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Preparation and value assignment of parabens and phenoxyethanol in cosmetic cream certified reference material

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Abstract: A new certified reference material has been developed by National Institute of Standards according to ISO Guides 30-35 and ISO 17034:2016 for preservatives in cosmetic cream to be used for validation and verification of analytical methods, proficiency testing and as internal quality control sample for quality assurance in testing laboratories. The material was prepared gravimetrically and mass fraction of each analyte was confirmed be validated liquid chromatographic method. The material along with analytes homogeneity and stability were completely evaluated. The certified value of phenoxyethanol, methyl, ethyl, propyl, and butyl paraben and their corresponding expanded uncertainties (k = 2.0) were found (0.500±0.017) %, (0.150±0.006) %, (0.051±0.003) %, (0.101±0.004) % and (0.050 ±0.003) %, respectively.

Keywords: Cosmetic cream; parabens; phenoxyethanol; value assignment; reference material.

1. Introduction

Nowadays, public awareness of the potential risk of chemicals and materials used in daily life has increased dramatically. The safety of raw materials used in cosmetic manufacturing has attracted much attention and chemicals such as parabens have been considered as unsafe materials in some countries because of their possible side effects on human health [1, 2]. In the cosmetic products, parabens are mainly used as preservatives due to broadness of their spectrum activity, antibacterial and fungicidal properties, stability over a wide pH range and low cost [3-5]. These materials have weak estrogenic activity [6, 7] and the excessive use could lead to adverse effects such as breast cancers and oxidative DNA damage [8-11]. Also, several studies have been conducted to confirm the endocrine disrupting activity of parabens with special emphasis on the presence of methyl paraben being reported in breast cancer tissues [12-14]. Butyl paraben and isobutyl paraben, are classified as allergens and have been shown to induce male reproductive disorders, male sexual developmental toxicity. So, their maximum permitted contents are regulated by legislation in many countries [15-16]. In response to safety of cosmetic products and its ingredients, the accurate measurement of parabens in cosmetic is particularly important. Therefore, various laboratories are focused on using matrix reference materials for validating their measurement procedures to improve the quality of results and meet the requirements of ISO/IEC 17025 standard for establishment of metrological traceability, validation and quality control requirements [17-20]. In response to customers' demands, NIS started to

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develop a series of cosmetic reference materials to measure heavy metals, preservatives and physical properties in different cosmetic matrices with high accuracy. The current reference material is characterized for methyl paraben, ethyl paraben, propyl paraben, butyl paraben and phenoxyethanol, the selection of these preservatives and concentration range of each individual preservative were based on market survey and customer needs. In this study, the process of preparation of cosmetic cream sample, characterization of analytes in the cream matrix reference material, establishing traceability to SI units, homogeneity assessment, stability evaluation and value assignment of the prepared reference material were completely described.

2. Experimental

2.1. Reagent and Chemicals

Phenoxyethanol (CRM 053), methyl (CRM 054), ethyl (CRM 055), propyl (CRM 056), and butylparaben (CRM 057) pure reference materials were developed by NIS and the purity was certified by mass balance in previous study [21]. The certified values of phenoxy ethanol, methyl, ethyl, propyl, and butylparaben and their corresponding expanded uncertainties are (996.82±0.68) mg/g, (998.48±0.71) mg/g, (997.80±1.36) mg/g, (998.20±0.51) mg/g and (998.35±0.89) mg/g, respectively. Analytical grade paraffin oil, vaseline, stearic acid, cetyl alcohol, triethanolamine, lanolin, ropyl glycol were purchased from Sigma-Aldrich (Germany). HPLC grade solvents, methanol, diethyl ether, ethyl acetate and acetone were purchased from Sigma-Aldrich (Germany) and used in all procedures without any further purification. High purity water was obtained through a Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.2. Equipment

Calibrated five digits analytical balance, model SE2 (Sartorius, Germany). Laboratory scale balance model MS32001L (Mettler Toledo, Switzerland). Digital shaking water bath, model SHKE7000-1CE (USA). Mechanical stirrer from Heidolph Instruments (Germany). Viscometer, model LVF (Brookfield, USA). pH meter model, Orion 4-Star Plus (Thermo Scientific, USA) equipped with epoxy electrode. Class A beakers and separating funnels with different volumes, 50, 100, 250, 500 and 1000 mL.

