An investigation of commonly used skin-lightening creams

by

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ABSTRACT

The use of skin-lightening products is a common practice, particularly in African countries. The reasons for this practice may be of aesthetic, medical, cultural or socio-economic origin. Skin-lightening creams may contain several active ingredients which can be absorbed through the skin and cause side effects, especially after prolonged application. The most common ingredients include hydroquinone and its derivatives, resorcinol, topical steroids of various potencies, and mercury-containing compounds. Exogenous ochronosis, postinflammatory hyperpigmentation, acne, striae, fish odour syndrome, and allergic dermatitis are the most reported dermatologic complications associated with lightening ingredients.

In this work, some commonly used skin-lightening creams available in some of South Africa's stores were investigated. The objective was to identify and quantify the organic active ingredients and heavy metals in these creams.

Forty-nine samples of skin-lightening creams were analysed to determine the levels of heavy metals (Cd, Co, Cr, Cu, Zn, Fe, Pb, Ni, As) and mercury. These elements were extracted from the sample matrices by using acid-assisted microwave digestion and the extracts were analysed by ICP-OES and CV-AAS, respectively. Among the 10 studied metals, only Cu and Fe were detected in all sample creams, whereas As, Cd, Co, and Ni were not detected in any of the cream samples. Cr and Pb were detected in three samples while Zn and Hg were found at relatively high levels in a few samples. The mean concentrations of detected metals ranged from 0.19 to 8.93 μg g^{-1} for Cu, 0.55 to 25007 μg g^{-1} for Fe, 1.73 to 12.56 μg g^{-1} for Cr, 2.98 to 15.72 μg g^{-1} for Pb, 79.97 to 12470 μg g^{-1} for Zn, and 2.48 to51.19 μg g^{-1} for Hg.

A high performance liquid chromatography (HPLC) method was developed for the determination of the organic active ingredients in nineteen creams samples. A UV spectrophotometer was employed to characterise the active ingredients within each sample on the basis of spectral analysis. The influence of the solvent for sample

preparation and the composition of the mobile phase for separation of the lightening agents have been investigated. Methanol was found to be a suitable extraction solvent. By using a C18 column (100mm×4.6mm) and an isocratic elution performed at 289 nm, 270 nm, and 240 nm, with a mobile phase (methanol: water, 55:45; v/v) pumped at 0.8 mL min^{-1} , ingredients such as hydroquinone (HQ), arbutin (ARB), niacinamide (NA), phenol (PO), kojic acid (KA), hydrocortisone valerate (HCV), and clobetasol propionate (CP) were identified in twelve products.

The quantitative results showed that the mean percentage concentrations of NA ranged from 0.035 to 1.170%, PO varied from 0.0025 to 0.920%, BQ varied from 0.008 to 0.036% and ARB varied from 0.030 to 0.063% (m/m). Furthermore, concentrations of 0.94%, 0.055% and 0.0031% (m/m) were found for HQ, CP and HCV, respectively. Levels of KA are not reported for the present study because of tailing in the HPLC.
Validation of the method was assessed by intra-day and inter-day reproducibility of standard, the linearity, detection limit, and recovery test. The linear correlation coefficient has been found to be greater than about 0.995, the acceptable recovery values range from 87.4 to 105.04 %, the comparable precisions for intra-day (R.S.D between 0.038 and 1.86%) and inter-day (R.S.D between 0.60 and 2.83%) variability demonstrate that the method was suitable for the analysis of skin-lightening creams.

It is clear from the present investigation that toxic compounds are being used as active ingredients or for other purposes in some skin-lightening creams. This is of concern because such products can expose users to health problem.

PREFACE

This dissertation presents the work performed by the author in the School of Chemistry and Physics, University of KwaZulu-Natal, Durban, from March 2012 to December 2013 and from July to December 2014, under the supervision of Prof Bice S. Martincigh.

The study is the original work by the author and has not been submitted in any form to any other university. Where use has been made of the work of others, it has been duly acknowledged in the text.

DECLARATION 1: PLAGIARISM

I, Clementine Niyibizi, hereby declare that

1. The research reported in this dissertation, except where otherwise indicated, is my original research.

2. This dissertation has not been submitted for any degree or examination at any other university.

3. This dissertation does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

4. This dissertation does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:
   a. Their words have been re-written but the general information attributed to them has been referenced.
   b. Where their exact words have been used, then their writing has been placed in italics and inside quotation marks, and referenced.

5. This dissertation does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the dissertation and in the reference sections.

Signed: 

DECLARATION 2: CONFERENCE PRESENTATION

The following has been presented in a conference:

C. Niyibizi and B.S. Martincigh, An investigation of the heavy metal content of commonly used skin-lightening creams, The 16th Biennual South African Chemical Institute Inorganic Chemistry Conference incorporating the Carman Physical Chemistry Symposium, Durban, South Africa, 30 June - 4 July 2013.

DEDICATION

I dedicate this work to my lovely son, George Ishema Nsengiyumva.

ACKNOWLEDGMENTS

I am indebted to God with whom everything is possible.

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Chapter 1

INTRODUCTION AND LITERATURE REVIEW

Pigmentation of the skin is directly or indirectly regulated by different physiological factors including melanin.[1,2] Melanin is the major dark pigment found in skin, hair, and eyes. It provides protection against harmful ultraviolet radiation that can cause photoaging and photocarcinogenesis.[3-5]

Studies have shown that the mean protective factor (PF) for ultraviolet light and ultraviolet light B (UVB, 280-320 nm) radiation of black epidermis is significantly higher than that of white epidermis.[5,7] About three to four times more ultraviolet A (UVA, 320-400nm) radiation reaches the upper dermis of whites than that of blacks.[7,9] Black-skinned people are less vulnerable to photoaging than white-skinned people.[9] Since the amount and type of synthesised melanin influences the skin complexion, either excessive melanin production or deficient melanin production can cause pigmentary disorders.[2]

Hyperpigmentation and hypopigmentation are pigmentary disorders that are extremely common in blacks, Hispanics, and Asian.[11] Moreover, acne vulgaris, eczema, contact dermatitis, fungal infections and other common dermatologic conditions are also unfavourable aesthetic skin problems that affect everybody.[12] In addition, a light and flawless skin complexion is considered as a major contributor of human beauty, attractiveness and welfare.[13] Consequently, various cosmetics and therapeutic treatments aimed at modulating melanogenesis have been used in order to control unwanted skin pigmentation.

Skin-lightening refers to the application of chemical agents in order to lighten the complexion of one's skin. It is also called skin-bleaching or skin-whitening. The use of

skin-lightening has become a universal phenomenon socially practiced by both women and men. For example, in African communities, light-brown skin type has been preferred since the colonial era.[13,14] Many surveys[15-17] agree that this practice originated from Africa's colonial and slave history where darker skin reflected dirty, unfair and wicked persons while light or white skin was associated with higher status, privileges, beauty and more intelligence.[14,18] Certainly, this segregation affected dark-skinned people and caused them to believe that they are inferior and less beautiful. Thus, it is understandable that some bleached their skin to try to attain the status associated with all manner of social and economic success. Hence, the cosmetic use of skin-lightening products became an increasingly popular practice as early as the 1930s and is currently widespread.[12]

Since the misconceived belief that darker skin is less beautiful is reflected in the practice of skin bleaching, it has led the cosmetic industry to manufacture various products that are designed to help dark-skinned people look lighter. These products often come in a variety of preparations including creams, lotions, gels, soaps, powder and pills. Hydroquinone, retinoids, resorcinol, mercury, and corticosteroids are the main active ingredients that have been reported in over-the-counter (OTC) skin-lighteners.[19-22]

Formulations of hydroquinone (4%), topical steroids and other skin-lightening agents such as tretinoin (0.1%), azelaic acid (20%) and kojic acid, are dermatologically used to treat acne and other pigmentary disorders such as postinflammatory hyperpigmentation and hypopigmentation, vitiligo, or melasma in Fitzpatrick skin types IV, V and VI.[11,23,24] Lightening agents act mainly by inhibiting the biosynthesis of melanin.[24] The mechanism of hydroquinone and mercury is based on the breakdown of melanin formation by competitively inhibiting the activity of the tyrosinase molecule.[25-27] An overview of the inhibitory mechanism of the top 20 whitening agents is given in Section 1.1.3.

Long-term exposure to skin-lightening agents has been associated with acute and chronic toxicity.[26] Hydroquinone causes contact dermatitis, exogenous ochronosis, and dyschromia.[29-31] Mercury is highly toxic and continued exposure can lead to neurological damage.[19] Continuous use of corticosteroids can cause cutaneous[32] ophthalmologic

and endocrinologic complications.[26,33-35] Besides, it has been demonstrated that diverse reactions such as kidney, blood vessel and nervous system damages, miscarriage, infertility in men and women, hormonal changes and behavioural problems can be induced by the exposure of other toxic metal-containing cosmetic products.[36] Reports[37-40] have shown the presence of heavy metals such as Cd, Cr, Cu, Hg, Ni, Pb in a variety of cosmetic products ranging from facial make-up (for example, lipsticks, eye shadows, mascara, eyeliners, face powder and foundation), sunscreen creams, hair dyes and shampoos. These reports have prompted us to determine the heavy metal content in the selected skin-lightening creams and to assess whether their concentrations conform to the existing regulatory limits.

The cosmetic safety regulation of the European Union (EU) and the US Food and Drug Administration[41] proposed that hydroquinone should not exceed 2% as an active ingredient in over-the-counter (OTC) skin-lightening products and a maximum concentration of 4% is allowed only by prescription. However, mercury is prohibited in any amount in cosmetics (Council Directive 76/768/EEC of 27 July 1976).[42] Despite widespread public health campaigns and government bans of these ingredients, the practice of skin-lightening is on-going and growing in African and Asian countries.

In this study inductively coupled plasma-optical emission spectroscopy (ICP-OES), cold vapour atomic absorption spectroscopy (CV-AAS) and high performance liquid chromatography (HPLC) were used to analyse the selected skin-lightening creams.

1.1 General overview of the practice of skin-lightening

Black skin represents the first ethnic group in the world with a higher natural protection against sun damage and skin aging due to the important role of melanin in absorbing ultraviolet light and blocking free radical generation.[27] But, why do many darker-skinned people not feel confident of their inherent skin texture? Studies argued that this practice had roots in the history of colonization and westernisation; as the Europeans dominated each part of the world, they integrated cultural beliefs that devalued darker skin and valued lighter skin.[15,17,18,43] Since this period, the establishment of racial and

psychological hierarchy based on distinctions of skin colour gained significance and importance within different communities around the world including Africa, Asia, North America and the Middle East.[14,16]

Over the past years, the execution of this hierarchy has been realised in Africa as light-skinned and some mixed-race descendants have occupied positions of power and economic advantage over darker-skinned populations. This discrimination has brought feelings of inferiority, poor identity, and low self-esteem within dark-skinned Africans.[43] Even in other countries where pigmentation among the inhabitants is quite varied, such as the USA, Brazil, and Asian communities, higher socioeconomic levels and a greater presence in leadership positions belong to lighter-skinned individuals.[13,17] Furthermore, in the post-colonial era, Western influences and the mass media continue to reinforce differences in racial skin colour by portraying lighter skin as beautiful (the ideals of beauty) and preferable over darker skin.[17]

In Asia, women were deeply influenced by the concept that a white complexion is powerful enough to hide a number of faults as stated by various idioms such as "white skin can conceal seven defects" (Japanese) and "white skin can cover thousand ugliness" (Chinese).[18] These ideals of whiteness motivated many Asian and African women to bleach their skin tone to obtain a light complexion.[12,43,44] Nowadays, the belief that light skin is a key criterion for positive outcomes, beautifulness, attractiveness, higher status, higher level of education and others, is still true in many societies.

The report of the World Health Organisation[44] indicated that the use of skin-lightening products in African countries is on-going and growing, among which women are some of the most widely represented users. The scores are high, for example up to 77% in Nigeria,[46] 52% in Senegal,[47] 44.3% in Burkina Faso,[48] 59% in Togo[46] and 25% in Mali.[49] This practice is reported also in South Africa[22], the Democratic Republic of Congo (DRC), Tanzania, Cameroon and Zambia.[43]

1.1.1 Biosynthesis of melanin (Melanogenesis and tyrosinase inhibitors)

The biosynthetic pathway for the formation of the smaller pigmented biopolymers, melanin, in human skin is called melanogenesis.[50] Melanin is produced in melanocyte cells localized in the outermost layer of the skin, the epidermis. Melanin is formed through the copper-based enzyme, tyrosinase, which catalyses the first two steps of melanin production: hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA) and the subsequent oxidation of dopa into dopa-quinone. Dopa-quinone is converted to dopachrome through enzymatic conversions by dopachrome tautomerase, also referred to as tyrosine-related protein 1 and 2 (TRP-1 and TRP-2), and then to dihydroxyindole or dihydroxyindole-2-carboxylic acid (DHICA) to form eumelanin. In the presence of cysteine or glutathione, dopaquinone is converted to cysteinyldopa or glutathionyldopa. Subsequently, pheomelanin is formed. Eumelanin is a dark brown-black insoluble polymer, whereas pheomelanin is a light red-yellow sulfur-containing soluble polymer. Figure 1.1 shows part of the chemical structures of eumelanin and pheomelanin. A combination of eumelanin and pheomelanin forms the melanin pigment.

Pheomelanin **Eumelanin**

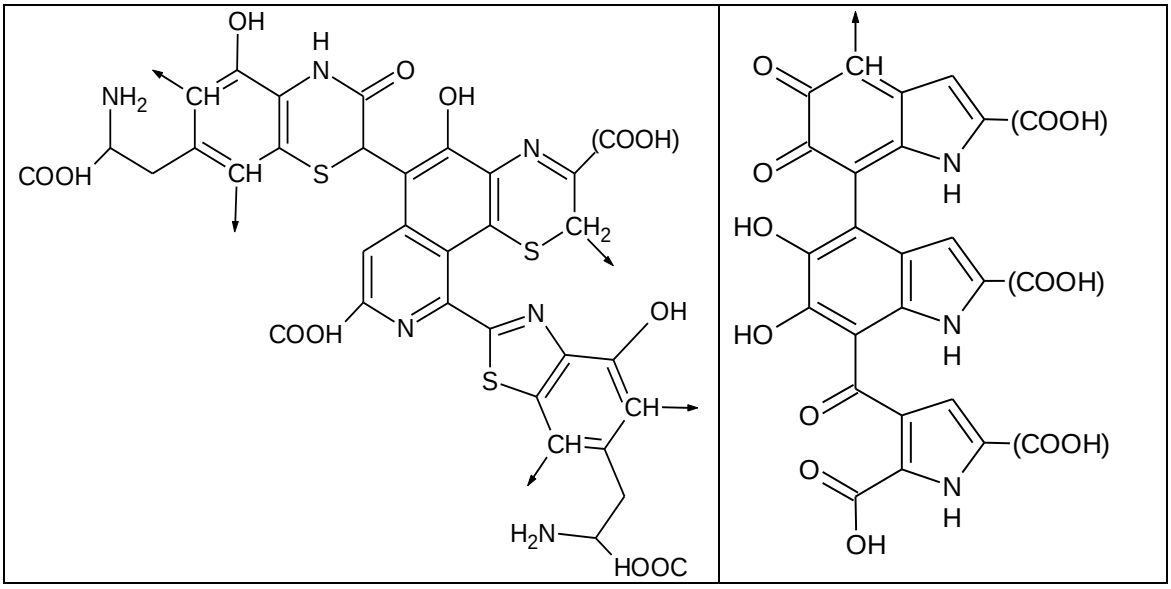


Figure 1.1: Part of the molecular structure of pheomelanin and eumelanin (↑ shows where the polymer continues) [http://www.photoprotection.clinuvel.com/node/204 *(Accessed on 27th July 2014)]*.

In addition to the activity of the tyrosinase enzyme, UV radiation and genes as well as hormones such as alpha melanocyte-stimulating hormone (α-MSH) and adrenocorticotropic hormone (ACTH) have also been shown to stimulate melanogenesis.[52] As noted above, tyrosinase is the enzyme that catalyses several oxidative reactions required for melanin synthesis from its precursor amino acid, tyrosine.[25] It is well-known for regulating melanogenesis. Figure 1.2 illustrates a simplified biosynthesis pathway of melanin. Hence, tyrosinase enzyme is the most targeted for the efficacious treatment of hyperpigmentary disorders.[51]

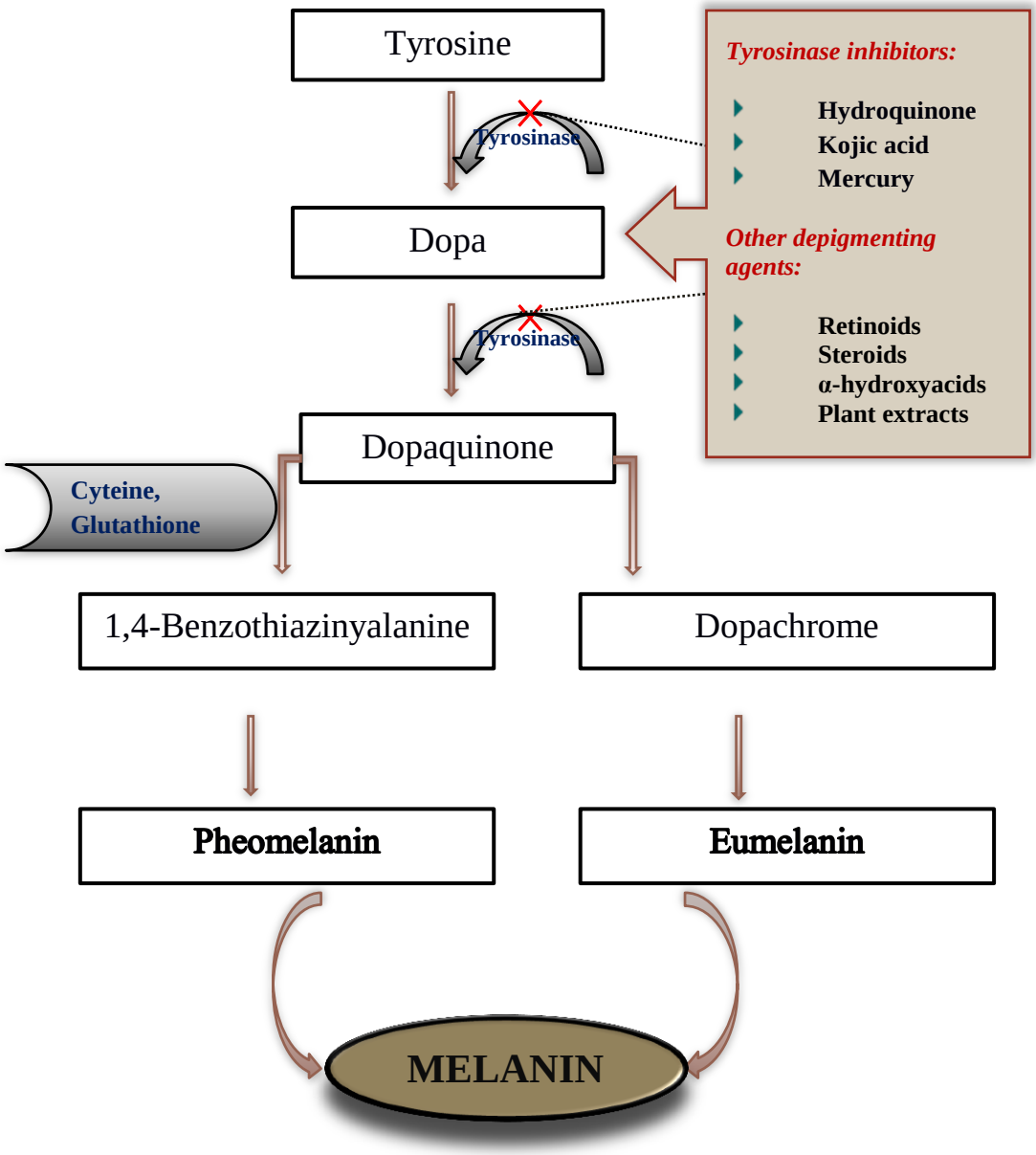


Figure 1.2: Simplified pathway of melanogenesis.[4]

Melanin play an important role in the absorption of free radicals generated within the cytoplasm and in shielding from ultraviolet radiation.[3,25] Moreover, the overproduction of melanin pigment causes hyperpigmentation of the skin while a decrease of melanin can result in hypopigmentation.

Despite the acknowledged mechanisms of melanoganesis, variations in the number, size, and aggregation of melanosomes within the melanocyte and keratinocyte result in the racial and ethnic differences in skin colour.[7,52] The distribution pattern of synthesised melanin in the epidermis determines the actual colour of the skin.[50] Parvez *et al.*[27] mentioned that a visible pigmentation in mammals results from the synthesis and distribution of melanin in the skin and hair bulbs.

Research[25] demonstrates that different steps of the melanogenesis pathway can also be interrupted depending on the mechanistic reaction of the inhibitor involved.[51] However, the inhibition of the enzymatic reaction of tyrosinase is still the most common approach to lighten the skin.[51,52] The active compounds aimed at inhibiting melanin production may be of synthetic sources or botanic sources. These compounds are currently being used in cosmetic products and traditional preparations.

Considering the mechanism of tyrosinase inhibitory activity, skin-lightening agents can be classified into three types, namely, competitive, non-competitive or mixed inhibitor.[4, 27] Commonly used skin-lightening agents are known as competitive inhibitors of tyrosinase. The competitive inhibitors are also divided into three categories: phenolic compounds, non-phenolic compounds, and combination formulas.[53] Hydroquinone (HQ) and its derivatives monobenzyl ether of hydroquinone (MBEH), phenol and its compounds such as 4-methoxyphenol (4-MEP), 4-isopropylcatechol, 4-hydroxyanisol and N-acetyl-4-S-cystaminylphenol (4-S-CAP) represent the first category.[54] The second category, non-phenolic compounds, includes kojic acid (KA), azelaic acid (AZA), N-acetylcystein, tretinoin, and L-ascorbic acid (AS).[53,54] The last category often involves a combination of HQ and various lightening active agents of different concentrations. The most commonly used combination formulas are Kligman's formula (HQ 5%, tretinoin 0.05–0.1%, and dexamethasone or betamethasone valearate 0.1% in hydro-

alcoholic base cream or ointment base), Pathak's formula (2% HQ, tretinoin 0.05–0.1% in hydro-alcoholic base cream or ointment base) and Westerhof's formula (N-acetylcysteine 3%, HQ 2%, hydrocortisone 1% in ointment base).[53]

Other active compounds, such as resorcinol and its derivatives (4-butylresorcinol, 4-phenylethylresorcinol, and 4-hexylresorcinol), mercury, and steroids have been shown to have a strong inhibitory effect on melanin synthesis.[26, 28, 51, 55] In addition to these, many plant extracts contain active compounds, mainly arbutin, aleosin, flavonoids, gentisic acid, niacinamide, licorice, and polyphenols that are also known to be inhibitors of melanogenesis, without melanoctyes cytotoxicity or mutagenicity.[56, 57] Section 1.1.3 gives more information about skin-lightening agents mostly used in cosmetics.

1.1.2 Skin types

The skin is a complex organ with complicated structures and mechanisms that connect it to the entire body. The skin is composed of three main layers: the epidermis, dermis and hypoderm.[58] The visible human skin colour results from the synthesis and distribution of melanin in the melanocyte cells, mainly located in the epidermis layer of the skin.[6, 50, 59] It is well known that the skin plays a role in thermoregulation, protection against pathogens, and a barrier function for maintaining proper hydration of the body. Paradoxically, the skin can be seen as a mirror that reflects the integumentary system and is a key criterion for visual evaluation of physical appearance, attractiveness and beauty.

1.1.2.1 Variability in skin tone

Human skin colour ranges in variety from the darkest brown to pinkish-white hues. The most commonly used scheme to classify a person's skin type is by their response to sun exposure. It correlates the colour of the skin with its dynamic ability to respond to UV light in terms of the degree of burning and tanning (see Table 1.1). Pale skin or white skin burns easily and tans slowly and poorly, whereas darker skin burns less and tans more easily. This system was developed by Thomas Fitzpatrick.[61]

Table 1.1: Sun-reactive skin types.[60]

Skin type	**Natural skin colour**	**Skin reactions to solar radiation**	**Examples**
I	Pale white	Always burns, never tans	People with blue eyes, often freckled
II	Fair white	Burns easily, tans mini-mally	People with red or blonde hair, blue eyes
III	Light brown	Burns moderately, tans uniformly	
IV	Moderate brown	Burns minimally, tans easily	People with dark brown hair, dark eyes, e.g. Orientals, Mediterranean
V	Dark brown	Rarely burns, tans darkly easily	East Indians, American Indians, Hispanics
VI	Black	never burns, tans pro-fusely	African and American Negroes, Australian Aborigines

Skin type I-III can be at risk of developing skin cancer following sun exposure whereas skin types IV-VI can be prone to an overactive production of melanin which can lead to melasma. A skin colour chart is shown in Figure 1.3. Therefore, all skin type I-III and IV-VI are advised to use appropriate sunscreen to protect themselves from harmful UV rays as extreme sun exposure can induce serious damage, uneven skin tone, premature ageing and possible skin cancers.

Figure 1.3: Skin colour chart (Professor B. Martincigh, Personal Communication, October, 2014).

Other skin classification systems consider factors such as lipid content and hydration of the skin, other than the sensitivity to sun light or the effect of UV radiation. Skin types may vary from normal, dry, oily to combination skin. As reported by Mercurio *et al*.,[63] skin is classified into six types: eudermic (healthy skin), oily, alipic, hydrated, dehydrated and combination. Consequently, the skin type can change over time. For instance, younger people are more likely than older people to have a normal skin type.

1.1.2.2 Racial differences

Understanding of various races and ethnic groups of *Homo sapiens* species entails the influence of many factors including biological or genetic traits.[6] Obvious physical differences exist in hair colour, texture, skin colour and facial features. Most studies evaluate racial/ethnic differences in skin structure, physiology and dermatologic disorders.[6, 61-64] Research into molecular genetics describes racial differences according to normal variation in the pigmentary traits of skin, hair and eye colour.[67] Berardesca[65] also noted that the colour of the skin is the main feature that characterises the race.

Considering the pigmentation of the skin, there are many racial and ethnic groups. For example, the black race or people with skin of colour. Subsequently, skin of colour is also divided into skin type IV, V and VI. The majority of this group include Africans, Afro-Caribbeans, Asians, and Native American and African Americans.[7] Hispanics constitute another group which is included in the non-Caucasian category. This includes people who are lighter in colour such as European Hispanics as well as Central American people and would therefore be classified as having Fitzpatrick skin types I through III.[11]

Other reviews consider racial variability in terms of different skin responses to cutaneous diseases. Blacks experience less skin irritation, as induced by either chemicals or UV light, compared to white persons.[66] Though skin cancer is the most common malignancy, the incidence is relatively low in black-skinned people (skin type IV-VI).[62, 65] The melanin content and melanosomal dispersion pattern in these people is thought to be responsible for providing protection from the carcinogenic effects of UV radiation. However, hyperpigmentation disorder (melasma) occurs frequently in black-skinned people and is reported to be more prevalent in Asians.[23, 69] More information concerning racial/ethnic differences regarding skin reactivity in pathologic and physiologic conditions is available.[5, 6, 65]

Considering the complexity of skin typing, skin disorders of any sort affect most people, regardless of bioethnic background or skin colour.[71]

For instance, skin may either appear blotchy, lighter, or darker than normal, or lack pigmentation. All these conditions occur when the body produces either too much or too little melanin.[71]

The desire for a fair tone is very common, particularly, among people with Fitzpatrick skin types IV to V. However, the need for efficacious and low undesirable side-effects of topical preparations in the treatment of uneven pigmentation has become a dermatological challenge worldwide, in view of the increasing incidents of exogenous ochronosis.[6] Attention is given to cosmetic products that claim to have skin-improvement properties such us moisturisers or emollients, sunscreens and skin-lighteners.

1.1.3 Skin-lightening agents

Skin-lightening agents are often applied to lighten uneven skin tone. For medical purposes these agents play an important role for the treatment of pigmentary disorders such as melasma, acne, age spots, and hyperpigmentation.[24, 48, 67, 70-72] These disorders greatly affect populations with skin phototypes IV, V and VI, like Africans, Asians and Hispanics or darker skin people living in different countries.[26, 73-75]

Hydroquinone, steroids and mercury are the ingredients widely used in skin-lightening products.[35] However, these agents expose users to certain additional cutaneous risks among which an increase of hyperpigmentation of the skin and many other health complications that reappear after discontinuation of therapy.[25] The following is a discussion of the most popular skin-lightening agents reported in the literature, their known mechanisms of action and potential side effects.

1.1.3.1 Hydroquinone and its derivatives

Hydroquinone (HQ) is a phenolic compound chemically used as a reducing agent, antioxidant, polymerisation inhibitor, and chemical intermediate in the synthesis of other antioxidant derivatives.[28] It has been a golden standard, dermatologically, used for treatment of pigmentary disorders of the skin such as melasma and postinflammatory

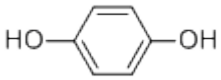
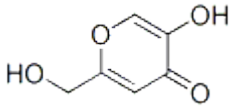
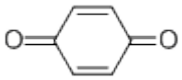
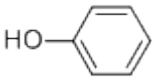
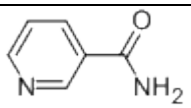
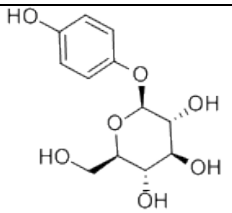
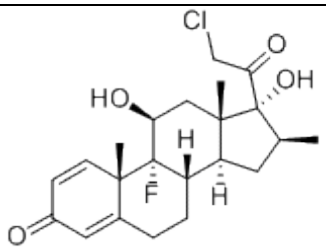
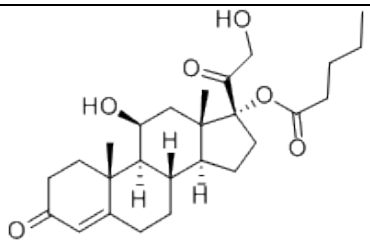
hyperpigmentation.[67] HQ occurs naturally in coffee beans, tea, red wine, wheat and leaves of berries. It is a commodity chemical also known under different synonyms such as 1,4-dihydroxybenzene, 1,4-diol/quinol, 1,4 benzenediol, p-benzenediol, β-quinol, p-hydroxphenol, p-dioxobenzene, hydrochinone or tecquinol. The structures of some common skin-lightening agents are shown in Table 1.2.

HQ is the most conventional active ingredient used in many skin-whitening products. It acts by competitively inhibiting melanin production by inhibiting sulfhydryl groups and acting as a substrate for tyrosinase. Other proposed mechanisms of action include the inhibition of DNA and RNA synthesis, the destruction of melanocyte and degradation of melanosomes.[27]

Hydroquinone is the most discussed agent in the literature for its toxic effects including neuropathy, exogenous ochronosis and leukoderma after long-term exposure. Consequently, the European Union has prohibited the use of hydroquinone in cosmetics since 2001.[18] The US Food and Drug Administration proposed that hydroquinone should not exceed 2% as an active ingredient in OTC skin-bleaching products.[41] In Nigeria, the National Food and Drug Agency of Nigeria (NAFDAC) had initially allowed a maximum of 2% hydroquinone in bleaching creams. However, due to the side-effects associated with long-term hydroquinone use and also lack of compliance with content and labelling requirements, all forms of bleaching agents were prohibited in cosmetics and toiletries.[25] Later on, the FDA released a statement proposing to consider all skin-bleaching drug products, whether currently marketed on prescription or OTC basis, to be new drugs requiring an approved new drug application for continued marketing.[77]

Since hydroquinone causes reversible inhibition of cellular metabolism by affecting both DNA and RNA synthesis,[4] its derivatives were considered as possible alternative agents. The most used hydroquinone derivatives, in skin-lightening products, are monobenzyl ether of hydroquinone (MBEH, monobenzone) and monomethyl ether of hydroquinone (MMEH, 4-hydroxyanisole, mequinol).[27,28, 30] However, both compounds were reported to induce highly melanocytotoxic effects.[28]

Table 1.2: Chemical structures of some common skin-lightening agents.

Active ingredient	**Chemical structure***
Hydroquinone	
Kojic Acid	
Benzoquinone	
Phenol	
Niacinamide	
Arbutin	
Clobetasol propionate	
Hydrocortisone valerate	

*Adapted from http://www.Chemicalbook.com/ (*Accessed on 05 December 2014*).

1.1.3.2 Phenol

Phenol is a carbolic acid (C_6H_5OH) used as a peel agent to eradicate heavy wrinkles and lines caused by chronic photodamage and solar lentigines.[78] It is absorbed fast through the skin into the blood stystem. Phenol can induce prolonged erythema and hypopigmentation.[5]

Other chemical peels used in cosmetics are salicylic acid and glycolic acid. Glycolic acid is commonly used in cosmetic products as a peeling agent that helps to alter the structure and content of the skin, increasing production of collagen. On the other hand, salicylic acid (o-hydroxybenzoic acid) is used as a keratolytic peeling agent and also as an anti-inflammatory. It is a non-competitive inhibitor of tyrosinase.[27]

Chemical peels are categorised as superficial peels (α-hydroxy acids, salicylic acids and Jessner's solution contains ≤ 25% trichloroacetic acid, TCA), medium-depth peels (phenol and 25-35% TCA) and deep peels (phenol and > 35% TCA).[27-29]

1.1.3.3 Kojic Acid

Kojic acid (5-hydroxy-2-hydroxymethyl-4-H-pyran-4-one) is a hydrophilic fungal metabolite obtained from *aspergillus* and *penicillium* species.[4] It is a potent tyrosinase inhibitor that functions by chelating copper at the active site of the enzyme and prevents the conversion of dopamine to its corresponding melanin.[52] Reports[30, 53, 66] have noted the efficacy of kojic acid in the treatment of melasma. However, it is a sensitiser that has been found to cause allergic dermatitis and erythema.[30] In one study kojic acid has been reported for its mutagenicity concerns.[27]

1.1.3.4 Resorcinol

Resorcinol is considered as antibacterial, anti-acne, and an effective topical disinfectant in concentrations of 1-3%.[77] It (14%) is often combined with salicylic acid (14%) and lactic acid (14%) to treat moderate to severe facial dyschromias, acne, oily skin, texturally rough skin, fine wrinkles and pseudofolliculitis.[5] Resorcinol and its derivatives,

rucinol and 4-n-butylresorsinol, are strong tyrosinase and TRP-1 inhibitors.[50] Resorcinol can induce thyroid dysfunction after a prolonged period of exposure.[80]

1.1.3.5 Arbutin

Arbutin is a derivative of hydroquinone, naturally isolated from bearberry fruit *(arctostaphylos uva ursi).* It decreases melanin content in melanocytes by a competitive inhibition of tyrosinase enzyme.[81] Although, arbutin has shown to be effective in treating solar lentigines and melasma, high concentrations can cause hyperpigmentation.[22]

1.1.3.6 Niacinamide

Niacinamide (nicotinamide, 3-pyridinecarboxamide) is the physiologically active amide, niacin (vitamin B3). Studies have shown that niacinamide is of dermatologic benefit in the treatment of melasma, and popular and pustular acne.[22,82] It prevents melanogenesis without inhibiting tyrosinase, but by decreasing melanosome transfer from melanoctyes to keratinocytes.[83]

1.1.3.7 Retinoids

Retinoids induce skin-lightening by accelerating epidermal turnover and, therefore, they are used as monotherapy and in combination with other topical agents for the treatment of melasma.[84] Retinoids, such as tretinoin, interrupt melanin synthesis by inhibiting UVB-induced tyrosinase and TRP-1 expression.[85] Topical retinoids are associated with local skin irritation, ertyhrema, dermatitis and severe peeling. Other effective retinoid agents reported are adapalene and isotretinoin.[84]

1.1.3.8 Plant extracts

Traditional herbal medicines and/or active compounds isolated from plants provide opportunities to develop a new approach to control pigmentary disorders. Studies have found that compounds, such as aloesin, hesperidin, licorice, flavonoids, gentisic acid and polyphenols, are more potent tyrosinase inhibitors of melanin than hydroquinone,

kojic acid, and arbutin.[4,56,57] Many of these plant extracts are believed not to cause potential effects like cytotoxicity or mutagenicity of melanocytes.[57]

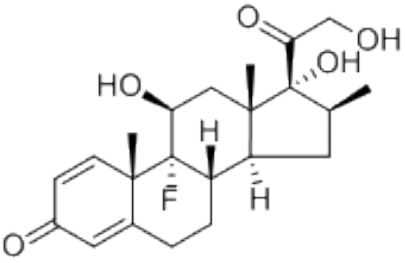
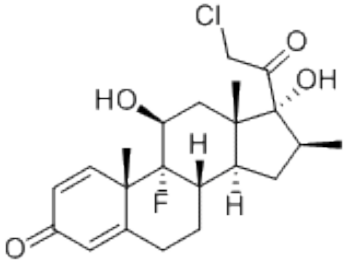
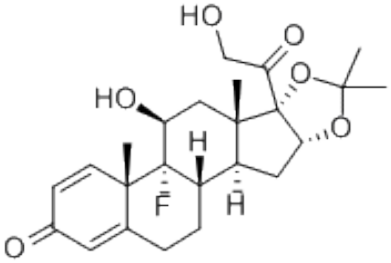
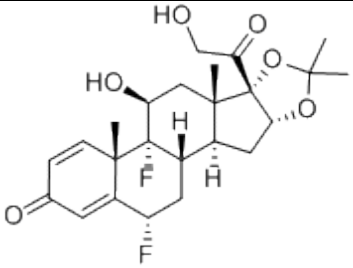
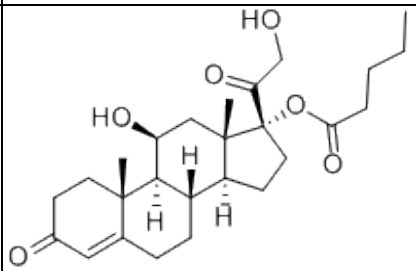
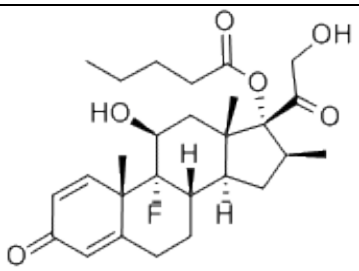
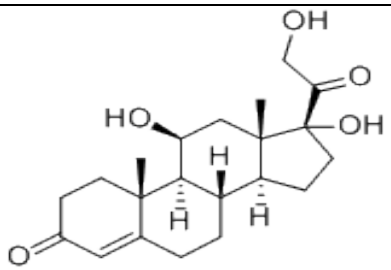
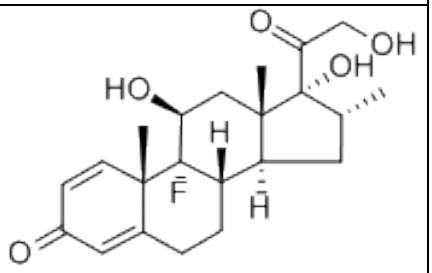
1.1.3.9 Steroids

Steroids (corticosteroids) are drug-based hormones widely used to control inflammation in the body. Steroids that are known for improving performance by increasing muscle mass and decreasing body fat are called anabolic steroids. These are commonly used in sport.[86] In dermatology, steroids are effective anti-inflammatory preparations used to control many inflammatory skin conditions including psoriasis, atopic dermatitis and eczema.[87, 88] Steroids are called systemic if they are taken by mouth or given by intramuscular injection, and topical steroids if they are applied on the skin. Topical steroids are divided into four groups: mild, moderate, potent and very potent.[88] Mild and moderate topical steroids rarely cause side-effects. Absorption through the skin of potent and very potent steroids can cause local and systemic effects. Indeed topical steroids are associated with complications, especially when used incorrectly. The most reported side-effects associated with long-term use and over dosage include increased acne, rosacea, burns, stretch marks, straie, decreased skin healing and numbness, skin atrophy and bruising.[89-91] It is also reported that dexamethasone and betamethasone may cause adverse effects on human health including obesity, hypertension and osteoporosis.[89,91]

Corticosteroids are used in bleaching creams as an active ingredient because of their ability of inhibiting cAMP and decreasing the precursor for melanocyte-stimulating hormone.[94] Table 1.3 lists topical steroids commonly used in skin-lightening products. Although these mechanisms lead to the reduction of melanin formation, sometimes steroids are combined with other lightening agents in order to increase their potency.[85]

Steroid formulations (0.025% to 0.5%, w/w) are prescribed and have been noted to improve therapy for psoriasis conditions.[93]

Table 1.3: Topical steroids mostly used in skin-lightening products.

