SAFETY ASSESSMENT OF SKIN-LIGHTENING FORMULATIONS COMMONLY AVAILABLE IN THE NIGERIAN MARKET

by

ABDULKADIR IBRAHIM MAHMOUD

Submitted in fulfilment of the academic requirements for the degree of
Master of Science in the School of Chemistry and Physics,
University of KwaZulu-Natal
Durban

December 2016

ABSTRACT

The use of skin-lightening products (SLPs) in an attempt to lighten the skin is a common practice among most African countries, with Nigeria making up the largest population in terms of sheer numbers. These products are aimed at modulating melanogenesis through their active ingredients by acting on the tyrosinase enzyme in order to control unwanted skin pigmentation. This in turn results in several health complications especially after their prolonged use. The major concern is that most of these SLPs contain active ingredients that are either banned or exceed regulation limits and do not comply with labelling requirements. This has resulted in the Nigerian Food and Drug Agency (NAFDAC) prohibiting all forms of bleaching agents in cosmetics and toiletries sold in Nigeria. Nevertheless, products containing skin-lightening agents are still widely available.

In this project, 35 skin-lightening formulations were sourced from Ilorin State in Nigeria. They were analysed in order to identify and quantify the organic active ingredients and heavy metals present in them.

All of the 35 skin-lightening formulations were investigated for the presence of some selected organic active ingredients. An appropriate extraction method was developed for these active ingredients, which included hydroquinone, benzoquinone, kojic acid, niacinamide, arbutin, clobetasol propionate, betamethasone dipropionate and clotrimazole. The extracts were analysed by reversed-phase high performance liquid chromatography (HPLC) with photodiode array detection in an isocratic mode.

Of the total samples analysed, 32 of the products were found to contain at least one or more of the active ingredients. Hydroquinone was detected in 15 samples in concentrations ranging from 0.017 to 7.096% (m/m). Some of the samples containing hydroquinone were also found to contain benzoquinone. Hydroquinone is unstable in some formulations and hydrolyses to benzoquinone. Of the six samples found to contain benzoquinone, its concentration ranged from 0.005 to 0.015% (m/m). Kojic acid was detected in nine samples in the range of 0.017 to 1.412% (m/m). Four samples were found to contain niacinamide. The concentration of niacinamide in the samples ranged from

0.029 to 1.827% (m/m). Arbutin was not detected in any of the products. Steroid compounds were detected in 13 of the studied samples. Eight samples were found to contain clobetasol propionate, two samples were found to contain both clobetasol propionate and clotrimazole, and three samples were found to contain betamethasone dipropionate. The concentrations of clobetasol propionate, betamethasone dipropionate and clotrimazole were found in the range of 0.007 to 0.035, 0.019 to 0.027 and 0.007 to 0.012% (m/m) respectively.

The HPLC methods developed were validated by intra-day and inter-day reproducibility of standards, linearity of calibration lines, limits of detection and quantification, and recovery tests. The linear correlation coefficients were found to be greater than 0.995, the acceptable recovery values ranged from 84.23 to 110.33%, and the percent relative standard deviation (%R.S.D.) for the intra-day precision ranged from 0.17 to 8.16% while that of the inter-day precision ranged from 0.14 to 4.78%. This shows that the methods developed are reproducible with good precision.

The second part of the study involved the analysis of the heavy metal content of the same products and also the assessment of the possible exposure risk due to the use of these products. The heavy metals investigated included As, Al, Cd, Co, Cr, Co, Fe, Mn, Ni, Pb, Zn and Hg. These elements were extracted into solution by acid-assisted microwave digestion. The extracts were analysed for the metallic elements by inductively coupled plasma-optical emission spectrometry and cold-vapour atomic absorption spectrometry for Hg. As, Cd and Co were not detected in any of the formulated SLPs. Al was detected in all the samples and the maximum concentration found was 0.354 $\mu g\ g^{-1}$. Fe, Mn, and Cu were detected in most of the samples at levels not exceeding 1.252 $\mu g\ g^{-1}$, 0.053 $\mu g\ g^{-1}$, and 0.056 $\mu g\ g^{-1}$ respectively. Zn was detected in all the samples but was relatively high in SLP 33 (76.33 $\mu g\ g^{-1}$). Cr was found in 21 samples at a maximum concentration of 0.217 $\mu g\ g^{-1}$. Pb was detected in all samples with concentrations not exceeding 0.026 $\mu g\ g^{-1}$ and nickel was detected in 26 samples at a maximum concentration of 0.433 $\mu g\ g^{-1}$. One sample (SLP 6) was found to have a relatively high level of mercury (69.86 $\mu g\ g^{-1}$). This level of mercury in a skin-lightening cream is alarming due to its high toxicity and that the presence of mercury in cosmetics is banned.

The systemic exposure dosage and margin of safety values for the studied SLPs show that the level of metal contents detected in these products are safe for use except in the case of one of the samples containing a relatively high level of Hg with a systemic exposure dosage of 2.07×10^{-2} mg kg^{-1} bw day^{-1} and a margin of safety value of 2.41. The World Health Organization proposed the provisional tolerable daily intake for mercury to be 7.14×10^{-4} mg kg^{-1} bw day^{-1} and the minimum margin of safety value of 100 for any heavy metal to be safe for use in skin-lightening products.