2.3. HPLC and Separation Conditions

One gram of sample was dissolved in 50 mL of methanol, stirred by magnetic stirrer for 10 min then, the methanol was directly filtered through a 0.45 µm nylon filter and collected in a 1 mL amber glass vial and 10 µL were then injected into Agilent 1100 HPLC integrated system equipped with a G1313A auto-injector and G1315B diode-array detector (DAD). The separation was carried out using a ZORBAX SB-C18 (150 mm × 4.6 mm, 5 µm) at flowrate of 1.0 mL/min using linear and step gradient from methanol and water as described in Table1. The chromatographic data was analyzed using Agilent chemstation B.02.01.

Table 1. Linear and step gradient program for chromatographic separation of parabens and phenoxyethanol

Time (min)	Methanol (%)	H_2O (%)
0	30	70
7	50	50
8	60	40
13	60	40
16	80	20
20	80	20
22	30	70

2.4. Material Preparation

Two kilograms of cosmetic cream bulk material were prepared from paraffin oil, vaseline, stearic acid, cetyl alcohol, triethanolamine, lanolin, propyl glycol raw materials. The materials were heated and mixed, the calculated amount of phenoxyethanol, methyl, ethyl, propyl, and butyl paraben reference materials were added to reaction vessel, and then the material was emulsified, homogenized with continuous stirring. The compositions of the cream were carefully selected matched with a real cosmetic cream. After pre- investigation of homogeneity, the material was packed in 20 g brown glass bottles sealed with screw caps with PTFE septa and capped tightly to avoid losing of water content. The bottles were labelled according to filling sequence, divided into five groups and stored at a controlled temperature of about -40°C for further studies.

2.5. Homogeneity Study

The evaluation of material homogeneity was conducted to investigate within and between bottles heterogeneity of phenoxyethanol, methyl, ethyl, propyl, and butylparaben in cream reference material. Stratified random sampling procedure was used to select five bottles from the batch. Five portions from each bottle were extracted and measured three times each by HPLC under the same conditions. All samples were measured randomly to avoid any possible trends regarding to measurement sequence. ANOVA was used to check material homogeneity and to estimate uncertainty due to heterogeneity of prepared material and distribution of analytes. Also, regression analysis was performed to detect possible trends regarding the filling sequence.

2.6. Stability Study

Classical stability study approach was applied to investigate stability of prepared material and analytes under the storage and transport conditions including controlled low and high temperature. [22-26]. Stratified random sampling approach was used to select twenty-one bottles from the whole patch for stability evaluation. The bottles were divided into three groups, each group consisted of seven bottles. The groups were stored at -20 ºC, 4 ºC and 40 ºC for one year. One sample from each group was measured in triplicates every two months at each temperature. The cream material was investigated for water content and microbiology to ensure its suitability. The data were investigated for outliers before application of linear regression analysis to test significance of slopes using a t test [22-26]

3. Results and Discussion

3.1. Homogeneity Assessment

The homogeneity of phenoxyethanol, methyl, ethyl, propyl, and butylparaben in prepared cream sample were fully investigated by HPLC after methanol extraction of about 1.0 g samples. The data from homogeneity study [Table 2] were analysed graphically [Figure 1] and statistically using ANOVA after outliers check using the Grubbs test to estimate uncertainty due to analytes heterogeneity. The uncertainty related to analytes heterogeneity (u_{bb}) was estimated from mean squares using equation 1 and 2.

$$u_{\mathrm{bb}} = \sqrt{\frac{MS_{\mathrm{between}} - MS_{\mathrm{within}}}{n}} \qquad (1)$$

$$u_{bb} = \sqrt{MS_{within}/n}.\sqrt[4]{2/(\nu MS_{within})} \quad (2)$$

Where $MS_{between}$ and MS_{within} represents between groups and within groups mean squares, respectively. Both equations were used to estimate uncertainty due to analytes heterogeneity for each analyte, the largest values were obtained from Eq. 2 because $MS_{between}$ are smaller than MS_{within} for all analytes, the selected values were combined with other sources to estimate expanded uncertainty [25-27].