Potency	**Chemical structure***
Very high	 Betamethasone 0.05%  Clobetasol propionate (0.05%)
High	 Triamcinolone acetonide (0.05%)  Fluocinonide (0.05%)
Moderate	 Hydrocortisone valerate (02%)  Betamethasone valerate 0.1%
Mild	 Hydrocortisone (0.5-2.5%)  Dexamethasone (0.1%)

*Adapted from http://www.chemicalbook.com/ (*Accessed on 05 December 2014).*

The OTC topical products containing topical steroids and some other substances with skin-lightening action have been banned by different countries of Africa. However, in spite of their exclusion, many anti-inflammatory products (creams and ointments) are legally exported from Europe to Africa.[90] Table 1.4 lists 20 most common skin-lightening ingredients and their inhibitory mechanism of melanogenesis.

1.1.3.10 Mercury

Mercury is well-known for its toxicity. It is distributed in the environment by natural and anthropogenic sources. Like other heavy metals, mercury can be taken into the body via inhalation, ingestion and skin absorption. Mercurous chloride, also known as calomel, mercuric chloride and mercuric oxide are the most important mercury compounds.[95] Mercurous chloride (Hg_2Cl_2) is used in antiseptic salves. Mercuric chloride ($HgCl_2$) is sometimes employed as a topical antiseptic in medicine whereas in agriculture it is used as a fungicide. In the chemical industry, it serves as a catalyst in the manufacture of vinyl chloride and as a starting material in the production of other mercury compounds. Mercuric oxide (HgO) provides elemental mercury for the preparation of various organic mercury compounds and some inorganic salts.

Two forms of mercury can be found in cosmetics. For example, organic mercury, such as thiomersal and phenyl mercuric salts, is used as a cosmetic preservative in eye make-up, cleansing products and mascara, while inorganic mercury (ammoniated mercury) and metallic mercury are used in some skin-lightening creams and soaps as active ingredients because of their ability to inhibit melanin pigment.[96]

Cases of mercury toxicity and dermatologic complications due to the use of skin-lightening creams have been reported.[25] The absorption of mercury through the skin can enhance its accumulation in tissues, and hence, impairs not only the skin but also various organs of the body including the kidneys, brain, central nervous system and the digestive system.[97] Mercury effects on the central nervous system often cause fatigue, weakness, headaches, depression and insomnia. Mercury can affect the skin, causing irritation, erythema, itching, reddening, rashes and cracking.[98] Mercury intoxication can

cause anemia, renal dysfunction and cataracts in newborns after it has been absorbed in the blood of a mother who uses products containing mercury during pregnancy and lactation.[34] Thus, mercury and its compounds are banned in many countries for use in cosmetic products.[75, 100]

Table 1.4: An overview of the melanogenesis inhibitory mechanism of 20 most common skin-lightening ingredients.

Agent[101]	**Mechanism**	**Reference**
Hydroquinone	Inhibits tyrosinase, DNA and RNA synthesis	27,67
Monobenzone	Competitive inhibitor of tyrosinase	27
Kojic acid	Chelating copper at the active site of the tyrosinase	50
Azelaic acid	Competitive and reversible inhibitor of tyrosinase	27
Arbutin	Inhibits tyrosinase and the oxidation of L-DOPA	57
Retinoids	Inhibits UVB-induced tyrosinase and TRP-1 expression	85
Niacinamide	Inhibits the transfer of melanosomes	101
N-acetyl-glucosamine	Interferes with pigment cell growth and proliferation	69
Corticosteroids	cAMP inhibition and decreases the precursor for melanocyte stimulating hormone	26,88
Mercury	Competitively combines with tyrosinase and prevents copper from binding at the active site.	50
Gentisic acid	Its alkyl esters noncompetitively inhibit tyrosinase	52
Mequinol	Competitive inhibitor of tyrosinase and destruction of pigment cells	23
Vitamin C	Suppression of melanin formation on purified tyrosinase and in melanocytes	101
Aloesin	Inhibits tyrosinase (noncompetitive) and DOPA oxidase activities	25
Hydroxystilbene	Reduction in MITF and tyrosinase promoter activities (non-competitive inhibitor)	50
Melatonin	Inhibits cAMP-driven processes in pigment cells	27
Soy protein	Inhibits the action of protease-activated receptor-2 cleavage and reduces keratinocyte phagocytosis	27
Glycolic acid	Inhibits melanin formation in human melanoma cells without affecting tyrosinase	25
Green tea	Competitively inhibits tyrosinase activities	55
Licorice	Competitively inhibits tyrosinase activities	57

cAMP: cyclic adenosine-3,5-monophosphate; **DOPA:** dihydroxyphenylalanine; **MITF**: microphthalmia transcription factor

1.1.4 Heavy metals

Heavy metals refer to those metals with a density at least five times greater than that of water (1 g cm^{-3}).[99] They occur naturally in the environment and become concentrated as a result of human activities. Common sources are mining, rocks, soil and water as well as the manufacture of pigments and other raw materials in all industries. Examples of metals classified as heavy metals include: antimony, arsenic, bismuth, cadmium, selenium, chromium, cobalt, copper, gallium, gold, iron, lead, manganese, mercury, nickel, platinum, silver, tellurium, thallium, tin, uranium, vanadium and zinc. Contamination by these metals is an environmental and health issue.

Heavy metals in various media have been widely studied because of their detrimental health effects and the fact that they are persistent and bioaccumulate.

1.1.4.1 Heavy metals in cosmetics

Cosmetics include any preparation intended to be rubbed, poured, sprinkled or sprayed on the human body for beautifying, promoting attractiveness, or altering the appearance of the skin, by the removal or correction of blemishes.[93] They can also be used to prevent skin diseases, to repair or hide skin imperfections, to cleanse, decorate, protect and treat hair, lips, nails or teeth.[100]

Common cosmetic formulations include skin-care creams, lotions, powders, sprays, perfumes, lipsticks, nail polishes, eye and facial makeup, hair colours, shampoos, soaps, deodorants, bath and shower oils, toothpaste, and sunscreens. Cosmetics are made from a range of organic or inorganic ingredients, which are deemed to be industrial chemicals. The US FDA has banned heavy metals and their compounds from being intentionally added to cosmetics, unless they occur as impurities.[41] However, the impurities are still a cause of concern in case they are not well controlled. For instance, heavy metals can be present in makeup because they are part of the raw ingredients used to make the product. On the other hand, some equipment used can occasionally contaminate the product during the manufacturing process. In this case, the impurities will not be considered as part of the cosmetic ingredients, even though they are present in high

concentrations.

Therefore, impurities should also be monitored as detrimental chemicals in many cosmetic products. People may think that these impurities are not important since these products are applied on the skin. However, since the skin is the thinnest and most highly permeable organ that covers all the body, it allows penetration of different substances to the bloodstream and thereby to the rest of the body.

1.1.4.2 Health impact of heavy metals in cosmetics

Some heavy metals have no function in the body and can be highly toxic. However, others are essential for maintaining human health. Iron, copper, zinc, cobalt and manganese are well known essential elements for biological systems. For example, iron is needed for the production of red blood cells and also for the transportation of oxygen from the lungs to the heart. Copper participates in biological oxidations through enzymes and proteins containing this element. Zinc helps wound healing, and is essential for normal growth of all tissues.[103] Cobalt is a part of vitamin B12, which serves to hold the vitamin molecule together and to make it function properly. At high levels cobalt can harm the lungs and heart or cause dermatitis.[104] Essential metals in the human body normally are needed at low concentrations; deficiency or excessive concentrations may cause various complications.[105]

Heavy metals enter the human body primarily via diet, air inhalation and skin exposure. The use of different cosmetics such as skin-lightening creams, facial make-up and nail polishes may represent another potential source of contamination as they are applied directly to the skin.[38, 93, 104-106] Long-term exposure to heavy metals can lead to their accumulation in organisms because they are hard to metabolise. Studies have shown that metal ions, when bound to proteins, enzymes, and nucleic acids get absorbed and interfere by forming complexes with carboxylic acids (–COOH), amines ($–NH_2$), and thiols (–SH) of proteins resulting in malfunctioning or death of the cells.[107-109] Symptoms and effects can vary according to the metal or metal-compound involved.[110]

Heavy metals have been found as impurities in several cosmetic products.[111] In some

instances, they have been intentionally used as cosmetic ingredients as colour additives, preservatives and/or active agents. Examples include mercury for inhibiting melanin production,[112] thiomerosal as a mercury-based preservative and lead acetate as a progressive hair dye,[113] red cinnabar (mercuric sulfide) as tattoo pigments, and cadmium as a deep yellow to orange pigment for lipsticks and face powders.[113]

Given that the health issues of heavy metals as deliberate cosmetic ingredients have been addressed, attention is turned to the presence of these substances as impurities. Lead, arsenic, cadmium, chromium and mercury are the most reported metals of primary toxicological concern in cosmetics.[104,107,113,114]

As mentioned previously, mercury is linked to kidney damage, and reproductive system and central nervous system disorders.[115] Lead has been proven to cause miscarriages, infertility in men and women, hormonal changes and behavioural problems.[35] Cadmium impairs kidneys, lungs, blood vessels, suppresses the immune system and causes bone degradation.[38, 39] Other heavy metals also present the same risk of toxicity to a biological system if allowed to accumulate over time in the human body.[35]

The European Directive 76/768/EEC banned Pb, Cd, Co, Cr, Ni and their compounds in cosmetic preparations.[36, 116] There is a general belief that even with the regulation of ingredients in cosmetic products, there are still health concerns regarding the presence of harmful materials within these preparations. Therefore, there is a need to investigate the concentration of toxic metals in some commonly used skin-lightening preparations.

In the present study, 10 elements: As, Cd, Co, Cr, Cu, Fe, Hg, Ni, Pb and Zn were quantitatively investigated in 49 skin-lightening creams by inductively coupled plasma- optical emission spectrometry.

1.2 Dermatological effects of skin-lightening products

As noted earlier, the active ingredients commonly used in many skin-lightening creams induce, with long-term application, many skin problems when used either at low or high

concentrations. The most reported skin problems are: exogenous ochronosis, allergic contact dermatitis, straie atrophicans, skin atrophy or thinning, acne, pigmented colloid millia, nail pigmentation, skin peeling and redness, patchy, erythrema, hyperpigmentation and hypopigmentation, folliculitis, Cushion's syndrome, pityriasis, bleach panda effect (heavy pigmentation around eyes), eczema, wrinkles, rosacea, and tinea. Some of the skin conditions recently diagnosed by dermatologists are shown in Figures 1.4 (a) - (f).

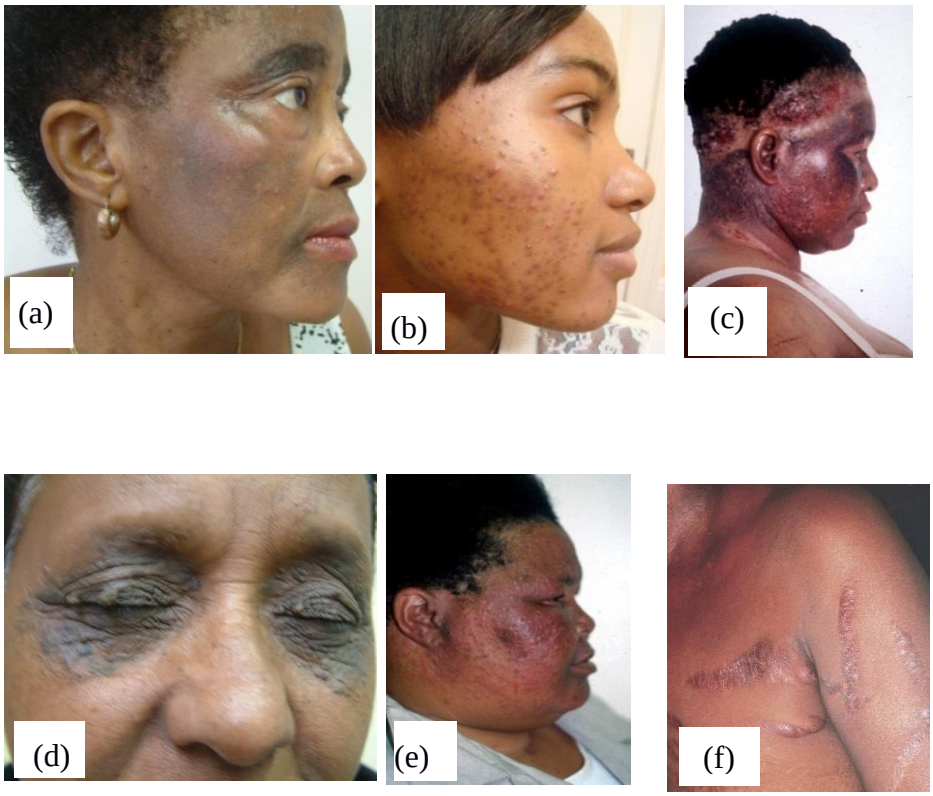


Figure 1.4: Skin conditions: (a) ochronosis and steroid dermatitis, (b) acne induced by topical application of steroid-based cream, (c) severe ochronosis, (d) periobiatal pigmentation, (e) steroid rosacea, and (f) steroid straie. (Source: Dr N Dlova, Nelson Mandela School of Medicine, University of KwaZulu-Natal)

1.3 Motivation, objectives and outline of the study

Everybody at some point has a problem with their own skin, whether it concerns pigmentation, texture, or any other skin problem. However, the practice of skin-

lightening in an attempt to beautify and achieve a moderate complexion may lead either to self-esteem or self-hatred. In view of the increasing incidence of undesirable side-effects, including exogenous ochronosis, contact dermatitis, dyschromia, nephrotic syndrome, and other potential health risks, that have been reported to be associated with long-term use of skin-lightening creams containing hydroquinone, mercury and steroid-based products, it is necessary to investigate whether hydroquinone and other toxic compounds are still among the ingredients of commercial skin-lightening creams.

In this study, we investigated some commonly used skin-lightening creams available in South Africa's stores. The main objective of the study was to identify and quantify the active ingredients as well as heavy metals in these products. It is well known that heavy metals can intentionally or unintentionally be added to cosmetics during the process of manufacturing.

An investigation of skin-lightening creams is important not only for the compliance of their active ingredients with the regulations, but also for the protection of the users and, in particular, for public awareness about the dangers of possible toxic ingredients of these skin-lighteners.

The analysis of skin-lightening cream samples for the organic active skin-lightening ingredients was carried out by using high performance liquid chromatography (HPLC). For the determination of mercury and heavy metals, cold vapour atomic absorption spectroscopy (CV-AAS) and inductively coupled plasma-optical emission spectroscopy (ICP-OES) were employed, respectively.

Chapter 2

EXPERIMENTAL

In this chapter, we present the analytical techniques employed during the investigation of commonly used skin-lightening creams (SLCs), including an overview of the instruments used, principles and experimental detail. This chapter also outlines the consumable materials and other equipment used for the preparation, characterisation, and quantification of the samples.

2.1 Materials

A total of 60 products were purchased from various shops and beauty stores in Durban, South Africa. During the collection of these products, we noticed that the package labels of most of them advertise and call for consumers to safely beautify their skin texture and/or enhance the colour of the skin. Collected sample creams were divided in two sets entitled Batch I and Batch II.

Batch I was selected for the analysis of heavy metals and Batch II was tested for the presence of organic active ingredients. All samples were stored out of sunlight and at room temperature in order to prevent photo-reaction and denaturation of their chemical constituents. A list and details of the products investigated in this project is given in Table 2.1. The chemicals used in this investigation as well as the manufactures and the grades are listed in Appendix A.

2.2 Equipment

The equipment used for qualitative and quantitative analysis is listed in Appendix B.

Table 2.1: Details of skin-lightening products investigated in this study.

BATCH I				
Code	**Brand name**	**Ingredients listed on packaging**	**Company**	**Country of manufacture**
SLC1	Amabala® Cream Asemini	-	IMC	South Africa
SLC2	Amabala® Cream X-TRA	-	IMC	South Africa
SLC3	Antiblemish & Fairness	-		South Africa
SLC4	Cosmotex BCo™ Original Skin Repair Cream	Pure tissue oil, herbal extracts, vitamin A, vitamin E, Vitamin B complex, moisturizing complex, ultraviolet sunscreen, vegetable oils	Champion Cosmetics	South Africa
SLC5	Beta Cream	Notice: Avoid sunlight exposure	Mthatha Plaza & Triangle Pharmacies	South Africa
SLC6	Bio Clear Nourishing Cream enriched with aloe vera & tea tree oil	Aloe vera and tea tree oil	*cmg* Trading	South Africa
SLC7	Bio Clear Vanishing Cream Oil Control For combination to oily skin	Pure plant extracts	*cmg* Trading	South Africa
SLC8	Bio-Oil	Paraffinum liquidum, triisononanoin, cetearyl ethylhexanoate, isopropyl myristate, retinyl palmitate, helianthus annus seed oil tocopheryl acetate, anthemis nobilis flower oil, lavandula angustifolia oil, rosmarinus officinalis leaf oil, calendula officinalis extract, glycerine soja oil, BHT, parfum, coumarin, eugenol, farnesol, purcellin oil, citronellol, amyl cinnamal, geraniol, limonene, linalool, CI26100, CI60725	Evolabs (pty) South Africa Ltd.	South Africa

SLC9	Caro light lightening beauty cream with carrot oil	Petrolatum, mineral oil, stearic acid, cetearyl alcohol, isopropyl miyristate, glycerin, methyl and propyl paraben, carrot oil, vitamin E (0.2%), hydroquinone (max. 2%), aqua, fragrance. Notice: Contains hydroquinone. Do not expose yourself to sunshine	Angel Cosmetics	Democratic Republic of the Congo
SLC10	Clear Essence skin smoothing crème with sunscreen	Deionized water, petrolatum, cetyl alcohol, isopropyl palmitate, stearic acid, ammonium lauryl sulfate, licorice extract, aspergillus ferment, ethoxydiglycol, glyceryl stearate, dimethicone, diazolidinyl urea, methyl paraben, propyl paraben, propylene glycol, sodium metabisulfite, octyl methoxycinnamate, lactic acid (an alpha hydroxy acid), trisodium EDTA, propylene glycol, BHA, propyl gallade, citric acid, ascorbic acid, fragrance. Notice: Not for use on children. If irritation occurs, stop use.	Bluefield Associates, Inc.	U.S.A.
SLC11	Cuticura Antiseptic Ointment	Phenol 0.160 g, precipitated sulphur 0.500 g, 8-hydroxyquinoline 0.050 g, perfumed base to 100 g	NOVARTIS South Africa (Pty) Ltd.	South Africa
SLC12	Cuticura Vanishing Cream for all skin types, oil reduction programme	Aqua, stearic acid, propylene glycol, glyceryl stearate, myristyl myristate, isopropyl palmitate, disodium edetate, potassium hydroxide, butylparaben, ethylparaben, isobutylparaben, methylparaben, phenoxyethanol, propylparaben, fragrance	NOVARTIS Consumer Health SA	South Africa
SLC13	Dabur Uveda complete fairness cream SPF 20	Extracts of Javetri, Kasturimanjal, Kesar and Raktachandan, glycerin, OMC, MBBT	Dabur India Ltd.	India
SLC14	Epimax	Cetomacrogol	Genop SA	South Africa.
SLC15	Eraser Plus anti-marks + fairness cream non-greasy	Each 100 g contains: Curcuma longa Linn 18.0 ml, Rosa centifolia Linn 4.0 ml, wheat germ oil 2.5 ml, Ocimum sanctum Linn 2.0 ml, citrus sinensis rind 2.0 ml, Azadirachta indica oil 2.0 ml, Santalum album Linn 1.0 ml, aloe vera 1.0 ml, Crocus sativus Linn 0.1 g, Non-greasy base q.s. Notice: Discontinue the use if skin irritation occurs	IPSA Labs (P) Ltd.	India

SLC16	Eraser UltraFair ayurvedic fairness cream	Each 100 g contains: Haldi oil 1 ml, neem 1 g, safed chandan 0.5 g, santra 2 g, gulab 2 g, aloe vera 2 g, kumkum 0.1 g, akhrot 1 g, jasmine 0.5 g non-greasy cream base q.s. Melanin disperser - Kumkum and Sandal, Moisturiser and sunscreen - aloe vera, Nourisher - Akhrot, Blemishes remover - Santra and jasmine, Improves complexion - Haldi and rose. Notice: Contains no bleach or harmful ingredients.	IPSA Labs (P) Ltd.	India
SLC17	Eskamel acne and pimple cream	Resorcinol 2 g, sulphur 8 g, hexachlorophene 0.25 g per 100 g.	Group Laboratories SA (Pty) Ltd. SmithKline Beecham	South Africa
SLC18*	Eucerin	10% Urea	Beiersdorf AG	Germany
SLC19*	Fair & Lovely advanced multi vitamin daily fairness expert	Water, palmitic acid and stearic acid, niacinamide, glycerin, cetearyl ethlhexanoate and isopropyl myristate, ethylhexyl methoxycinnamate, butyl methoxydibenzoylmethane, hydroxystearic acid, sodium ascorbyl phosphate, tocopheryl acetate, allantoin, pyridoxine hydrochloride, cetyl alcohol, dimethicone, titanium dioxide and aluminium hydroxide and stearic acid, phenoxyethanol, methylparaben, propylparaben, potassium hydroxide, titanium dioxide and dimethicone, disodium EDTA, CI 77491 (and) isopropyl titanium triisostearate (and) triethoxysilylethyl polydimethylsiloxyethyl dimethicone, CI 15510, CI 17200, perfume. Notice: Contains no harmful ingredients or bleach.	Unilever Pakistan Ltd.	Pakistan
SLC20*	Paris Fair & White Eclaircissante Whitening Cream	Aqua (water), paraffinum liquidum (mineral oil), cetearyl alcohol, cera microcristallina, petrolatum, polysorbate-60, glycerin, sorbitan stearate, stearic acid, parfum (fragrance), triethanolamine, caprylic/capric triglyceride, diacetyl boldine, propylparaben, methylparaben, carbomer, myrtrimonium bromide, methylchloroisothiazolinone, benzyl salicylate, cinnamyl alcohol, limonene, eugenol, alpha isomethyl lonone, hexyl cinnamal, isoeugenol, butylphenyl methylpropional, linalool.	Labo. DERMA	France
SLC21*	Faiza Beauty Cream	-	Poonia Brothers	Pakistan

SLC22	Fem Bleach Cream	Hydrogen peroxide, propylene glycol, stearic acid, glyceryl mono stearate, cetomacrogol-1000, isopropyl myristate, disodium EDTA, glycerin, Brij 786, cetosteryl alcohol, ortho-phosphoric acid, disodium maleate, water.	FEM Care Pharma Ltd.	India
SLC23	Fem perfumed blue activator powder	Persulphate, starch, colloidal silicon dioxide, di-sodium EDTA, mineral oil, ZLSS carbonate, metal stearate, colour blue, perfume.	FEM Care Pharma Ltd.	India
SLC24	Fem post-bleach cream	Stearic acid, cetyl alcohol, isoprppyl myristate, sodium stearoyl lactylate, glycerin, propylene glycol, methyl paraben, propyl paraben, triethanolamine, titanium dioxide, perfume, water q.s.	FEM Care Pharma Ltd.	India
SLC25	Fem pre-bleach gel	Hydrogen peroxide, carbomer, glycerin, methyl paraben, propyl paraben, metamethoxy benzamide, disodium EDTA, tetrasodium pyrophosphate, sodium hexametaphosphate, triethanolamine, citric acid, disodium maleate, water.	FEM Care Pharma Ltd.	India
SLC26*	Gentle Magic cream	-	Du Marc cc	South Africa
SLC27	He Man complexion lotion	-	Adcock Ingram	South Africa
SLC28	Hypoderm Day Cream	Vitamin A & C derivative	Cosmetic Warehouse	South Africa
SLC29	Hypoderm Night	Retinol, Alpha hydroxyl acids, vanilla extracts, ascorbyl palmitate	Cosmetic Warehouse	-
SLC30	Lemon Lite Complexion Cream with real lemon extract, Vitamin A, E & F Complex, SPF5	Water, stearic acid, glycerin, ethylhexyl methoxycinnamate, triethanolamine, sodium borate, cetyl alcohol, isopropyl myristate, ethyl linoleate, tocopheryl acetate, Helianthus annuus, retinyl palmitate, retinyl acetate, methyl paraben, propyl paraben, Citrus Medica Limonum (lemon) extract, fragrance (parfum), FD and C yellow 5 (CI 19140), citral, limonene	Tiger Consumer Brands Ltd	South Africa
SLC31*	Royal New You anti blemish fairness cream with green tea extract	Saffron, Neem extract, Green tea extract	Sara Lee	South Africa

SLC32	Palmer's Cocoa Butter Formula® with Vitamin E, softens, smoothes & relieves dry skin, smoothes marks, tones skin	Water (aqua), theobroma cacao (cocoa) extract, glyceryl stearate, petrolatum, propylene glycol, glycerin, cocos nucifera (coconut) oil, mineral oil (paraffinum liquidum), behentrimonium methosulfate, cetearyl alcohol, cetyl alcohol, theobroma cacao (cocoa) seed butter, elaeis guineensis (palm) oil, dimethicone, tocopheryl acetate, fragrance (parfum), hydroxyethylcellulose, PEG-8 stearate, methylparaben, propylparaben, benzyl alcohol, yellow 5 (CI 19140), orange 4 (CI 15510).	E.T. Browne Drug Co., Inc.	USA
SLC33*	Pure Cream	Purified water, propylene glycol, glycerin, cetyl alcohol, ceteareth 20, ricinus communis seed oil, hydrogenated castor oil, beeswax, copernicia cerifera wax, caprylic/capric triglyceride, isopropyl myristate, cetostearyl alcohol, polysorbate 20. Preserved with ethylhexylglycerin and phenoxyethanol.	Reitzer Pharmaceuticals (Pty) Ltd.	South Africa
SLC34	Pure Perfect Parfait(24)	Aqua (water), butylene glycol, squalane, caprylic/capric triglyceride, PEG-60 almond glycerides, kojic acid dipalmitate, saccharomyces/xylinum black/tea ferment, arbutin, propylene glycol, diacetyl boldine, dimethylmethoxy chromanyl palmitate, caesalpinia spinosa oligosaccharides, caesalpinia spinosa gum, tocopherol, phenoxyethanol, aminomethyl propanol, caprylyl glycol, acrylates/C10-30 alkyl acrylate crosspolymer, glycerin, hydroxyethylcellulose,parfum (fragrance), benzyl salicylate, butylphenyl methylpropional, citronellol, geraniol, hexyl cinnamal, hydroxyisohexyl 3-cyclohexene carboxaldehyde, limonene, linalool, CI 17200 (Red 33).	Pure Perfect cc	South Africa
SLC35	Pure Perfect Parfait(25)	Aqua (water), butylene glycol, squalane, caprylic/capric triglyceride, PEG-60 almond glycerides, kojic acid dipalmitate, saccharomyces/xylinum black/tea ferment, arbutin, propylene glycol, diacetyl boldine, dimethylmethoxy chromanyl palmitate, caesalpinia spinosa oligosaccharides, caesalpinia spinosa gum, tocopherol, phenoxyethanol, aminomethyl propanol, caprylyl glycol, acrylates/C10-30 alkyl acrylate crosspolymer, glycerin, hydroxyethylcellulose,parfum (fragrance), benzyl salicylate, butylphenyl methylpropional, citronellol, geraniol, hexyl cinnamal, hydroxyisohexyl 3-cyclohexene carboxaldehyde, limonene, linalool, CI 17200 (Red 33).	Pure Perfect cc	South Africa
SLC36	Pure Perfect Parfait(6)	Aqua (water), butylene glycol, squalane, caprylic/capric triglyceride, PEG-60 almond glycerides, kojic acid dipalmitate, saccharomyces/xylinum black/tea ferment, arbutin, propylene glycol, diacetyl boldine, dimethylmethoxy chromanyl palmitate, caesalpinia spinosa oligosaccharides, caesalpinia spinosa gum, tocopherol, phenoxyethanol, aminomethyl propanol, caprylyl glycol, benzyl salicylate, butylphenyl methylpropional, citronellol, geraniol, hexyl cinnamal, hydroxyisohexyl 3-cyclohexene carboxaldehyde, limonene, linalool, CI 17200 (Red 33).	Pure Perfect cc	South Africa

SLC37	Pure Perfect Parfait (7)	Aqua (water), butylene glycol, squalane, caprylic/capric triglyceride, PEG-60 almond glycerides, kojic acid dipalmitate, saccharomyces/xylinum black/tea ferment, arbutin, propylene glycol, diacetyl boldine, dimethylmethoxy chromanyl palmitate, caesalpinia spinosa oligosaccharides, caesalpinia spinosa gum, tocopherol, phenoxyethanol, aminomethyl propanol, caprylyl glycol, benzyl salicylate, butylphenyl methylpropional, citronellol, geraniol, hexyl cinnamal, hydroxyisohexyl 3-cyclohexene carboxaldehyde, limonene, linalool, CI 17200 (Red 33).	Pure Perfect cc	South Africa
SLC38	Pure Perfect Parfait (8)	Mulberry extract, lactic acid, safflower oil, stearic acid, carbomer, ploydecene, glycerol stearate, benzoyl alcohol, methlisothiazolinone, undecylenoyl phenylalanine (SEPIWHITE MSH), kojic acid, licorice root extract, dimethicone, cetearyl alcohol, macadamia (vitamin E), fragrance	Pure Perfect cc	South Africa
SLC39	Queda Amabala day and night cream plus skin conditioner now with sun protector	Notice: Avoid excessive exposure to sunlight while using this product	Adcock Ingram	South Africa
SLC40	Quick Clear Blemish Cream	-		South Africa
SLC41	Replay Skin Care Day Cream	SPF 15 Notice: Mild stinging may occur at first. Discontinue use if irritation occurs.	Dry Skin Research (Pty) Ltd.	South Africa
SLC42	Replay Skin Care Night Cream	Notice: Mild stinging or itching may occur. If this persists or other irritation occurs discontinue use.	Dry Skin Research (Pty) Ltd.	South Africa
SLC43	Rico lemon X-TRA skin lightening complexion cream	Aqua (water), glyceryl stearate, cetearyl alcohol, glycerin, dipropylene glycol, betula alba, glycyrrhiza glaba, citrus limonum, sambucus nigra, tilia cordata, morus nigra, isopropyl myristate, sodium lactate, fragrance (parfum), ethylhexyl methoxycinnamate, tocopheryl acetate (vitamin E), allantoin, ascorbic acid, sodium lauryl sulfate, sodium metabisulfite, disodium EDTA, methylparaben, propylparaben, p-chloro-m-cresol. Notice: If skin irritation occurs discontinue use. Not suitable for sunburn prevention. This crem should not be used by children under the age of 12 without the advice of a doctor.	Rico Skin Care Ltd.	EU

SLC44	R-Lactin	Purified water, propylene glycol, glycerin, cetyl alcohol, ceteareth 20, ricinus communis seed oil, hydrogenated castor oil, beeswax, lactic acid triethanolamine, caprylir-ic/capric triglyceride, isopropyl myristate, cetostearyl alcohol, polysorbate 20. Preserved with ethylhexylglycerin and phenoxyethanol.	Reitzer Pharmaceuticals (Pty) Ltd.	South Africa
SLC45	Shirley medicated cream	Natural albumen	Shirley Chemical Corp.	Taiwan
SLC46	Skinspor GS	-	Mthatha Pharmacy	-
SLC47	Susa Amabala Vanishing Cream with oil control ingredients	Water (aqua), stearic acid, propylene glycol, niacinamide, cetearyl alcohol, sodium C8-16 isoalkylsuccinyl, soy protein sulfonate, cyclomethicone, lauryl methacrylate/glycol dimethacrylate crosspolymer, triethanolamine, fragrance (parfum), ethylhexyl dimethyl PABA, DMDM hydantoin, tocopheryl acetate, menthol, camphor, amyl cinnamal, butylphenyl methylpropional, citral, citronellol, coumarin, geraniol, hexyl cinnamal, hydroxisohexyl 3-cyclohexene carboxaldehyde, limonene, linalool	Amka Products	South Africa
SLC48	Unknown Sample	-		-
SLC49*	Yoko/acne melasma Cream	Hydroquinone Free, Candellila wax, Carnauba wax, castor oil, salicylic acid, tocopheryl acetate, titanium dioxide, licorice extract, co-enzyme Q10	Siam Yoko Co., Ltd.	Thailand
BATCH II				
SLC50	*e* Beauty Vanishing Cream for long lasting oil and shine control, advanced formula with vitamins E and B3 for a beautiful complexion, normal to oily skin types	Aqua, stearic acid, niacinamide, glycerine, propylene glycol, silica, ethyhexyl adipate, dimethicone, carbomer, ceto stearyl alcohol, ethylene glycol monostearate, tocopheryl acetate, parfum, potassium hydroxide, methylparaben, propylparaben, disodium EDTA, DMDM hydantoin.	The E Manufacturers (Pty) Ltd.	South Africa

SLC51	Naturoriché Skin Life Cream Turmeric Cream	Aqua, stearic acid, aloe barbadensis (aloe vera), Ocimum Sanctum (Tulsi), Rubia Cordifolia (Manjishta), berberis species (Daru Haldi), Curcuma long extract (Haldi), tumeric oil, glycerin (glycerol), cetyl alcohol, methylpara hydroxy benzoate, propyl para hydroxy benzoate, EDTA, butyl hydro toluene, dimethicone (silicone oil), vitamine E, potassium hydroxide, parfum, colour-Tartazine Yellow (CI No. 19140), sunset yellow FCF. Notice: Before using on face, test cream on a small section of arm or leg, if irritation occurs discontinue use. Contact physician.	Hesh Pharma	India
SLC52	Seven Herbal Ubtan with sunscreen makes the skin radiant and fair	Pure herbs	C.P.H.L.	Pakistan
SLC53	Mod Girl Fairness Snow with sunscreen multi-protection SPF 15	SPF 15	Max-Zealer Cosmetics (Pvt) Ltd.	Pakistan
SLC54	Skin White Golden Beauty Cream	No steroids, no mercury, no side effects Natural beeswax, petrolatum, zinc oxide, titanium dioxide, kojic palmitate, arbutin/plant and flower extracts, octyl methoxy cinnamate, gold pigment, color and fragrance.	Skincare Company	Pakistan
SLC55	Movate Cream	100 g contains: Clobetasol propionate 0.050 g, Excipients: propylene glycol, titanium dioxide, carbomer, purified water q.s.	Esapharma srl Pharmaceutical Laboratory	Italy
SLC56	G & G Lightening Beauty Cream with D.S.N. 56 for all skin types with sun rays filters	Aqua (water), stearyl alcohol, petrlatum, (paraffin), paraffinum liquidum (mineral oil), kojic acid, artosphylos UVA-URSI (Bearberry extracts), isopropyl myristate, glycerin, 1% octylmethoxycinnamate,AHA (citric, gluconic, glycolic, lactic, malic, salycilic, tartaric acids), cyclomethicone, sodium metabisulfite, fragrance, Simondsia, Chinensis (Jojoba oil),triticum vulgare (wheat germ oil), propyl paraben, cetyl alcohol, , methyl paraben, polysorbate 60, 0.5% butylmethoxydibenzoylmethane, BHT, tocopheryl acetate, 3% D.S.N. 56 (kojic acid and bearberry extracts). Notice: In case of irritation, stop application. Do not use for children under 12 years old.	N.P. Gandour	Ivory Cost

SLC57	Eraser Anti Marks Cream	Each 1000 g contains: wheat germ oil 2.5 g, curcuma longa linn 18.0 g, azadirachta indica oil 2.0 g, santalum album linn 1.0 g, Ocimum sanctum linn 2.0 g, citrus sinensis rind 2.0 g, rosa centifolia linn 4.0 g, aloe vera 1.0 g, crocus sativus linn 0.1 g, cream base q.s. Notice: Contains no bleach or harmful ingredients. Discontinue use if skin irritation occurs	IPSA Labs (P) Ltd.	India
SLC58	Bio Claire Lightening Body Cream without Hydroquinone	Water, mineral oil, petrolatum, lanolin, stearic acid, cetyl-stearylic, alcohol, isopropyl myristate, tocopheryl acetate, BHT, methyl paraben, propyl paraben, sodium lauryl sulfate, sodium metabisulfite, sodium sulfite, citric acid, propylene glycol, arctostaph-ylos, bearberry extracts, mulberry extracts, licorice extracts, lemon extracts, kojic acid, fragrance. Notice: If skin is irritated sto use. Do not use for children under 12 years old.	NP Gandour	Ivory Cost
SLC59	Even Tone Cream	-	-	India
SLC60	Dr James Flower Bleach-ing Cream	Tricosan, allantoin, Vitamin E	What is Green Co. Ltd.	Thailand

Batch I: Sample products analysed for heavy metal content.

Batch II: Sample products analysed for organic skin-lightening active ingredients.

*Were also analysed for organic skin-lightening active ingredients by HPLC.

2.3 Extraction of heavy metals in skin-lightening creams

With the development of modern analytical chemistry, the speed, precision and accuracy of sample analysis have been improved considerably. These means are based not only on the instrumentation but also on the treatment of the matrix of the sample. Most samples with a complex matrix are difficult to handle during the extraction process. Reports have revealed that the matrix of cosmetic creams is not simple because it consists of an emulsion containing a mixture of organics and inorganics.[117] This mixture is mainly composed of water, oil or a silicone phase.[104] Oil often refers to an overall mixture of chemicals including proteins, waxes and alkaloids, which demand more consideration throughout the sampling processes. The methods reported in various studies use acid digestion, calcination and ultrasound-assisted emulsification as sample treatment.[37-39,104,106,118] Sometimes, even with these treatments, complete solubilisation of a sample is not feasible. The residuals (metallic oxides and pigments) that can affect the analysis must be removed by means of filtration or centrifugation.[104,119] In the present study, acid digestion techniques were employed for the dissolution of heavy metals in skin-lightening creams, subsequent to sampling and sample preparation, in order to completely transform the analytes into solution that can be introduced into the ICP-OES or CV-AAS in liquid form.

2.3.1. Open acid digestion

It was desirable to first carry out a preliminary test in order to assess the safety and efficiency of the mineralisation method. Consequently, open acid digestion and closed acid digestion (microwave digestion) were conducted on five different cream samples. Thereafter, the digestion technique with the optimum conditions was applied to all cream samples.

The open acid digestion method known as wet digestion and the dry ashing procedure were performed in order to extract metals from the matrix of the samples. The procedures are described below.

Dry-ashing approach

Dry ashing in an electric furnace was conducted on the selected sample creams.[38] A sample mass of 0.5 g of each skin-lightening cream was accurately weighed into a fused silica crucible. To avoid charring of certain samples that can happen when subjected to high temperature in the furnace, samples were left to dry overnight in an oven at 90 ºC. The sample was then heated in an electrical furnace at 600 ºC for three hours to obtain a carbon free ash. The ash was allowed to cool down to room temperature in a desiccator and the resultant mass (approximately 0.18 g) was recorded for each sample. For the next stage, all the accurately weighed ash was dissolved in 3 mL of hot conc. HNO_3 and then the mixture was accurately diluted to 50 mL in a volumetric flask with double-distilled water.

Wet digestion approach

Wet digestion of the samples was carried out by using a hot plate. A mass of 0.25 g of each cream sample was directly placed in a beaker into which 10 mL of conc. HNO_3 was added. The mixture was heated at 130 ºC for one hour in a fume hood. After cooling, the solution was transferred into a 50 mL volumetric flask and diluted to volume with double-distilled water. The results of both dry-ashing and the wet digestion approaches are discussed in Chapter 3, Section3.1.1.