It is evident from the results of the present study that banned and toxic ingredients, such as hydroquinone, mercury and very potent steroids, like clobetasol propionate and betamethasone dipropionate, are still found in skin-lightening products in the Nigerian market in spite of the NAFDAC prohibition. This is worrisome as it can expose users to serious health issues considering the chronic use of such products by unsuspecting consumers.

PREFACE

The experimental work described in this dissertation was carried out in the School of Chemistry and Physics, University of KwaZulu-Natal, Durban, from February 2015 to December 2016, under the supervision of Professor Bice S. Martincigh.

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others it is duly acknowledged in the text.

DECLARATION 1: PLAGIARISM

I, Abdulkadir Ibrahim Mahmoud, 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 thesis and in the References sections.

Signed: ……

DECLARATION 2: CONFERENCE PRESENTATION

The following poster was presented at a conference:

Abdulkadir Mahmoud and Bice S. Martincigh, A rapid HPLC method for the simultaneous determination of steroids in skin-lightening creams, 42nd National Convention of the South African Chemical Institute (SACI), Durban, South Africa, 29th November – 4th December 2015.

DEDICATION

I dedicate this work to my beloved mother, Late Hajiya Muslimat Mahmoud.

ACKNOWLEDGEMENTS

I am thankful to God whom by his will made this work possible.

I am very grateful to my supervisor Professor Bice Martincigh for her countless support and guidance throughout this degree. It took me no time to realise what a special supervisor I got when I came on board.

I am also grateful to Mrs Anita Naidoo and Miss Unathi Bongoza for the instrumentation training and other technical assistance they gave me. I was amazed by their kind treatment. I am equally thankful to Mr Kishore Singh for his uncountable assistance and Mr Ajay Bissessur for helping with the AA lamp I used for the mercury determination.

I am indebted to a host of my research group members whose help was invaluable during my study. The brotherly treatment and laboratory guidance given to me by Dr Oluwaseun Oyetade and Dr Ovokeroye Abafe will always be remembered. I am equally grateful to Dr Moses Ollengo for explaining some aspects of my work. I extend my gratitude to every other member of my research group for the wonderful times and the kindly advice.

I must sincerely say thank you to Mr Isaac Ofori for his useful suggestions and assistance.

I owe special gratitude to my parents, Professor I.S. Mahmoud and, in particular, my beloved mother Late Hajiya Muslimat Mahmoud for giving meaning to my life with her endless support. I pray that God may continue to have mercy on her soul. I am also grateful to my grandmother whose prayers and words of wisdom have kept me going. I am so indebted to my wonderful siblings Captain Ahmad Mahmoud, Habibullah Mahmoud and Safinat Onipede for their unconditional support. To my uncles and aunties, I say a big thank you, particularly Hajiya Nurat Arebge for her motherly guidance.

I am grateful to my cousins Al-amin and Jamila and my good friends Ibrahim Akinbo, Kazeem Abdulazeez and Ibrahim Sani for checking on me all the time during this degree.

Finally, I would like to thank the National Research Foundation (NRF) and the University of KwaZulu-Natal for financial support for the duration of my degree.

TABLE OF CONTENTS

ABSTRACT i

PREFACE iv

DECLARATION 1: PLAGIARISM v

DECLARATION 2: CONFERENCE PRESENTATION vi

DEDICATION vii

ACKNOWLEDGMENTS viii

TABLE OF CONTENTS ix

LIST OF FIGURES xiii

LIST OF TABLES xv

Chapter 1 1

INTRODUCTION AND LITERATURE REVIEW 1

1.1 A general overview of skin-lightening practices 2

1.2 The human skin 4

1.2.1 Variation in skin color 5

1.3 Melanin and pigmentation 7

1.3.1 Biosynthesis of melanin 8

1.3.2 Melanin pathway inhibitors (Tyrosinase inhibitors) 10

1.4 Skin-lightening agents 12

1.4.1 Hydroquinone 13

1.4.2 Benzoquinone 15

1.4.3 Kojic acid 16

1.4.4 Niacinamide 18

1.4.5 Arbutin 18

1.4.6 Glycolic acid 18
1.4.7 Mequinol 19
1.4.8 Azelaic acid 19
1.4.9 Retinoids (Topical) 19
1.4.10 Resorcinol 20
1.4.11 Aleosin 20
1.4.12 Soy proteins 21
1.4.13 Flavonoids 21
1.4.14 Steroids 21
1.4.15 Mercury 23
1.5 Clotrimazole 24
1.6 Heavy metals in cosmetics and their health complications 26
1.7 Dermatological effects of skin-lightening products 28
1.8 Methods of analysis of skin-lightening agents 29
1.9 Methods of analysis of heavy metals 30
1.10 Quantitation methods 32
1.11 Problem statement 33
1.12 Motivation 33
1.13 Aim and objectives 34