Table 2. Results of homogeneity study for phenoxy ethanol and parabens in cosmetic cream reference material

Analyte	Bottles					$MS_{between}$	MS_{within}	*P*-value	u_{bb}
	B1	**B2**	**B3**	**B4**	**B5**				
Phenoxy Ethanol	0.496	0.502	0.504	0.493	0.496	3.62E-05	6.886E-05	0.718068	0.00311
	0.485	0.496	0.497	0.496	0.501				
	0.509	0.504	0.502	0.515	0.495				
	0.491	0.493	0.483	0.503	0.507				
	0.503	0.506	0.503	0.504	0.516				
Methyl Paraben	0.150	0.148	0.149	0.153	0.155	5.86E-06	7.04E-06	0.520402	0.00100
	0.150	0.152	0.151	0.151	0.149				
	0.150	0.149	0.151	0.148	0.151				
	0.148	0.151	0.147	0.146	0.150				
	0.153	0.158	0.151	0.148	0.153				
Ethyl Paraben	0.049	0.051	0.050	0.051	0.050	5.6E-07	1.96E-06	0.883764	0.00053
	0.049	0.052	0.052	0.049	0.052				
	0.049	0.051	0.048	0.051	0.049				
	0.051	0.049	0.052	0.052	0.051				
	0.051	0.048	0.051	0.049	0.051				
Propyl Paraben	0.099	0.101	0.103	0.103	0.099	4.3E-06	4.34E-06	0.435251	0.00078
	0.101	0.097	0.100	0.101	0.101				
	0.102	0.102	0.103	0.098	0.100				
	0.100	0.100	0.100	0.099	0.100				
	0.102	0.096	0.101	0.106	0.101				
Butyl Paraben	0.050	0.050	0.052	0.052	0.051	1.06E-06	0.00000126	0.515295	0.00042
	0.050	0.050	0.050	0.050	0.048				
	0.050	0.051	0.049	0.051	0.051				
	0.050	0.050	0.050	0.050	0.050				
	0.051	0.051	0.053	0.050	0.048				

According to the homogeneity test results illustrated in Table 2, no statistically significant variability was found between bottles based on *P*-value and *F* test. *P*-value is higher than 0.05 and *F* calculated is smaller than *F* critical for all analytes.