2.3.2. Microwave-assisted digestion

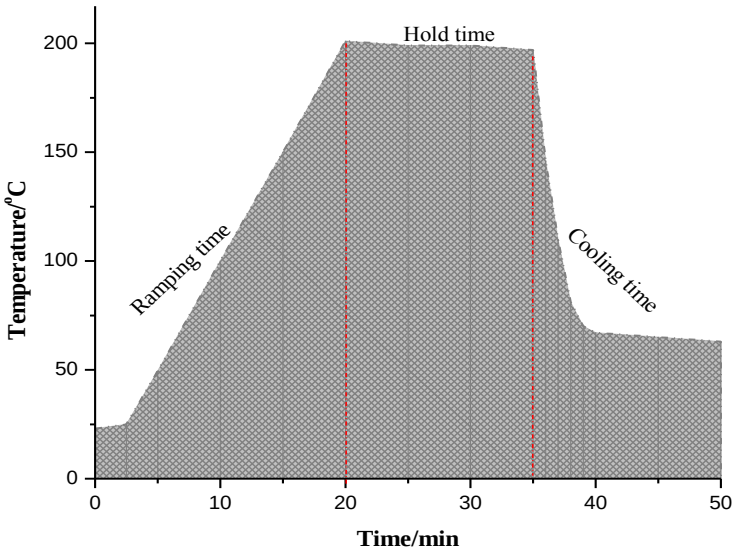
Microwave irradiation has allowed scientists to systematically study the decomposition mechanism of a variety of matrices in closed-vessels. Decomposition of most samples can be achieved with the aid of mineral acids, primarily HNO_3, HCl, HF, H_2SO_4 and other liquid reagents such as hydrogen peroxide. Microwave digestion involves irradiation with electromagnetic waves with a high frequency electric field to achieve fast heating of the sample through the direct absorption of microwave energy by substances. The higher temperature gained results in a dramatic increase in the reaction kinetics that allows the decomposition of most sample matrices.

Depending on the matrix of the sample, a combination of acids such as HNO_3, HCl and/or HF, has been reported to provide better recoveries for digestion of some cosmetics such as eye shadows, eyeliner and lipsticks in a closed system.[117] However, when this combination was used for the skin-lightening creams in this work, it was prone to explode. Consequently, in this study, a strong oxidising agent, HNO_3, was chosen to digest skin-lightening creams. The extraction of all selected heavy metals was then accomplished by a Mars 6 microwave digestion system (CEM Corporation, USA).

A sample mass of 0.250 g of each skin-lightening cream was accurately weighed into a high pressure resistant TFM-vessel, to which 10 mL of conc. HNO_3 was added. After pre-digestion of samples for 15 minutes in uncovered microwave vessels placed in the fume hood, the vessels were sealed and subjected to microwave irradiation by using the program shown in Figure 2.1a.

During the ramp period, the temperature was gradually increased from room temperature to 201 ºC over 20 minutes. The vessels were held at this temperature for 15 minutes and then cooled down to ± 65 ºC over 15 minutes. After cooling to room temperature, the vessel modules were disassembled. Both lids and vessels were washed with double-distilled water while collecting the digests. The digested samples were then filtered through filter paper (Whatman №1) into 50 mL volumetric flasks and diluted to volume with double-distilled water. It was necessary to filter all samples in order to ensure that any residue, which could result in data fluctuation, was removed before analytical measurement. Samples and blanks were digested in triplicate. Both samples and blanks were stored in plastic bottles.

(a)



(b)

Figure 2.1 (a) The outline of the program used for sample digestion. (b) Photograph of the microwave device and the XP-1500 TFM reaction vessels used.

2.4 The determination of heavy metals in skin-lightening creams

Skin-lightening creams were analysed for As, Cd, Co, Cr, Cu, Fe, Hg, Ni, Pb and Zn by inductively coupled plasma-optical emission spectrometry (ICP-OES). This technique was preferred for analysis because it allows multi-element analysis and supports a wide linear calibration range. Afterwards, a very sensitive method for the atomic absorption analysis of mercury cold-vapour atomic absorption spectrometer (CV-AAS) was also used for measuring trace amounts of mercury detected by ICP-OES.

All glassware and plastic-ware needed for analysis were placed overnight in dilute nitric acid (10% HNO_3) and washed with warm soapy-water, and then rinsed with distilled water before use.

2.4.1 Inductively coupled plasma-optical emission spectrometry

Inductively coupled plasma-optical emission spectrometry (ICP-OES) is an analytical technique used for the detection of trace metals. It is a type of emission spectroscopy that uses the inductively coupled plasma to produce excited atoms and ions that emit electromagnetic radiation at wavelengths characteristic of a particular element. The intensity of emission is indicative of the concentration of the element within a sample.

The ICP-OES is composed of two parts: the ICP torch and the optical spectrometer. The ICP torch consists of three concentric tubes made of quartz. The copper coil that surrounds the top end of the torch is connected to the radio frequency (RF) generator.

The inductively coupled plasma used in OES consists of a high temperature discharge generated by flowing a conductive gas (argon) through a magnetic field induced by a coil that surrounds the tubes carrying the gas. Argon gas is typically used to create the plasma.

More specifically, when the torch is turned on, an intense electromagnetic field is created within the coil by the high power radio frequency signal flowing in the coil. The argon gas flowing through the torch is ignited with a Tesla unit that creates a brief discharge arc through the argon flow to initiate the ionization process. The argon gas is ionized in the intense electromagnetic field and flows in a particular rationally symmetrical pattern towards the magnetic field of the RF coil. Stable, high temperature plasma is then generated as the result of the inelastic collisions created between the neutral argon atoms and the charged particles. The characteristic emission lines of the atoms and ions are dispersed by a monochromator or polychromator and the intensity of these lines is detected, measured and compared to the intensities for known standards to provide quantitative results.

An ICP-OES spectrometer is generally equipped with a concentric nebuliser where sample solutions are changed into mist, the source used to generate the high temperature plasma required to atomise and ionise the analytes, the basic spectrometer which has the task of separating the emission lines generated by the sample and a detection system with data handling to measure and interpret the emitted light (Figure 2.2).

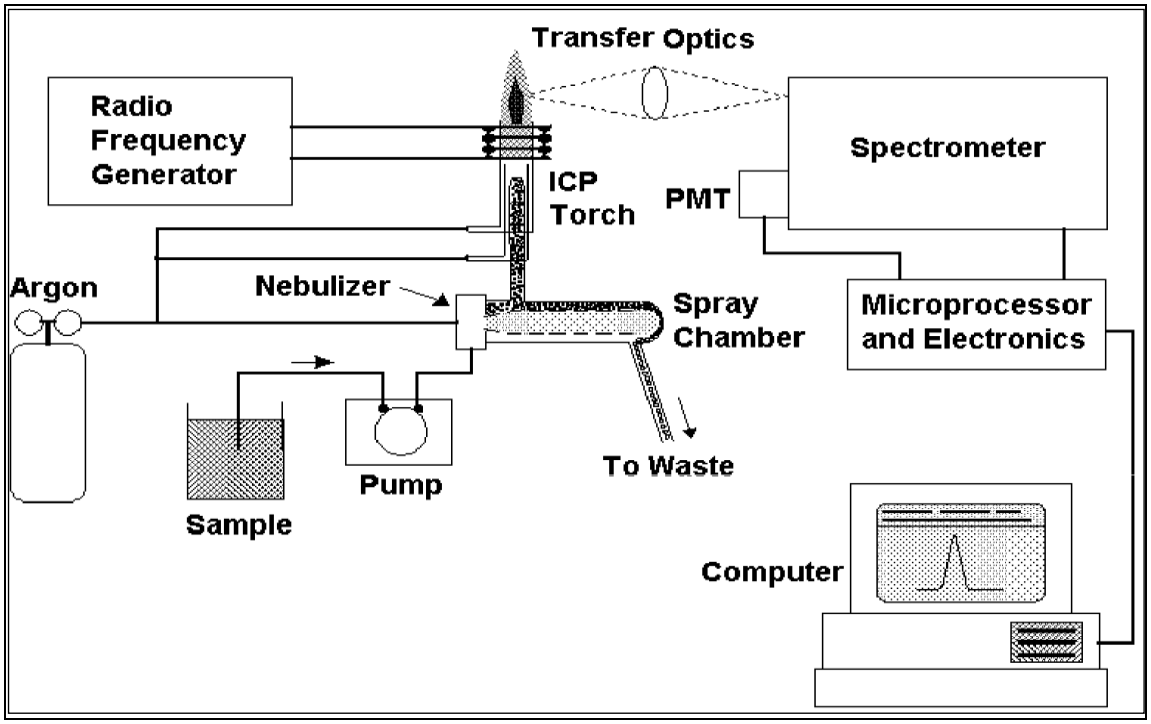


Figure 2.2: Schematic representation of an ICP-OES instrument.[122]

The ICP-OES is a powerful instrument used for analysis of a variety of sample matrices including environmental, pharmaceutical, industrial and biological samples.

2.4.1.1 Preparation of standard solutions

High purity 1000 mg L^{-1} stock solutions of Cr, Cu, Fe, Zn and Pb were used in order to prepare standard solutions necessary to determine the calibration curves. From the stock solutions, a 1 mg L^{-1} working stock standard solution was prepared by transferring an aliquot of 0.1 mL of each metal ion solution into a 100 mL volumetric flask and then 10 mL of conc. HNO_3 was added. The mixture was diluted to volume with double-distilled water. Nitric acid was added to mimic the matrix effects of the sample and standard solutions. Further dilutions were made with double-distilled water to give solutions desirable for the construction of calibration curves.

A total of five standard solutions, with different concentrations, were prepared for each element. The concentration ranges were 0.02 - 0.1 mg L^{-1} for Cr, Cu, Pb and 0.01 - 0.1 mg L^{-1} for Fe and then 0.1 - 0.5 mg L^{-1} for Zn.

Standard solutions of As, Cd, Co and Ni were not prepared. A rough scan of the samples could not confirm the presence of these metal ions in any of the samples. Blank solutions were made up with double-distilled water and an appropriate amount of nitric acid was included.

2.4.1.2 Sample analysis for heavy metal determination

A Perkin-Elmer Optima 5300 DV ICP-OES was employed to determine concentrations of heavy metals present in the skin-lightening creams. The instrument conditions were: generator power, 1000 W; plasma gas flow rate, 12 L min^{-1}; nebuliser pressure, 3 bars and sample flow rate, 1 mL min^{-1}.

All the digested cream samples were first scanned before preparation of standard solutions. This scan confirmed either the presence or absence of targeted elements within

the samples. Three different analytical wavelengths were preselected for each element; then one of these wavelengths, with high intensity, low detection limits and minimal impact on the background of an emission line was chosen for further use for quantitative analysis. The wavelength used for each of the metal ions analysed in this project is shown in Table 2.2.

Standard solutions and samples were allowed to aspirate for 60 seconds before sampling and the instrument was programmed for sampling each solution five times and the average was recorded. The calibration data and the corresponding curves obtained from the analysis are shown in Appendices C and D.

Table 2.2: Emission wavelengths used for the analysis of heavy metals by ICP-OES.

Metal	**Emission wavelength/nm**
Arsenic	193.696
Cadmium	228.802
Chromium	267.716
Cobalt	238.892
Copper	324.752
Iron	259.939
Lead	220.353
Mercury	253.652
Nickel	231.604
Zinc	213.857

2.4.2 Cold-vapour atomic absorption spectrometry

Atomic absorption spectrometry is a technique used to determine the concentration of a particular metal ion within samples.[123] Unlike ICP-OES, no plasma and torch are required for AAS. However, the AAS technique uses a flame (air/acetylene or nitrous oxide) or a graphite furnace to atomise samples. The sample is atomised in a flame through which radiation of a specific wavelength, from a hollow cathode lamp or an

electrodeless discharge lamp, is passed. The amount of light energy absorbed by free ground-state atoms is the measure of the concentration of the selected metal.

For metals, free ground-state atoms do not exist at room temperature, without heating the sample to break the bonds linking atoms into molecules. The only notable exception to this is mercury. Free mercury atoms can exist at room temperature and, therefore, mercury can be measured by atomic absorption without a heated sample. This is so-called "cold-vapour atomic absorption spectroscopy" (CV-AAS).[124]

In the cold-vapour technique, mercury is chemically reduced to the free atomic state by allowing the sample to react with a strong reducing agent like stannous chloride ($SnCl_2$) or sodium borohydride ($NaBH_4$) in a closed reaction system.[108] The volatile free mercury is then driven from the reaction flask by bubbling air or an inert gas through the solution. Mercury atoms are carried in the gas stream through the tubing connected to a quartz cell, which is placed in the light path of the spectrometer. The CV-AAS technique is important due to its low detection limits in the single digit part per trillion (ppt) range and an abundance of analytical methods which allow for the measurement of mercury in almost any sample matrix.

To perform mercury analysis with an atomic absorption spectrometer, a manually-operated accessory such as the Perkin-Elmer MHS 15 Mercury/Hydride System is required for vapour generation. Figure 2.3 depicts the pneumatic system of the MHS 15 unit. The MHS 15 unit comprises a reactor assembly (a reaction flask, reservoir bottle and carrier gas pipelines) and a quartz tube atomiser (QTA) assembly. The MHS 15 must be well connected to the gas supply and the spectrometer.

During the analysis of mercury, by using $SnCl_2$ as the reducing agent, the inert gas from the pressure reducer flows through restrictor *F2* and through line *b* to the changeover valve, and then travels through line *e* to the reaction flask. However, the gas pressure applied by means of ports *1* to *4* and then via line *g*, forces the transfer of $SnCl_2$ reductant solution through line *h* into the reaction flask. Mercury vapour is then transported by the combined gas streams through the transfer tube *f* to the QTA, where its atomic absorption is measured.

(a)

(b)

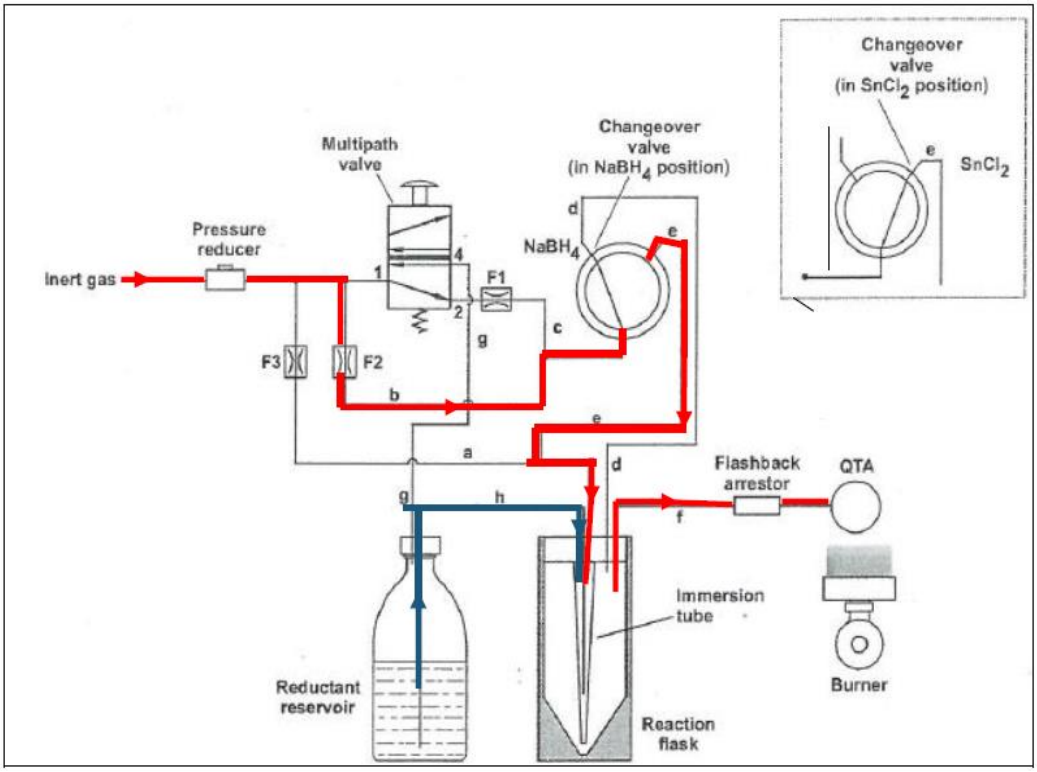


Figure 2.3 (a) A photograph of the atomic absorption spectrometer. (b) Schematic representation of the mercury hydride pneumatic system (adapted from the MHS 15 practical manual).

The reduction of mercury to the elemental state by stannous chloride (in acidic solution) can simply occur by means of a sequence of reactions as has been summarised by Sainio *et al*.[108]

$$2Hg^{2+} + Sn^{2+} + 2Cl^{-} \rightarrow Hg_2Cl_2 + Sn^{4+}$$
$$Hg_2Cl_2 + Sn^{2+} \rightarrow 2Hg + Sn^{4+} + 2Cl^{-}$$

2.4.2.1 Preparation of reagents and standard solutions

The reductant solution, 5% $SnCl_2.2H_2O$, was prepared by dissolving 50 g of the salt in 100 mL of hot conc. HCl. This solution was then diluted to 1 L with distilled water. To prepare a stock solution of 1000 mg L^{-1} mercury, a mass of 0.135 g mercury chloride ($HgCl_2$) was accurately weighed and dissolved in 25 mL of distilled water and the solution was diluted to 100 mL in a volumetric flask with distilled water. Thereafter, a 1 mg L^{-1} mercury working stock standard solution was prepared by pipetting 100 µL from the stock solution into a 100 mL volumetric flask and diluted to volume with distilled water. This solution was used to construct the calibration graph. The dilute nitric acid (10% HNO_3) solution was also prepared for use during all analyses.

2.4.2.2 Sample analysis for mercury determination

Mercury analysis was carried out with a Perkin-Elmer Analyst 100 atomic absorption spectrometer fitted with a hallow cathode lamp operated at a wavelength of 253.7 nm. A Perkin-Elmer MHS 15 Mercury/Hydride System was also connected to the instrument.

During analysis, 10 mL of dilute acid (10% HNO_3) was placed into the reaction flask, to which aliquots of 100, 200, 300, 400, 500, 600 µL of a 1 mg L^{-1} mercury working stock solution was added sequentially to generate the calibration standards. These aliquots corresponded to 100, 200, 300, 400, 500, 600 ng of mercury. When the reaction flask was attached to the reactor assembly, the extractor was then switched on and the argon gas was also allowed to flow through the immersion tube placed into the reaction flask. The reductant ($SnCl_2$) was automatically transferred from the reagent reservoir into the

reaction flask to reduce mercury (either in the standard or sample solution). The stream of inert gas was required to promote mixing of the solutions and to drive out mercury vapour through the immersion tube to the QTA. The reaction flask and the outside of the immersion tube were rinsed with dilute acid after every single measurement. As the mercury atoms passed into the QTA, the measured absorbance gradually increased and then decreased as the mercury was depleted. The highest absorbance detected for each measurement was recorded. Absorbance readings for standard solutions were used to plot the calibration graph. Standard solutions were analysed in triplicate and digested samples were analysed five times.

2.4.3 Validation of quantitative methods

The suitability of the two methods, ICP-OES and CV-AAS, used to determine the heavy metal content in skin-lightening creams was evaluated by determining the instrumental limits of detection (LODs) and quantification (LOQs), linearity, precision and accuracy.

Due to the lack of a cream-based certified reference material, the accuracy of the methods was determined by means of recovery tests. Therefore, a known amount of each element under study was added into a cream that was free from the spike element. A mass of 0.20 g of cream was accurately weighed, transferred into the reaction vessels and spiked with the following solutions: 0.05 mg L^{-1} of Cr, 0.045 mg L^{-1} of Cu, 0.025 mg L^{-1} of Hg and 0.03 mg L^{-1} of Pb; these were added into SLC33 while 0.4 mg L^{-1} of Zn and 0.25 mg L^{-1} of Fe were added into SLC4. Thereafter, 10 mL of conc. HNO_3 was added to the solutions and allowed to predigest. The digests were filtered, diluted to a volume of 50 mL with double-distilled water and analysed according to the analysis conditions of the parent-samples. The recovery results are reported in Chapter 3, Section 3.2.3.

The precision of the methods was expressed as the relative standard deviation (RSD) of the measurements. The limits of detection and quantitation (LOQ) for both ICP-OES and CV-AAS methods were determined based on the standard deviation amongst responses and slope of the regression equation of a curve constructed at lower concentration levels as discussed by Bunhu, 2006.[125] The linearity was evaluated by the least

squares regression method. The LOD was calculated as $3S_{y/x}\,b^{-1}$ and the LOQ as 3.33 × LOD, where $S_{y/x}$ is the standard deviation of the slope and b is the slope of the relevant calibration line. The calibration data and graphs as well as the residual plots are shown in Appendices C and D.

2.5 Determination of active organic agents by high performance liquid chromatography

Chromatography and spectrophotometry are the major techniques described in the literature for the determination of various lightening agents in cosmetic products.[124-126] For instance, a rapid and sensitive reversed-phase high performance liquid chromatography (RP-HPLC) method has been developed for the separation of phenol, hydroquinone and six preservatives,[130] as well as phenol, resorcinol, α–hydroxy acids and salicylic acid in creams and lotions.[130] Furthermore methods for the analysis of arbutin either in skin-whitening products or plant extracts have been developed using HPLC,[129] gas chromatography (GC)[132] and micellar liquid chromatography.[131] Desiderio *et al.*[132] quantified hydroquinone and its ethers in cosmetics by capillary electrochromatography. Chisvert *et al.*[135] also described an uncommon but interesting gas chromatography-mass spectrometric method for the simultaneous determination of kojic acid, azelaic acid, arbutin, resorcinol and hydroquinone in cosmetic products. In some cases, UV-visible spectrophotometry has been successfully used for the determination of HQ as a single active agent.[136-137] However, the technique is exposed to interferences that can cause poor resolution since peaks overlap with an increase in the number of analytes in the matrix.[126] In general, chromatography has been the powerful method for the determination of organic ingredients in cosmetic products including skin-lightening creams.[138, 139]

Chromatography is a technique by which a mixture is separated into its components. This process is based on the distribution equilibrium of the components in a mixture between two phases. The stationary phase which is a solid adsorbent or other packing material immobilised in column with special chemical functionality and the mobile phase, which transports the mixture through the system. The mobile phase can be a gas, a liquid or a supercritical fluid, hence, the two major separation techniques are gas chromatography (GC) and liquid chromatography.[140] Although gas chromatography is

widely used, it is limited to compounds that have a significant vapour pressure at temperatures up to about 200 ºC. Thus, compounds of high molecular weight and high polarity cannot be separated by gas chromatography unless they are derivatised prior to injection.

As mentioned previously, since the matrix of cosmetic creams contains a variety of ingredients that need to be analysed appropriately, liquid chromatography has been a powerful chromatographic method used for the separation of whitening agents,[31, 141, 142] particularly, in creams,[143] and so was considered as the appropriate technique for separating and identifying different compounds in these creams. It has the advantage based on its unique versatility in terms of column and mobile phase interactions or mechanisms of separation and selectivity. HPLC allows one to accomplish very difficult separations at low levels of detection, high precision, and accuracy. The following section describes the major components of the HPLC and the principle of separation in detail.

2.5.1 Description of instrumentation

The HPLC instrument typically has five main units, which are the mobile phase reservoir, pump, column, injector system, and detector. In addition, the recorder and waste units are also needed. Figure 2.4 shows the major components of a HPLC system.

The mobile phase reservoir holds the solvent that intends to carry the samples into the column. The column is a stainless steel tube which is packed with spherical solid particles needed to effect the separation. This packing material is called the stationary phase because it is held in place by the column hardware. An injector system or autosampler is able to introduce the sample into the continuously flowing mobile phase stream. The detector is responsible for identifying and generating a signal of each eluted component against a background of mobile phase

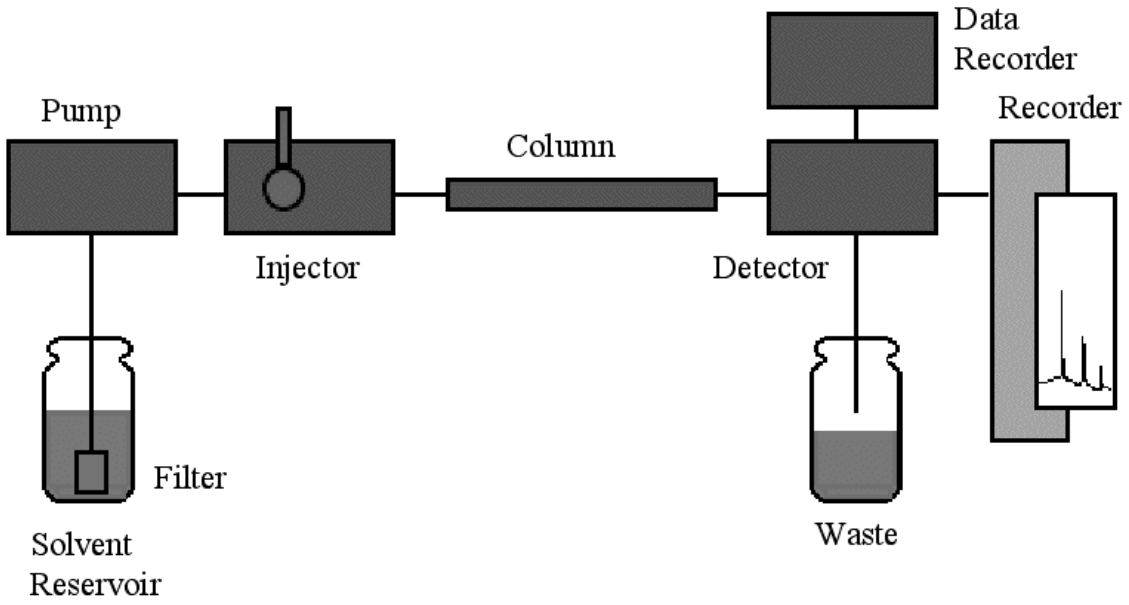


Figure 2.4: Components of an HPLC system.

(http:// www.waters.com/waters/nav.htm?cid=10048919, *Accessed on 8th July, 2014*).

Different types of detectors can be used in HPLC depending on the properties of the compounds being analysed. A UV detector or photodiode array detector (PDA), fluorescence detector [10] and refractive index detector (RI) are the most available for use in HPLC. A photodiode array detector is commonly used for a wide variety of substances that can absorb light within the ultraviolet to visible region (190-900 nm). The PDA makes possible simultaneous monitoring of more than one absorbing component at different wavelengths. This provides the benefit of time-saving and reduction of solvents.

Other detectors, such as the fluorescence detector (FL), the differential refractive index detector (RI), the electrochemical detector (ECD) and the evaporative-light-scattering detector (ELSD), are also available to meet different sample requirements.[140] A mass spectrometer is the ultimate detector for liquid chromatography (LC-MS) that significantly enhances the application range for HPLC in terms of sensitivity and selectivity. This type of detection is based on fragmentation of molecules by electric fields and separation on the basis of the mass to charge ratio of fragmented molecules.

The solvent delivery system or pump mainly serves to move the solvent through the column with a constant and high-pressure flow. The pump controls both isocratic and gradient flow modes. In isocratic mode, the composition (polarity) of the mobile phase remains constant throughout the run, while a gradient mode can change the proportions of solvents during the analysis.

The recorder reads out the information given from the detector. It interprets and records detected compounds by means of peaks and produces chromatograms as relevant. The waste unit serves to carry undesirable solutions given from the detector. Tubing lines and fittings are also necessary for interconnection of the pump, injector, column and detector components in order to form an entire channel for the mobile phase, sample, and separated compounds. The following sections give a description of the procedures used in the quantitative analysis of the organic active ingredients contained in the skin-lightening preparations investigated in this study.

2.5.2 Identification of skin-lightening active ingredients

Characterisation of active ingredients in various products was done based on UV spectra and the retention time of pure analytes and sample products. This was achieved by first recording spectral information of analytes (active ingredients) by means of UV-visible spectrophotometry. Furthermore, UV-visible spectra of pure analytes, measured by a UV-visible spectrophotometer, were compared with those obtained from the PDA detector in HPLC in order to confirm the identity of each compound. To identify components in a particular sample product, only HPLC-UV-visible spectra of the pure analytes were compared with those of sample analytes. On the other hand, the retention times of pure analytes and those of sample active ingredients were also verified.

This section presents a brief background information on UV-visible spectrophotometry and equipment as well as the spectral information of the various active agents detected in the tested sample creams.

2.5.2.1 Theory of UV-visible absorption spectrophotometry

UV-visible spectroscopy is routinely used for the qualitative determination of highly conjugated organic compounds, biological macromolecules, and transition metal ions. Organic compounds absorb light in the UV or visible regions of the electromagnetic spectrum. For a fixed path length, UV-visible spectroscopy can be used to determine the concentration of an absorbing species in a solution by using the Beer-Lambert law, which states that the absorbance of a solution is directly proportional to the concentration of the absorbing substances in the solution and the pathlength, which can be written symbolically as:

$$A = \log_{10}(I_o/I) = \varepsilon cL,$$

where A is the measured absorbance, I_o is the intensity of the incident light at a given wavelength, I is the transmitted intensity, L is the pathlength through the sample, c is the concentration of the absorbing substance, and ε is the molar absorptivity constant that designates a fundamental molecular property of a given substance.

Different substances absorb different wavelengths of light, and this can be used to identify the presence of particular functional groups in organic compounds.

2.5.2.2 Instrumentation

A UV-visible spectrophotometer consists of three major components: the source, dispersive system which is ordinarily integrated in a monochromator, and a detector. These components are typically combined in the same instrument to enable chemical analysis. An absorption spectrophotometer measures the way that the light absorbed by a compound varies across the UV and visible spectrum. Figure 2.5 illustrates a schematic representation of a double-beam spectrophotometer.

Two types of light sources, namely, a tungsten halogen lamp and a deuterium lamp, are commonly used in this spectral domain. An incandescent lamp made from a tungsten filament housed in a glass envelope is used for the visible portion of the spectrum, for

wavelengths longer than 350 nm while a deuterium lamp is needed for the UV portion of short wavelengths (< 350 nm). The sample cell contains a solution of the substance needed for test that can be placed in the optical path before or after the monochromator and the recorded spectra can be treated by using a number of different computer algorithms. The detector is usually a photomultiplier tube and the wavelength selector is a grating or prism monochromator. More information on the UV-visible spectrophotometer is available.[144, 145]

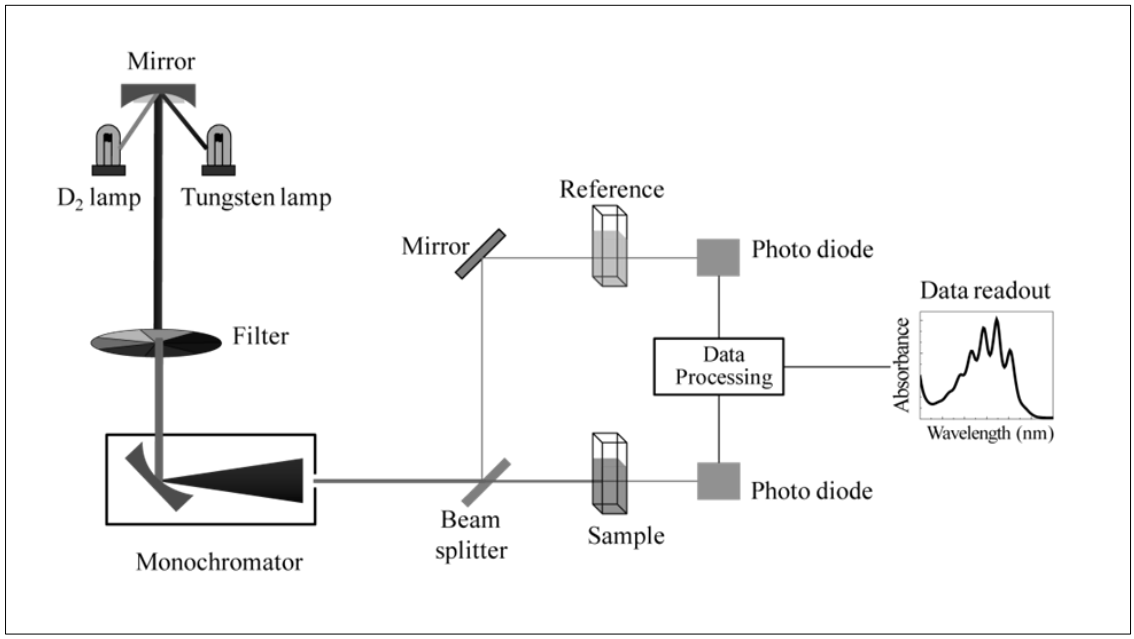


Figure 2.5: Schematic of a double-beam UV-visible spectrophotometer. (http://en.wikipedia.org/wiki/Ultraviolet%E2%80%93visible-spectroscopy, *Accessed on 15th November, 2014*).

A double-beam Perkin Elmer Lambda 25 UV-visible spectrophotometer was used to measure the maximum absorbance of skin-lightening active ingredients. Data were recorded and presented in the form of spectra. The UV-visible absorption spectra of the active skin-lightening ingredients studied are given in Section 2.5.2.3.

2.5.2.3 Determination of UV absorbance of skin-lightening active agents

The UV absorption bands of lightening agents were obtained over the wavelength range of 190 nm to 400 nm, by using 1cm pathlength quartz cuvettes and deionised water as the blank in the reference beam. The maximum absorbance was detected at 246 nm for BQ, 222 and 289 nm for HQ, 211 and 270 nm for phenol, 214 and 262 nm for NA, 223 and 286 nm for ARB, 218 and 269 nm for kojic acid, 242 nm for HCV, and 239 nm for CP. The spectra are shown in Figures 2.6 - 2.13 with the respective concentrations of each active ingredient used. All active ingredients (pure standards) were dissolved in water to obtain these spectra.

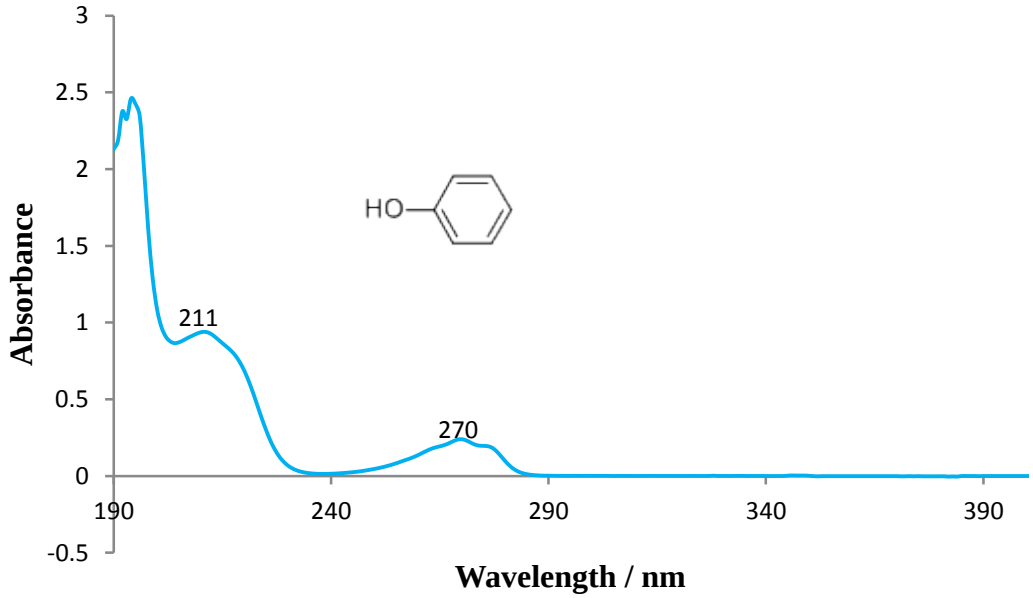


Figure 2.6: UV absorption spectrum of phenol (1.09×10^{-3} M) in deionised water. The maximum absorbance for the long wavelength peak was observed at 270 nm.

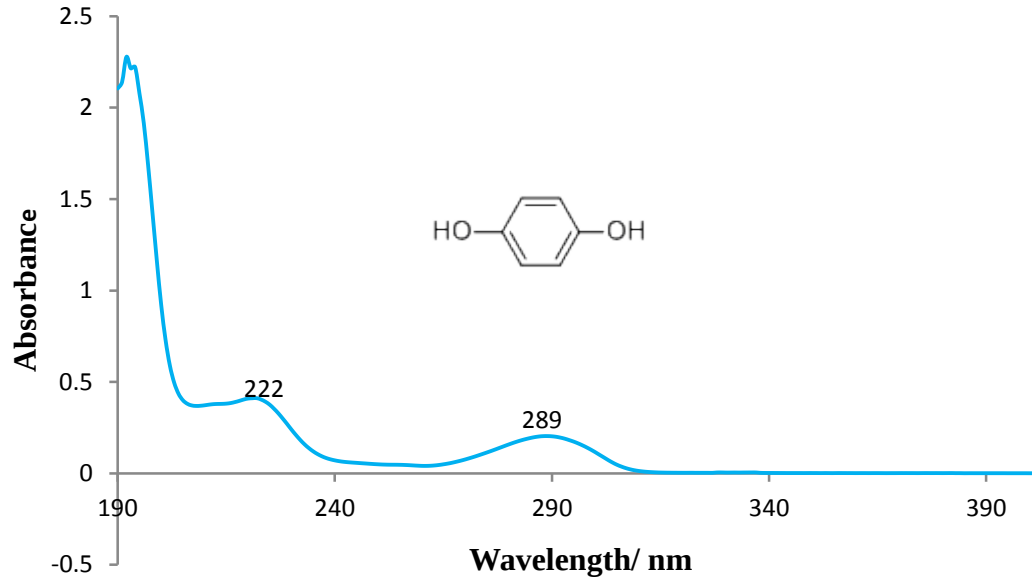


Figure 2.7: UV absorption spectrum of hydroquinone (1.09×10^{-3} M) in deionised water. Maximum absorbance for the long wavelength peak was observed at 289 nm.

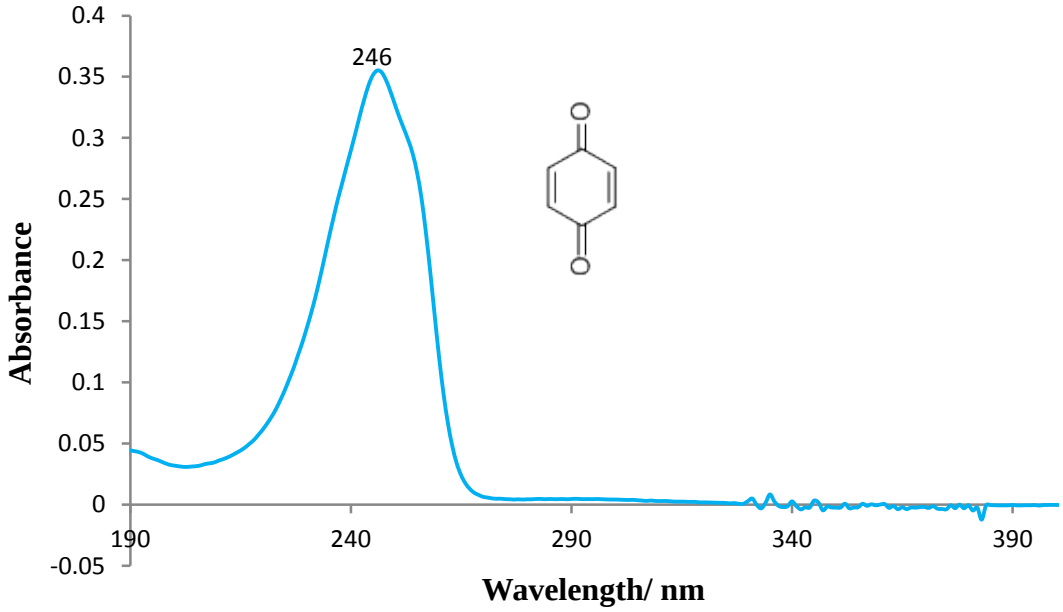


Figure 2.8: UV absorption spectrum of benzoquinone (1.09×10^{-3} M) in deionised water. Maximum absorbance for the long wavelength peak was observed at 246 nm.

