of zinc pyrithione, hydrolyzed keratin, disodium cocoyl glutamate and cocamide DEA to BF1 resulted in lower concentration values of the preservatives when compared to the base formulation (Table 4).

Table 4. Quantification of the preservative 3 days after preparation.

Samples	% (w/w) MI	% (w/w) CMI	% (w/w) CMI/MI
BF1	0.000097	0.000785	0.000919
BF1 + Zinc Pyrithione	0.000214	0.000394	0.000608
BF1 + Behentrimonium Chloride	0.000244	0.001002	0.001246
BF1 + Hydrolyzed Keratin	0.000200	0.000697	0.000897
BF1 + Disodium Cocoyl Glutamate	0.000198	0.000673	0.000870
BF1 + Cocamide DEA	<LQ [1]	<LQ [1]	
BF2, BF2 + zinc pyrithione, BF2 + Behentrimonium Chloride, BF2 + Hydrolyzed Keratin, BF2 + Disodium Cocoyl Glutamate and BF2 + Cocamide DEA	<LQ [1]	<LQ [1]	

[1] LQ MI = 0.0001187% w/w and LQ CMI = 0.0003562% w/w.

The addition of cocamide DEA had greater influence since the quantification was lower than the LQ for both analytes.

The samples were fortified with a known concentration of Kathon[TM] CG. The fortification consisted on the addition of 1 µL of Kathon[TM] CG to 1 g of shampoo, which represents the addition of 0.0015% CMI/MI mixture. The results are presented in Table 5.

Fortification with Kathon[TM] CG allowed the achievement of acceptable recovery percentages for the base formulation BF1 and the formulations added of the ingredients zinc pyrithione, behentrimonium chloride, hydrolyzed keratin and disodium cocoyl glutamate. The quantification of BF1 + cocamide

DEA was lower than the LQ for both analytes and the recovery percentage was lower than 80%, which can be explained by an immediate interaction between cocamide DEA and CMI/MI or due to a limitation in the quantification method in this particular matrix.

Table 5. Fortification of samples with Kathon™ CG.

Samples	% of Recovery
BF1 (fort)	117.2
BF1 + Zinc Pyrithione (fort)	116.3
BF1 + Behentrimonium Chloride (fort)	109.1
BF1 + Hydrolyzed Keratin (fort)	118.6
BF1 + Disodium Cocoyl Glutamate (fort)	106.7
BF1 + Cocamide DEA (fort)	74.4
BF2, BF2 + Zinc Pyrithione, BF2 + Behentrimonium Chloride, BF2 + Hydrolyzed Keratin, BF2 + Disodium Cocoyl Glutamate and BF2 + Cocamide DEA	<70%

The value obtained for BF1 (9 ppm) was lower when compared to the value obtained for the formulation similar to BF1 in which sodium laureth sulfate was replaced for water (12 ppm).

However, it shall be considered that the resulting formulation has much less viscosity comparing to BF1, which could be facilitating the extraction procedure and consequently influencing the concentration obtained.

Three days after preparation, the quantification of preservatives in the BF2 formulations and BF2 formulations with ingredients added was below the LQ and recovery percentages of quantification with Kathon™ CG were lower than 70%.

The CMI/MI quantification on the day of preparation of the base formulation BF2 presented a value of 0.001112% and after 3 days both analytes were below LQ. On the day of preparation of BF2 the recovery percentage of Proclin™ 150 fortification was 109%. However, after 3 days the fortification of the same formulation with Kathon™ CG presented a recovery percentage lower than 70%. These results suggest that there is an immediate interaction between ingredients that rapidly reduces the CMI/MI concentration.

On the other hand, the content of CMI/MI and the percentage recovery in the BF1 formulation three days after preparation presented very slight variations from the quantification on the day of preparation.

Only those formulations whose recovery percentages were between 80% and 120% were analyzed for preservative stability after storage.

The extraction procedure is critical to breaking the interaction between the analytes and a matrix. Previous studies have reported difficulties in the extraction process of high water content matrices due to the high polarity and solubility of MI in water [39].

One commercial shampoo was analyzed in order to verify if the previously optimized procedure of extraction and quantification allows the quantification of CMI/MI in even more complex matrices. The sample was fortified with 0.0015% w/w of Kathon™ CG.