Preparation of cosmetic cream reference material

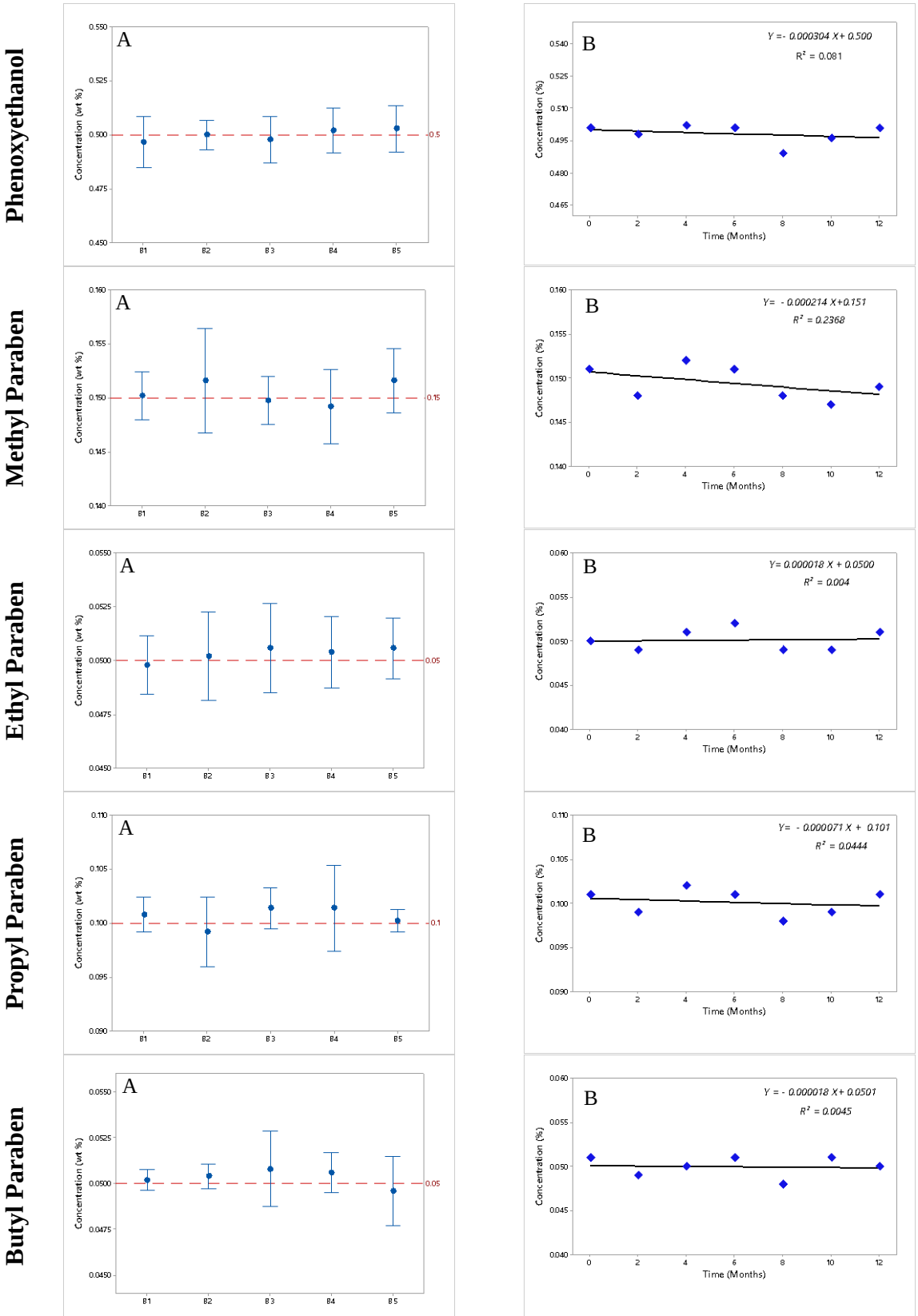

Figure 1. A) Graphical representation of homogeneity study and **B)** linear regression plots for long-term stability study during 12 months of storage at -20 ºC for phenoxyethanol and parabens in cosmetic cream reference material

3.2. Stability Assessment

Classical stability study approach was applied to investigate stability of prepared material and analytes under the storage and transport conditions including controlled low and high temperature. [22-26]. Stability study was performed over a period of 12 months at two months intervals. Samples were stored at -20 ºC, 4 ºC and 40 ºC. Seven bottles of samples were stored at each temperature for 2, 4, 6, 8, 10 and 12 months. After the predetermined storage periods, the samples were analysed for phenoxyethanol, methyl, ethyl, propyl, and butylparaben in triplicate. Stability data were screened for stragglers and outliers by applying Mandel's h and k statistics at confidence levels of 95 % and 99 %, respectively.