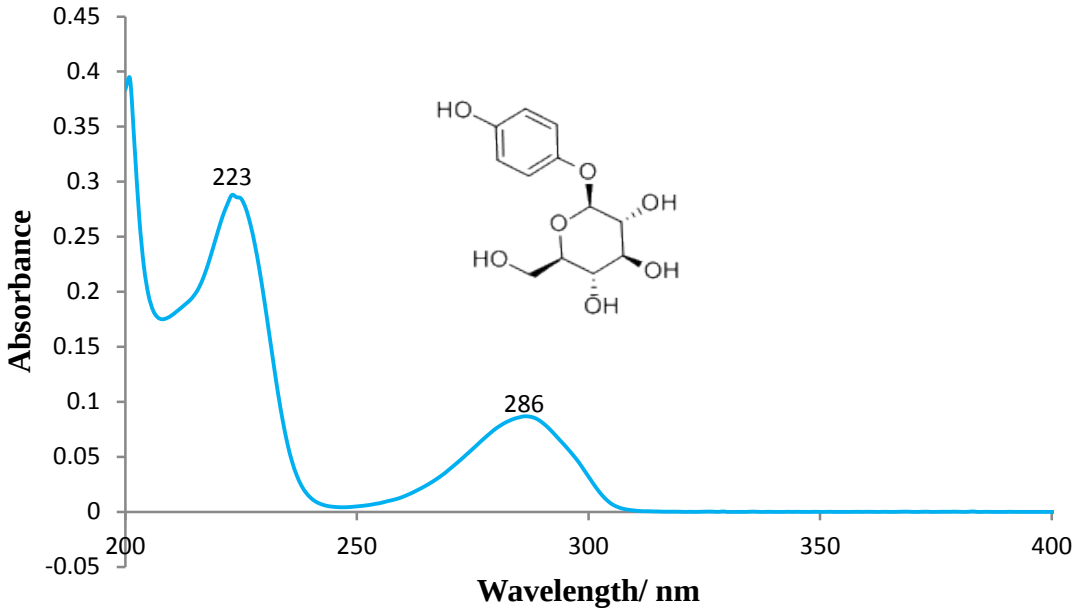


Figure 2.9: UV absorption spectrum of arbutin (6.071×10^{-4} M) in deionised water. Maximum absorbance for the long wavelength peak was observed at 286 nm.

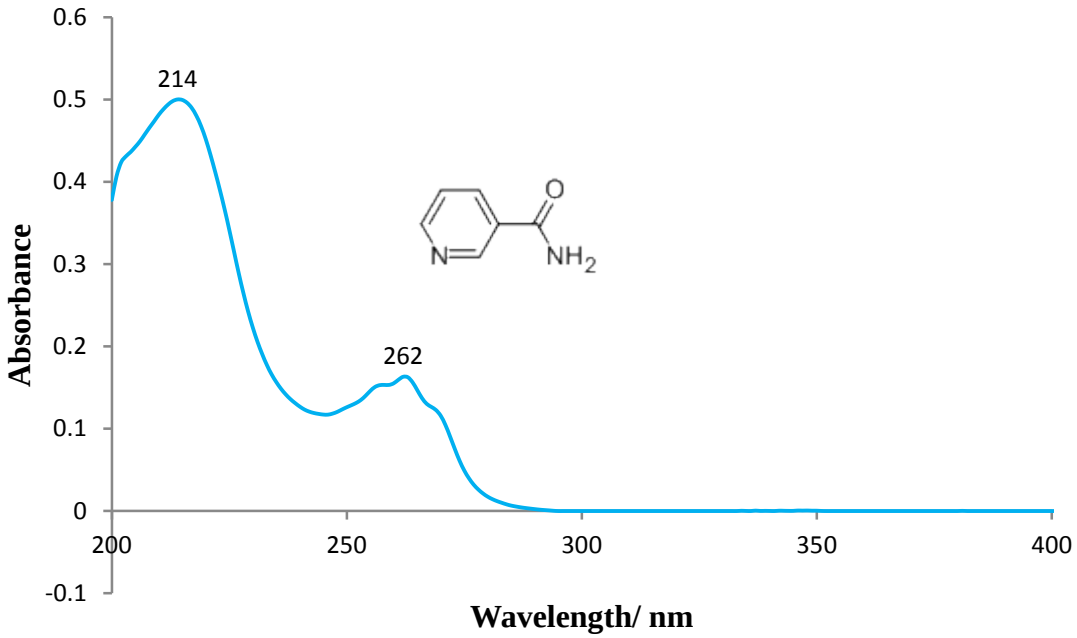


Figure 2.10: UV absorption spectrum of niacinamide (8.059×10^{-4} M) in deionised water. Maximum absorbance for the long wavelength peak was observed at 262 nm.

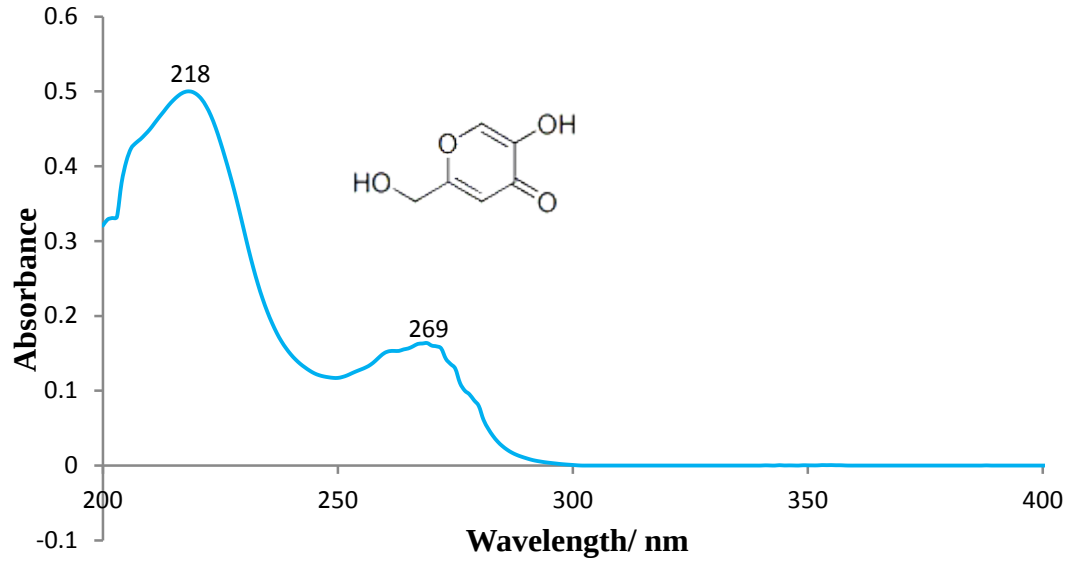


Figure 2.11: UV absorption spectrum of kojic acid (1.531×10^{-4} M) in deionised water. Maximum absorbance for the long wavelength peak was observed at 269 nm.

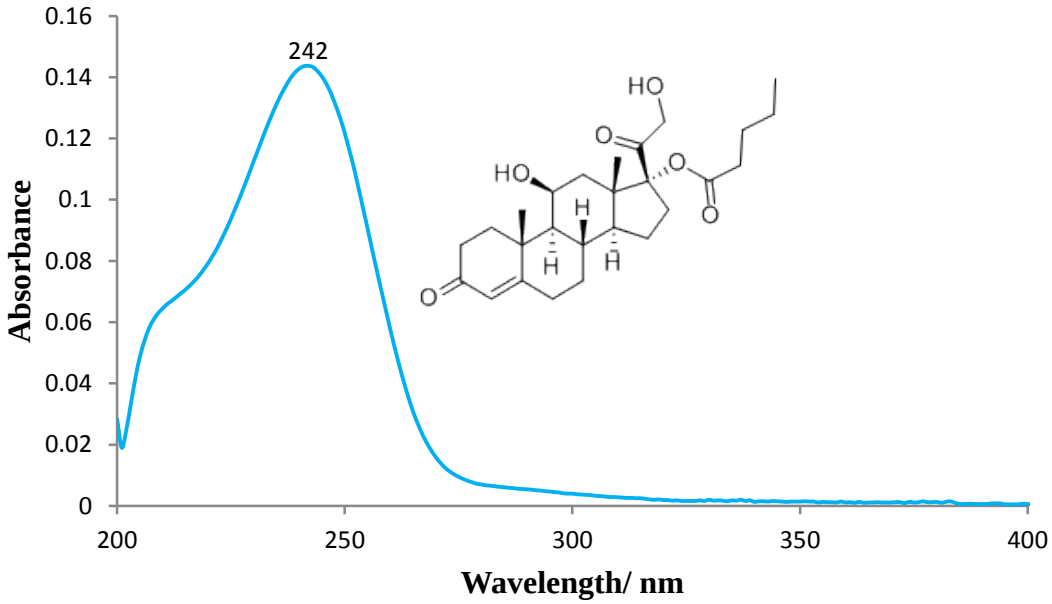


Figure 2.12: UV absorption spectrum of hydrocortisone valerate (1.395×10^{-3} M) in deionised water. Maximum absorbance for the long wavelength peak was observed at 242 nm.

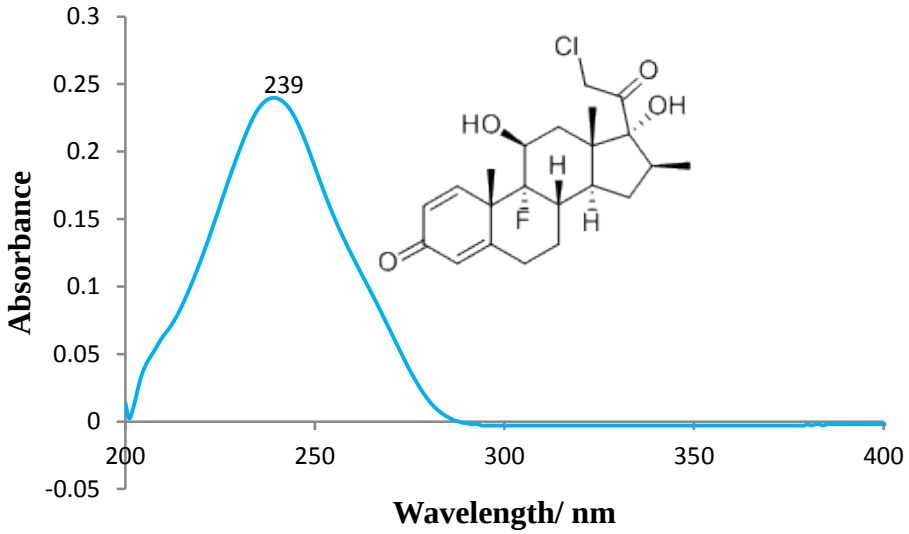


Figure 2.13: UV absorption spectrum of clobetasol propionate (1.395×10^{-3} M) in deionised water. Maximum absorbance for the long wavelength peak was observed at 239 nm.

2.5.3 Development of high performance liquid chromatography method

Several precautions were taken in the day to day operation of the analytical HPLC method in order to consistently obtain the best possible results. A brief summary is given below.

All organic solvents were of HPLC-grade, and ultrapure water obtained from a Milli-Q® unit was used to prepare all solutions. The mobile phase was prepared daily and filtered through Millipore 4.5 µm HV organic aqueous filters for removal of any particulates. The mobile phase was degassed with high purity helium at a rate of 100 mL min^{-1} for 5 minutes prior to use. The degassing process was to prevent a change in PDA signal-to-noise ratio caused by light scattering that can be due to air bubbles in the eluents. The required volumes of methanol and water eluents were separately measured out with measuring cylinders and premixed to avoid a negative volume of mixing or gas evolution in the HPLC mixing chamber.

To stabilise the baseline by ensuring that the entire flow system was fully saturated with the mobile phase, the HPLC pumps were left on overnight pumping at a rate of 0.1 mL min^{-1}. The PDA detector and the column were also allowed to equilibrate for approximately 30 minutes before injection. This minimised the problems of pressure fluctuations and noisy baseline which can cause inconsistencies in retention time.

Samples were filtered prior to injection by using Millex HV solvent resistant syringe filters to remove any particulate matter. Blank injections were run in between samples to check that the injection port, needle, and column were clean.

In the following sections, the techniques used for optimising the chromatographic condition are discussed in detail. The separation and extraction of active skin-lightening agents was first studied during the development of the HPLC analytical method. Sections 2.5.3.1 and 2.5.3.2 describe how these two important factors were investigated. The suitability and reproducibility of the quantitative method developed was checked to evaluate inconsistencies in the results.

2.5.3.1 Determination of optimum separation method

As mentioned in (Section 2.5), reversed-phase is the most suitable HPLC method for the determination of a multi-component mixture of skin-lightening products. Based on this method, chromatographic separations were performed with a Brownlee C18 (4.6 mm × 100 mm, 5 μm) column. It is believed that organic molecules have hydrophobic regions in their structures and effectively interact with the stationary phase in the reversed-phase mode.[9,10] The mobile phase in reversed-phase chromatography is more polar than the stationary phase,[139] hence, hydrophilic molecules in the mobile phase will tend to elute faster than hydrophobic molecules.[9] Since the mobile phase in reversed phase chromatography contains a polar solvent, separation of the detected whitening agents was carried out by varying the polarities of the mobile phase and its flow rate.

By using an isocratic elution mode, several mobile phases were tried including methanol and water (60:40, v/v), methanol and water (50:50, v/v), methanol and water (55:45, v/v), methanol and water (45:55, v/v), acetonitrile and water (50:50, v/v), methanol and 0.2% acetic acid water (45:55, v/v), in order to obtain the best separation of the studied active organic ingredients in various commercial lightening creams. Mixtures of standard solutions were used for this assay. The flow rate was alternatively changed from 0.5 mL min^{-1} to 1.2 mL min^{-1}.

2.5.3.2 Determination of optimum extraction method

A preliminary extraction test was carried out in order to determine a suitable solvent for the extraction of the active ingredients contained in the skin-lightening creams. The extraction method for the sample preparation was studied on five lightening agents and the analysis was done by HPLC on a Phenomenex C18 (4.6 mm × 250 mm, 5µm) column.

About 1.0 g of a cream containing no lightening agents was weighed into a glass flask, and small amounts of a mixture containing the same concentration of each of the lightening agents: HQ, BQ, PO, ARB and CP, were immediately added and then diluted with 10 mL of each extractant (solvent) mentioned below. This solution was stirred for 5 min, brought to a total volume of 25 mL, ultrasonicated in a water bath at 40 °C for 20 min and allowed to cool down before injection. A 100% methanol, 100%, acetonitrile, a mixture of 80% methanol in water, 60% methanol in water, 50% methanol in water and a mixture of 45% acetonitrile and 45% methanol in water were used as extractants. The ultrasonication parameters used were determined according to a modified method reported in the literature.[19] The method states that a 0.25 g mass of the cream was dissolved into 10 mL acetonitrile and the mixture was sonicated in a hot water bath at 40 °C for 15 min. In this work, a mass of 1.0 g of the cream sample was dissolved into 10 mL methanol and the mixture was placed in an ultrasonic bath and held there at 40 °C for 20 min.

2.5.3.3 Preparation and analysis of standard solutions

A 1000 mg L^{-1} stock solution of each studied analyte was prepared by dissolving 0.025 g of each standard in 25 mL of methanol. After adding the solvent to the proper amount of the standard in a volumetric flask, the mixture was kept in an ultrasonic bath for 10 min, to ensure that all solutes were completely solubilised. Three standard stock solutions for each active ingredient were made and stored at −20 °C for a period not longer than a week. A 100 mg L^{-1} standard working stock solution was prepared by diluting and mixing aliquots from the stock solutions. A set of working standard stock solutions was prepared. This included HQ, BQ, PO, ARB, CP, HCV and NA. From the working standard stock solution, serial dilution was performed by using the mobile phase.

Seven different concentrations of standard solutions were prepared from the working standard solutions in the range of 1.25 – 25 mg L^{-1} for HQ and PO, 0.625 – 20 mg L^{-1} for BQ, ARB and NA, 0.391 - 25 mg L^{-1} for CP and HCV. Aliquots of standard solutions were transferred into vials and subjected to HPLC analysis in order to generate calibration curves. Calibration curves were used for the quantitation of active ingredients in skin-lightening products.

During the analysis, the autosampler was programmed to perform three injections per standard solution, and with blanks injections in between to avoid any carry-over from previous injections into the column. The injection volume was 10 µL and the flow rate was at 0.8 mL min^{-1}. The detector was set to monitor the compounds at 240 nm, 270 nm, and 289 nm.

A Shimadzu Prominence HPLC system (Shimadzu Corporation, Japan) was employed to conduct all the analyses. The chromatographic system consisted of: LC-20 AD binary pumps, DGU-20A3 degasser, LC-20AD/UFLC XR fraction collector, SIL-20A/UFLC-XR autosampler, SPD-M20A diode array detector, and LC/Lab Solution system software.

2.5.3.4 Preparation of sample solutions and analysis

A mass of 1.0 g of each cream sample was accurately weighed into a 25 mL volumetric flask and diluted with 10 mL methanol, then shaken vigorously for 10 min and filled to volume with methanol. After ultrasonication in water bath at 40 °C for 20 min, sample extracts were allowed to cool down to room temperature. All sample solutions were filtered through a millipore 0.45 μm syringe filter to remove all particulate matter that could clog the column and aliquots were transferred into injection vials for HPLC analysis. Three replicates were prepared for each cream sample. During analysis, the autosampler was programmed so that it performed three injections per sample and sample injections were always interspaced with blank injections as was done for the analysis of standard solutions. The results of the quantified skin-lightening products are presented in Section 3.3.2.3.

Samples and standard solutions were analysed using Brownlee C18 (4.6 mm × 100 mm) column with a mobile phase composed of methanol:water (55:45, v/v). The flow rate was 0.8 mL min^{-1}, the wavelengths used were 240, 270 and 289 nm and the maximum run time was 12 minutes.

2.5.3.5 Recovery analysis

As a cream-based certified reference material are not available, a sample cream (SLC33) containing no whitening ingredients was used as a validation sample in the recovery test. This cream was previously screened, in order to check if the spike analytes were part of its ingredients. On the assumption that concentration levels of pure standard in a validation cream will approximately reach 2.5 and 10 mg L^{-1}, two spike solutions of different concentrations (62.5 and 250 mg L^{-1}) were prepared for each of the analytes of interest (HQ, PO, BQ, ARB, NA, CP and HCV). Spiking was done by adding these solutions to sample cream (SLC33).

A mass of 1.0 g of validation sample (SLC33) was accurately weighed into 25 mL volumetric flask, 1mL of spike solution was immediately added to this cream and the mix-

ture was diluted with 10 mL methanol, then shaken vigorously for 10 min and filled to volume with methanol. After ultrasonication process, spiked sample solutions were filtered through a Millipore 0.45 μm syringe filter and the aliquots were transferred into injection vials for HPLC analysis. The spiked cream samples were analysed in triplicate following the method described in Section 2.5.3.4.

2.5.3.6 Data analysis

The data were evaluated by using the Microsoft Excel add-in package called AnalysisToolPak. Calibration curves were determined by means of a simple linear regression equation, $y = a + bx$, with null intercept,[125] where *y* is the peak area, *x* is the concentration of analyte, *b* is the slope, and *a* is the intercept. Assuming that $a = 0$ at 95% confidence interval, only *b* needs to be determined to establish the calibration line taking into account the error in each of y_0, *a* and *b*, and thereby to produce an estimated standard deviation Sx_0 for the point estimate $x_0 = (y_0-a)/b$.

The limit of detection was calculated as thrice the standard deviation of the calculated values for the lowest concentration of each analyte. Thus, LOD was calculated as $3S_{y/x}$ b^{-1}($S_{y/x}$ is the standard deviation of the slope and b is the slope of the calibration line) and LOQ was calculated as 3.33 × LOD.[146]

The percentage of the analyte in the product was determined by using the formula

$$\% = \frac{C_s \times V_s \times D_f \times S_p}{W_s}$$

where *Cs* is the concentration of the component of interest from the standard curve (mg/ml), *Vs* is the volume of the sample extract, *Df* is the dilution factor (where applicable), W_s is the weight of the cream sample, and *Sp* is the standard purity (%).

Chapter 3

RESULTS AND DISCUSSION

This chapter discusses the data obtained from the experimental techniques used during the course of the investigation as detailed in Chapter 2. It consists of two major parts, described below:

The first part considers the results obtained from the investigation of the heavy metal content of 49 skin-lightening creams. This was achieved by means of the spectroscopic techniques, ICP-OES and CV-AAS. The second part discusses the chromatographic (HPLC) and UV/Vis spectrophotometry results obtained for the qualitative and quantitative determination of the active ingredients of 19 skin-lightening creams.

3.1 Extraction of heavy metals in skin-lightening creams

In order to solubilise the heavy metals in the samples, different extraction techniques as described in Sections 2.3.1 and 2.3.2 were evaluated.

3.1.1 Open acid digestion

The extraction of heavy metals in the skin-lightening creams presented a number of challenges during the sample pretreatment process. As can be seen in the preliminary experiments detailed in Section 2.3.1, the dry-ash process of each tested cream sample resulted in approximately 0.18 g (32%) of ash. This meant that 64% of the constituents of the cream sample were decomposed. The lost part of the sample was linked to the presence of organic substances and/or some metals, such as mercury and others which are volatile at low temperature. On the other hand, the acid digestion carried out on a hot plate failed to reach a very high temperature and generated a final sample solution containing residual materials in the shape of gums and powders which could cause blockages in the tubing of the ICP-OES. This wet digestion method showed poor disso-

lution of the sample matrix. Besides, these two methods were time-consuming and involved tedious experimental work. Therefore, the digestion of samples was attempted via microwave-assisted digestion.

3.1.2 Microwave-assisted sample digestion

Since the cream samples were not conveniently processed by dry-ashing and wet digestion, they were then irradiated in closed vessels with acid-assisted microwave digestion. This method provided a complete break-down and better solubilisation of the organic materials for all samples within a short period of time. However, microwave digestion can pose a high risk of explosions.

This was observed when the method used by Na-Ri Cha *et al.*[119] for microwave digestion of different cosmetics with a combination of H_2SO_4 and HNO_3 acids were used. Also, when a combination of HNO_3 and HCl/HF acids was used for digestion of the skin-lightening creams in the present study, the vessels exploded at temperatures below 150 °C. However, with nitric acid alone, samples were able to reach a maximum temperature of 201 °C without risk of explosions.

The temperature attained in a closed system promoted a fast decomposition of oils, surfactants, oxide species, and metallic solid particles of the analytes into their elemental forms without sample loss and contamination. Overall, digest solutions were quite clear despite the presence of residual materials found in a few samples in the form of powder or suspensions, which could be removed by filtration. The clear digested solutions obtained showed the successful solubilisation of sample matrices.

Indeed, microwave-assisted nitric acid digestion resulted in reasonable recoveries during the extraction of heavy metals in skin-lightening creams in this study. The recovery data are shown in Table 3.3. To achieve successful mineralisation of cosmetics by microwave-digestion each step must be performed vigilantly in order to minimise hazards.

3.2 Determination of mercury and heavy metals in skin-lightening creams

Levels of mercury and other heavy metals in skin-lightening creams were determined by using a Perkin-Elmer MHS 15 mercury hydride system and a Perkin-Elmer Optima 5300 DV ICP-OES respectively. The ICP-OES and CV-AAS were simple and accurate methods for quantifying heavy metals in samples, however, the methods encountered challenges, such as interferences, which typically occur for elemental analysis with ICP-OES.

3.2.1 Linearity of calibration curves for heavy metal analysis

Calibration graphs were obtained by using an external standard calibration method. For each standard solution, three replicates were prepared and measured in the same way to generate three data points. A detailed description of the standards preparation procedure is given in Section 2.4.1.1. Calibration graphs were constructed from the data obtained for each standard solution. The calibration data can be found in Tables C1-1 to C1-6 of Appendix C and the calibration graphs and residuals plots are shown in Figures D1 to D12 of Appendix D.

An analysis of the residuals in the regression showed that they were randomly dispersed around the horizontal axis; hence, a good fit for a linear regression model. These were performed by using the Analysis ToolPak package of Microsoft Excel. The values of the linear regression analysis are shown in Table 3.1.

The slope and standard error of the calibration lines for each metal were used to calculate the limit of detection and the limit of quantitation (LOQ).[130] The LOD was calculated as $3S_{y/x}\,b^{-1}$ and the LOQ as 3.33 × LOD, where $S_{y/x}$ is the standard deviation of the slope and b is the slope of the calibration line. The correlation coefficients for all the calibration graphs used in the quantitation of Cr, Cu, Fe, Pb, and Zn were not less than 0.995 ($R^2 > 0.995$); the method had limits of detection less than 0.06 mg L^{-1} and limits of quantification less than 0.17 mg L^{-1} for these elements.

Table 3.1: Linear regression of calibration data for heavy metals.

Parameters	Cr	Cu	Fe	Pb	Zn
Calibration range/10^{-1} mg L^{-1}	0.2-1.0	0.2-1.0	0.1-1.0	0.2-1.0	1.0-5.0
Slope (b)/10^{4} mg L^{-1}	8.363	32.823	5.726	7.38	4.107
Standard error of slope/10^{3}	0.666	1.587	0.169	0.123	0.202
Coefficient of determination (R^2)/10^{-1}	9.991	9.997	9.999	9.961	9.997
LOD/10^{-2} mg L^{-1}	2.39	1.45	0.88	5.01†	1.48
LOQ/10^{-2} mg L^{-1}	7.96	4.83	2.94	1.67	4.92

- LOD calculated as $3S_{y/x}\,b^{-1}$($S_{y/x}$ is the standard deviation of the slope and b is the slope of the calibration line) and LOQ calculated as 3.33 × LOD.
† LOD/10^{-3} mg L^{-1}

3.2.2 Linearity of calibration curves for mercury analysis

A typical regression line between the absorbance signal and the mercury concentration was described by the equation: $A = 8.093 \times 10^{-4}\ C_{Hg}$ (ng) with a coefficient of determination of 0.996. The LOD was less than 0.025 ng and the LOQ was less than 0.08 ng. Table 3.2 displays the results of the linear regression analysis for mercury.

Table 3.2: Linear regression of calibration data for the analysis of mercury.

Parameters	Value
Calibration range/ng	100-600
Slope (b)/10^{-4} ng	8.093
Standard error of slope/10^{-6}	6.272
Coefficient of determination (R^2)	0.999
LOD/10^{-2} ng	2.32
LOQ/10^{-2} ng	7.74

- LOD was calculated as $3S_{y/x}\,b^{-1}$($S_{y/x}$ is the standard deviation of the slope and b is the slope of the calibration line) and LOQ was calculated as 3.33 × LOD.

3.2.3 Validation of quantitation methods

Due to the lack of a cream-based certified reference material, the accuracy of the methods used for quantitation of metals was checked throughout by performing recovery tests. Test was carried out by spiking skin-lightening samples with known amounts of the element of interest as described in Section 2.4.3. The amounts added and the resulting recoveries are reported in Table 3.3.

The mean percentage recoveries achieved ranged from 97.6 % to 106.19 % for all the elements analysed. The RSD values for recoveries were below 3.0% for Cr, Fe, Hg, Pb and Zn, while Cu had a value not higher than 7.17%. Accordingly, the recovery tests confirmed that the matrices of the cream samples were consistently mineralised.

Table 3.3: Recovery data for heavy metals quantified in skin-lightening creams (n = 3).

Element	Spiked amount /μg g^{-1}	Found /μg g^{-1}	Recovery/%	Standard Deviation/μg g^{-1}	RSD/%
Cr	12.374	12.561	101.57	0.27	2.11
Cu	11.133	11.014	98.97	0.79	7.17
Fe	61.597	60.12	97.6	0.17	0.28
Hg	6.024	6.363	105.61	0.18	2.87
Pb	7.207	7.653	106.19	0.16	2.05
Zn	95.507	96.921	101.51	2.01	2.07

3.2.4 Levels of mercury and heavy metals in selected skin-lightening creams

This study investigated the level of ten heavy metals (As, Cd, Co, Cr, Cu, Fe, Hg, Ni, Pb and Zn) in 49 skin-lightening creams sold in South Africa. The results showed that six metals (Cr, Cu, Fe, Hg, Pb and Zn) could be detected.

Table 3.4 shows the distribution of heavy metals in the studied samples. As seen, arsenic, cadmium, cobalt and nickel were undetectable in all the skin-lighteners while copper was detected in all samples with concentrations varying from 0.19 to 8.93 μg g^{-1}. The highest copper concentration was found in sample SLC11 and the lowest concentra-

tion was found in sample SLC27. Copper compounds and CuO nanoparticles are used as antimicrobial agents in cosmetic and dermatological preparations,[147, 148] besides Polefka[105] reported that several commercial cosmetic products are based on copper–peptides that have been proven to calm irritated skin, improve skin elasticity and firmness, repair photo-damaged skin and reduce facial wrinkles. These could be responsible for the presence of copper in this study. However, topically applied CuO nanoparticles have been reported to induce inflammatory cytokine secretion and necrosis especially in the epidermis.[147]

Chromium was detected in eight products and lead was present only in three products. The concentrations of Cr and Pb ranged from 1.73 to 12.56 μg g^{-1} and 2.98 to 15.72 μg g^{-1}, respectively. The product SLC4, which contained a high content of Cr, also had the highest level of Pb that is above the WHO and Canadian Health's acceptable limits. Table 3.4 shows a comparison of the toxic metal levels detected in certain skin-lightening creams investigated with some permissible standards for cosmetics.

Iron was found in 33 samples (i.e. 67.3% of the samples that were tested). The iron concentrations varied from 0.55 to 25007 μg g^{-1}, most samples having contents between 3.48 and 46.26 μg g^{-1}. Remarkably, higher concentrations of 3860.9 μg g^{-1} (0.39% m/m), 5666.6 μg g^{-1} (0.57% m/m) and 25007 μg g^{-1} (2.5% m/m) were obtained in SLC17, SLC39 and SLC31, respectively. High levels of Fe (6.15%) were also reported in local eye shadows sold in Nigeria,[39] although, Ullah *et al.*[110] found Fe (0.25%) in cosmetic creams manufactured in Pakistan. Iron oxide has been used in colour cosmetics to mask minor imperfections of the skin. Keeping in view of its function in the human body, there is no significant health hazard reported on the topical application of iron and iron compounds.[39, 105] In contrast, the essential metals are toxic when present in higher concentrations in human body.[38,116,149]

Zinc was detected in less than five products and the concentrations found ranged from 79.97 to 12470 μg g^{-1} which corresponds to the range of 0.008 to 1.2% (m/m). Zinc salts and compounds have been approved by the FDA for over-the-counter (OTC) uses as safe and effective topical skin protectants. ZnO and TiO_2 are likely mineral sun-

screens approved to reduce skin penetration by UVB radiation and provide protection against UVA radiation.[105] The European laws banned cosmetic formulations containing more than 0.5% zinc pyrithione while other zinc salts are allowed up to 1% (calculated as zinc) in cosmetics.[42]

Table 3.4: Comparison of toxic metal levels with some permissible standards for cosmetics.

Sample name	**As/ µg g^{-1}**	**Cd*/ µg g^{-1}**	**Cr*/ µg g^{-1}**	**Hg*/ µg g^{-1}**	**Ni/ µg g^{-1}**	**Pb*/ µg g^{-1}**
SLC1	-	-	3.73	-	-	-
SLC2	-	-	2.29	-	-	2.98
SLC4	-	-	12.56	-	-	15.72
SLC12	-	-	-	2.49	-	-
SLC17	-	-	-	-	-	9.15
SLC27	-	-	4.41	-	-	-
SLC36	-	-	7.72	-	-	-
SLC39	-	-	1.73	2.94	-	-
SLC43	-	-	-	7.57	-	-
SLC45	-	-	-	51.19	-	-
SLC46	-	-	10.71	-	-	-
SLC47	-	-	4.73	-	-	-
Health Canada[150]	**3.0**	**3.0**		**3.0**		**10.0**
WHO[18,45]	**10.0**	**0.3**		**1.0**		**10.0**

*****banned in cosmetics by FDA[41]
WHO (World Health Organization)
- Not detected

The concentration of zinc (1.2% m/m) in sample, SLC45, was above the acceptable limit. In addition, the mercury content in this product exceeded the permissible limit established by the WHO (< 1.0 ppm),[18] and the Health Canada limit (3.0 ppm).[150] Countries and international organisations have regulated toxic metals for cosmetic use.

Mercury was detected in four products: SLC12, SLC39, SLC 43, and SLC45 with levels of 2.49, 2.94, 7.57, and 51.19 µg g^{-1} respectively. Higher concentrations of mercury in some cosmetic creams have been reported. For instance, Al-Saleh *et al.*[18] has found 95.75 µg g^{-1} in skin-lightening creams made in Syria, Peregrino *et al.* also detected an

extremely high mercury content of 878 and 36000 μg g^{-1} in Mexican facial whitening creams. Mercury, through skin absorption, can enter and accumulate in body tissues and attack the brain, immune system, kidneys and the ovaries. This can cause psychological, neurological, immunological and other problems in humans.[20] Engler *et al.*[151] reported that application of skin-lightening creams containing mercury during pregnancy or lactation expose the developing foetus or infants to high risk.

Table 3.5: Levels of heavy metals detected in different samples of skin-lightening creams analysed in this study (n = 3). (SLC-Skin-lightening cream, LOD-Limit of detection, - Not detected)

Sample Code	Mean concentration ± SD/µg g^{-1}						
	Cr	**Cu**	**Fe**	**Hg**	**Ni**	**Pb**	**Zn**
SLC1	3.73 ± 0.10	2.13 ± 0.06	-	-	<LOD	<LOD	-
SLC2	2.30 ± 0.11	2.79 ± 0.01	39.22 ± 1.73	-	-	2.98 ± 0.16	<LOD
SLC3	<LOD	1.70 ± 0.14	3.93 ± 0.10	-	<LOD	<LOD	<LOD
SLC4	12.56 ± 0.07	2.71 ± 0.15	8.45 ± 0.43	-	-	15.72 ± 0.72	-
SLC5	-	0.93 ± 0.05	-	-	-	<LOD	<LOD
SLC6	<LOD	5.38 ± 0.51	7.94 ± 0.45	-	-	<LOD	<LOD
SLC7	<LOD	5.92 ± 0.51	-	-	-	<LOD	-
SLC8	-	2.24 ± 0.16	7.53 ± 0.39	-	-	<LOD	<LOD
SLC9	<LOD	0.57 ± 0.04	46.26 ± 4.18	-	-	<LOD	-
SLC10	<LOD	6.68 ± 0.15	22.19 ± 1.03	-	-	-	<LOD
SLC11	<LOD	8.93 ± 0.56	4.25 ± 0.27	2.49 ± 1.21	-	<LOD	<LOD
SLC12	-	1.40 ± 0.16	1.58 ± 0.12	-	<LOD	<LOD	<LOD
SLC13	<LOD	4.61 ± 0.24	-	-	-	<LOD	<LOD
SLC14	-	0.93 ± 0.08	7.94 ± 0.52	-	-	<LOD	<LOD
SLC15	<LOD	2.18 ± 0.15	9.60 ± 0.18	-	-	<LOD	<LOD
SLC16	<LOD	1.80 ± 0.14	25.07 ± 1.24	-	-	<LOD	<LOD
SLC17	<LOD	2.88 ± 0.23	3861. ± 228.8	-	<LOD	9.15 ± 0.07	<LOD
SLC18	-	2.34 ± 0.01	-	-	-	<LOD	<LOD
SLC19	<LOD	0.97 ± 0.08	16.36 ± 1.13	-	<LOD	<LOD	<LOD
SLC20	<LOD	4.70 ± 0.34	3.48 ± 0.30	-	-	<LOD	<LOD
SLC21	<LOD	6.07 ± 0.17	90.93 ± 2.12	-	-	<LOD	<LOD
SLC22	<LOD	1.33 ± 0.06	-	-	-	<LOD	<LOD

Sample code	Mean concentration ± SD/µg g^{-1}						
	Cr	**Cu**	**Fe**	**Hg**	**Ni**	**Pb**	**Zn**
SLC23	<LOD	1.10 ± 0.08	-	-	-	<LOD	<LOD
SLC24	<LOD	1.56 ± 0.02	-	-	-	<LOD	<LOD
SLC25	<LOD	1.76 ± 0.02	2.60 ± 0.15	-	-	<LOD	<LOD
SLC26	<LOD	1.74 ± 0.05	12.95 ± 0.42	-	-	<LOD	-
SLC27	4.41 ± 0.03	0.19 ± 0.02	45.84 ± 2.94	-	-	<LOD	<LOD
SLC28	-	2.67 ± 0.13	10.19 ± 0.23	-	-	<LOD	<LOD
SLC29	-	3.62 ±0.29	10.48 ± 0.72	-	-	<LOD	<LOD
SLC30	<LOD	2.70 ± 0.13	7.024 ± 0.39	-	-	<LOD	<LOD
SLC31	<LOD	0.97 ± 0.13	25007±906.6	-	<LOD	<LOD	<LOD
SLC32	<LOD	0.28 ± 0.25	56.32 ± 0.71	-	<LOD	<LOD	<LOD
SLC33	-	1.64 ± 0.02	-	-	-	N.D.	-
SLC34	-	1.54 ± 0.16	-	-	-	<LOD	<LOD
SLC35	<LOD	1.94 ± 0.16	-	-	-	<LOD	<LOD
SLC36	7.73 ± 0.10	5.40 ± 0.11	-	-	-	<LOD	-
SLC37	<LOD	1.27 ± 0.06	-	-	-	<LOD	<LOD
SLC38	-	2.71 ± 0.31	-	-	-	<LOD	<LOD
SLC39	1.74 ± 0.10	4.79 ± 0.25	5666. ± 11.85	2.94 ± 1.58	-	<LOD	<LOD
SLC40	-	2.85 ± 0.30	112.1 ± 5.34	-	-	<LOD	<LOD
SLC41	<LOD	1.13 ± 0.02	-	-	-	<LOD	79.97 ± 4.14
SLC42	<LOD	1.06 ± 0.06	0.55 ± 0.04	-	<LOD	<LOD	7148. ±154.1
SLC43	<LOD	2.66 ± 0.12	9.76 ± 0.84	7.58 ± 1.80	-	<LOD	<LOD
SLC44	-	2.12 ± 0.29	-	-	-	<LOD	<LOD
SLC45	<LOD	3.56 ± 0.66	61.06 ± 4.88	51.19 ± 12.25	-	<LOD	12470 ± 420
SLC46	10.71 ± 0.09	1.65 ± 0.11	3.60 ± 0.38	-	-	<LOD	-
SLC47	4.73 ± 0.01	3.47 ± 0.02	11.83 ± 0.63	-	-	<LOD	<LOD
SLC48	<LOD	3.26 ± 0.11	10.80 ± 0.27	-	-	<LOD	<LOD
SLC49	<LOD	2.43 ± 0.01	887.8 ± 2.82	-	-	<LOD	<LOD

3.3 Determination of skin-lightening organic active ingredients

It was desirable to first develop a method that defines the chromatographic conditions suitable for characterisation and separation of lightening active ingredients quantification. The following sections discuss in detail the results of the experiments conducted for determining the organic active ingredients contained in the skin-lightening creams chosen for this study.

3.3.1 Method development and optimisation of chromatographic conditions

The optimisation and validation of the instrumental conditions were performed by using an HPLC system (Shimadzu Prominence) consisting of a binary solvent manager, autosampler and photodiode array detector. The output signal was monitored and processed by using Shimadzu LCsolution software. Chromatography and resolution of skin-lightening active ingredients was performed on a Brownlee C18 4.6 mm × 100 mm, 5.0 μm column with an isocratic elution method.

3.3.1.1 Determination of optimum wavelength for UV detector

A detection wavelength which provides high sensitivity and better resolution of analytes was used for the determination of detected agents. The UV spectra and the maximum absorption peak of each studied lightening active agent were first obtained by means of a UV-visible spectrophotometer. The representative spectra were given in Chapter 2, Figures 2.6-2.13. The maximum UV absorbance is at 246 nm for BQ, at 242 nm for HCV, 289 nm for HQ, at 239 nm for CP, at 270 nm for phenol, at 262 nm for NA, at 269 for KA and at 286 nm for ARB. Standard solutions were prepared in deionised water. These UV spectra and the maximum absorbance for the long wavelength peak were used to confirm the UV spectra generated by the PDA detector in HPLC. Figure 3.1 confirms the identity of the UV absorbance of the detected active ingredients obtained from both of the instruments: HPLC equipped with PDA detector and UV-visible spectrophotometer.