Chapter 2 35

EXPERIMENTAL 35
2.1 Materials 35
2.2 Equipment 35
2.3 HPLC analysis of skin-lightening agents 47
2.3.1 Theory of HPLC 47
2.3.2 Components of an HPLC system 48
2.3.3 Identification of skin-lightening agents 50
2.3.3.1 Theory and instrumentation for UV-visble spectrophotometry 50

2.3.3.2 Determination of UV absorbance of skin-lightening agents 53
2.3.4 HPLC method development 58
2.3.4.1 Determination of optimum separation method 59
2.3.4.2 Determination of optimum extraction method 60
2.3.4.3 Standard solution preparation and analysis 61
2.3.4.4 Sample preparation and analysis 62
2.3.4.5 Recovery analysis 63
2.3.4.6 Inter-day and intra-day analysis 64
2.4 Analysis of heavy metal content 64
2.4.1 Extraction of heavy metals 65
2.4.1.1 Microwave-assisted digestion 66
2.4.2 Inductive coupled plasma-optical emission spectrometry 67
2.4.2.1 Preparation of standard solutions of heavy metal 69
2.4.2.2 Determination of heavy metals 69
2.4.3 Cold-vapour atomic absorption spectrometry 71
2.4.3.1 Preparation of reagents and standard solutions 72
2.4.3.2 Determination of mercury 73
2.4.4 Validation of heavy metal and mercury analyses 74
2.5 Data analysis 76

Chapter 3 80

RESULTS AND DISCUSSION 80

3.1 HPLC method development for the determination of organic skin-lightening agents 80
3.1.1 Optimum detection wavelengths 80
3.1.2 Identification of skin-lightening agents 81
3.1.3 Optimum separation conditions 82
3.1.4 Optimum extraction solvent for sample preparation 90

3.2 Validation of HPLC analytical method 91

3.2.1 Linearity of calibration curves and limits of detection and quantitation 91

3.2.2 Accuracy 94

3.2.3 Precision 95

3.3 Amount of skin-lightening agents detected in skin-lightening products 98

3.4 Validation of ICP-OES and CV-AAS methods 105

3.4.1 Linearity of calibration plots for heavy metal analysis 105

3.4.2 Accuracy 107

3.5 Levels of mercury and heavy metals detected in skin-lightening products 109

3.6 Sytemic exposure dosage and margin of safety 111

Chapter 4 119

CONCLUSIONS AND RECOMMENDATIONS 119

REFERENCES 124

APPENDIX A: MATERIALS 141

APPENDIX B: EQUIPMENT 143

APPENDIX C: CALIBRATION DATA FOR SKIN-LIGHTENING AGENTS 145

APPENDIX D: CALIBRATION GRAPHS AND RESIDUAL PLOTS FOR SKIN-LIGHTENING AGENTS 149

APPENDIX E: HPLC CHROMATOGRAMS OF STANDARD MIXTURES OF SKIN-LIGHTENING AGENTS AND SKIN-LIGHTENING PRODUCTS 157

APPENDIX F: RAW DATA FOR SKIN-LIGHTENING PRODUCTS 179

APPENDIX G: CALIBRATION DATA FOR HEAVY METALS 182

APPENDIX H: CALIBRATION CURVES AND RESIDUAL PLOTS FOR HEAVY METALS 186

LIST OF FIGURES

Figure 1.1. Synthesis of melanins

Figure 1.2: Chemical structures of common skin-lightening agents

Figure 1.3: Chemical structures of topical steroids mostly present in skin-lightening products

Figure 1.4: Chemical structures of some imidazole fungicides

Figure 1.5: Photographs of patients diagnosed with various skin conditions arising from the improper use of skin-lightening products

Figure 2.1: Components of an HPLC instrument

Figure 2.2: Relative energies of orbitals and the types of electronic transitions

Figure 2.3: Schematic of a double-beam UV-visible spectrophotometer

Figure 2.4: A photograph of the PerkinElmer Lambda 25 double-beam UV-visible spectrophotometer used for measuring the UV spectra of the selected SLAs

Figure 2.5: UV spectrum of 5 mg L^{-1} clobetasol propionate (CP) dissolved in methanol

Figure 2.6: UV spectrum of 5 mg L^{-1} betamethasone dipropionate (BD) dissolved in methanol

Figure 2.7: UV spectrum of 10 mg L^{-1} clotrimazole (CT) dissolved in methanol

Figure 2.8: UV spectrum of 5 mg L^{-1} hydroquinone (HQ) dissolved in methanol

Figure 2.9: UV spectrum of 5 mg L^{-1} benzoquinone (BQ) dissolved in methanol

Figure 2.10: UV spectrum of 5 mg L^{-1} kojic acid (KA) dissolved in methanol

Figure 2.11: UV spectrum of 5 mg L^{-1} niacinamide (NC) dissolved in methanol

Figure 2.12: UV spectrum of 5 mg L^{-1} arbutin (ARB) dissolved in methanol

Figure 2.13: A photograph of the Shimadzu Prominence UFLC$_{XR}$ instrument used for the determination of skin-lightening agents in this study