Table 3. Results of stability study at -20 ºC, 4 ºC and 40 ºC for phenoxy ethanol and parabens in cream reference material

Analyte	-20 ºC			4 ºC			40 ºC		
	Slope	S	*P*-Value	Slope	S	*P*-Value	Slope	S	*P*-Value
Phenoxy Ethanol	-0.00030	0.000457	0.536	-0.000696	0.000137	0.004	-0.00125	0.000168	0.001
Methyl Paraben	-0.00021	0.000172	0.268	-0.000393	0.000105	0.013	-0.00077	0.000193	0.011
Ethyl Paraben	0.000018	0.000126	0.892	-0.000036	0.000080	0.673	-0.00025	0.000070	0.016
Propyl Paraben	-0.000071	0.000148	0.650	-0.000232	0.000115	0.099	-0.00063	0.000113	0.003
Butyl Paraben	-0.000018	0.000119	0.887	-0.000054	0.000131	0.701	-0.00064	0.000161	0.010

Linear least square regression lines were constructed using stability data as a function of testing time, the observed slopes were tested for significance using two tailed t test at 95% confidence interval ($\alpha = 0.05$). The slopes and statistical parameters of stability testing at -20 ºC, 4 ºC and 40 ºC are illustrated in Table 3. On the basis of the results, the storage of prepared material is recommended at -20 ºC as shown in Figure 1. The uncertainty due to analytes instability estimated from regression line slope is included in the combined uncertainty of the assigned value [22-26]. The data given in the Table 3 indicate a sufficient stability of samples stored at -20 ºC. The stability of the cream material and analytes will be monitored by further testing over the period of material availability to maintain confidence in the status of the prepared material.

3.3. Preparation and Characterization of Reference Material

The cosmetic cream was prepared from paraffin oil, vaseline, stearic acid, cetyl alcohol, triethanolamine, lanolin, propyl glycol pure materials. The materials were heated, mixed and stirred strongly, the material was accurately weighed and the calculated amount of phenoxyethanol, methyl, ethyl, propyl, and butylparaben reference materials were added into bulk material to provide concentration of 0.500 %, 0.150%, 0.050%, 0.100% and 0.050%, respectively. Then the material with preservatives was emulsified, homogenized by vigorous stirring. The physical–chemical characteristics selected to investigate cream matrix were pH, viscosity and microbiology. The selected parameters are mainly used to detect any change in the cream matrix. The physical–chemical characteristics selected to investigate cream matrix were pH, viscosity and microbiology. The selected parameters are mainly used to detect any change in the cream matrix. The initial values of the pH and viscosity were 7.3 and 38000 CPS, respectively. The microbiology testing was subcontracted to accredited laboratory complying with ISO/IEC 17025 requirements. According to ISO 17516:2014 [28], the total aerobic microbial count (TAMC) and total yeast/ mold count (TYMC) in cosmetic cream should be less than 100 cfu/g and 10 cfu/g, respectively. The initial values and results obtained over a period of 12 months were within acceptable levels. The composition and properties of prepared cream sample are similar to materials used by customers and tested by cosmetic testing laboratories. The prepared concentrations were confirmed and verified by HPLC validated method The validation scheme was designed to evaluate precision, Trueness, linearity, LOD and LOQ of target concentration of all analytes according to ISO 17025 standard, ISO 5725 and IUPAC harmonized guidelines [17,

29-30]. The validation results illustrated in Table 4 proved that, the method was found to be very sensitive and suitable for accurate quantification of phenoxy ethanol and parabens in cosmetic cream. Additional validation of the test method was effectuated by inter-laboratory comparison between NIS and Johnson & Johnson, Analytical Development, R&D, Val de Reuil, France. The data obtained during comparison confirmed Intra-laboratory validation results.

Table 4. Calibration parameters and results of precision (RSD in %), trueness (recovery in %), LOD and LOQ for phenoxy ethanol and parabens chromatographic test method

	Calibration range (mg/mL)	**Linearity**	**Precision (RSD)**	**Trueness (%)**	**LOD (mg/mL)**	**LOQ (mg/mL)**
Phenoxy Ethanol	0.50-1.50	0.9998	0.29	100.37	0.002	0.006
Methyl Paraben	0.10-0.50	0.9999	0.62	101.52	0.001	0.003
Ethyl Paraben	0.05-0.20	0.9999	1.50	100.57	0.001	0.003
Propyl Paraben	0.10-0.30	0.9999	0.90	100.14	0.001	0.003
Butyl Paraben	0.05-0.20	0.9999	1.06	100.86	0.001	0.003