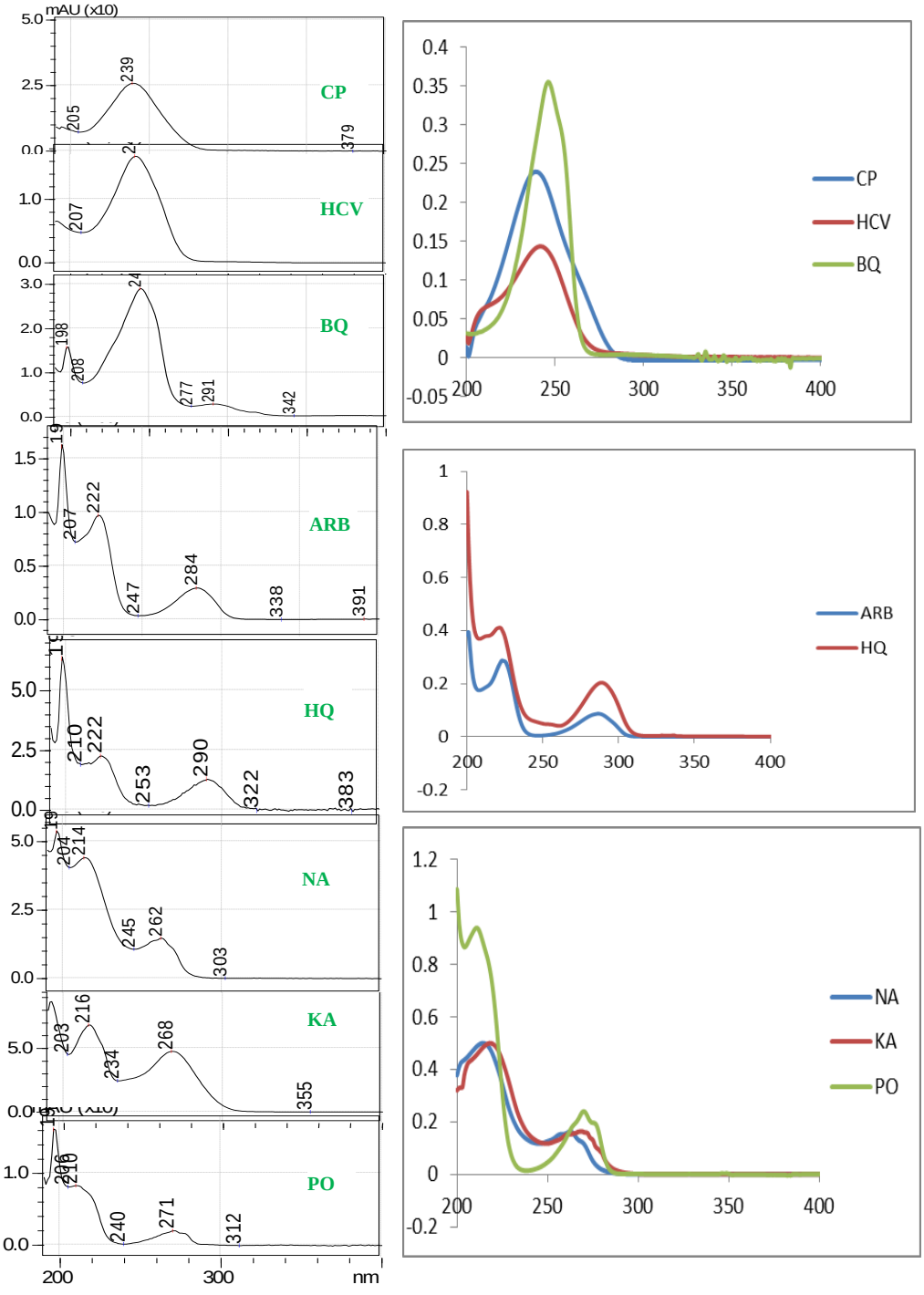


Figure 3.1: Comparison between PDA-HPLC UV spectra (left) vs UV-Vis spectrophotometer spectra (right).

The UV detector showed a high sensitivity in detecting PO, KA and NA at 270 nm, HQ and ARB at 289 nm, and BQ, CP, and HCV at 240 nm. At 270 nm, clear peaks for all compounds in the HPLC-chromatograms were observed; however, but the highest sensitivity was noticed only for PO, KA and NA. Therefore, in order to improve the quantitation of these ingredients, three UV absorption wavelengths (240, 270, and 289 nm) were considered during the analysis of both standards and samples.

In order to identify skin-lightening organic active ingredients: HPLC-UV-visible spectra and the retention time's representative for the pure analytes and those for detected analytes in sample product were considered

3.3.1.2 Identification of active ingredient in skin-lightening product

To identify components in a particular sample product, only HPLC-UV-visible spectra of the pure analytes were compared with those of sample analytes. Furthermore, the retention times of pure analytes and those corresponding to their related compound in sample active ingredients were also verified. Table 3.6 indicates the variability of the retention times for each active ingredient quantified in skin-lightening creams.

Table 3.6: Variability in retention time of detected active ingredients (n = 9).

Active ingredient	Retention time variability/min
HQ	1.45-1.49
BQ	1.72-1.74
PO	2.45-2.49
ARB	1.28-1.33
NA	1.39-1.43
CP	8.22-8.30
HCV	5.0-5.3

KA was removed from the standard mixture.because it consistently produced a peak that tailed thereby affecting the resolution of other compounds. Consequently, a standard solution containing KA only was used to characterise this agent. The retention time of KA was 1.46 min. Figure 3.2 shows the chromatogram for the elution of KA.

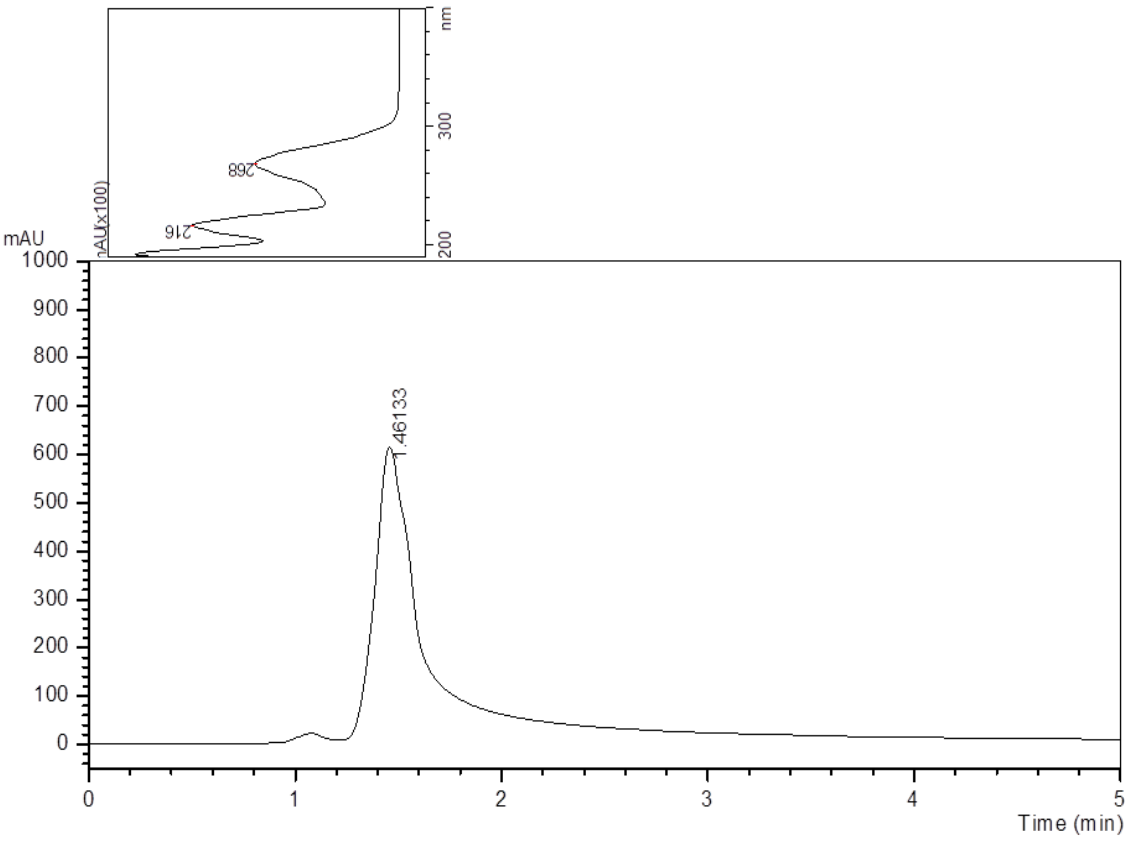


Figure 3.2: HPLC chromatogram of KA standard solution (2.5 mg L^{-1}). The retention time is at 1.46133 minutes. The detection wavelength was 270 nm.

3.3.1.3 Determination of optimum separation of the skin-lightening agents

Most of the skin-lightening active ingredients are very polar molecules. For polar analytes, a polar solvent, such as water, methanol or acetonitrile, is usually used as a constituent of the mobile phase for a HPLC separation effected on a C18 column. However, the co-elution of these polar analytes is a challenge for their simultaneous analysis.

It is well-known that a good separation condition should satisfy the need that the analysed peaks have baseline separation with adjacent peaks within a short analysis time.

Therefore, to obtain the chromatograms with the good separation, mobile phases with different compositions of methanol in water, detection wavelengths and flow rates varying between 0.5-1.2 mL min^{-1} were investigated.

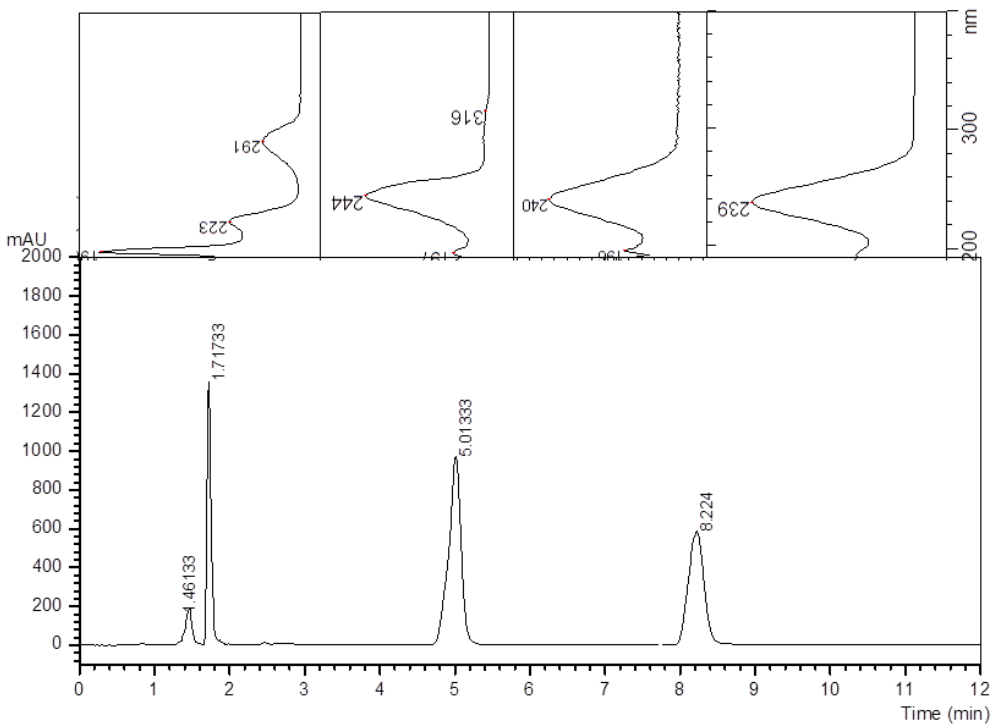
When a mobile phase consisting of 60% methanol, and above, in water was used at a flow rate of 0.8, 1 and 1.2 mL min^{-1} peaks either co-eluted or clustered together, while at 0.5 mL min^{-1} peaks broadened. In the reverse, when 40% methanol and below was used, only CP and HCV could be separated from the other components and the retention times were also increased with the reduction of the methanol percentage in the mobile phase.

On the other hand, elution time of the components was decreased as the polarity of the mobile phase was decreased by adding more organic solvent (methanol). Actually decreasing the mobile phase polarity reduces the hydrophobic interaction between the solute and stationary phase resulting in desorption. This implies also that the more hydrophobic the molecule the more time it will spend on the stationary phase in RP-HPLC.

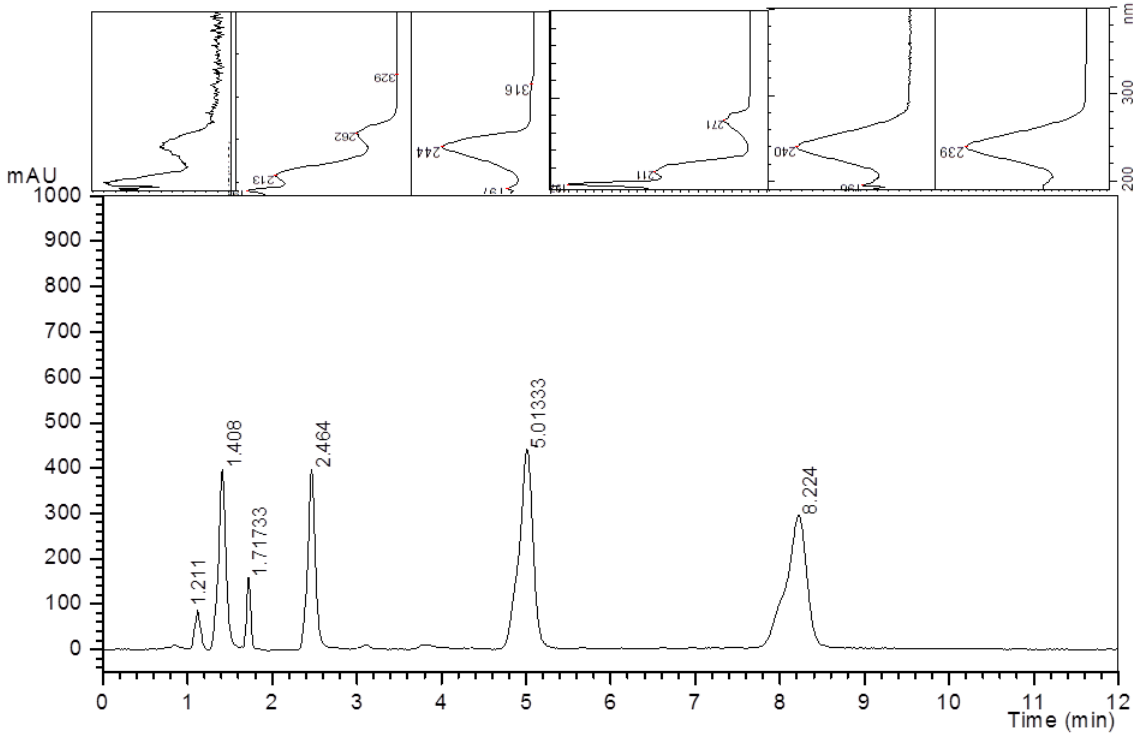
By using mobile phases of 45% methanol, 50% methanol, and 50% acetonitrile in water, the resolution of the peaks was better at 0.8 mL min^{-1}. However, HQ and NA were poorly separated, while late eluting components had broadened peak shapes. When acetic acid was added to these eluents in order to keep acidic groups in the analytes protonated and thereby improve the peak shapes, similar results were found and the time for the analysis was still long.

Finally, the seven ingredients were successfully separated by using a composition of 55% methanol and 45% water as the mobile phase. The flow rate was suitable at 0.8 mL min^{-1} and the total run time was within 10 min. In addition, the column temperature was kept at 25 °C. Chromatograms in Figure 3.3 show the separation of the active ingredients: HQ, BQ, PO, ARB, NA, HCV and CP.

(A)


mAU
nm
Time (min)
1.46133
1.71733
5.01333
8.224
291
223
316
244
240
239

(B)


mAU
nm
Time (min)
1.211
1.408
1.71733
2.464
5.01333
8.224
329
262
213
316
244
271
211
240
239

(C)

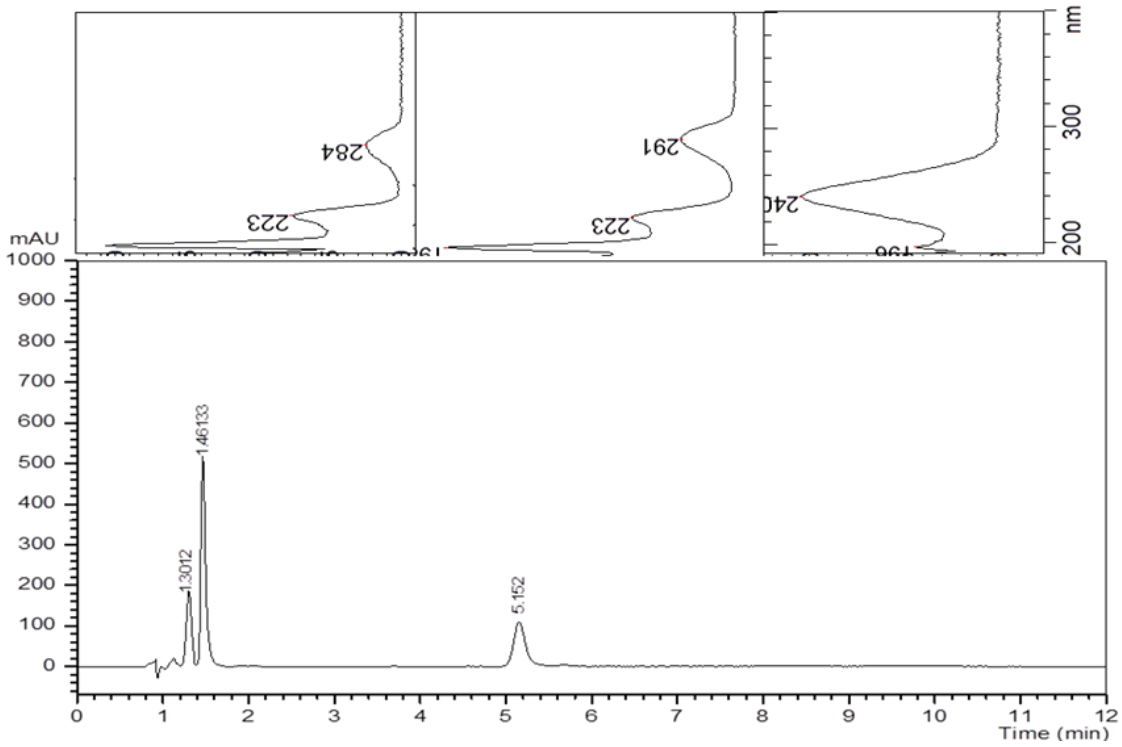


Figure 3.3: Chromatograms for separation of active ingredients: HQ, BQ, PO, ARB, NA, HCV and CP. Chromatogram (A) shows the separation of BQ, HCV, and CP detected at 240 nm, chromatogram (B) shows the separation of NA, PO, HCV, and CP detected at 270 nm and chromatogram (C) indicates the separation of ARB and HQ detected at 289 nm. The chromatographic conditions used were: Brownlee C18 column, mobile phase, MeOH-H_2O 55:45% (v/v), injection volume - 10 µL, flow rate 0.8 mL min^{-1}.

3.3.1.4 Determination of extraction solvent and sample preparation

In order to obtain quantitative extraction of the active ingredients from the cream samples, variables involved in the procedure such as solvent, extraction method and extraction time were optimized.

The extraction solvent was optimized for sample preparation by using different solvents including acetonitrile, methanol, 80% methanol, 60% methanol, 50% methanol and a mixture of 45% methanol-45% acetonitrile in water.

Peak area and peak height can directly reflect the extraction efficiency of the studied components. The results showed that with the increase of methanol content, the peak areas of the components increased and peak height also showed a slight increase. It has been reported in the literature[152,153] that clobetasol propionate and other steroids can be effectively extracted using acetonitrile while other reports used methanol as the extractant of choice[90,143]

When acetonitrile was used as the extraction solvent, the peak areas and peak height did not change significantly compared with those observed with methanol. However, the peak areas and peak heights were obviously reduced when using the mixture of acetonitrile, methanol and water. From the extraction efficiency of the different solvents (see Figure 3.4), it is clear that, when 100% methanol was used, the peak areas of the components reached the highest values. Therefore, after comparing the influence of each solvent, pure methanol was selected as the most suitable extraction solvent.

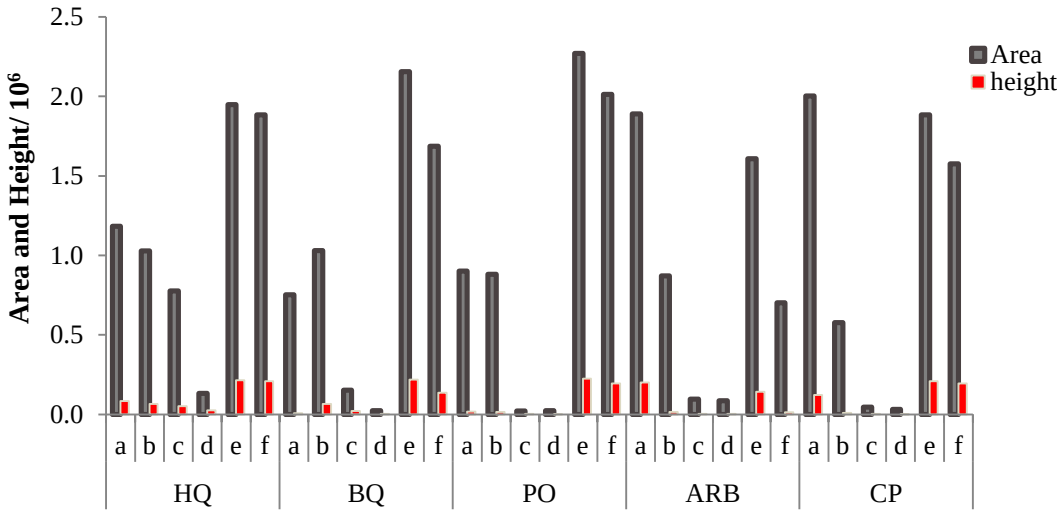


Figure 3.4 Effect of different solvents on the extraction of HQ, BQ, PO, ARB, and CP. *a*) methanol:water (80:20, v/v), *b)* methanol:water (60:40, v/v), *c)* methanol:water (50:50, v/v), *d)* acetonitrile:methanol:water (45:45:10, v/v), *e)* methanol (100%), *f)* acetonitrile (100%).

The extraction was better when the samples were extracted with 25 mL methanol for a minimum time of 20 min by using an ultrasonic bath set at 40 °C. Furthermore, all

cream samples were extracted in methanol and gave acceptable recoveries despite the presence of some preservatives (methyl paraben and propyl paraben) which could likely interfere with the active ingredients. Methyl and propyl parabens were detected in most samples.

3.3.2 Validation of HPLC analytical method

The characteristics for consideration during validation of the analytical procedures have been discussed.[138] For the validation of the HPLC quantitation method employed in the determination of organic active ingredients in different skin-lightening samples, linearity, accuracy, precision, and the detection limit were evaluated.

3.3.2.1 Linearity of calibration curves and limits of detection

The linearity was assessed based on calibration curves obtained with an external standard method. For each curve, three injections were performed for each concentration. Seven different concentrations were prepared for each ingredient. The calibration curves were determined in triplicate. Furthermore, regression equations were used for quantitation of the seven whitening ingredients in the cream samples. Calibration data used to construct the calibration curves are given in Tables C2-1 to C2-8 of Appendix C while their corresponding calibration curves as well as the residual plots are listed in Figures D13- D29 of Appendix D.

The calibration curves were constructed by plotting the peak area against the concentration. Linear regression analysis (at 95% confidence level) of the calibration data for each active ingredient was performed by using the Microsoft Excel add-in package called Analysis ToolPak. The linearity was confirmed from the correlation coefficient R^2 values. As shown in Table 3.7, in all cases, peak areas and analyte concentrations were found to be linearly related with a correlation coefficient greater than 0.997 ($R^2 > 0.997$) over the range tested for each of the analysed whitening ingredients. The LOD values found in this study were in the range of 0.001 - 0.0065 mg L^{-1} and the LOQ values ranged from 0.014 to 0.054 mg L^{-1}.

Table 3.7: Results of linear regression analysis of calibration curves for skin-lightening active ingredients analysed by HPLC.

Parameters	**HQ**	**BQ**	**PO**	**ARB**	**NA**	**CP**	**HCV**
Maximum absorption/nm	291	246	273	286	262	239	246
Detection wavelength used/nm	289	240	270	289	270	240	240
Calibration range/mg L^{-1}	1.25-25	0.625-20	1.25-25	0.625-20	0.625-20	0.391-25	0.391-25
Slope (b)/10^4 mg L^{-1}	1.2072	7.053	1.055	3.090	1.408	2.350	2.484
Standard error of slope	17.364	136.177	19.479	6.755	48.326	90.672	133.996
Correlation Coefficient (R^2)/10^{-1}	9.998	9.989	9.998	9.993	9.994	9.991	9.985
LOD/10^{-3} mg L^{-1}	4.32	5.79	5.54	6.56	10.3	11.6	16.2
LOQ/10^{-2} mg L^{-1}	1.44	1.93	1.84	1.97	3.43	3.85	5.39

3.3.2.2 Accuracy

The accuracy of the quantitation method was checked by a recovery test. The results are summarised in Table 3.8. Known amounts of the pure active ingredients were added to a cream that did not contain any of these lightening ingredients and analysed in triplicate. Concentration of each ingredient was subsequently calculated by using the corresponding regression equation of the calibration curve. The resulting recoveries ranged from 87.4 to 105.04 % and the relative standard deviation (R.S.D) values obtained ranged from 0.45 to 4.82 %. No interference was observed at either low or high concentrations. However, the mean percentage recovery achieved for HQ was below 90%. Since hydroquinone is easily oxidised into benzoquinone, this could possibly explain the low recovery obtained.

Table 3.8: Recoveries of the seven skin-lightening active ingredients (n = 3).

Active ingredients	Spiked amount/mg/L^{-1}	Found/mg L^{-1}	Recovery/%	R.S.D./%
HQ	2.5	2.26 ± 0.06	90.47	2.60
	10	8.71 ± 0.26	87.14	2.98
BQ	2.5	2.46 ± 0.07	98.25	2.80
	10	10.10 ± 0.20	101.02	1.94
PO	2.5	2.43 ± 0.02	97.35	0.63
	10	10.09 ± 0.05	100.88	0.45
NIA	2.5	2.45 ± 0.09	97.81	1.62
	10	9.66 ± 0.07	96.62	0.73
ARB	2.5	2.63 ± 0.07	105.04	2.77
	10	9.86 ± 0.15	98.58	1.54
CP	2.5	2.40 ± 0.2	95.94	0.99
	10	9.37 ± 0.45	93.71	4.82
HC V	2.5	2.45 ± 0.07	98.15	2.96
	10	9.69 ± 0.16	96.93	0.93

Recovery (%) = (mean found concentration/spiked concentration) × 100

3.3.2.3 Precision

The precision of the method was calculated as the relative standard deviation (R.S.D.) of assays containing the seven skin-lightening active ingredients in different ranges of concentration (low, medium and high). The authentic standard solutions were prepared and subjected to HPLC analysis for assessing the intra-day and inter-day variability. The quantity of each analyte was obtained from the corresponding calibration curve. The intra-day variability was examined within one day by five replicate injections and the results showed that the R.S.D. was in the range of 0.038–1.86 %. The inter-day precision was calculated from nine determinations over three consecutive days for each concentration. The R.S.D. values ranged from 0.60 to 2.83 % for inter-day variability (see Table 3.9). For both the intra- and inter-day studies the accuracy was at acceptable levels and ranged between 91.8-102.8%.

3.3.3 The contents of organic active ingredients in skin-lightening creams

The developed method was applied to different skin-lightening products for the determination of active ingredients such as NA, HQ, BQ, ARB, PO, CP, and HCV. The list of ingredients contained in each product is shown in Table 2.1. Table 3.10 shows the results of the mean percentage concentrations (% m/m) of different active ingredients detected in the skin-lightening creams in this work.

The results of this study showed that out of the nineteen samples analysed, twelve creams contain at least one of these seven agents. The peak areas obtained for the active ingredients are given in Appendix F and the representative HPLC chromatograms of each cream sample are shown in Figures E5-E16 of Appendix E.

Three creams contained NA. The mean concentrations recorded were 0.25, 1.17 and 1.04% (m/m), for SLC20, SLC21 and SLC31, respectively. For medical purposes, NA is used to treat pustular acne and it can be used up to 5%.[154] A cream, SLC54, contained 0.94% (m/m) of HQ and 0.008 % (m/m) of BQ. BQ was also detected in cream sample, SLC51. The content of HQ in this cream is below the maximum limit concentration (2%, m/m) permitted by the World Health Organization [44].

Table 3.9: Analytical results of intra-day and inter-day variability.

Active ingredient	Retention time/min	Concentration/ mgL^{-1}	Intra-day (n = 5)			Inter-day (n = 9)		
			Found	R.S.D./%	Accuracy/%	Found	R.S.D./%	Accuracy/%
HQ	1.45-1.46	2.0	1.89 ± 0.02	1.30	94.5	1.84 ± 0.05	2.50	91.8
		6.0	5.92 ± 0.11	1.86	98.7	5.75 ± 0.08	1.40	95.8
		24.0	23.85 ± 0.16	0.71	95.2	22.5 ± 0.36	1.58	93.7
BQ	1.73-1.74	2.0	2.04 ± 0.07	0.94	101.9	1.96 ± 0.05	2.81	97.8
		6.0	5.91 ± 0.03	0.58	98.5	5.88 ± 0.05	0.89	97.9
		18.0	18.02 ± 0.06	0.32	100.1	17.79 ± 0.31	1.73	98.8
PO	2.45-2.49	2.0	1.99 ± 0.05	0.90	99.8	1.98 ± 0.01	0.60	99.2
		6.0	6.17 ± 0.06	0.87	102.8	6.2 ± 0.08	1.37	100.4
		24.0	24.26 ± 0.01	0.038	101.1	23.91 ± 0.29	1.71	99.6
ARB	1.28-1.33	2.0	2.05 ± 0.01	0.35	102.4	1.98 ± 0.06	2.83	98.4
		6.0	5.87 ± 0.03	0.51	97.9	6.02 ± 0.07	1.30	96.5
		18.0	17.78 ± 0.12	0.67	98.7	23.91 ± 0.12	0.66	99.6
NA	1.39-1.43	1.5	1.41 ± 0.01	0.49	94.0	1.40 ± 0.03	2.50	93.4
		5.0	4.63 ± 0.039	0.94	92.6	4.65 ± 0.06	1.39	92.9
		15.0	14.64 ± 0.07	0.45	97.6	14.43 ± 0.15	1.04	96.2
CP	8.22-8.30	0.5	0.47 ± 0.05	0.49	94.4	0.47 ± 0.01	1.40	93.8
		5.0	4.84 ± 0.04	0.94	96.8	4.85 ± 0.04	0.81	97.0
		20.0	19.76 ± 0.04	0.45	98.8	19.66 ± 0.18	0.92	98.3
HC V	5.0-5.3	0.5	0.48 ± 0.04	1.10	96.6	0.48 ± 0.01	1.08	95.1
		5.0	4.78 ± 0.02	0.81	95.7	4.76 ± 0.05	1.04	95.2
		20.0	19.39 ± 0.04	0.19	96.9	19.52 ± 0.13	0.67	97.6

Accuracy (%) = (mean of found concentration/theoretical amount) × 100
R.S.D.(%)=(S.D./mean concentration) × 100

For dermatological therapy hydroquinone is an important agent for treating hyperpigmentation. The use of HQ in cosmetics has been banned by many countries due to its toxicity. A report has revealed that HQ, benzene and/or benzene-ring related molecules, for example BQ and PO, may induce marrow toxicity and carcinogenesis.[155] The risks of long-term effects of topical application induce brown discoloration of the skin and fingernails, an asymptomatic hyperpigmentation of the face with gradually progressive blue-black macular patches and exogenous ochronosis.

ARB was detected in two creams (one containing CP) and the mean concentrations found were 0.063% in SLC49 and 0.003% (m/m) in SLC59. Studies have demonstrated that arbutin is safer and less cytotoxic whitening agent compared with HQ.[156]

PO was found in four creams with levels of 0.092%, 0.261%, and 0.0025% (m/m) for SLC51, SLC57 and SLC60, respectively. PO was not quantified in the fourth cream sample (SLC58) because of the presence of KA.

KA was also detected in two samples SLC56 and SLC58. Due to its tailing factor, optimised chromatographic conditions failed to separate KA from other compounds. KA presented a serious peak tailing which could interfere and produce bias in the quantitation of other compounds. Consequently, levels of KA were calculated neither in SLC56 nor in SLC58.

Steroid compounds were observed in two creams: one cream (SLC59) contained 0.055% of CP and another cream (SLC52) had 0.0031% of HCV. Topical steroids are effective anti-inflammatory preparations used to control eczema and many other skin conditions.[87] Concentrations of CP and HCV detected in the studied creams are consistent with the maximum medically used concentration (0.05%) of most steroids agents.[89, 143] Clobetasol propionate and hydrocortisone are associated with local side-effects including straie, redness, scaling and epidermal thinning as well as systemic side-effects such as dysfunction of the hypothalamus, Cushing's syndrome and neuropathy.[91, 152, 153] Also many other side-effects have been reported.[157]

Table 3.10: Levels of detected active ingredients (n = 3).

Sample Code	Active ingredient detected	Concentration ± SD /mg L^{-1}	Mean concentration/% (w/w)
SLC18	-	-	-
SLC19	-	-	-
SLC20	NA	18.554 ± 1.95	0.232
SLC21	NA	18.723 ± 0.713	1.170
SLC26	-	-	-
SLC31	NA	1.474 ± 0.10	0.035
SLC33	-	-	-
SLC49	ARB	2.524 ± 0.26	0.063
SLC50	-		-
SLC51	BQ	7.320 ± 0.82	0.036
	PO	18.40 ± 0.78	0.920
SLC52	HCV	1.226 ± 0.79	0.003
SLC53	-	-	-
SLC54	HQ	15.044 ± 0.58	0.940
	BQ	1.560 ± 0.14	0.008
SLC55	-	-	-
SLC56	KA*	-	-
SLC57	PO	10.445 ± 0.79	0.261
SLC58	PO*	-	-
	KA*	-	-
SLC59	ARB	1.204 ± 0.16	0.003
	CP	4.418 ± 0.31	0.055
SLC60	PO	1.023 ± 0.13	0.0025

* Not quantified in the product
- Not detected

Chapter 4

SUMMARY AND CONCLUSIONS

This research has investigated some of the most popular skin-lightening creams, commercially available on South African markets. The sample products were manufactured in different countries: about 40% were from in South Africa, about 18% from India, about 10% from Pakistan, about 10% from Europe and USA, and about 6% from East Africa. For about 15% of the sample products, the countries of manufacture were not known.

The aim of the study was to identify and quantify active ingredients and heavy metals that might be contained in commonly used skin-lightening creams in order to provide information that can guide everyone in choosing safe cosmetic skin products.

The analysis of the skin-lightening creams was carried out in two parts. The first part involved the determination of heavy metals by using ICP-OES and/or CV-AAS. The second part concerned the determination of organic active ingredients by means HPLC.

Determination of heavy metals by means of ICP-OES and/or CV-AAS

All screened skin-lightening creams were found to be free from cobalt, cadmium, arsenic, and nickel. This is good news to the users of these products because they will not face problems of allergic contact dermatitis caused by nickel and cobalt.[108,118]

Iron and copper have been detected, but at low levels (at about 0.5 mg g^{-1}), except for one case in which iron exceeds 20 mg g^{-1}. The presence of iron and copper at low levels is not of concern to the users since these are essential elements recommended for the human diet at low concentrations.[158] This, however, does not imply that we recommend the inclusion of iron or copper in cosmetic creams as it might boost, through the skin permeation, their accumulation and lead to high levels in the human body.

Metals such as lead and chromium have been found at levels not higher than 16 μg g^{-1}, in less than ten creams. Lead and chromium are used as colour additives in cosmetics. Exposure to lead can result in high blood pressure, kidney damage, infertility, and miscarriages.[37] Chromium causes allergy contact dermatitis, in particular, Chromium (VI) is a known carcinogen.[102] One of the three sample creams that was found to contain mercury had a relatively high concentration of 51.2 μg g^{-1}. This result is a cause for concern because the inclusion of any form of mercury, in skin-lighteners is currently forbidden because of its toxicity.

Determination of organic active ingredients by means of HPLC

The following active ingredients were detected in the 19 creams that were analysed by means of HPLC: hydroquinone, niacinamide also called Vit B3, arbutin, benzoquinone, phenol, clobetasol propionate, and hydrocortisone valerate. Quantitative results indicate that about 63% of the analysed creams had at least one or two of the above-mentioned active ingredients.

Hydroquinone was found in one cream only with a concentration of 0.94% (m/m) that is below the WHO limit of 2% (m/m) but its toxicity may not be ignored. Benzoquinone was detected in two creams of which one contains hydroquinone and the other one contained phenol. Phenol was found in four creams. Arbutin was detected in two creams and niacinamide in three creams. To the best of our knowledge, there is no limit on the concentration of arbutin and niacinamide in cosmetic products. Thus, concentrations obtained for these ingredients cannot be judged as high or low. The determined mean concentration ranges were: 0.0025-0.261%, 0.004-0.036%, 0.003-0.0063%, and 0.0035-1.17% for phenol, benzoquinone, arbutin and niacinamide, respectively. Steroids, namely clobetasol and hydrocortisone, were detected in two creams and were found to be compliant with therapeutic levels (≤ 0.05% w/w) for ointment creams.

Levels of the active organic ingredients detected in skin-lightening creams are low and should probably have no significant side-effect for short periods of application;

however, the user who applies any of these creams may experience some effects such as, skin discoloration, redness, acnes and systemic diseases particularly after repeated and long-term exposure.

In view of the findings of this study (Table 4.1) and the toxicological effects associated with lead (Pb), chromium (Cr), mercury (Hg), hydroquinone (HQ), clobetasol propionate (CP), and hydrocortisone valerate (HCV), efforts should be made in all countries in order to abolish inclusion of these agents or any toxic ingredient in commercial skin-lightening creams. Efforts should also be made to educate the users and the general public on the possible dangers associated with the skin-lightening practices.

We have collected and investigated a few samples of creams that are sold in South Africa, but there are hundreds of brands that are available from around the world. It is worth noting that although many of the most important skin-lightening creams have now been reformulated with new ingredients, they may still contain harmful ingredients that can induce long-term side effects including permanent skin damage. Therefore, we recommend that before buying any skin-care products or any other cosmetic products, the user must double check the labelling, and fully understand whether the product is really safe. People are usually advised not to use skin-lightening creams. For skin conditions that do require such treatment, should only be used under supervision of a medical practitioner.

A future study can determine the concentrations of parabens, such as methyl, ethyl, propyl and butyl-paraben, used as preservatives in skin-lightening products. Methyl paraben was present in most of the skin-lightening creams tested in this study. Similarly, a previous study found high concentrations of parabens in different cosmetic creams.[163] This shows the prevalence of parabens in cosmetics. Concerns over the toxicity of preservatives are well-known.[129,164,165]

Table 4.1: Detection overview of metal and organic active ingredient contained the investigated skin-lightening creams.

Sample code	Metal	Organic active ingredient
SLC2	Cr, Cu, Fe, Pb	-
SLC4	Cr, Cu, Fe, Pb	-
SLC11	Cu, Fe, Hg	-
SLC17	Cu, Fe, Pb	-
SLC20	Cu, Fe	NA
SLC21	Cu, Fe	NA
SLC27	Cr, Cu, Fe	-
SLC31	Cu, Fe	NA
SLC39	Cr, Cu, Fe, Hg	-
SLC43	Cu, Fe, Hg	-
SLC45	Cu, Fe, Hg, Zn	-
SLC46	Cr, Cu, Fe	-
SLC47	Cr, Cu, Fe	-
SLC49	Cu, Fe,	ARB
SLC51	-	BQ, PO
SLC52	-	HCV
SLC54	-	HQ, BQ
SLC56	-	CP
SLC57	-	PO
SLC58		KA
SLC59	-	KA, PO
SLC60	-	ARB

- Not detected

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Appendix A

MATERIALS

The different chemicals, including analytical grade reagents and other consumable materials, used during the study are listed below.

A1 Chemicals used for the determination of active skin-lightening agents by HPLC

Chemical	Supplier
Acetonitrile (HiperSolvTM, HPLC grade)	BDH Chemicals, Ltd
Methanol (HPLC grade)	Merck
Millipore water Elix 5 UV	Millipore
Hydroquinone (99.09%)	Fluka
Phenol (99.5%)	Fluka
Benzoquinone (99.5%)	Fluka
Resorcinol (99%)	Riedel-de Haën
Niacinamide (99.9%)	Supelco Analytical
Kojic acid (98%)	Fluka
Arbutin (96%)	Sigma-Aldrich
Clobetasol dipropionate (98%)	Sigma-Aldrich
Hydrocortisone valerate (98.7%)	Sigma-Aldrich

A2 Chemicals used for the determination of heavy metals by ICP-OES and CV-AAS

Chemical	Supplier
Tin chloride dihydrate ($SnCl_2.2H_2O$)	Associated Chemical Enterprises
Mercury chloride ($HgCl_2$)	BDH Laboratory Reagents
Analytical grade, Cadmium 1000 ppm	Fluka Chemicals
Analytical grade, Chromium 1000 ppm	Fluka Chemicals
Analytical grade, Iron (Fe) 1000 ppm	Fluka Chemicals
Analytical grade, Lead (Pb) 1000 ppm	Fluka Chemicals
Analytical grade, Zinc (Zn) 1000 ppm	Fluka Chemicals
Analytical grade, nitric acid (≥ 69%)	Riedel-de Haën
Analytical grade, hydrochloric acid (55%)	Sigma Aldrich

Appendix B

INSTRUMENTATION AND EQUIPMENT

The instruments and all equipment used to carry-out the analyses are listed below.