The fortification with Kathon™ CG allowed 83.3% of recovery, so the method is able to quantify in very complex matrices.

The product information of Kathon™ CG mentions the minimum concentrations of the preservative that inhibit the growth of some bacteria and fungi [18].

The concentrations obtained (Table 6) are below the minimum inhibitory concentrations indicated for most microorganisms, however it is necessary to consider that isothiazolinones were not the only preservatives listed in the label of this shampoo. The real impact of low concentrations on the preservative performance can only be evaluated through microbiological testing [12,16].

Table 6. Quantification of the CMI/MI mixture in a commercial shampoo and fortification with Kathon[TM] CG.

Sample	% (w/w) MI	% (w/w) CMI	% (w/w) CMI/MI
4A	0.0003	<LQ [1]	0.0007
4B	0.0002	<LQ [1]	0.0006
4 (fort)	0.0006	0.0013	0.0019

[1] LQ MI = 0.0001187% w/w and LQ CMI = 0.0003562% w/w.

3.3. Study of the Storage Conditions' Influence on the Stability of Isothiazolinones

3.3.1. Organoleptic Characteristics

BF1 formulations presented fewer changes in appearance compared to BF2 formulations. The only formulation that retained its characteristics after storage under both conditions was the base formulation BF1.

3.3.2. Quantification after Storage

BF1 was the only that showed CMI quantification after the storage period under both conditions (room temperature and 40 °C; Table 7).

Table 7. Preservative quantification after storage at room temperature and 40 °C.

	5 Months at Room Temperature			2 Months at Room Temperature Followed by 3 Months at 40 °C		
	% (w/w) MI	% (w/w) CMI	% (w/w) CMI/MI	% (w/w) MI	% (w/w) CMI	% (w/w) CMI/MI
BF1	0.000221	0.000927	0.001148	0.000190	0.000792	0.000983
BF1 + Zinc Pyrithione	0.000334	<LQ [1]	0.000690	0.000369	<LQ [1]	0.000725
BF1 + Behentrimonium Chloride	0.000189	<LQ [1]	0.000545	0.000168	<LQ [1]	0.000524
BF1 + Hydrolyzed Keratin	0.000238	<LQ [1]	0.000594	0.000325	<LQ [1]	0.000681
BF1 + Disodium Cocoyl Glutamate	0.000183	<LQ [1]	0.000539	<LQ	<LQ [1]	0.000475
BF1 + Cocamide DEA	0.000174	<LQ [1]	0.000530	0.000121	<LQ [1]	0.000477

[1] LQ MI = 0.0001187% w/w and LQ CMI = 0.0003562% w/w.

For formulations BF2 with added ingredients and in all BF1 formulations, the CMI concentration was below the LQ.

The degradation of the CMI in the formulations containing added ingredients is evidenced by the analysis of the results obtained in the lot analyzed 3 days after preparation (Table 4), which show that for all formulations the CMI concentration is higher than the LQ. Although the comparison is carried out between different lots, the overall data show that there is an effective influence of the ingredients added to the formulation on the degradation of the CMI and that this occurs mainly after storage. The greatest susceptibility to degradation observed for CMI (compared with MI) is in accordance with the literature, since in the CMI molecule there is an additional electrophilic center, responsible for greater instability [35].

It was found that the concentration of MI and CMI in the base formulation was lower for the formulation stored at 40 °C compared to the formulation stored exclusively at room temperature.

Regarding the analyses of the BF1 + disodium cocoyl glutamate, after storage at 40 °C, the MI content was below the LQ, unlike that observed after storage exclusively at room temperature. This

formulation proved to be the most susceptible to storage at higher temperatures since it was not possible to quantify any of the analytes in this stress condition.

The "added" ingredients were selected by their theoretical risk of interference with the chemical stability of the preservative and their frequent use in shampoos.

The thiol groups present in hydrolyzed keratin and zinc pyrithione can perform nucleophilic attacks and open the thiazolinone ring. Zinc pyrithione has antidandruff and antiseborrheic properties and hydrolyzed keratin is a skin conditioning and antistatic ingredient. These ingredients were added to the base formulations.

The addition of these two ingredients to BF1 resulted in lower MIC / MI concentration after preparation and after storage when compared to the base formulation BF1, indicating that these ingredients are responsible for a degradation of the preservative.