Figure 2.14: A photograph of the Mars 6 microwave digestion device and the XP-1500 TFM reaction vessels used for digestion of skin-lightening samples in this study

Figure 2.15: Schematic of an ICP-OES instrument

Figure 2.16: A photograph of the PerkinElmer Optical 5300 DV Simultaneous ICP-OES device used for determination of heavy metals in this study

Figure 2.17: Schematic of the PerkinElmer MHS 15 mercury hydride pneumatic system

Figure 2.18: A photograph of the PerkinElmer MHS 15 mercury hydride system connected to the PerkinElmer Analyst 200 atomic absorption spectrometer instrument and gas supply during mercury determination in this study

Figure 3.1: Chromatogram for the separation of active ingredients CP, BD and CT

Figure 3.2: Comparison of the UV–visible spectra of other SLAs with those of the unknown analytes

Figure 3.3: Chromatogram for the separation of active ingredients NC, KA, HQ, BQ and ARB

Figure 3.4: Optimisation of four different solvents for the extraction of CP, BD, CT, HQ, BQ, KA, NC, and ARB

Figure 3.5: Prevalence of skin-lighting agents detected in the investigated products

Figure 3.6: Levels of SLAs detected in the studied SLPs compared to their cost

LIST OF TABLES

Table 1.1: Fitzpatrick sun-reactive Skin Types

Table 1.2: Some tyrosinase inhibitors derived from synthetic and natural sources

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

Table 2.2: The wavelength of maximum absorption for each skin-lightening agent obtained by UV-visible spectrophotometry

Table 2.3: Emission wavelengths used for analysis of heavy metals by ICP-OES

Table 2.4: Details of the solution preparation in 50 mL volumetric flasks for validation of heavy metal and mercury analyses

Table 3.1: Retention times and detection wavelengths for the skin-lightening agents

Table 3.2: Results of the linear regression analysis of the calibration curves for the skin-lightening active ingredients analysed by HPLC

Table 3.3: Percentage recoveries of skin-lightening active ingredients

Table 3.4: Determination of precision for intra-day and inter-day variability

Table 3.5: Levels of active ingredients detected

Table 3.6: Results from the linear regression analysis of the calibration data for heavy metals

Table 3.7: Results from the linear regression analysis of the calibration data for the determination of mercury

Table 3.8: Recovery data for heavy metal quantification in skin-lightening products

Table 3.9: Levels of heavy metals detected in different samples of skin-lightening products analysed in this study

Table 3.10: Systemic exposure dosage values of metals in skin-lightening products for 100% dermal absorption

Table 3.11: Margin of safety values of metals in skin-lightening products for 100% dermal absorption

Chapter 1

INTRODUCTION AND LITERATURE REVIEW

Skin-lightening, or in other words, skin-whitening, or skin-bleaching, has become a global concern. Among the possible ways of achieving a lighter skin is through the use of cosmetic skin-lightening products (SLPs), which can take many forms, including creams, ointments, solutions, and gels. The active ingredients in these products act on the tyrosinase enzyme to prevent melanin formation. Melanin, a major dark pigment found in the skin, hair and eyes, serves to provide protection against harmful ultraviolet radiation capable of causing photoaging and photocarcinogenesis (1, 2). Consequently, these products are aimed at modulating melanogenesis in order to control unwanted skin pigmentation such as freckles, melasma, age spots, acne, scars, or discoloration related to hormones, and most of all to lighten naturally dark skin. However, the use of SLPs does come with some risk. Several active ingredients in SLPs have been shown to be effective and non-detrimental, while some have proven to be toxic or have a questionable safety profile, adding to the controversy surrounding their use and impact on certain ethnic groups.

In Nigeria, the use of SLPs to lighten skin is common especially among women. The perception of a light skin signifying beauty, grace and high social ranking, encourages most women to indulge in the use of SLPs. The use of these products cuts across all sociodemographic characteristics, including religious groups, rich or poor, single or married, literate or illiterate. But, unfortunately, most of these women end up indulging in SLPs that bleach the skin, and they neglect any form of injury to their bodies or threat to their health. A myriad of these products contain different kinds of skin-lightening agents (SLAs) that after prolonged use may eventually affect the users' health through dermal absorption. These include formulations containing mercury, corticosteroids, and hydroquinone. However, the list of SLAs has now expanded as a result of some manufacturers introducing new chemicals of unknown safety profile such as kojic acid, niacinamide, arbutin, triethanolamine and many more. In addition, there have been reports (3-6) on the presence of heavy metals, such as Cd, Cr, Cu, Ni, and Pb, in a variety

of cosmetic products including SLPs. Since heavy metals bioaccumulate, they too can exert toxic health effects.

The source of danger arises as a result of over exposure to certain chemicals. Hydroquinone, the gold standard in skin-lightening, is known to cause contact dermatitis, exogenous ochronosis and dyschromia (7-9). Prolonged use of corticosteroids is associated with cutaneous, ophthalmologic and endocrinologic complications (10-13). Continuous exposure to mercury can lead to neurological damage (14). Moreover, it has been established that diverse health effects, such as kidney, blood vessel and nervous system damage, miscarriage, infertility in men and women, hormonal changes and behavioural problems, can be induced by exposure to toxic metals (15).