B1 Instruments

Perkin Elmer Lambda 25 UV/VIS dual beam spectrophotometer

Shimadzu Prominence HPLC equipped with:

DGU-20A3 Prominence Degasser,
A & B LC-20AD/UFLC XR Prominence Liquid Chromatograph,
SIL-20A/UFLC-XR Prominence Autosampler,
SPD-M20A Prominence Diode Array Detector,
CTO-20A Prominence Column Oven and
CBM-20A/UFLC Prominence Communication Bus Module

Perkin-Elmer optima 5300 DV ICP-OES

Perkin-Elmer™ Atomic spectrometer coupled with MHS 15 Mercury Hydride System

B2 Equipment

CEM Mars 6 Microwave digester with a turntable containing 12 reaction vessels (XP-1500)
Phenomenex HPLC column; Luna RP-C18 (250 mm × 4.6 mm, 5.0 µm)
Brownlee HPLC column; C18 (100 mm × 4.6 mm, 5.0 µm)
Ultrasonic bath (Ultra Manufacturing Company)
Millipore Milli-Q water purification system
Millipore Millex hydrophilic PFTE 0.45 µm syringe filters
Millipore Durapore® 0.45 µm membrane solvent filters.

Appendix C

CALIBRATION DATA

This section presents in tables, the calibration data of all heavy metals and organic active ingredients studied in this investigation**.**

C1 Calibration data for heavy metals

TableC1-1: Calibration data for the determination of copper (Cu).

Concentration /mg L^{-1}	0.02	0.04	0.06	0.08	0.1
1	6108.4	13210.6	20197.8	25676.2	32709.2
2	6718.8	12917.5	19376.3	26835	32293.8
3	6418.1	13076.2	19464.2	27032.3	33041.2
Mean	6415.1	13068.1	19679.4	26514.5	32681.4
Std Dev	305.1862	146.7165	451.1	732.6717	374.4998
RSD/%	4.757327	1.122708	2.292089	2.763286	1.145912

Table C1-2: Calibration data for the determination of chromium (Cr).

Concentration /mg L^{-1}	0.02	0.04	0.06	0.08	0.1
1	1669.8	3279.9	5294.1	6645.2	8651.1
2	1589.9	2996.6	4951.7	6428.9	8199.1
3	1653.4	3351	5071.5	6762.1	8452.5
Mean	1637.7	3209.2	5105.8	6612.1	8434.2
Std Dev	42.20036	187.4969	173.753	169.0308	226.553
RSD/%	2.576806	5.842529	3.403073	2.556406	2.686112

Table C1-3: Calibration data for the determination of lead (Pb).

Concentration /mg L^{-1}	0.02	0.04	0.06	0.08	0.1
1	159.1	284.3	495.9	539.3	676.1
2	157.5596	315.1192	472.6788	609.2384	717.8
3	152.8	278.7	470.9	590.5	773.8
Mean	156.4865	292.7064	479.8263	579.6795	722.5667
Std Dev	3.28422	19.61097	13.94865	36.20301	49.02411
RSD/%	2.098724	6.699878	2.90702	6.24535	6.784718

TableC1-4: Calibration data for the determination of iron (Fe).

Concentration /mg L^{-1}	0.01	0.02	0.04	0.06	0.08	0.1
1	581.2	1150.2	2253.7	3442.5	4567.3	5723.7
2	574.8	1171.9	2396.9	3538.2	4516.8	5697.5
3	569.1	1138.1	2240.6	3601.2	4324.5	5591.1
Mean	574.6	1153.4	2297.07	3527.3	4469.53	5670.77
Std Dev	6.05	17.13	86.76	79.91	128.12	70.23
RSD/%	1.05	1.48	3.77	2.26	2.86	1.24

Table C1-5: Calibration data for the determination of mercury (Hg).

weight /ng	100	200	300	400	500	600
1	0.088	0.166	0.225	0.326	0.389	0.503
2	0.081	0.162	0.25	0.322	0.401	0.483
3	0.101	0.157	0.233	0.33	0.418	0.477
Mean	0.09	0.162	0.236	0.326	0.403	0.488
Std Dev	0.010	0.005	0.013	0.004	0.015	0.014
RSD/%	11.276	2.789	5.410	1.223	3.619	2.792

Table C1-6: Calibration data for the determination of zinc (Zn).

Concentration /mg L^{-1}	0.1	0.2	0.3	0.4	0.5
1	4178.1	8091.4	12414.7	15989.7	20902.3
2	4300.6	8309.1	12401	16732.8	20813.6
3	3995.9	7998.8	11981.3	15992.2	20502.7
Mean	4158.2	8133.1	12265.6	16238.2	20739.5
Std Dev	153.3	159.3	246.3	428.3	209.8
RSD/%	3.68	1.95	2.01	2.63	1.01

C2 Calibration data for the organic active skin-lightening ingredients

Table C2-1: Calibration data for the determination of hydroquinone (HQ).

Concentration /mg L^{-1}	1.25	2.5	5	10	15	20	25
1	15001	30650	60855	120198	180841	240577	300200
	15022	30052	60120	120004	180149	240013	300893
	15045	30124	60585	120344	180115	240821	301081
2	15178	30681	61162	121839	180802	241914	300580
	15105	30675	60028	121631	190031	241754	300730
	14999	30020	60034	126562	190048	241358	304471
3	14921	29996	60023	120327	180675	241039	300469
	14998	29945	59992	120398	180214	240112	301218
	14974	30015	60100	120045	180009	240333	300543
Mean	15027	30239.8	54290.4	121260.9	182542.7	240880.1	301131.7
Std Dev	75.5	325.2	435.4	2098.2	4261.5	691.3	1291.2
RSD/%	0.5	1.1	0.8	1.7	2.3	0.3	0.4

Table C2-2: Calibration data for the determination of phenol (PO).

Concentration /mg L^{-1}	1.25	2.5	5	10	15	20	25
1	13562	26515	53456	107770	160554	211889	261445
	13655	26276	53517	102871	158956	212409	271878
	13271	26125	52738	106428	160903	211604	269796
2	12953	25993	51814	106812	155722	209089	259398
	12982	26063	51896	103564	155206	210125	257121
	12887	25834	51844	103769	155812	207329	261947
3	13399	26059	52976	106052	159053	210805	263244
	13465	26404	53061	106002	158189	211620	268885
	12955	26992	53083	106744	158013	210527	265006
Mean	13236.5	26251.2	52709.4	105556.9	158045.3	210599.7	264302.2
Std Dev	297.4	348.4	685.3	1712.7	2084.9	1590.5	4994.0
RSD/%	2.2	1.3	1.3	1.6	1.3	0.8	1.9

Table C2-3: Calibration data for the determination of benzoquinone (BQ).

Concentration /mg L^{-1}	0.625	1.25	2.5	5	10	15	20
1	42987	82400	175916	355338	724648	1052193	1420534
	42229	82676	174050	353523	722852	1050472	1428770
	40748	83464	175384	351397	720103	1040883	1430014
2	42424	83765	172257	343906	700285	1042453	1424335
	42542	83841	173338	343814	700786	1052378	1425073
	41809	81716	172153	342401	698026	1043124	1427903
3	43187	86634	178509	321881	691287	1056251	1427907
	43407	86744	169460	327628	697284	1055021	1400156
	43623	86614	179876	317161	697910	1047843	1400922
Mean	42550.7	84206	174549.2	339672.1	705909	1048958	1420624
Std Dev	894.3	1962.6	3264.0	14078.4	12805.7	5670.1	11732.6
RSD/%	2.1	2.3	1.9	4.1	1.8	0.5	0.8

Table C2-4: Calibration data for the determination of arbutin.

Concentration /mg L^{-1}	0.625	1.25	2.5	5	10	15	20
1	2014	3879	7743	15244	30617	46020	61532
	2248	3852	7710	15245	30541	45727	61381
	2052	3919	7724	15488	30982	45983	60833
2	2092	3669	8193	15988	31429	47007	61143
	2069	3700	8099	16974	31307	47090	60833
	2017	3783	8615	16102	31757	46252	61731
3	2100	3712	7596	16192	30591	47772	61362
	2093	3795	7618	16665	31093	47103	61036
	2008	3755	7689	16915	30965	47395	61685
Mean	2077	3784.9	7887.4	16090.3	31031.3	46705.4	55155.6
Std Dev	73.5	85.4	343.4	671.4	414.9	721.5	340.2
RSD/%	3.5	2.3	2.1	4.2	1.3	1.5	0.6

Table C2-5: Calibration data for the determination of niacinamide (NA).

Concentration /mg L^{-1}	0.625	1.25	2.5	5	10	20
1	8252	16676	32648	65994	131618	277728
	8338	16327	32465	65668	136205	274528
	8148	16676	32522	66468	136534	272988
2	8843	17666	35073	70747	141494	287728
	8804	17680	35338	70007	144564	284528
	8888	17657	35754	70684	147429	282988
3	8599	17984	35569	70106	142279	284658
	8848	17401	35488	71139	149052	286790
	8792	17849	36127	71531	145227	282455
Mean	8612	17324	34554	69149	141600	281599
Std Dev	290	603	1534	2384	5770	5294
RSD/%	3	3	4	3	4	2

Table C2-6: Calibration data for the determination of clobetasol dipropionate (CP).

Concentration /mg L^{-1}	0.391	0.781	1.562	3.125	6.25	12.5	25
1	8218	17654	37432	69540	129763	268839	611642
	9235	19475	36950	73901	144802	295604	591208
	8064	17990	36032	71575	149315	306303	602606
2	9319	18616	37232	74464	148928	297857	595715
	9308	18257	37051	74103	144061	299785	592882
	9128	18025	37515	74610	144911	295778	588243
3	9203	18517	37034	71068	138137	276275	590886
	9258	19832	36539	71792	138522	270443	579255
	9213	19026	36805	72676	139937	275307	574081
Mean	8994	18599	36954	72637	142042	287355	591835
Std Dev	488	723	458	1763	6155	14403	11248
RSD/%	5	4	1	2	4	5	2

Table C2-7: Calibration data for the determination of hydrocortisone valerate (HCV).

Concentration /mg L^{-1}	0.391	1.562	3.125	6.25	12.5	25
1	7952	31455	69540	157668	307658	642696
	8047	31923	73901	156549	305932	642673
	8319	31601	71575	156814	305635	647904
2	8392	30869	74464	138944	299651	613419
	8312	30253	74103	140016	279473	617517
	8341	30856	74610	139952	289689	610045
3	8309	32477	71068	141983	301341	636034
	8701	33505	71792	149252	309913	628027
	8511	35958	72676	146564	305860	630125
Mean	8320	32100	72637	147527	300572	629827
Std Dev	223	1738	1763	7847	9905	13745
RSD/%	3	5	2	5	3	2

Appendix D

CALIBRATION GRAPHS AND RESIDUAL PLOTS

This section presents the calibration graphs and the residual plots used for the determination of heavy metals and active organic ingredients in skin-lightening creams analysed in this investigation. For ICP-OES, instrument conditions were: generator power, 1000 W; plasma gas flow rate, 12 L min^{-1}; nebulizer pressure, 3 bars and sample flow rate, 1 mL min^{-1}.

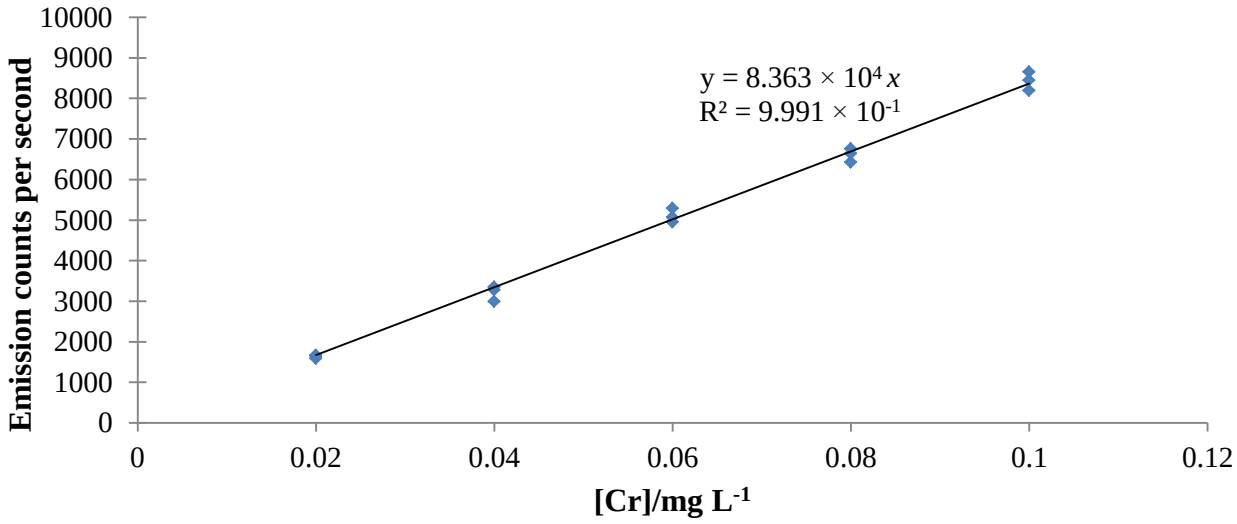

Figure D1: ICP-OES calibration graph for the determination of chromium. The analytical wavelength was 267.716 nm.

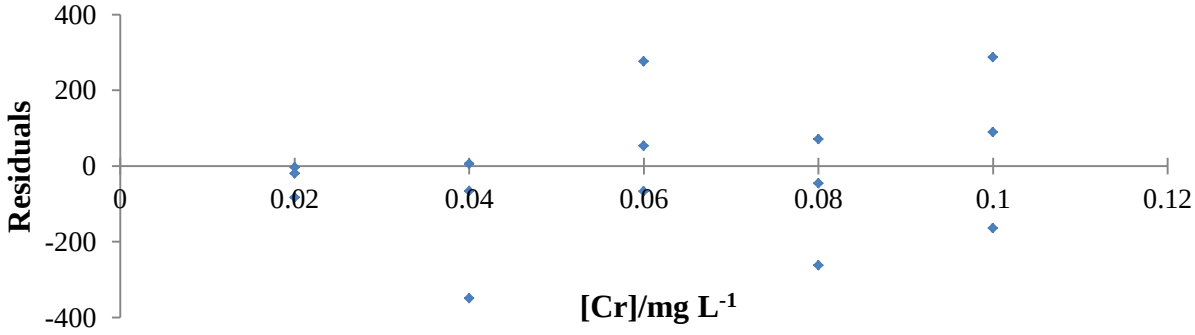

Figure D2: Residual plot for the calibration graph of chromium.

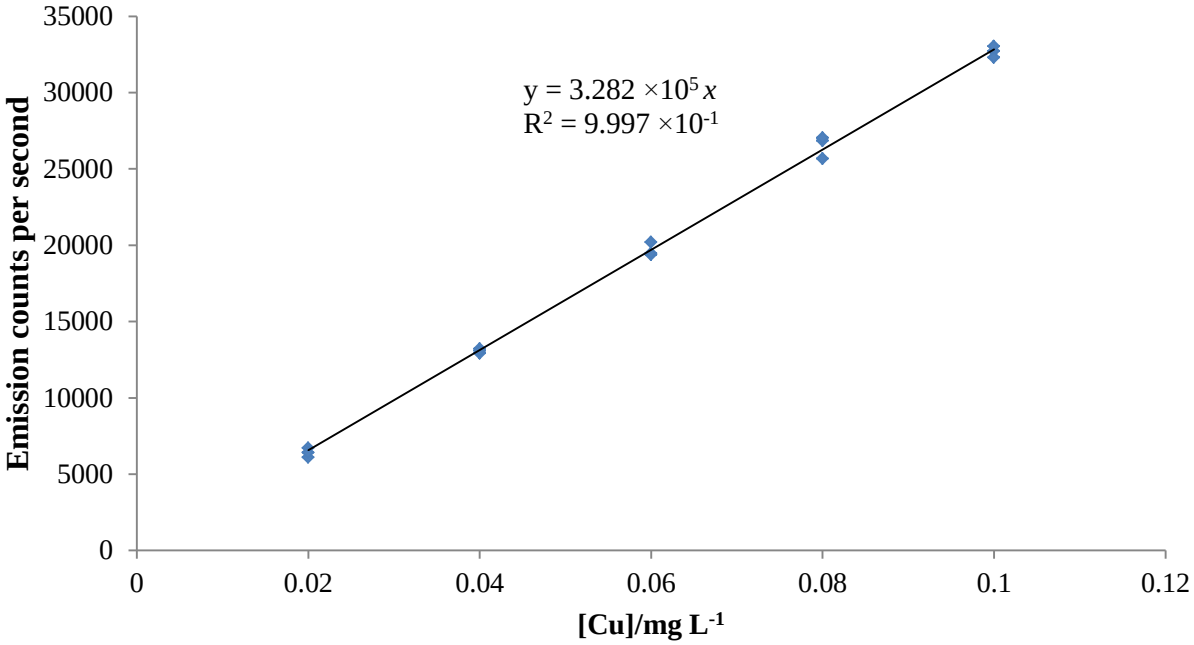

Figure D3: ICP-OES calibration graph for the determination of copper. The analytical wavelength was 324.752 nm.

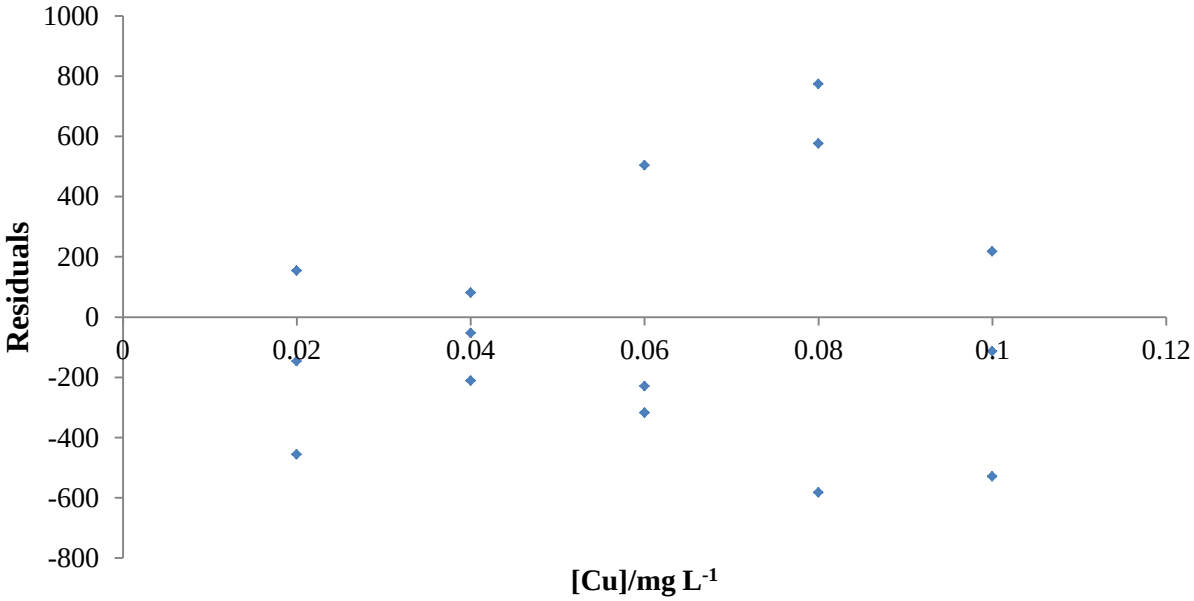

Figure D4: Residual plot for calibration graph of copper.

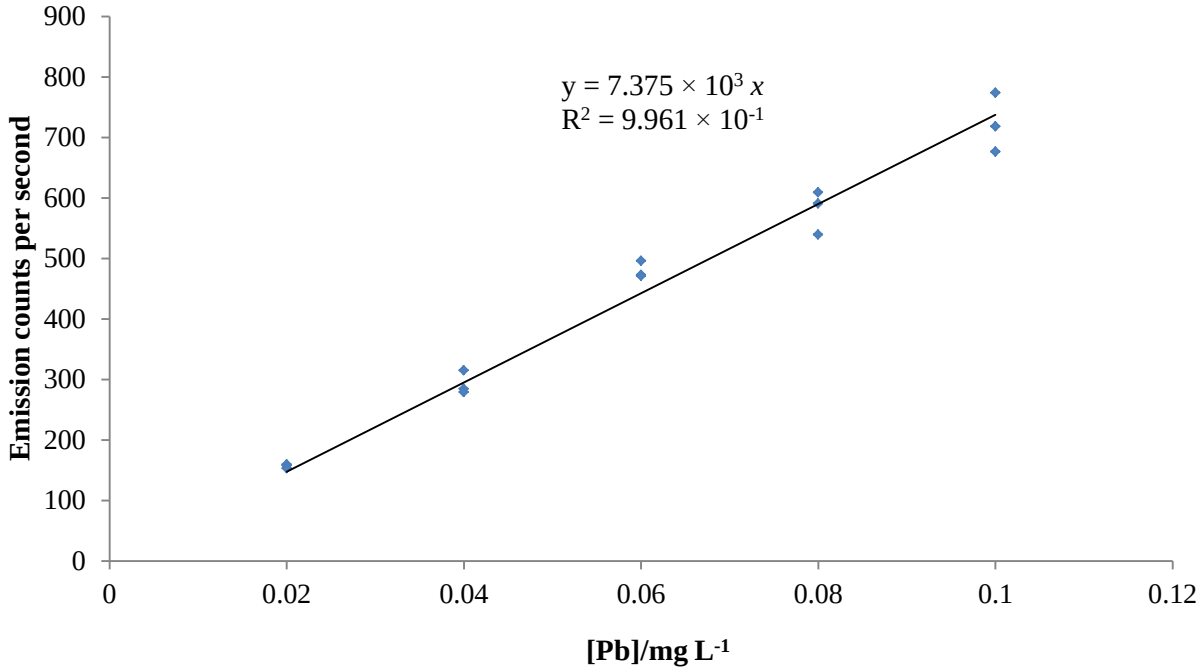

Figure D5: ICP-OES calibration graph for the determination of lead. The analytical wavelength was 220.353 nm.

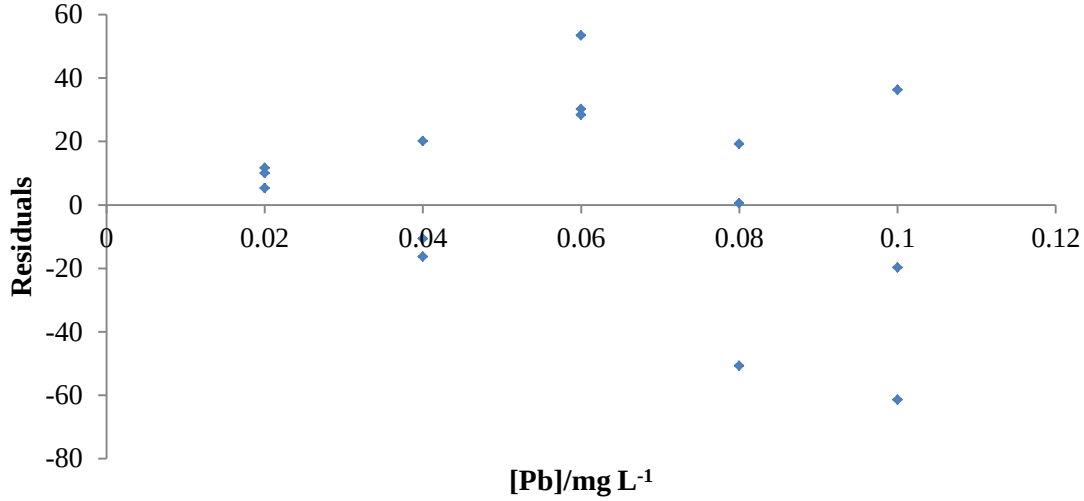

Figure D6: Residual plot for the calibration graph of lead.

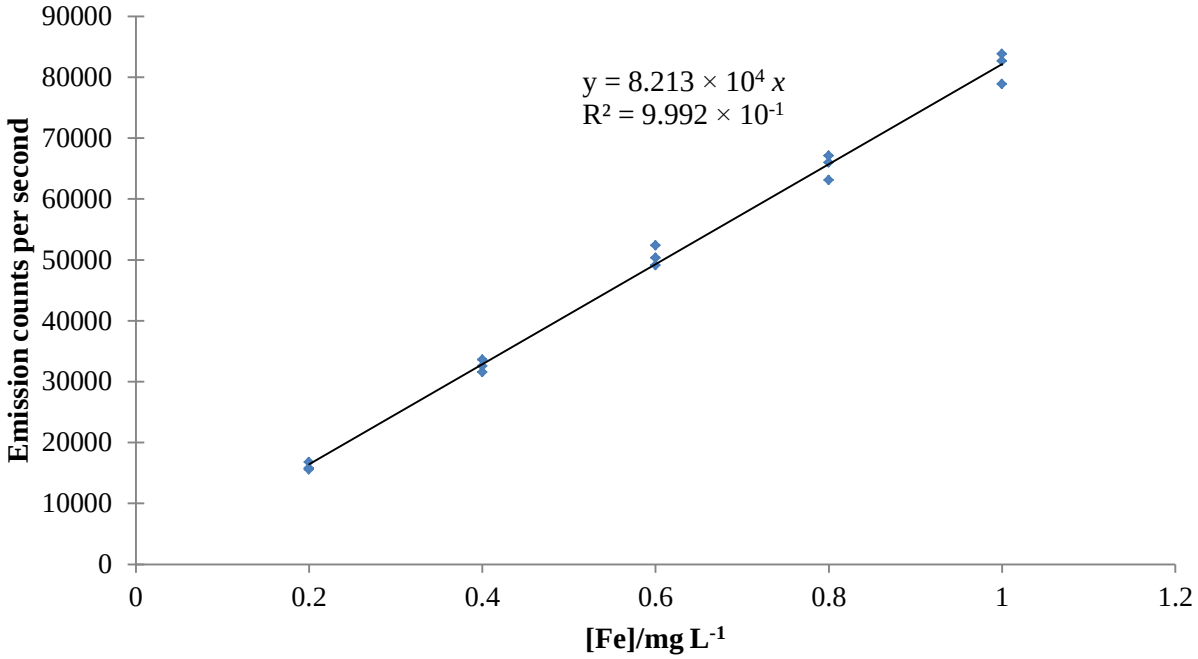

Figure D7: ICP-OES calibration graph for the determination of iron. The analytical wavelength was 259.939 nm.

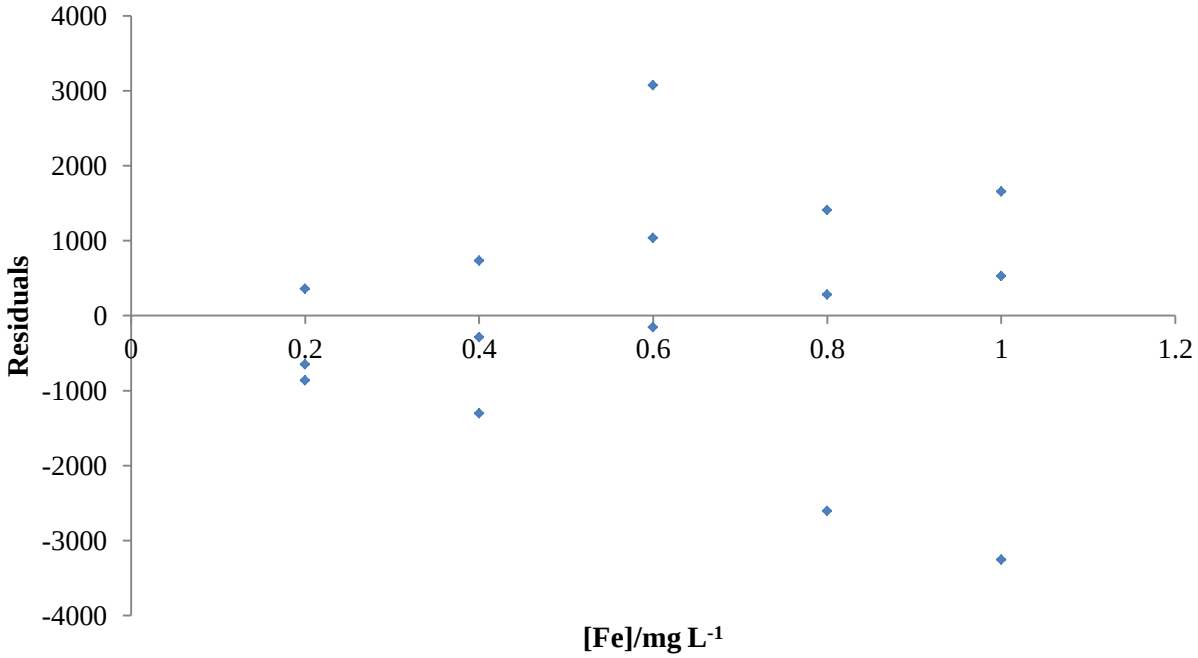

Figure D8: Residual plot for the calibration graph of iron.

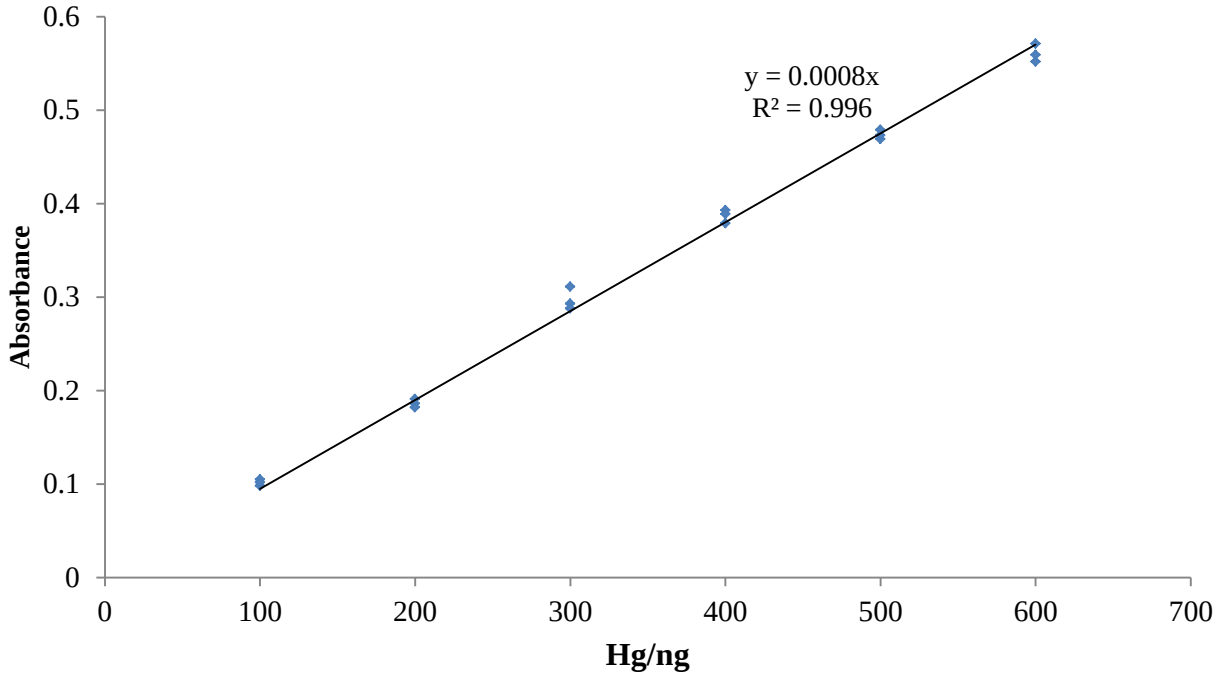

Figure D9: CV-AAS calibration graph for the determination of mercury. The analytical wavelength was 253.652 nm.

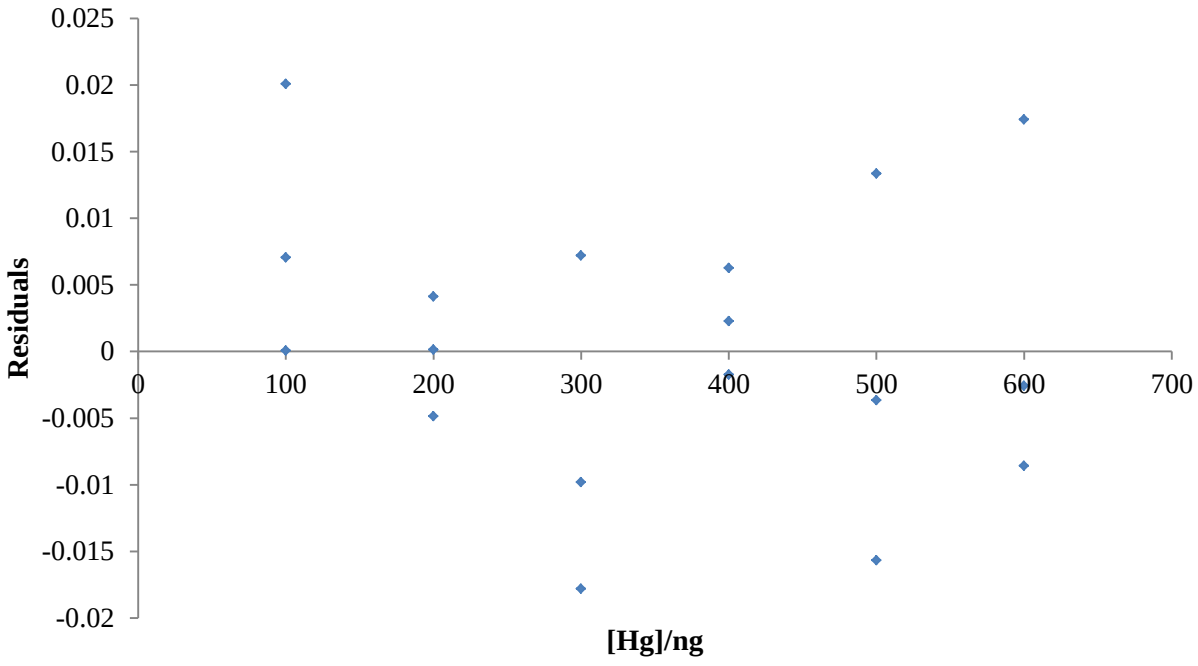

Figure D10: Residual plot the calibration graph of mercury.

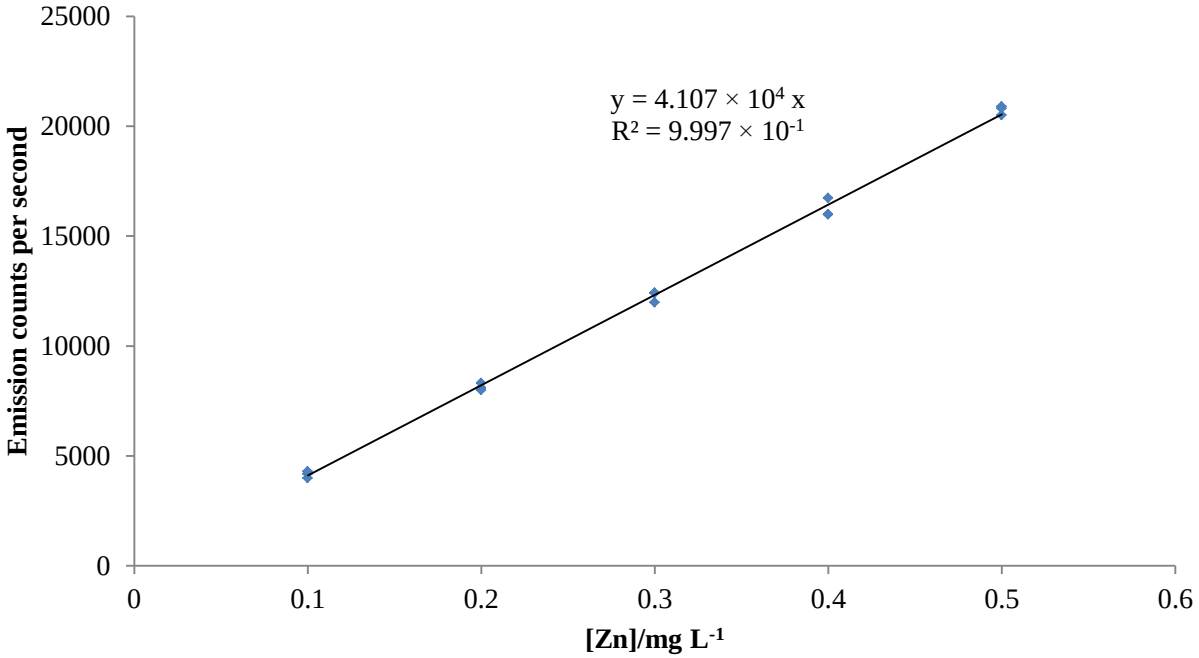

Figure D11: ICP-OES calibration graph for the determination of zinc. The analytical wavelength was 213.857 nm.

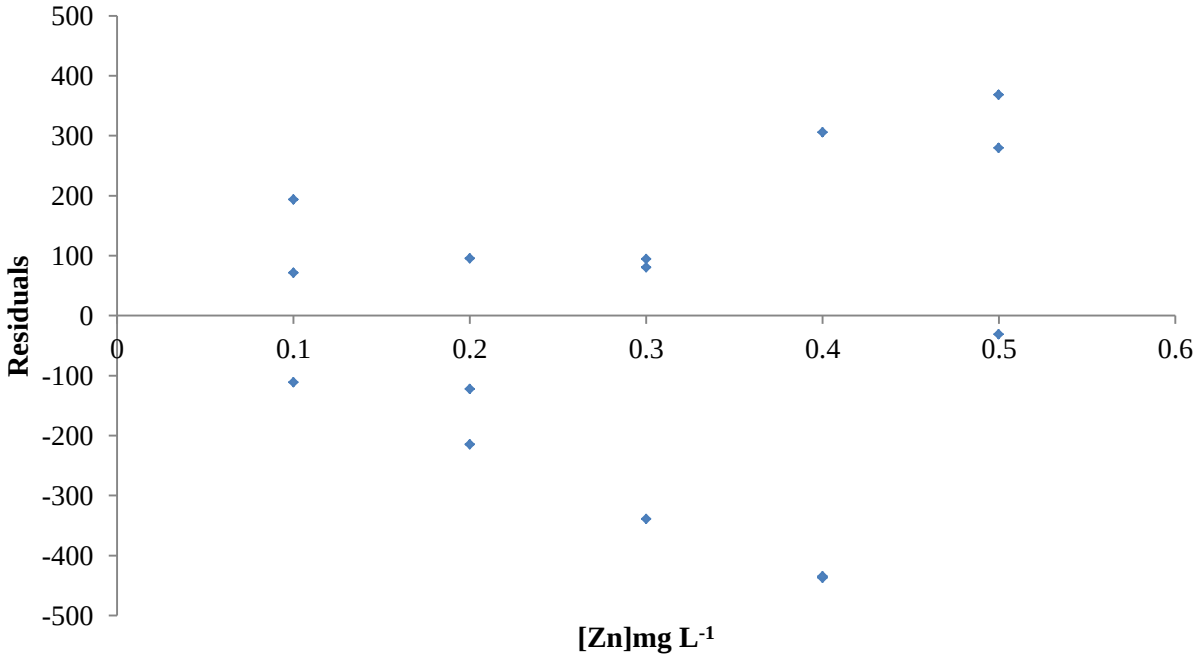

Figure D12: Residual plot for the calibration graph of zinc.