The product information of Kathon^{TM} CG indicates that amines and amine derivatives have a detrimental effect on the stability of isothiazolinones, and this drawback can be attenuated by lowering the pH to below 7, which is the case for most shampoos. Thus, amines and amine derivatives (behentrimonium chloride, disodium cocoyl glutamate and cocamide DEA) were added to the shampoo base formulation, and the pH adjusted to an optimal range below pH 7. The results allow us to conclude that the addition of disodium cocoyl glutamate to BF1 formulation results in the decrease of CMI/MI contents when compared to the base formulation BF1 by interaction between ingredients that starts soon after incorporation and increases over time [18].

Some reducing agents are detrimental to isothiazolinone stability. Sulfated and sulfonated surfactants often contain residual sulfite or bisulfite, which can react with CMI/MI [18]. The base formulation BF1 contains sodium laureth sulfate.

The possibility of experimental interference related to dealing with very low preservative concentrations and also to the fact that the laboratory lot has been prepared in a small size cannot be ruled out, which may be related with some losses through the preparation process.

The results of Table 7 indicate a lower decrease of the preservative concentration after storage for formulations BF1 and BF1 + zinc pyrithione.

The Kathon^{TM} CG product information indicates that the preservative stability at room temperature is highly dependent on the formulation and the high temperature favors degradation [18].

The ingredient that caused the lowest decrease in preservative concentration after storage was zinc pyrithione and the most relevant reduction was observed for BF1 + behentrimonium chloride. The base formulation BF1 was the most stable after storage under both conditions.

The maximum temperature tested in this study was 40 °C. Temperatures above 50 °C are known to be detrimental to the stability of the preservative. Kathon™ CG was incorporated into the formulations when they cooled below 40 °C. Small differences in preservative concentration were observed between the samples stored at room temperature and the samples stored at 40 °C. The greatest decrease caused by storage at elevated temperatures in the CMI/MI concentration was found in the base formulation BF1. The formulation BF1 + disodium cocoyl glutamate proved to be the most susceptible to the effect of stress conditions since it was not possible to quantify any of the analytes after storage at 40 °C, contrary to storage at room temperature.

4. Conclusions

This study highlights the challenging quantification of CMI/MI complex formulations such as hair products related with extraction and the analytical method itself. Since cosmetic ingredients are frequently mixtures of substances, the matrix of final cosmetics is extremely complex, making it difficult to assay and allowing the occurrence of interactions between substances.

Therefore, validation of the analytical method for the particular matrix to be analyzed stands as an important step, although it is frequently difficult in practice since the matrix without the analyte is not easily accessible.

Furthermore, the results herein presented with both matrices show that these preservatives interfere with several ingredients frequently used in shampoo formulations, suggesting that there is an immediate interaction between ingredients that rapidly reduces the CMI/MI concentration and leads to a decrease in the concentration of preservative over time. Considering this risk of decreased concentration of preservatives over time is essential to assure the quality of such cosmetic products.

References

1. Regulation (EC) No. 1223/2009 of the European parliament and of the Council of 30 November 2009 on cosmetic products. Available online: https://eur-lex.europa.eu/legal-content/PT/ALL/?uri=CELEX%3A32009R1223 (accessed on 31 December 2019).

2. Ficheux, A.S.; Wesolek, N.; Chevillotte, G.; Roudot, A.C. Consumption of cosmetic products by the French population. First part: Frequency data. *Food Chem. Toxicol.* **2015**, *78*, 159–169. [CrossRef] [PubMed]

3. Neste, D.V.; Shaker, G. Interations with hair and scalp. In *Handbook of Cosmetic Science and Technology*; Marcel Dekker Inc.: New York, NY, USA, 2001; pp. 575–602. ISBN 0-8247-0292-1.

4. Draelos, Z.D. Shampoos, conditioners, and camouflage techniques. *Derm. Clin.* **2013**, *31*, 173–178. [CrossRef] [PubMed]

5. Bhushan, B. *Biophysics of human hair*; Springer: Heidelberg, Germany, 2010; pp. 1–18. ISBN 978-3-642-15900-8.

6. Draelos, Z.D. Essentials of Hair Care often Neglected: Hair Cleansing. *Int. J. Trichology* **2010**, *2*, 24–29. [CrossRef] [PubMed]

7. Trueb, R.M. Shampoos: Ingredients, efficacy and adverse effects. *J. Dtsch. Dermatol Ges.* **2007**, *5*, 356–365. [CrossRef]

8. Milstein, S.R.; Bailey, J.E.; Halper, A.R. Definition of Cosmetics. In *Handbook of Cosmetic Science and Technology*; Marcel Dekker Inc.: New York, NY, USA, 2001; pp. 5–13. ISBN 0-8247-0292-1.