The method based on extraction of phenoxyethanol and parabens by methanol and separation on the reversed phase C18 column ZORBAX SB-C18 (150 mm × 4.6 mm, 5 µm) with absorbance detection at 254 nm. The separation of phenoxyethanol and all parabens were achieved in 17 minutes with good baseline resolution (R >1.5) as shown in Figure 2. The results of chromatographic measurements are 0.500%, 0.150%, 0.051%, 101%, and 0.050% for phenoxyethanol, methyl, ethyl, propyl, and butylparaben, respectively. These results are in a good agreement with the gravimetrically prepared concentration, this agreement confirms validity of the preparation process.

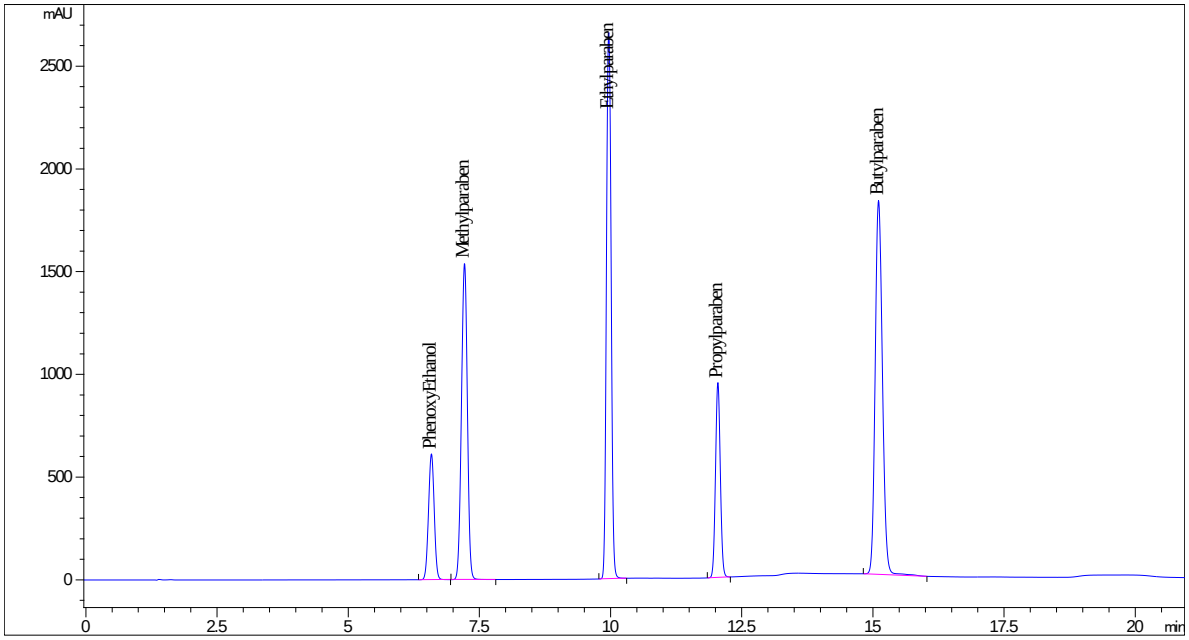

Figure 2. Liquid chromatographic chromatogram of phenoxy ethanol and parabens in cosmetic cream by ZORBAX SB-C_{18} column and UV detection.

3.4. Metrological Traceability and Value Assignment

The metrological traceability of reference values to the SI units was achieved by the use of primary pure reference materials characterized by mass balance approach and calibrated balances directly traceable to the mole and international kilogram, respectively. The value assignment of the reference material was based on purity and masses of primary reference materials, and uncertainties of RMs and balances. Estimated uncertainties are derived from the uncertainty components due to uncertainties of certified values u_{CRM}, material preparation (u_{mp}), characterization (u_{ch}), analytes homogeneity (u_{bb}), short and long terms stability (u_{sts}, u_{lts}) as illustrated in Figure 3. Expanded uncertainty is expressed as two times the root of the sum of the squares of u_{ch}, u_{bb}, u_{st} (u_{sts}, u_{lts}), u_{CRM}, and u_{mp} at a confidence level of approximately 95 % [31-33].