The cosmetic safety regulations of the European Union (EU) have prohibited the use of hydroquinone in cosmetics, while the United States Food and Drug Administration (US FDA) proposed a maximum of 1.5-2.0% hydroquinone as an active ingredient in over-the-counter cosmetics (16). In addition, the US FDA have also stated that mercury should be less than 1 $\mu g\ g^{-1}$ under good production practice resulting from unavoidable impurities (16). In spite of several efforts made by various health bodies to increase awareness of the dangers of skin-bleaching and governmental bans of harmful ingredients, the practice of skin bleaching and the use of potentially harmful ingredients in SLPs does not seem to slow down, rather it is ongoing and growing in most African countries and around the world.

In this study, a number of SLPs were investigated and analysed in an attempt to identify and quantify the active organic SLAs and heavy metals in them.

1.1 A general overview of skin-lightening practices

From time immemorial and among the different races of people, and in various places around the world, a myriad of concoctions have been used to lighten the skin. According to Blay (17), the practice of skin-lightening actually began in Europe and stems from Queen Elizabeth I's efforts to make her skin appear ghostly white, and nearly transparent. This extremely pale skin became known as the Elizabethan ideal of beauty. These ideals were later carried over to the Americans by female European settlers.

Another common propaganda that lead to the practice of skin-lightening was based on the dichotomy of white versus black and light versus dark as they were, and are still, viewed as pure and opposite to each other. Glenn (18) explains that the concept of colourism in southern Africa is among the negative inheritance of European colonialism. The white supremacy ideology that the European colonists brought associated blackness in natives with primitiveness, lack of civilisation, unrestrained sexuality, pollution and dirt. This served as a potent type of threat against black people and also the group of darker skinned or brown people around the world (18). In order to escape these negative associations in the form of discrimination and concrete oppression, ill-informed black people strived to attain a light skin through bleaching and various other ways.

Blay (17) gives the most common reasons provided by Ghanaian and Tanzanian women for using SLPs. They include to get rid of blemishes and to mitigate the effects of the sun, to appear and feel clean, to look beautiful, white and European, to please a partner, grab attention, or attract potential mates, and to impress peers, to appear sophisticated and modern and to gain economic and social mobility.

With the rise of the internet, companies involved in the skin-bleaching market have become even more connected and have new easy ways for marketing and distribution of their products to their consumers via informational networks. Mostly, skin-lighteners are manufactured in some countries and exported or smuggled into others. For instance, the distribution of mercury soap has been illegal in the EU since 1989, but its manufacture has remained legal as long as it is for export only. Although, the government and manufacturers in those countries know the dangers of mercury, they only focus on making a quick profit at the expense of people's health in other nations (18).

According to studies carried out by medical researchers on the prevalence of skin-lightening practices among African women, they estimate that 25% of women in Bamaki, Mali; 35% in Pretoria, South Africa; 52% in Dakar, Senegal; and a staggering 77% of women traders in Lagos, Nigeria (12, 19-21) intentionally use SLPs for cosmetic reasons. In Asia, India is reported to consume the highest tonnage of bleaching products and in terms of sheer numbers, Indians make up the largest skin bleaching market. In the Philippines, 50% of the population are reported to use skin-lighteners, and in places like Japan, China, Taiwan, and Korea users of skin-lighteners are reported to be within 20 -

50%. Likewise, in some parts of Latin America and in the USA, women of all races, including Europeans, have long legacies of skin-lightening practices (18). These statistics reiterate that the practice of skin-lightening is not a regional problem, but rather a global issue of significant concern.

1.2 The human skin

The skin is a complex organ, which covers the entire body surface as a flexible shield with the primary function to protect the organism and act as a permeability barrier to the external environment. In terms of chemical composition the skin is made up from about 70% water, 25% proteins and 3% lipids. The remainder includes minerals, nucleic acids, glycosamines, proteoglycans and other chemicals (22). The skin serves numerous protective functions (23):

- A chemical barrier in two directions: controlling the loss of water, electrolytes, and other body constituents while barring the entry of harmful or unwanted compounds from the environment.
- A radiation barrier against ultraviolet (UV) light as a result of the production of melanin by the melanocytes in the basal layer upon ultraviolet light stimulation.
- A microbiological barrier preventing the penetration of microorganisms through the intact stratum corneum.
- The skin is responsible for regulating the temperature of the body at approximately 37°C.

The skin is made up of two layers: the outer epidermis and the underlying dermis. They form a barrier that ranges in thickness from 0.8 mm on the palms and soles to 0.06 mm on the eyelids (23). The basement membrane is what separates the epidermis and the dermis.

The epidermis, as the outermost layer of the skin, provides the initial barrier of protection from the invasion of foreign substances into the body (24). The epidermis consists of a stratified squamous epithelium with an underlying basement membrane. The most

important cells of the epidermis are the keratinocytes. Other constituents of the normal epidermis are Langerhans cells, melanocytes, Markel cells and sporadic T-lymphocytes.