9. Manual of the working group on cosmetic products (Sub-group on borderline products) on the scope of application of the cosmetics regulation (EC) No 1223/2009. February 2016. Available online: https://ec.europa.eu/docsroom/documents/29002 (accessed on 31 December 2019).

10. Gavazzoni Dias, M.F.R.; de Almeida, A.M.; Cecato, P.M.R.; Adriano, A.R.; Pichler, J. The Shampoo pH can Affect the Hair: Myth or Reality? *Int. J. Trichology* **2014**, *6*, 95–99. [CrossRef]

11. Updated Guidelines for the Cosmetics Industry based on the 7th Amendment to the Cosmetics Directive. In Proceedings of the Cosmetics Europe: Cosmetic ingredient labelling in the European Union. September 2006. Available online: https://www.cosmeticseurope.eu/download/Y3VSQkcrMERhNnlRTWExblFZc2hWZz09 (accessed on 31 December 2019).

12. Smaoui, S.; Hlima, H.B. Effects of parabens and isothiazolinone on the microbiological quality of baby shampoo: The challenge test. *Biocontrol Sci.* **2012**, *17*, 135–142. [CrossRef]

13. Siegert, W. A Comparison to other methods to evaluate the efficacy. *SOFW-Journal* **2012**, *138*, 44–53.

14. Harvey, P.W.; Darbre, P. Endocrine disrupters and human health: Could oestrogenic chemicals in body care cosmetics adversely affect breast cancer incidence in women? *J. Appl. Toxicol.* **2004**, *24*, 167–176. [CrossRef]

15. Thiemann, A.; Janichen, J. The formulator's guide to a safe cosmetic preservation. *Pers. Care* **2014**, 39–43.

16. Department of Health and Human Services. U.S. food and drug administration center for food safety and applied nutrition. In Proceedings of the Cosmetic Microbiological Safety Issues, Linda Katz, Patricia Hansen, Etan Yeshua, Jay Ansell, Phil Geis, David Stainberg, Richard Whiting, Washington, DC, USA, 30 November 2011.

17. Gombault, L.N. Sodium benzoate et MIT. Presented at Université du Quebéc à Quicoutimi, Saguenay, QC, Canada, 10 December 2014.

18. Dow Company. Product information of KathonTM CG Preservative. Available online: http://msdssearch.dow.com/PublishedLiteratureDOWCOM/dh_0988/0901b80380988b37.pdf?filepath=biocides/pdfs/noreg/253-02698.pdf&fromPage=GetDoc (accessed on 31 December 2019).

19. Berthet, A.; Spring, P.; Vernez, D.; Plateel, G.; Hopf, N.B. Ex vivo human skin permeation of methylchloroisothiazolinone (MCI) and methylisothiazolinone (MI). *Arch. Toxicol.* **2017**, *91*. [CrossRef]

20. Rodrigues-Barata, A.R.; Conde-Salazar, L. Methylisothiazolinone and Methylchloroisothiazolinone: New Insights. *EMJ Dermatol.* **2014**, *2*, 101–105.

21. Williams, T.M. The Mechanism of Action of Isothiazolone Biocides. *PPChem.* **2007**, *9*, 14–22.

22. Yazar, K.; Johnsson, S.; Lind, M.L.; Boman, A.; Liden, C. Preservatives and fragrances in selected consumer-available cosmetics and detergents. *Contact Dermat.* **2011**, *64*, 265–272. [CrossRef] [PubMed]

23. Boyapati, A.; Tam, M.; Tate, B.; Lee, A.; Palmer, A.; Nixon, R. Allergic contact dermatitis to methylisothiazolinone: Exposure from baby wipes causing hand dermatitis. *Australas J. Dermatol.* **2013**, *54*, 264–267. [CrossRef]

24. Scientific Committee on Consumer Safety SCCS. Opinion on methylisothiasolinone (P94). Presented at 4th Plenary Meeting, Luxembourg, 12 December 2013.