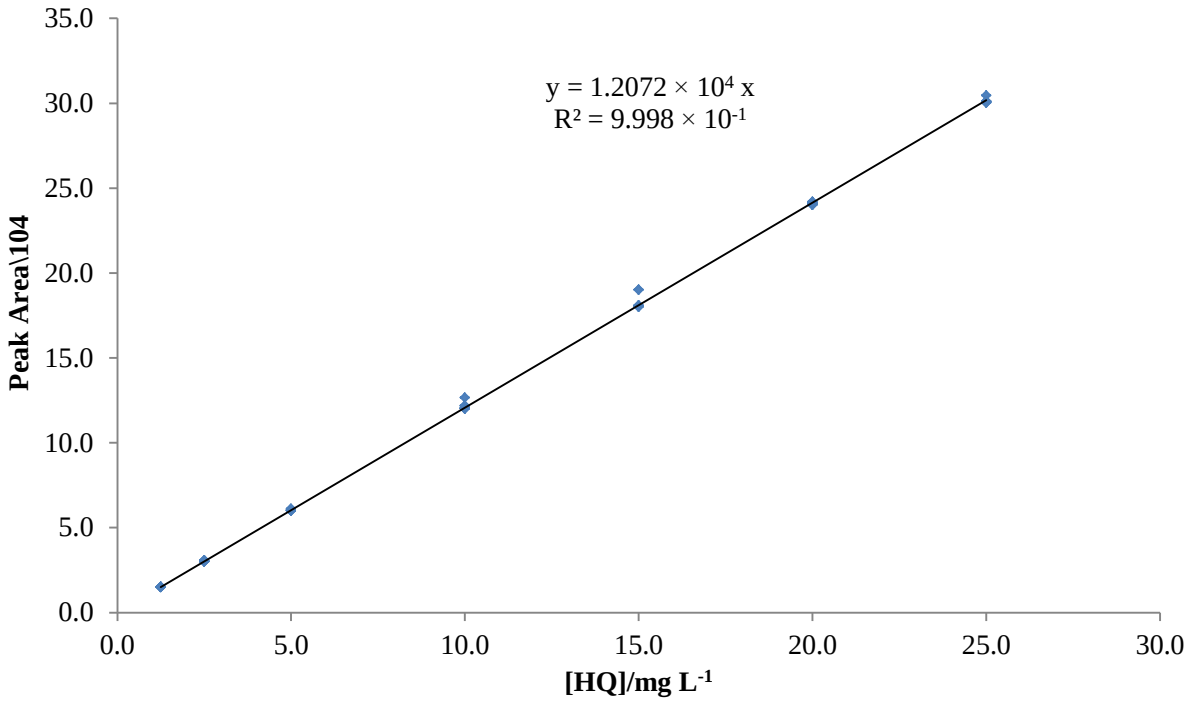

Figure D13: HPLC calibration graph for the determination of HQ. The detection wavelength was 289 nm.

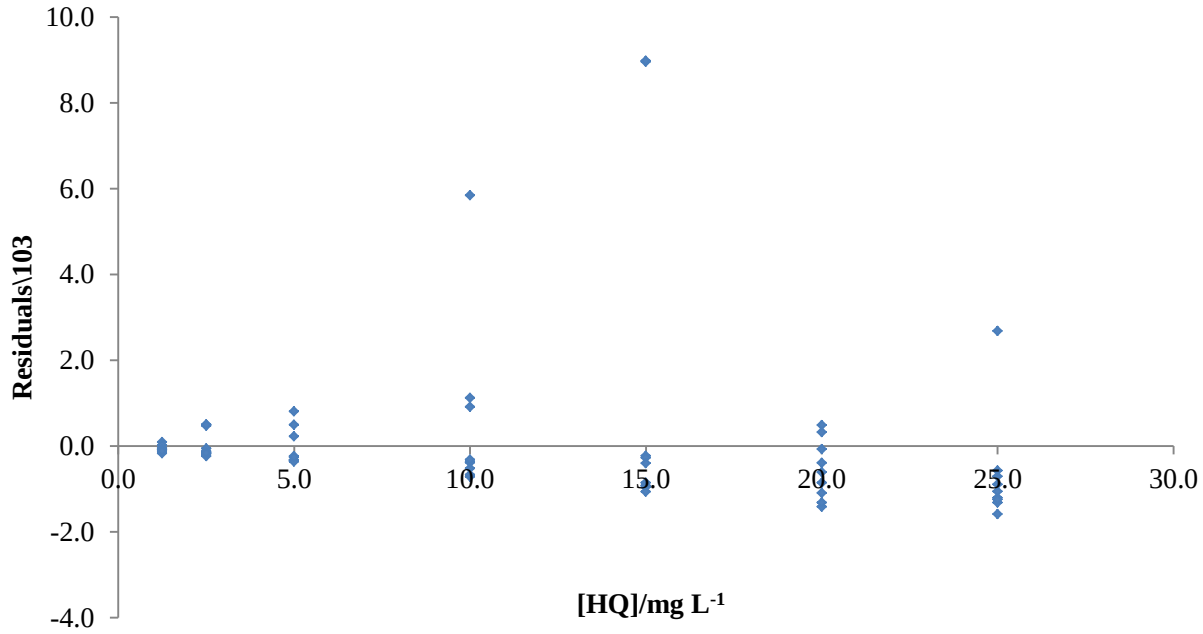

Figure D14: Residual plot for the calibration graph of HQ.

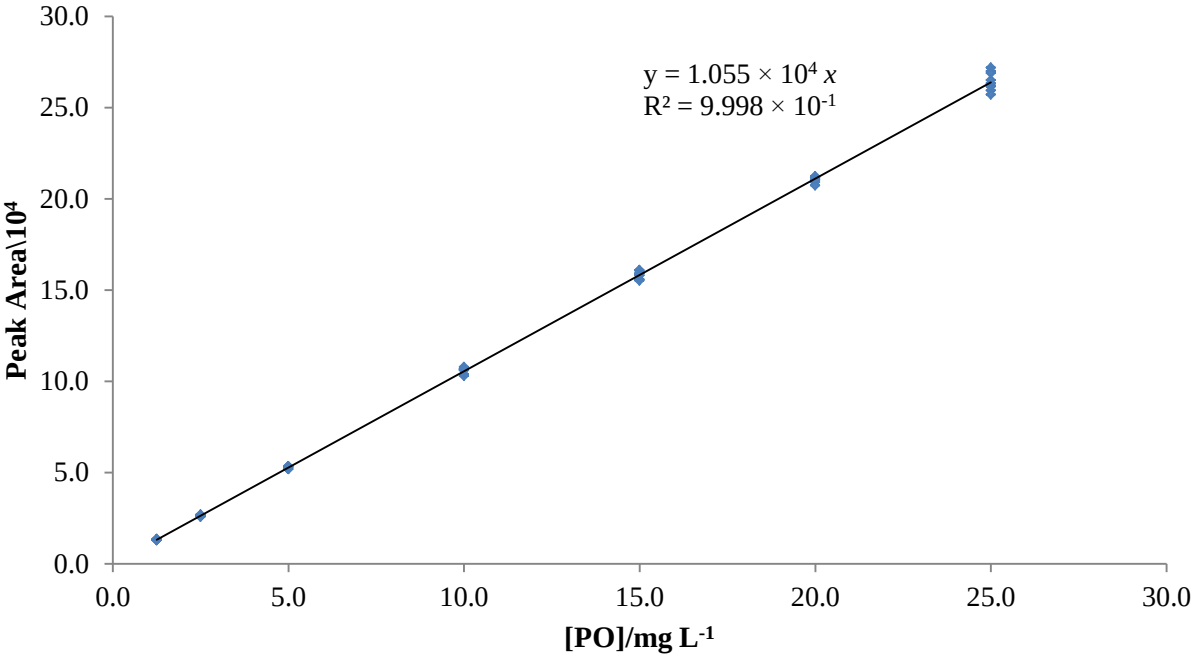

Figure D15: HPLC calibration graph for the determination of PO. The detection wavelength was 270 nm.

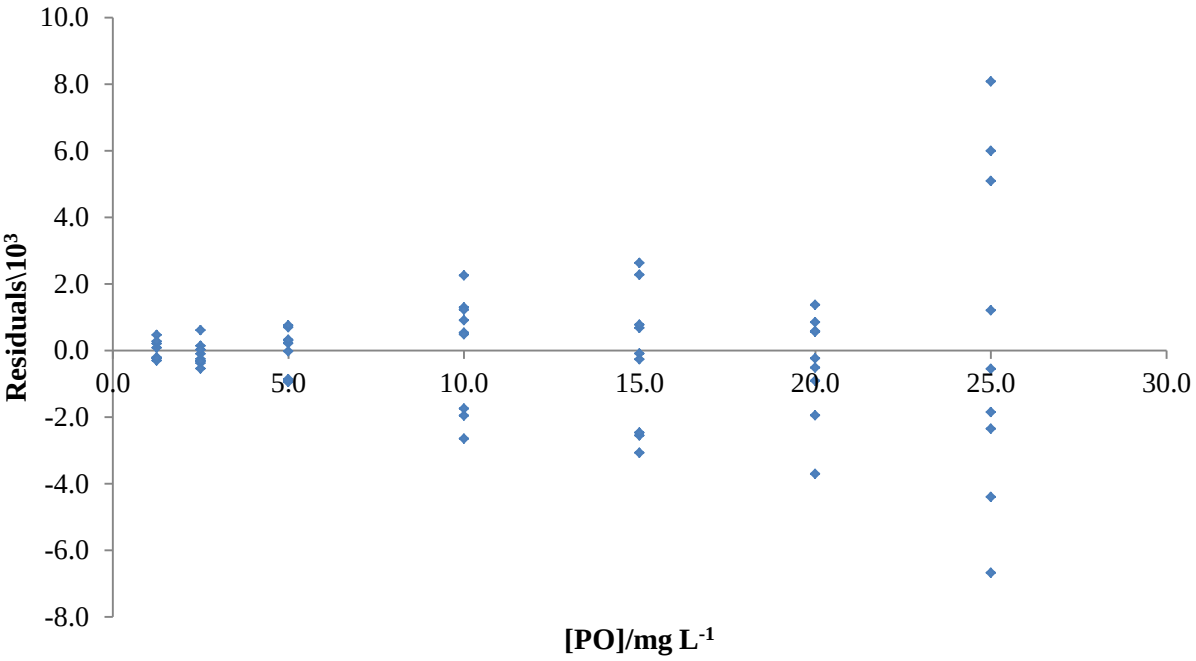

Figure D16: Residual plot for the calibration graph of PO.

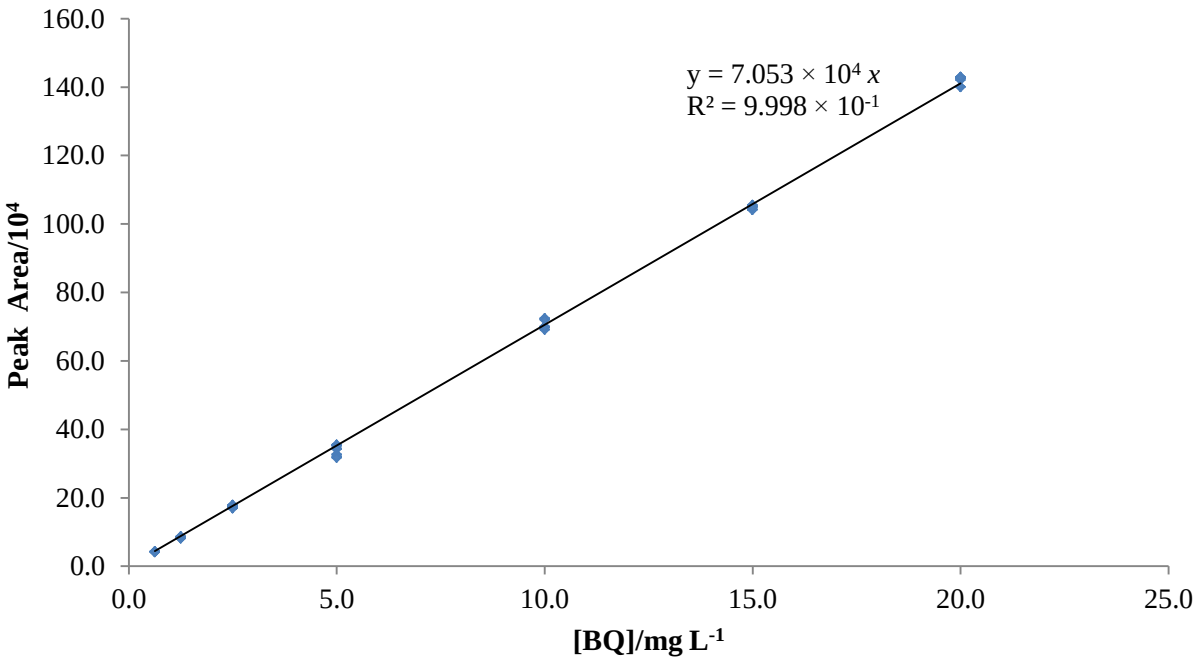

Figure D17: HPLC calibration graph for the determination of BQ. The detection wavelength was 240 nm.

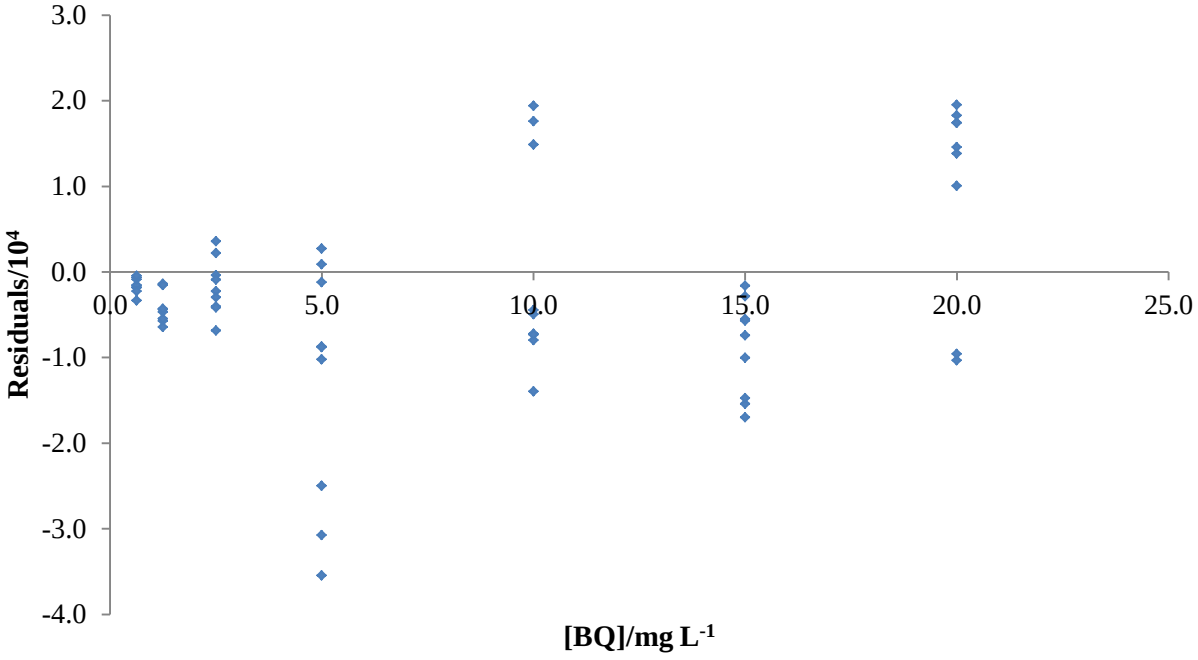

Figure D18: Residual plot for the calibration graph of BQ.

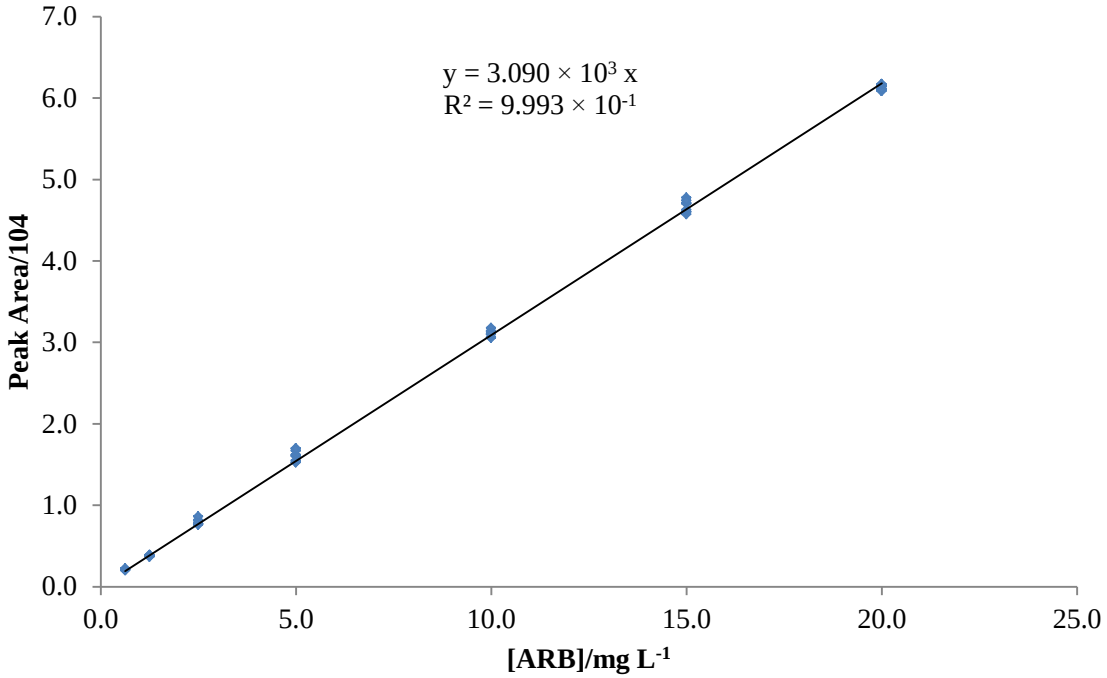

Figure D19: HPLC calibration graph for the determination of ARB. The detection wavelength was 289 nm.

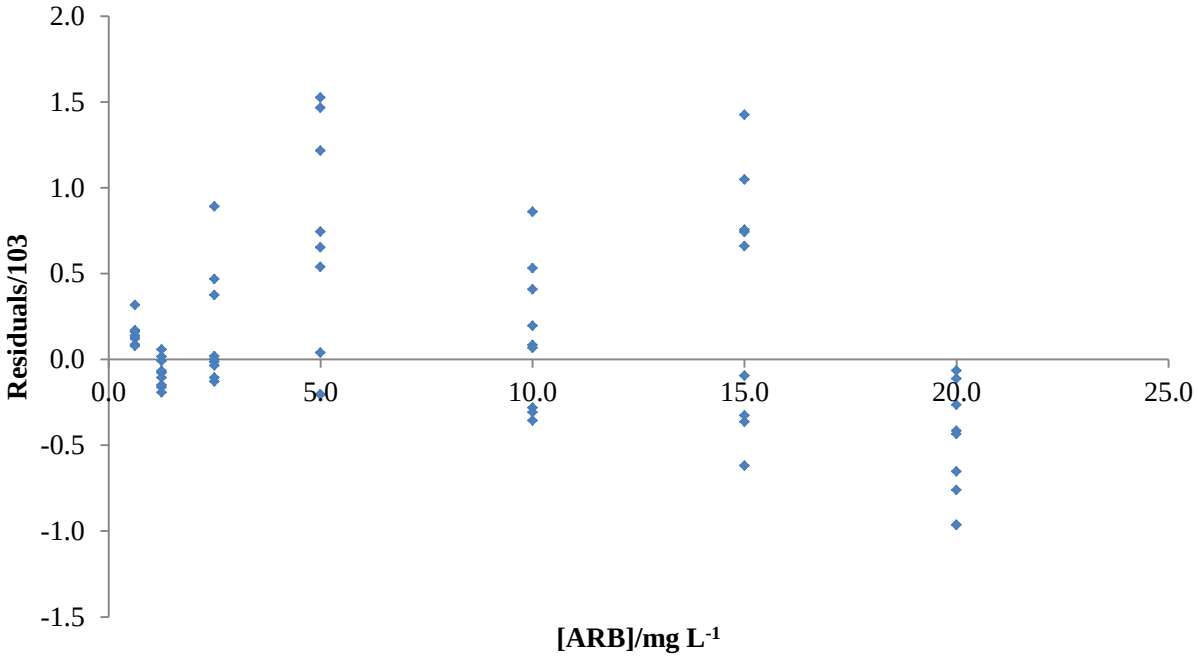

Figure D20: Residual plot for the calibration graph of ARB.

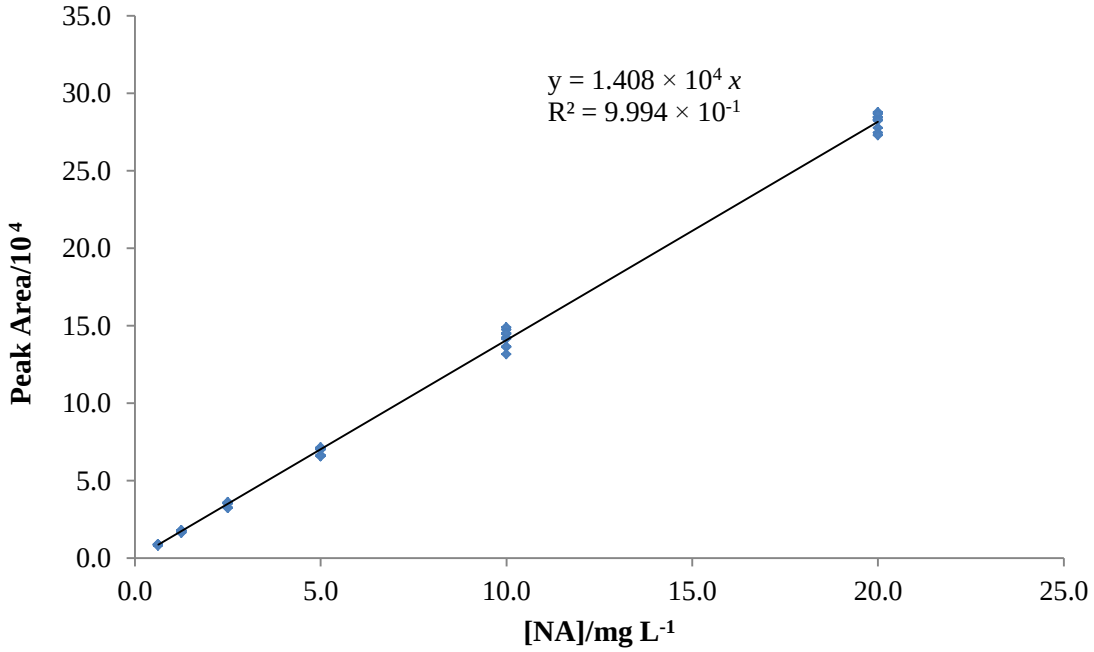

Figure D21: HPLC calibration graph for the determination of NA. The detection wavelength was 270 nm.

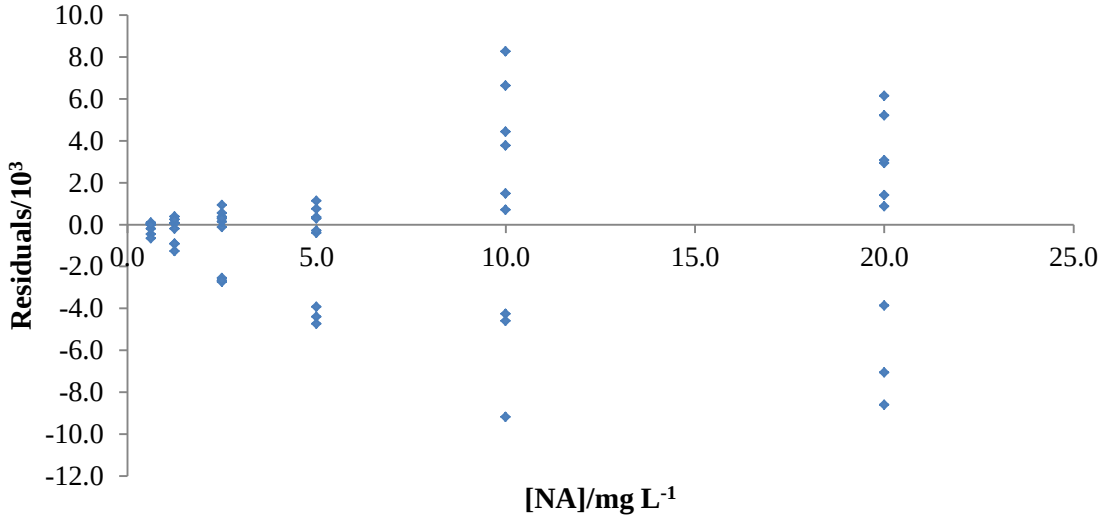

Figure D22: Residual plot for the calibration graph of NA.

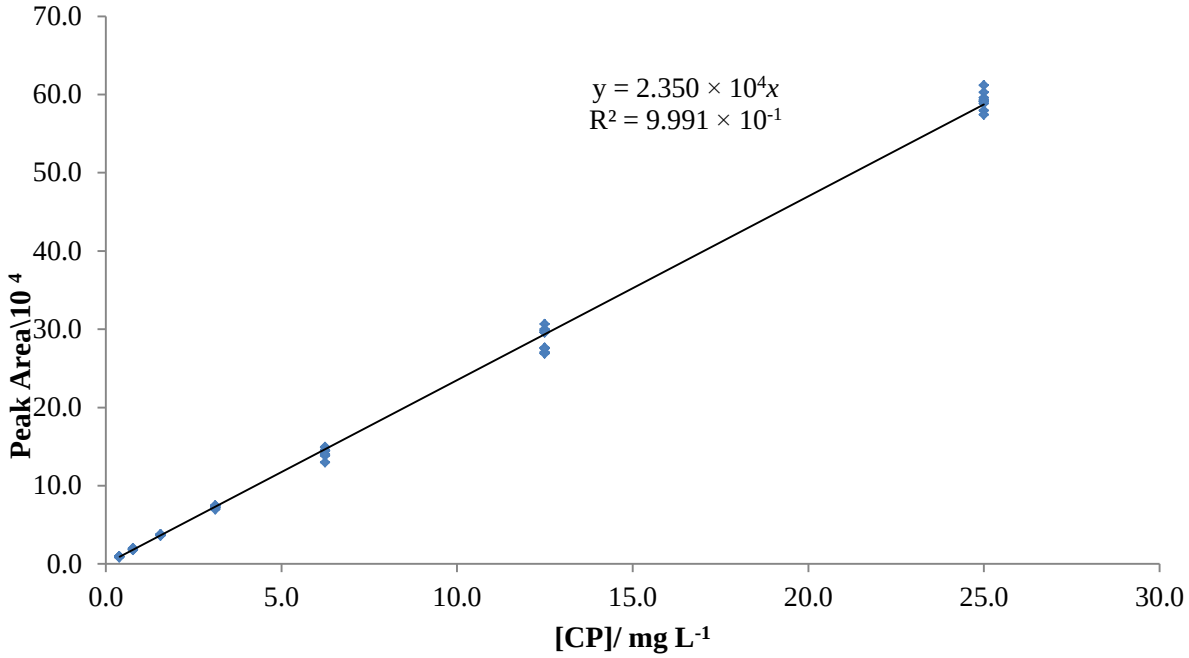

Figure D23: HPLC calibration graph for the determination of CP. The detection wavelength was 240 nm.

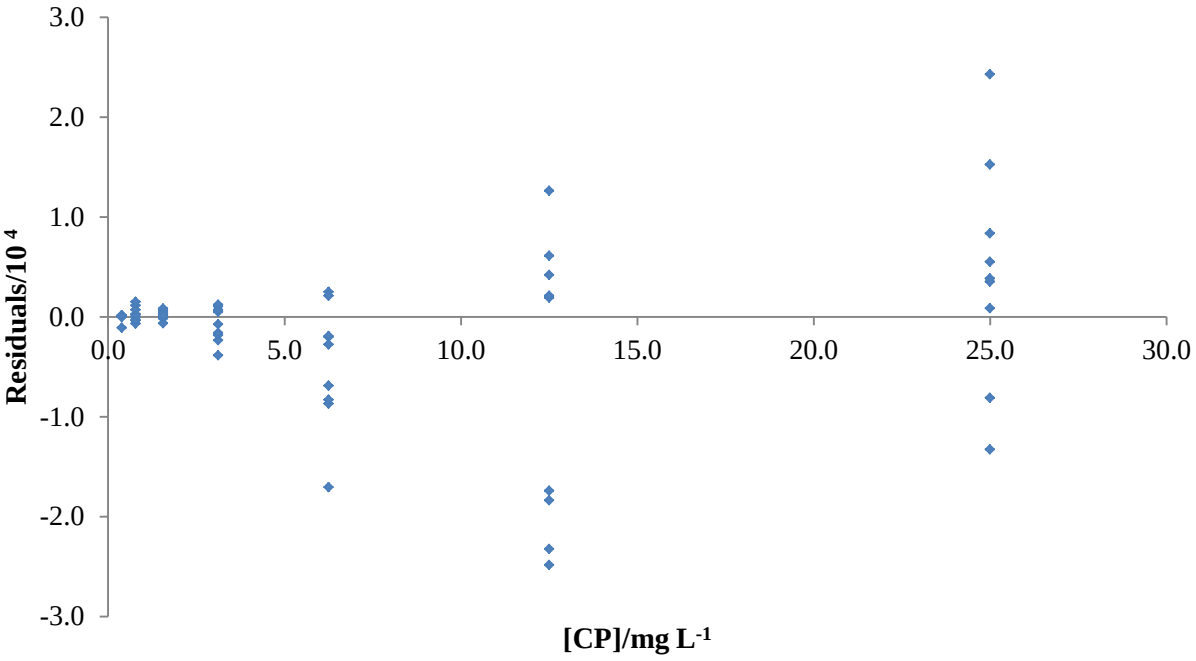

Figure D24: Residual plot for the calibration graph of CP.

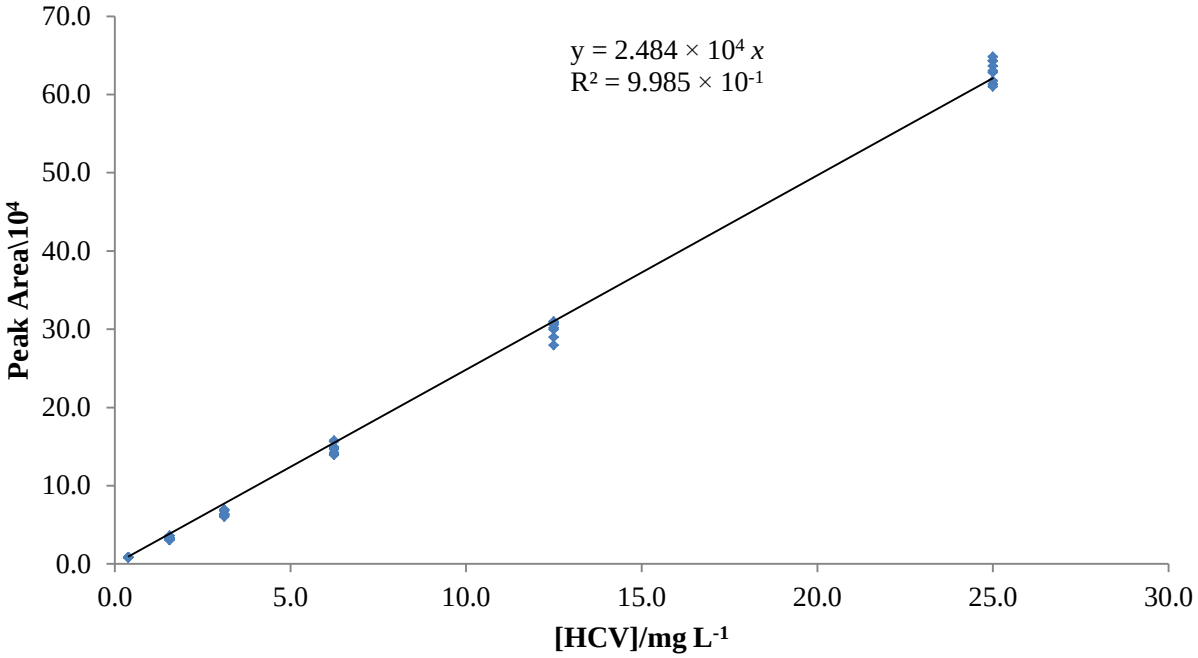

Figure D25: HPLC calibration graph for the determination of HCV. The detection wavelength was 240 nm.

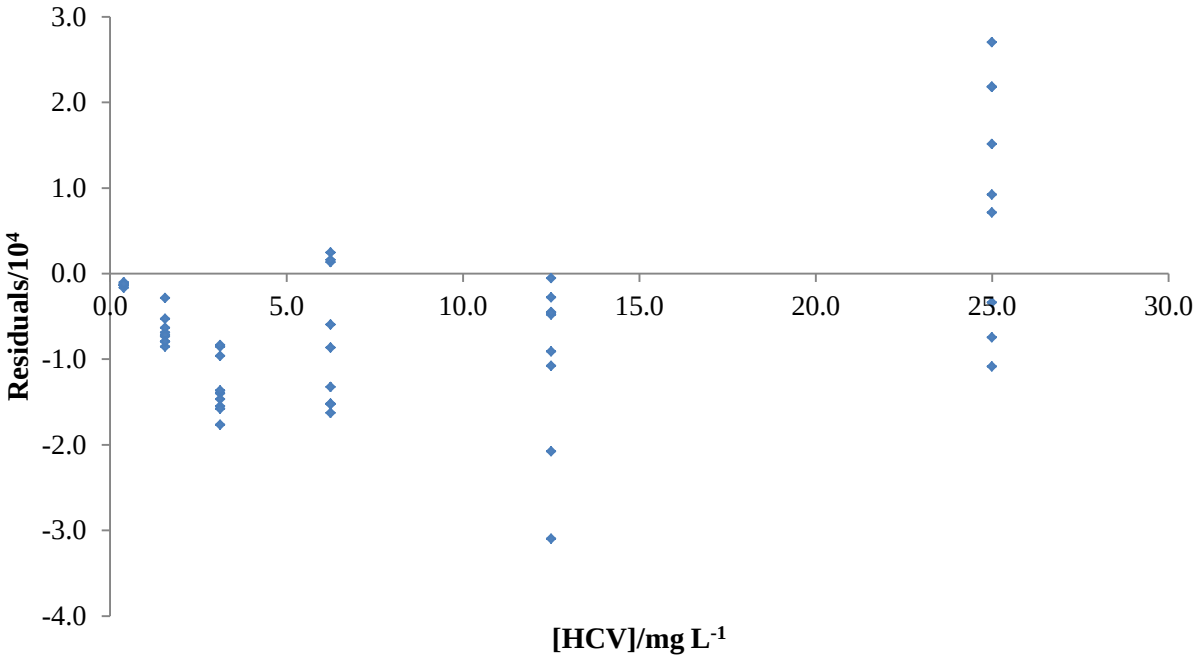

Figure D26: Residual plot for the calibration graph of HC

Appendix E

CHROMATOGRAMS FOR SKIN-LIGHTENING CREAMS

I. Chromatograms show the separation of a standard mixture of skin-lightening active ingredients. The chromatographic conditions used were: Brownlee C18 column mobile phase, MeOH-H_2O 55:45% (v/v), injection volume: 10 µL, flow rate 0.8 mL min^{-1}, and detection wavelength: 240, 270, or 289 nm.

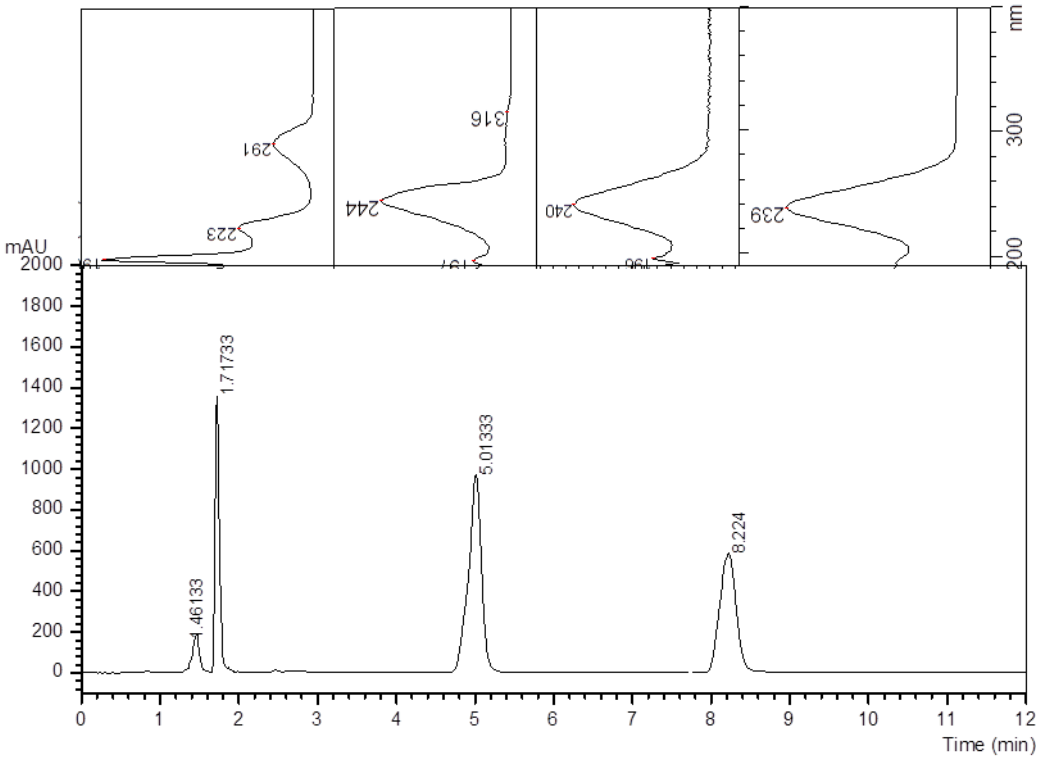

Figure E1: Typical chromatogram for the separation of a standard mixture of skin-lightening active ingredients at 240 nm. The order of elution is HQ, BQ and HCV and CP. The retention time is at 1.46133, 1.71733, 5.0133 and 8.224 minutes, respectively.

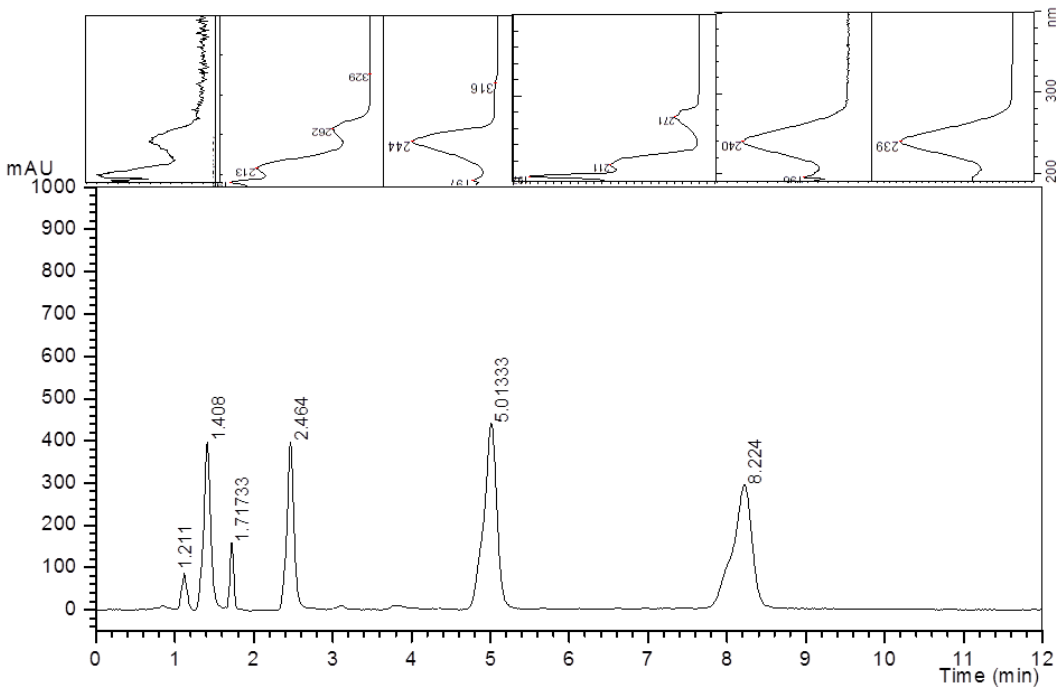

Figure E2: Typical chromatogram for the separation of a standard mixture of skin-lightening active ingredients at 270 nm. The order of elution is NA, BQ, PO, HCV and CP. The retention time is at 1.408, 1.71733, 2.464, 5.0133 and 8.224 minutes, respectively.