The dermis, which is 2-3 mm thick, contains numerous structures including blood vessels, nerves, hair follicles, smooth muscle, glands and lymphatic tissue. The main cell types of the dermis are fibroblasts, macrophages, and mast cells. Fibroblasts are responsible for secreting collagen, elastin, and proteoglycans that give the support and elasticity of the skin. Both the cells and the matrix have an influence on the rate and extent of differentiation of the keratinocytes and the formation of the epidermal layer (25, 26).

Below the dermis is a subcutaneous layer, which is the third layer and composed mainly of adipocytes (fat cells) arranged in lobules, fibroblasts, lymphocytes and mast cells. Although this layer is not technically part of the skin, it plays an integral role by acting as a heat insulator, mechanical cushion and stores readily available high energy chemicals(23, 26).

1.2.1 Variation in skin colour

The human skin ranges in variety from the darkest brown to the lightest pinkish-white hues. Primarily, the evolvement of skin pigmentation in humans is to regulate the amount of ultraviolet radiation (UVR) penetrating the skin and, thus, modify its biochemical effects (27). There is a direct correlation between the geographical distribution of UVR and the distribution of indigenous skin pigmentation around the world. Areas that receive a higher amount of UVR, generally located closer to the equator, tend to have a darker-skinned population. Areas that are far from the tropics and closer to the poles have a lower intensity of UVR, which is reflected in the lighter skinned population (28).

A person's natural skin colour has an impact on their reaction to the sun. Generally, darker skin colour burns less and has a better ability to tan, whereas pale skin or white skin burns easily and tans slowly and poorly (29). The biggest difference resulting from sun exposure is visible in moderately brown skin, the change is dramatically visible as tan lines, where part of the skin which is tanned is delineated from unexposed skin (30). The Fitzpatrick classification of skin-photo types based on sunlight sensitivity is shown in Table 1.1.

Table 1.1: Fitzpatrick sun-reactive skin types (31).

Skin type	**Natural skin colour**	**Skin reaction to solar radiation**	**Examples**
I	Pale white	Always burns, peels and never tans.	People with blue eyes, often freckled. Northern European/British.
II	Fair white	Burns easily, peels and tans minimally.	People with red or blonde hair, blue eyes. European/Scandinavian.
II	Light brown	Burns moderately, tans uniformly.	People with brown eyes, dark hair. Southern or central European.
IV	Moderate brown	Burns minimally, tans easily.	People with dark brown hair, dark eyes. Mediterranean, Asian or Latino.
V	Dark brown	Rarely burns, tans easily and substantially.	People with dark eyes, dark hair. East Indian, Native American, Latino or Africa.
VI	Black	Almost never burns, tans readily and profusely.	Dark eyes, dark hair. African or Aboriginal ancestry.

Skin-types I-III are melanocompromized. These skin types have a risk of developing skin cancer following sun exposure. On the other hand, skin types IV-VI, which are melanocompetent, have a tendency to an overactive production of melanin following sun exposure, which can lead to melasma. Therefore, all skin-types advisedly should use appropriate sunscreens to protect themselves from harmful UV rays due to the serious damage induced by extreme sun exposure, such as uneven skin tone, premature ageing, and possible skin cancers.

Other skin classification systems are based on factors such as lipid content and hydration of the skin, rather than the sensitivity to sunlight or the effect of UV radiation. In the report by Mercurio et al. (32), they scientifically classify skin into six types based on secretory characteristics. These include eudermic (healthy skin), oily, alipic, hydrated

and combination. Skin-types can change over time. An instance is younger people being more likely to have a normal skin than older people.

1.3 Melanin and pigmentation

Melanin is a large bio-aggregate composed of subunits of different pigment species formed by oxidation and cyclisation of the amino acid tyrosine. Skin complexion and UV-sensitivity is determined by the amount and type of epidermal melanin (33-35). Melanin exists in two main chemical forms, eumelanin, a dark pigment found abundantly in the skin of heavily pigmented individuals, and pheomelanin, a light-coloured sulfated pigment resulting from the incorporation of cysteines into melanin precursors (36). The efficiency of eumelanin to block UV photons is greater than that of pheomelanin, therefore the more eumelanin in the skin, the less UV-permeable is the epidermis (37). The levels of pheomelanin are similar between dark-skinned and light-skinned individuals, and it is the amount of epidermal eumelanin that determines skin complexion, UV sensitivity and cancer risk (38).

Over production or too little production of melanin brings about uneven pigmentation known as skin pigmentation disorder. Overproduction of melanin brings about hyperpigmentation, often referred to as melasma, chloasma or solar lentigines (39). Melasma is a general term describing darkening of the skin. Chloasma is generally used to describe skin discolouration caused by hormones. These hormonal changes are usually the result of pregnancy, birth control pills or oestrogen replacement therapy. Solar lentigines are the technical term for darkened spots on the skin caused by the sun. Solar refers to sunlight and lentigines describes a darkened area of skin. These spots are most common in adults with a long history of unprotected sun exposure. Hyperpigmentation, aside from sun exposure and hormones, can also be trigged by skin damage such as remnants of blemishes, wounds or rashes. This is usually observed with darker skin tones (40).