25. Comission Regulation (EU) No. 2016/1198 of 22 july 2016 amending Annex V to Regulation (EC) No 1223/2009 of the European Parliament and of the Council on cosmetic products. Available online: https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A32016R1198 (accessed on 31 December 2019).

26. Usatine, R.P.; Riojas, M. Diagnosis and Management of contact dermatitis. *Am. Fam. Physician* **2010**, *82*, 249–255.

27. Latheef, F.; Wilkinson, S.M. Methylisothiazolinone outbreak in the European Union. *Curr. Opin. Allergy Clin Immunol.* **2015**, *15*, 461–466. [CrossRef]

28. Pestana, C.; Gomes, R.; Pinheiro, V.; Gouveia, M.; Antunes, I.; Goncalo, M. Main Causes of Occupational Allergic Contact Dermatitis: A Three Year Study in the Center of Portugal. *Acta. Med. Port.* **2016**, *29*, 449–455.

29. Gonçalo, M. Methylisothiazolinone in rinse-off products: Additional fuel to the world epidemics of allergic contact dermatitis to isothiazolinones. *Br. J. Dermatol.* **2015**, *173*, 11. [CrossRef]

30. Goncalo, M.; Goossens, A. Whilst Rome burns: The epidemic of contact allergy to methylisothiazolinone. *Contact Dermat.* **2013**, *68*, 257–258. [CrossRef]

31. Geier, J.; Lessmann, H.; Schnuch, A.; Uter, W. Recent increase in allergic reactions to methylchloroisothiazolinone/methylisothiazolinone: Is methylisothiazolinone the culprit. *Contact Dermat.* **2012**, *67*, 334–341. [CrossRef]

32. Urwin, R.; Wilkinson, M. Methylchloroisothiazolinone and methylisothiazolinone contact allergy: A new 'epidemic'. *Contact Dermat.* **2013**, *68*, 253–255. [CrossRef] [PubMed]

33. Commmision Regulation (EU) N° 1003/2014 of 18 September 2014 amending Annex V to Regulation (EC) No 1223/2009 of the European Parliament and of the Council on cosmetic products. Available online: https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A32014R1003 (accessed on 31 December 2019).

34. Commision Regulation (EU) N° 2017/1224 of 6 July 2017 amending Annex V to Regulation (EC) No 1223/2009 of the European Parliament and of the Council on cosmetic products. Available online: https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A32017R1224 (accessed on 31 December 2019).

35. The scientific committee on cosmetic products and non-food products intended for consumers. Update of entry n° 39 of annex VI to directive 76/768/EEC on cosmetic products: Misture of 5-chloro-methyl-isothiazolin-3(2H)-one and 2-methylisothiazolin-3(2H)-one. Presented at 24th Plenary Meeting, Brussels, Belgium, 24–25 June 2013.

36. Rastogi, S.C. Kathon CG and comestic products. *Contact Dermat.* **1990**, *22*, 155–160. [CrossRef] [PubMed]

37. Rastogi, S.C.; Jensen, G.H.; Petersen, M.R.; Worsoe, I.M.; Christoffersen, C. *Preservatives in Skin Creams*; Ministry of Environment and Energy: Copenhagen, Denmark, 1999; ISBN 87-7772-503-4.

38. Baranowska, I.; Wojciechowska, I. The Determination of Preservatives in Cosmetics and Environmental Waters by HPLC. *Pol. J. Environ. Stud.* **2013**, *22*, 1609–1625.

39. Alvarez-Rivera, G.; Dagnac, T.; Lores, M.; Garcia-Jares, C.; Sanchez-Prado, L.; Lamas, J.P.; Llompart, M. Determination of isothiazolinone preservatives in cosmetics and household products by matrix solid-phase dispersion followed by high-performance liquid chromatography-tandem mass spectrometry. *J. Chromatogr. A.* **2012**, *1270*, 41–50. [CrossRef] [PubMed]

40. Sigma Aldrich, Certificate of Analysis Proclin 150. Available online: https://www.sigmaaldrich.com/catalog/product/supelco/48061?lang=zh&region=CN (accessed on 31 December 2019).

41. Instituto Português de Acreditação. *Guia de Acreditação de Laboratórios Químicos*; Instituto Português de Acreditação: Caparica, Portugal, 2011; pp. 1–12.