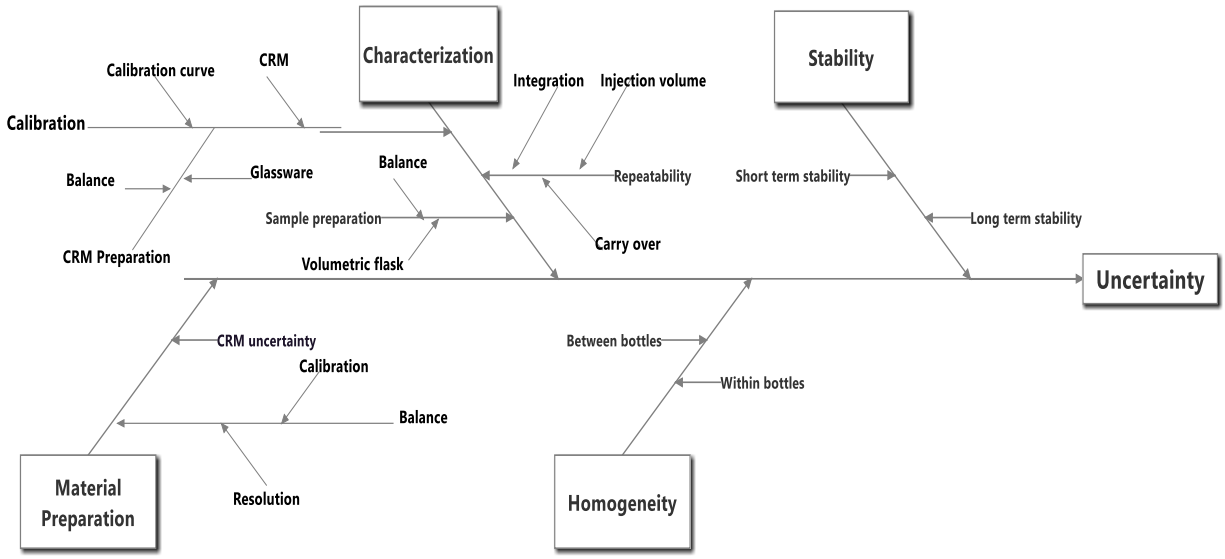

Figure 3. Uncertainty sources for certification of for phenoxyethanol and parabens in cosmetic cream reference material

The certified values are calculated by combining the nominal values from gravimetric preparation and data from liquid chromatographic method after treatment of outliers by Grubbs statistical test. The certified value of phenoxyethanol, methyl, ethyl, propyl, and butylparaben and their corresponding expanded uncertainties (k = 2.0) were found (0.500±0.017) %, (0.150±0.006) %, (0.051±0.003) %, (0.101±0.004) % and (0.050 ±0.003) %, respectively.

4. Conclusions

A complete gravimetric method has been developed to prepare reference material for preservatives in cream. The concentration of phenoxyethanol and parabens was confirmed be validated liquid chromatographic method. The results presented in this study demonstrate that both gravimetric and chromatographic methods have good agreement which confirms the absence of any systematic errors. The results from long-term stability showed that the phenoxyethanol and parabens content and cream matrix under the storage condition -20 ^{o}C are stable within its expanded uncertainty for one year. The certified and their corresponding expanded uncertainties (k = 2.0) values were found (0.500±0.017) %, (0.150±0.006) %, (0.051±0.003) %, (0.101±0.004) % and (0.050 ±0.003) %, for phenoxyethanol, methyl, ethyl, propyl, and butylparaben, respectively. Due to lack of availability of

preservatives in cream reference materials, these reference materials will be useful tool for cosmetic testing laboratories in compliance with ISO/IEC 17025 requirements.

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