The inability of melanocytes to produce melanin or properly transport melanosomes results in hypopigmentation. Vitiligo is an example of hypopigmentation, which is characterised by patches of unpigmented skin (often surrounded by a heavily pigmented

border). Vitiligo affects all skin types and is generally considered a cosmetic condition, but it can cause significant psychological distress, particularly to some black patients (41).

Despite the controversy surrounding the use of SLPs, they have shown to effectively correct specific zones of abnormal high pigmentation on the skin. Thus, SLPs can help remove a tan or discoloration due to some pigments in the top layer of the skin, but cannot make a dark person fair (42).

1.3.1 Biosynthesis of melanin

The initial step of melanin biosynthesis begins from either the hydroxylation of $_{L}$-phenylalanine to $_{L}$-tyrosine (noncompulsory step, operative *in vivo*) or directly from $_{L}$-tyrosine which is then hydroxylated to $_{L}$-dihydroxyphenylalanine ($_{L}$-DOPA), which is an obligatory step both *in vitro* and *vivo*. $_{L}$-DOPA serves as a precursor to both melanins and catecholamines, acting along separate pathways (Figure 1.1). Oxidation of $_{L}$-DOPA to dopaquinone, represents the next step, which is common to both eu- and pheomelanogenic pathways (43).

Further transformation of dopaquinone to leukodopachrome, followed by a series of oxidoreduction reactions with the production of the intermediates dihydroxyindole (DHI) and DHI carboxylic acid (DHICA), that undergo polymerization to form eumelanin, is referred to as eumelanogenesis (44, 45). Pheomelanogenesis also begins with dopaquine; this is conjugated to cysteine or glutathione to yield cysteinyldopa and glutathionyldopa, which further transform into pheomelanin (43, 44). Eu- and pheomelanin are contained in mixed melanin. $_{L}$-DOPA generation of catecholamines requires its enzymatic decarboxylation, hydroxylation, and methylation to produce dopamine, norepinephrine, and epinephrine, respectively. All of these catecholamines can potentially convert into neuromelanin through several oxidation/reduction reactions *in vitro* (Figure 1.1) (35). *In vivo*, the only primary precursors to pigment are dopamine and cysteinyldopamine (46-48). Thus, melanin pigments have a similar arrangement of several units linked by carbon-carbon bonds, but the chemical composition, structural and physical properties differ from each other (43, 44).