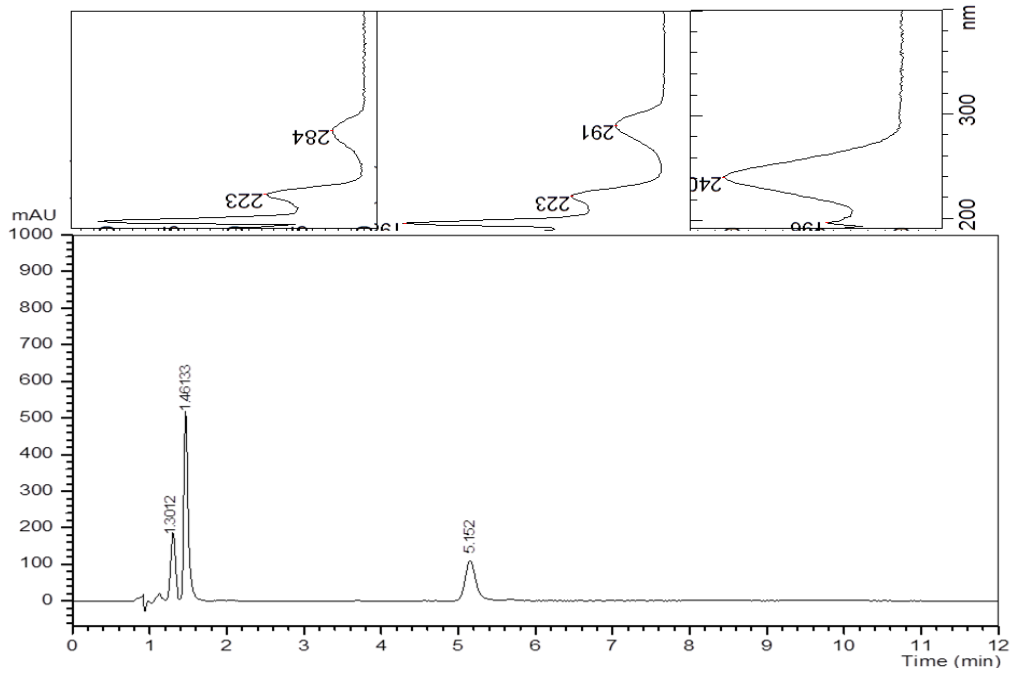

Figure E3: Typical chromatogram for the separation of a standard mixture of skin-lightening active ingredients at 289 nm. The order of elution is ARB, HQ and HCV. The retention time is at 1.3012, 1.46133 and 5.0133 minutes, respectively.

II. Chromatograms of skin-lightening cream samples (SLC). The chromatographic conditions used were: Brownlee C18 column mobile phase, MeOH-H_2O 55:45% (v/v), injection volume - 10 µL, flow rate 0.8 mL min^{-1}, and detection wavelength – 240, 270, or 289 nm.

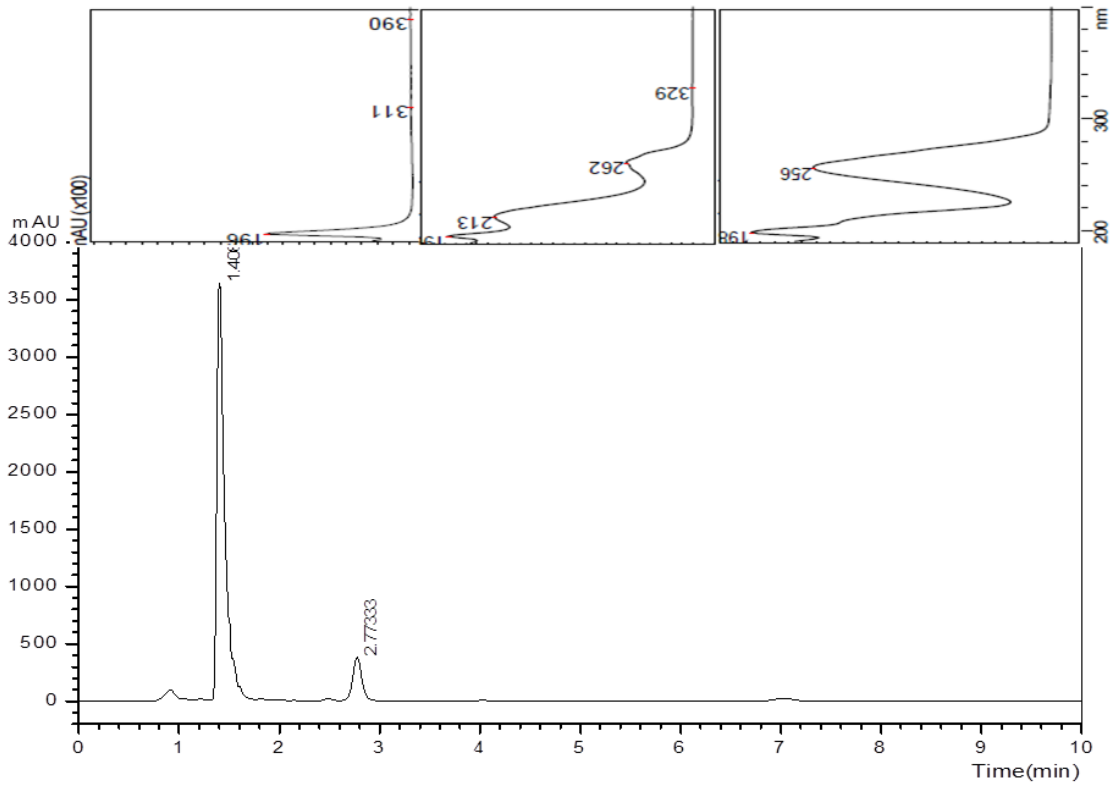

Figure E5: chromatogram of SLC20. The active ingredient is NA. The retention time is at 1.408 minutes. The detection wavelength is 270 nm.

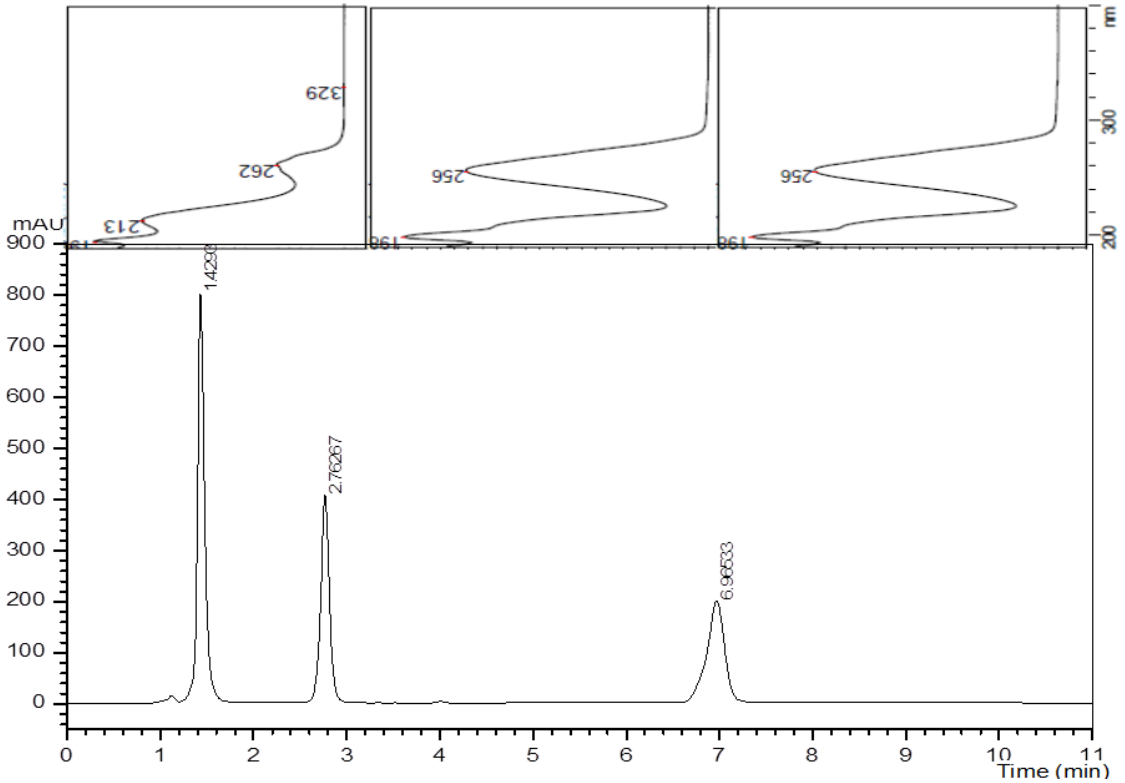

Figure E6: chromatogram of SLC21. The active ingredient is NA. The retention time is at 1.4298 minutes. The detection wavelength is 270 nm.

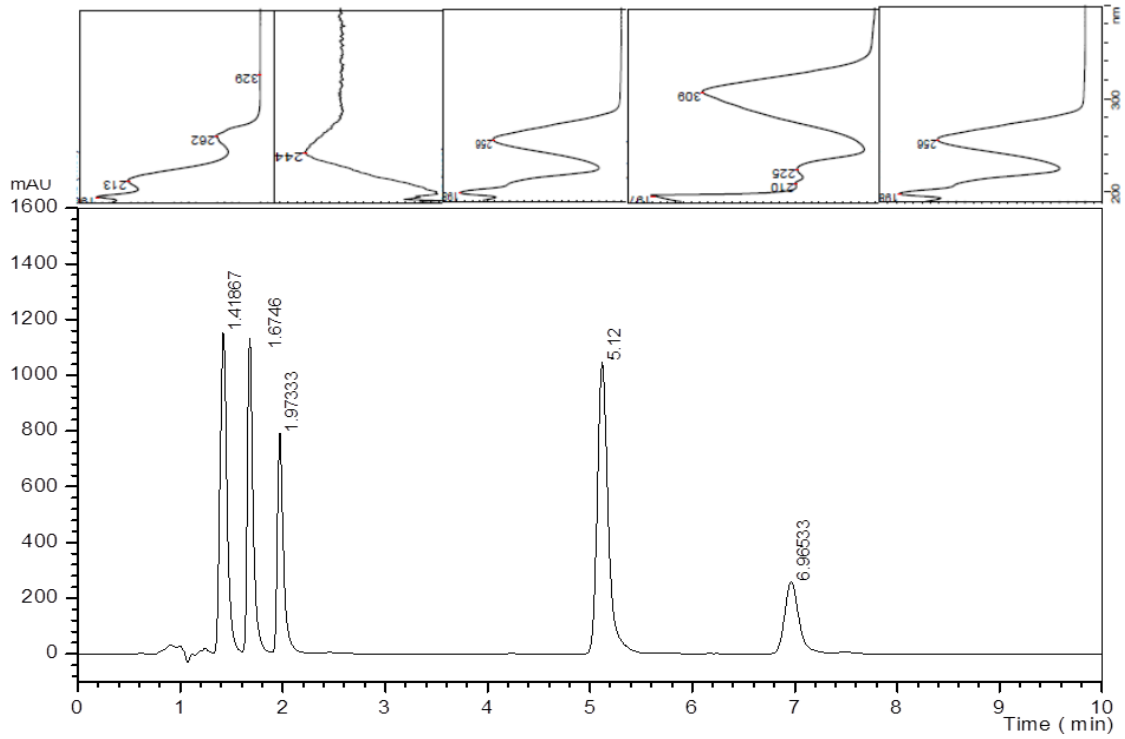

Figure E7: chromatogram of SLC31. The active ingredient is NA. The retention time is at 1.4188 minutes. The detection wavelength is 270 nm.

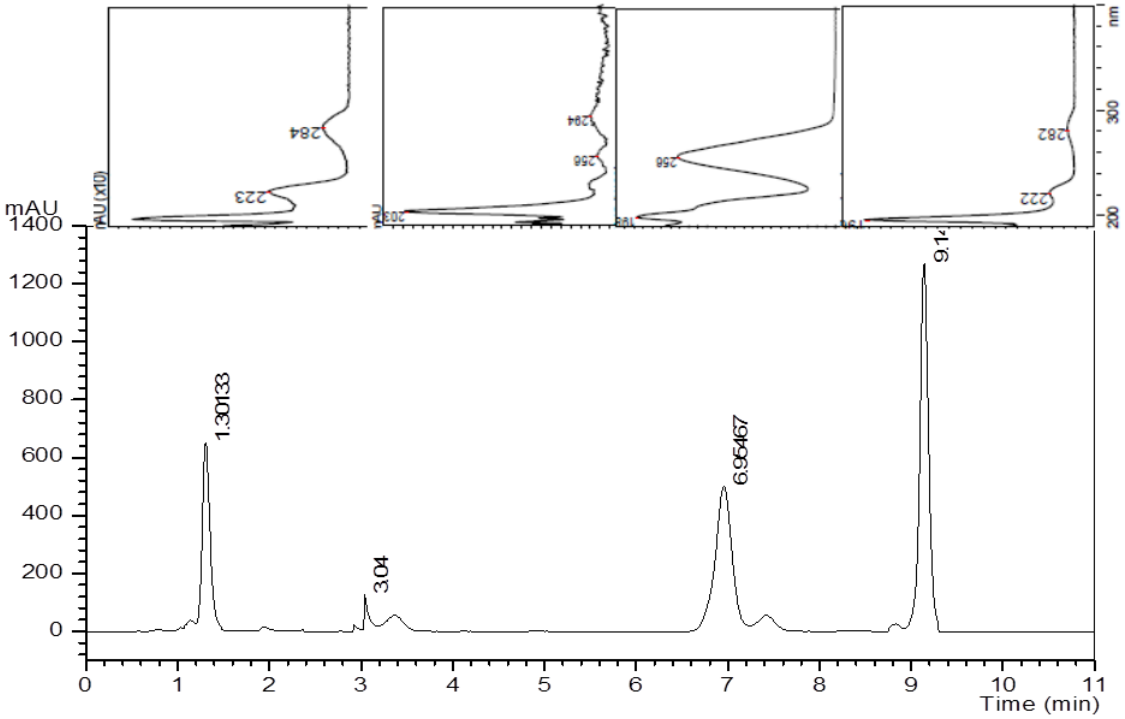

Figure E8: chromatogram of SLC49. The active ingredient is ARB. The retention time is at 1.30133 minutes. The detection wavelength is 289 nm.

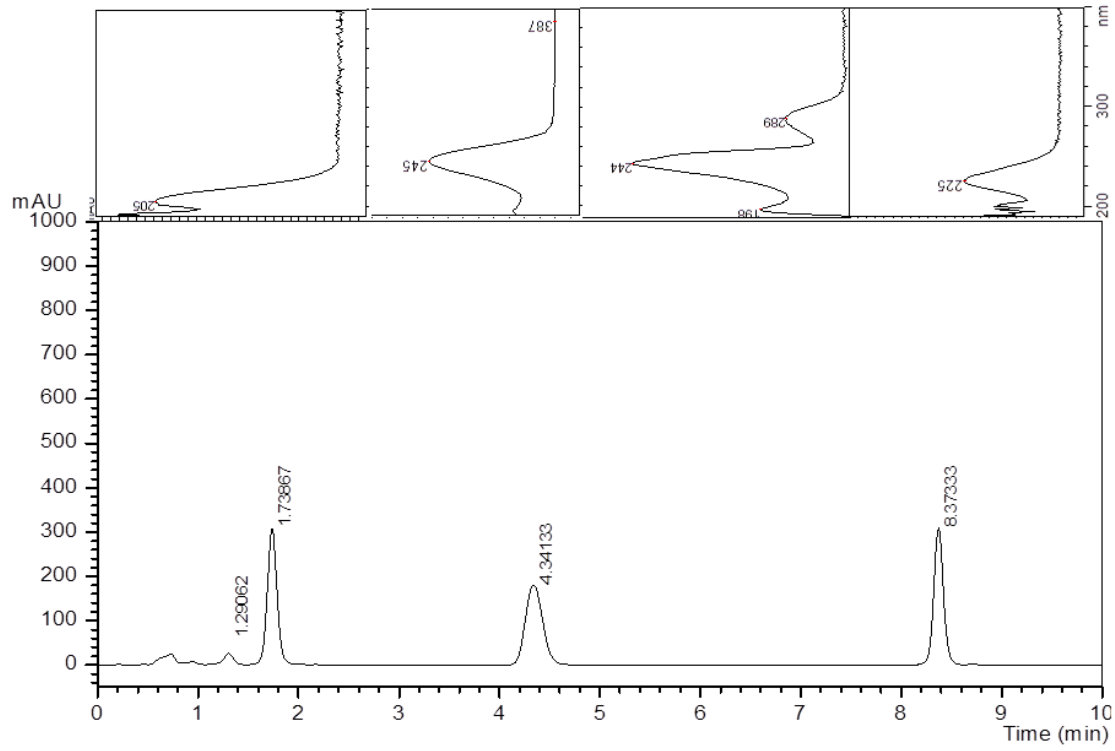

Figure E9: chromatogram of SLC51. The active ingredient is BQ. The retention time is at 1.73867 minutes. The detection wavelength is 240 nm.

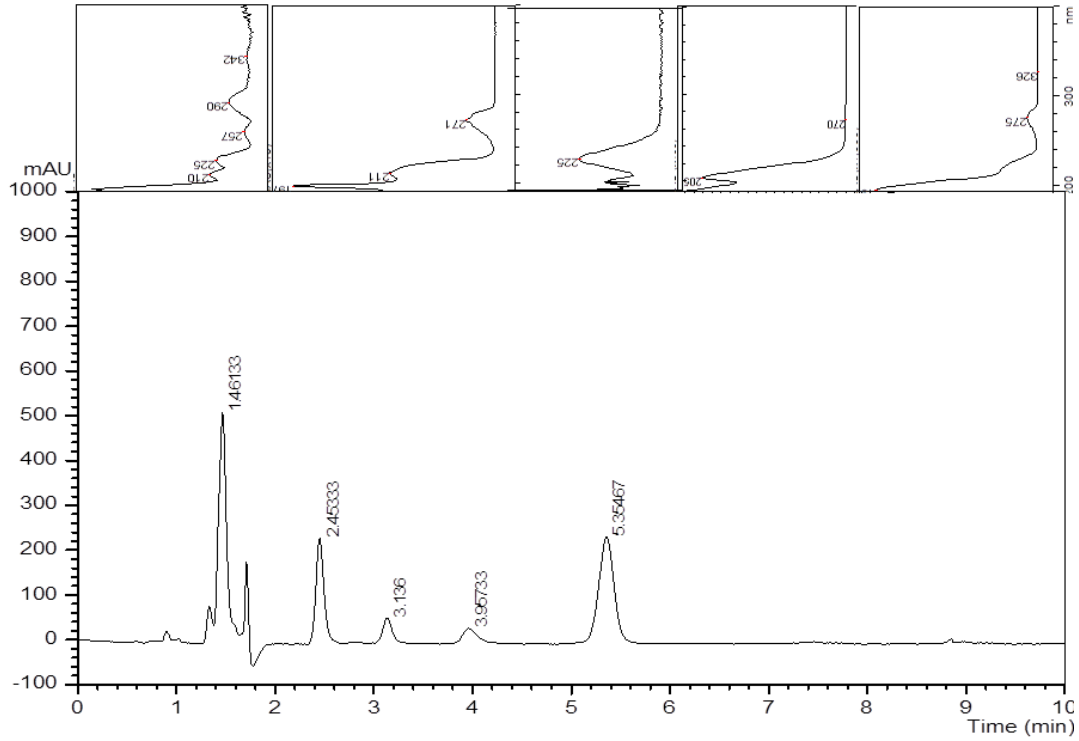

Figure E10: chromatogram of SLC51. The active ingredient is PO. The retention time is at 2.45333 minutes. The detection wavelength is 270 nm.

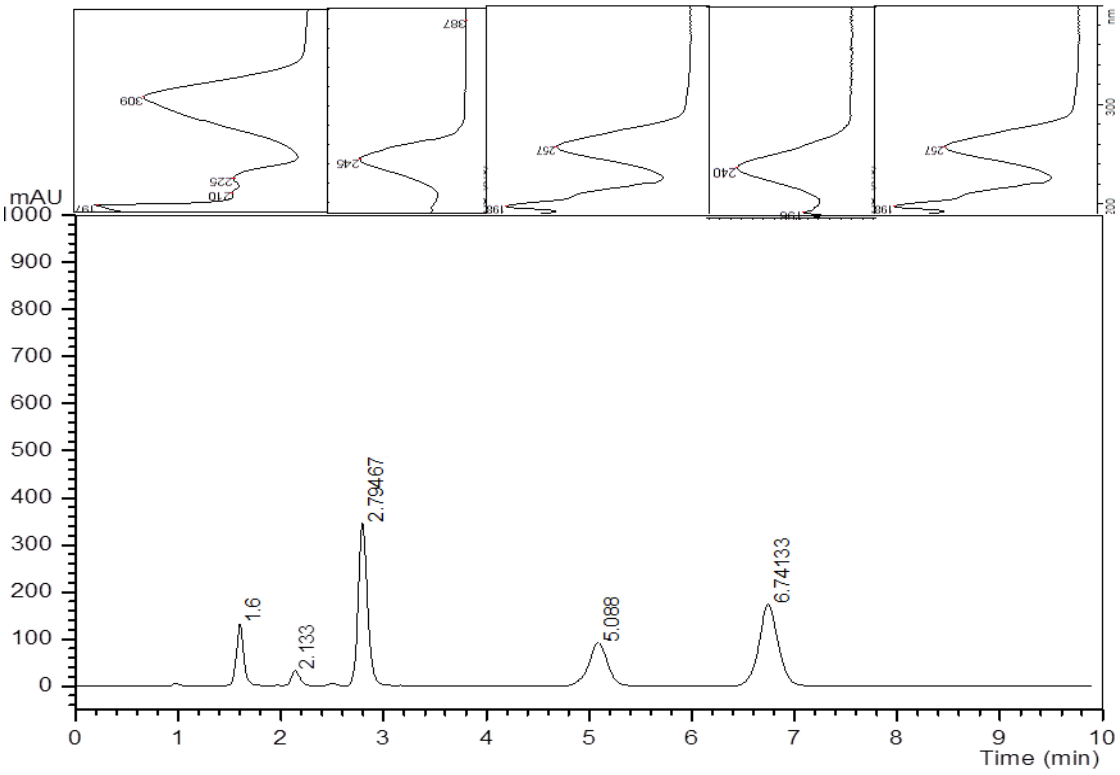

Figure E11: chromatogram of SLC52. The active ingredient is HCV. The retention time is at 5.088 minutes. The detection wavelength is 240 nm.

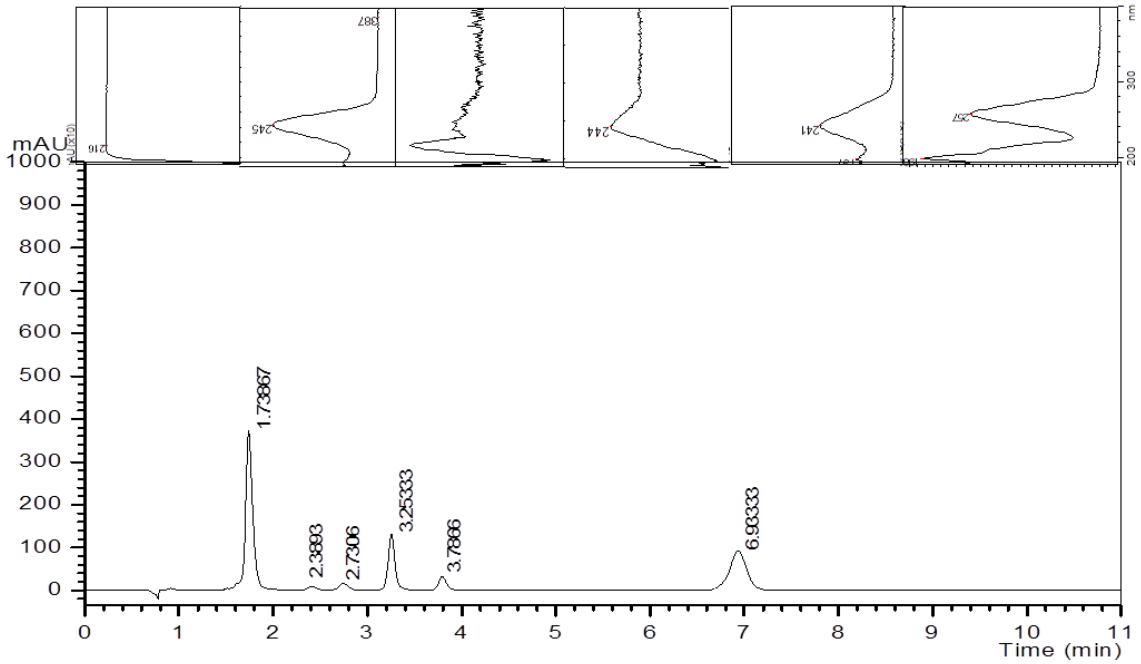

Figure E12: chromatogram of SLC54. The active ingredient is BQ. The retention time is at 1.73887 minutes. The detection wavelength is 240 nm.

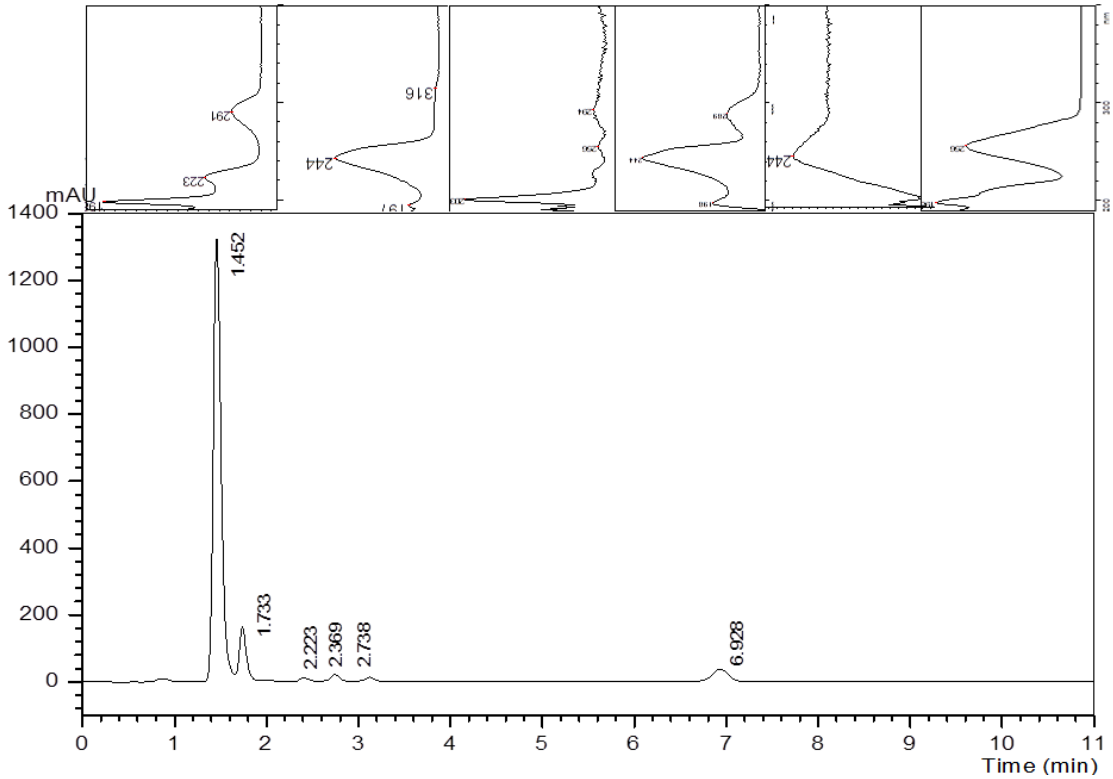

Figure E13: chromatogram of SLC54. The active ingredient is HQ. The retention time is at 1.452 minutes. The detection wavelength is 289 nm.

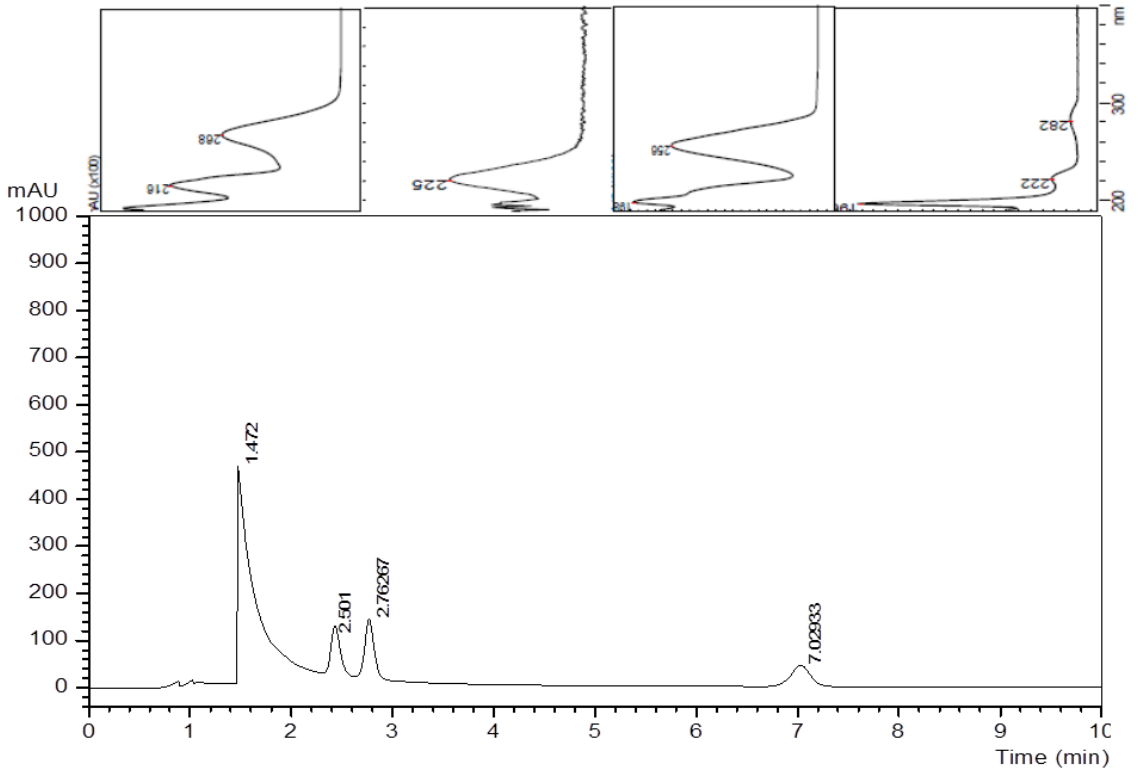

Figure E14: chromatogram of SLC56. The active ingredient is KA. The retention time is at 1.472 minutes. The detection wavelength is 270 nm.

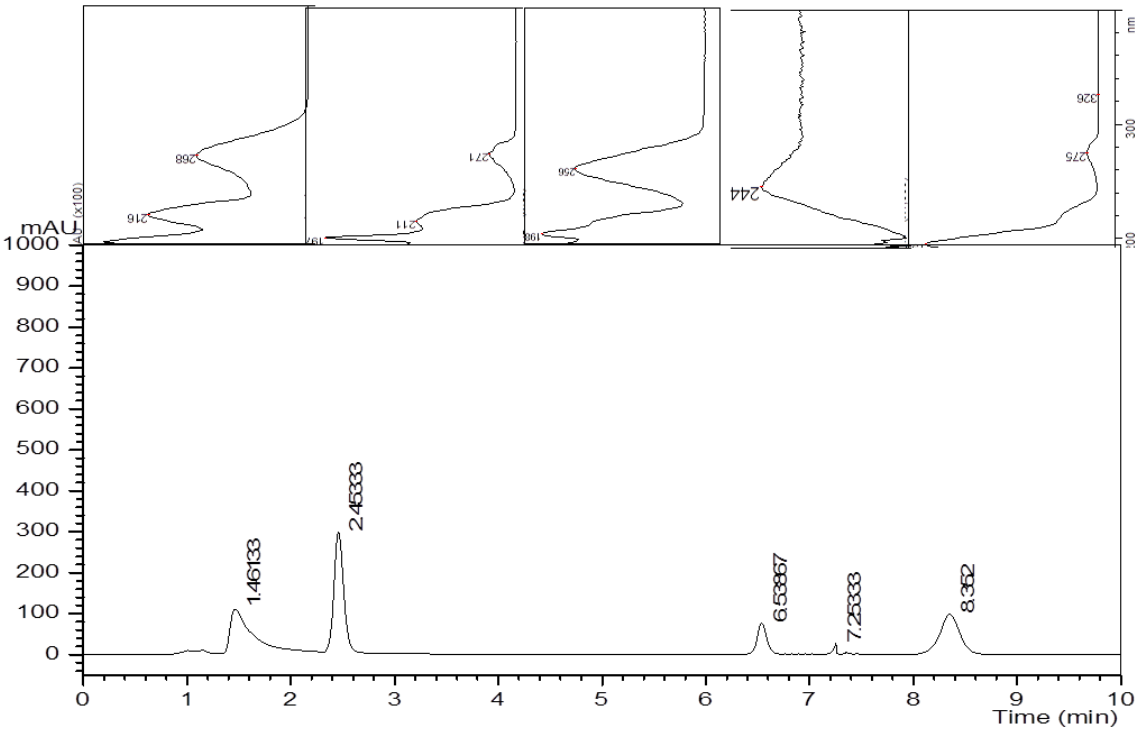

Figure E15: chromatogram of SLC58. The order of elution is KA and PO. The retention time is at 1.4613, and 2.4533 minutes, respectively, for active ingredient. The detection wavelength is 270 nm.

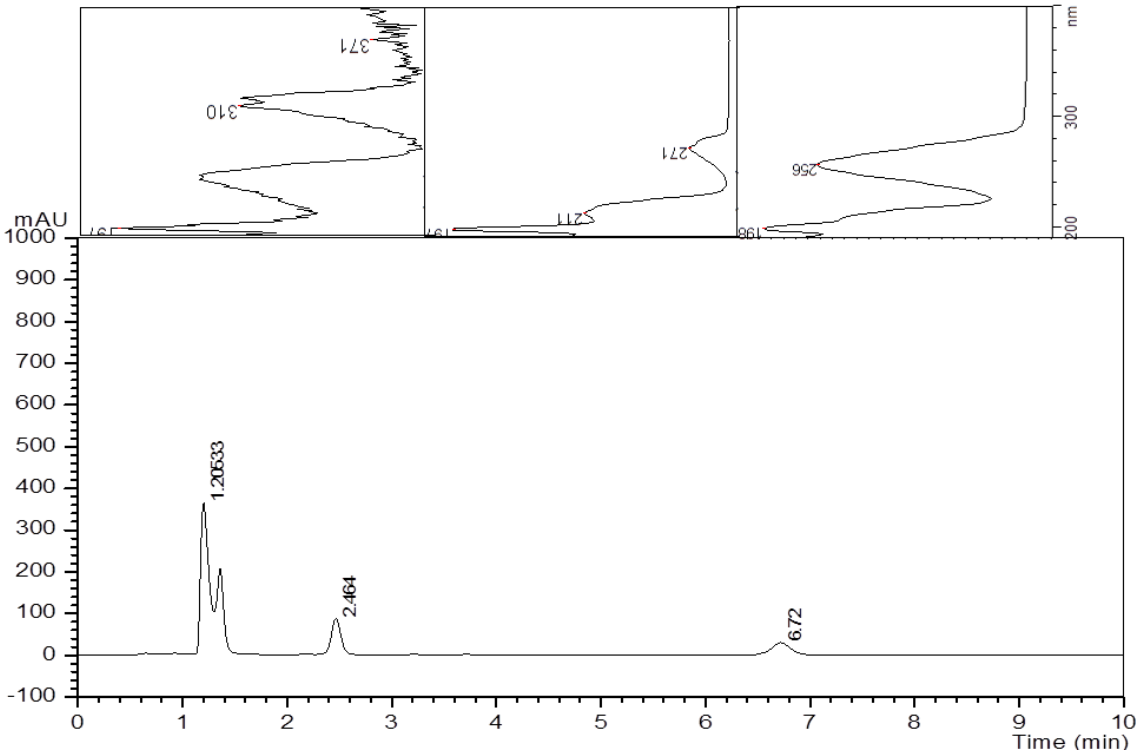

Figure E16: chromatogram of SLC60. The active ingredient is PO. The retention time is at 2.464 minutes. The detection wavelength is 270 nm.

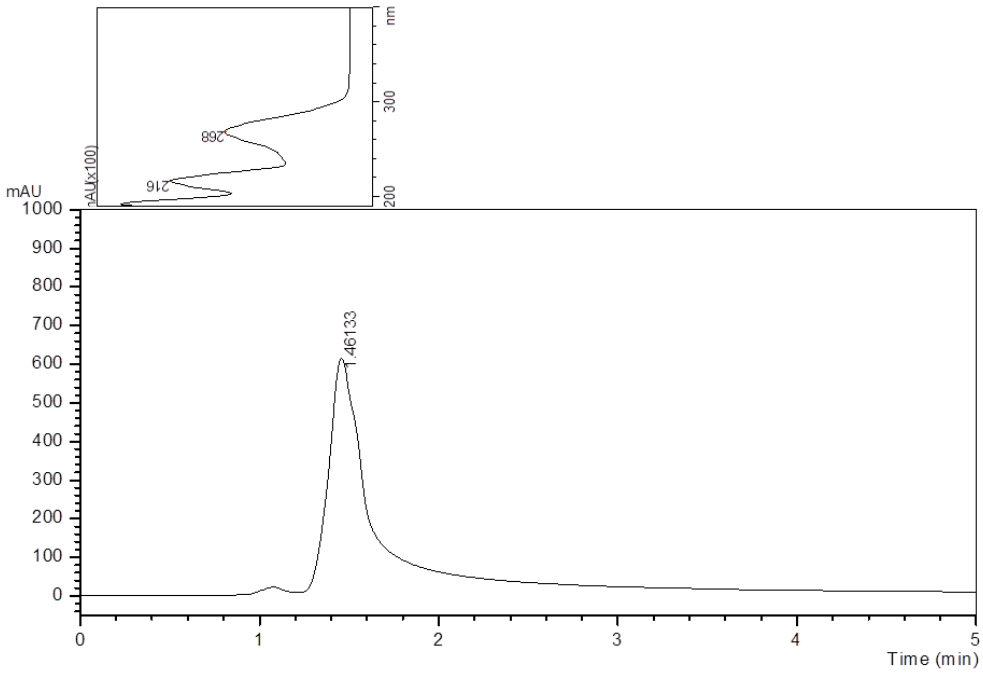

Figure E17: chromatogram of KA standard solution. The retention time is at 1.46133 minutes. The detection wavelength is 270 nm.

Appendix F

RAW DATA FOR SKIN-LIGHTENING CREAMS

The following tables list the peak areas obtained for the organic active ingredients in the different skin-lightening creams analysed by HPLC.

SLC20	**SLC21**	**SLC31**	**SLC49**	**SLC51**		**SLC52**
NA	NA	NA	ARB	BQ	PO	HCV
291917	252803	20697	7786	507816	194120	31801
253658	251672	18723	7753	516932	197812	31583
262144	249230	19295	7896	486210	174830	31023
222281	266741	22653	8532	459665	200330	28898
246583	270186	20765	8910	580252	202520	31156
228183	262713	23197	8702	426872	199465	33690
301256	273017	20136	6846	496321	192812	26983
286532	270033	21362	6986	572568	189657	29214
258670	276125	19956	6783	600125	195587	29683
Df 1:5	**Df 1:25**			**Df 1:2**	**Df 1:20**	

Df – dilution factor

SLC54		**SLC57**	**SLC59**		**SLC60**
HQ	BQ	PO	ARB	CP	PO
191297	112273	111652	3908	102369	10048
182551	109312	118596	4136	93982	10320
175456	110282	97864	4011	98588	13120
187173	102079	120556	4325	110923	10220
185042	111402	116593	3918	115551	9264
188129	133678	114218	3900	111042	9463
172492	100890	100982	2896	105663	10358
171985	101049	101062	3015	98679	11638
180413	109284	110208	3392	97668	12712
Df 1:25	**Df 1:2**	**Df 1:10**		**Df 1:5**	