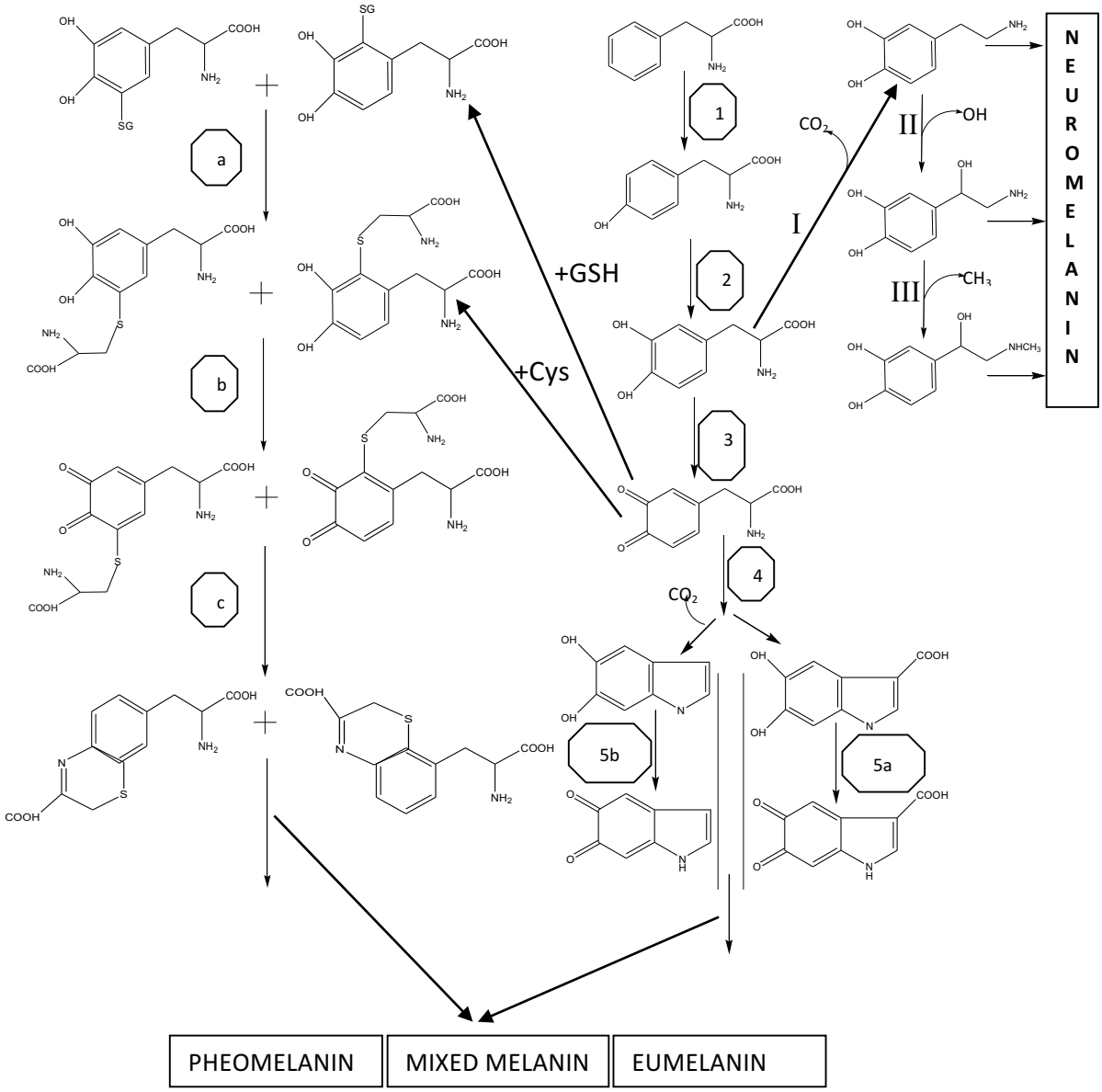


Figure 1.1. Synthesis of melanins. Adapted from (35).

GSH, glutathione; Cys, cysteine. 1: phenylalanine hydroxylation; 2: tyrosine hydroxylation; 3: DOPA oxidation; 4: dopachrome tautomerization; 5a: DHICA oxidation; 5b: DHI oxidation; a: hydrolysis of glutathionyldopa; b: oxidation of cysteinyldopa; c: intramolecular cyclization of cysteinyldopaquinone; I: DOPA decarboxylation; II: hydroxylation of dopamine; III: methylation of norepinephrine.

1.3.2 Melanin pathway inhibitors (tyrosinase inhibitors)

Different steps of the melanin pathway can be interrupted, depending on the mechanistic reaction of the inhibitors involved (49). The most common approach in skin-lightening is the inhibition of the enzymatic reaction of tyrosinase (49, 50). The active compounds used in cosmetic products and traditional preparations to inhibit melanin production are either of synthetic, natural or botanic sources.

SLAs can be classified into four types depending on the mechanism of tyrosinase inhibition. These include competitive inhibitors, uncompetitive inhibitors, mixed type (competitive/uncompetitive) inhibitors and non-competitive inhibitors (51). Table 1.2 lists several tyrosinase inhibitors and gives their classification. A competitive inhibitor tends to combine with a free tyrosinase and therefore hinders substrate binding. A competitive inhibitor could be tyrosinase-substrate analogues, copper ion chelators, or derivatives of $_{L}$-tyrosinase or $_{L}$-Dopa. An uncompetitive inhibitor binds only to the tyrosinase-substrate complex. The mixed-type inhibitor binds with both a free tyrosinase and the tyrosinase-substrate complex. The equilibrium binding constants for free tyrosinase and the tyrosinase-substrate complex differ for most mixed-type inhibitors. For non-competitive inhibitors, the free tyrosinase and the tyrosinase-substrate complex can bind with the same equilibrium constant (51).

Table 1.2: Some tyrosinase inhibitors derived from synthetic and natural sources.*

Synthetic sources		**Natural sources**	
Inhibitor	**Type of inhibition**	**Inhibitor**	**Type of inhibition**
Benzoic acid	Mixed	Arbutin	Competitive
Benzaldehyde	Noncompetitive	Aloesin	Noncompetitive
Cupferron	Competitive	Anacardic acid	Competitive
Cinnamaldehyde	Noncompetitive	Anisaldehyde	Noncompetitive
Cinnamic acid	Mixed	Anisic acid	Uncompetitive
Captopril	Noncompetitive	Agaritine	Uncompetitive
Citral	Noncompetitive	Cumic acid	Noncompetitive
Dimethyl sulfide	Competitive	Cuminaldehyde	Noncompetitive
Methimazole	Mixed	*p*-Coumaric acid	Mixed
Kojic acid	Mixed	(-) Epicatechin-3-*O*-gallate (ECG)	Competitive
L-Mimosine	Competitive	(-) Epigallocatechin-3-*O*-gallate (EGCG)	Competitive
Tiron	Competitive	3,4-Dihydroxycinnamic acid	Noncompetitive
Tropolone	Competitive	Oxyresveratrol	Noncompetitive
2-Methoxycinnamic acid	Noncompetitive	Kaempferol	Competitive
3-Methoxycinnamic acid	Noncompetitive	*Trans*-cinnamaldehyde	Competitive
4-Methoxycinnamic acid	Noncompetitive	4-Hydroxy-3-methoxycinnamic acid	Noncompetitive
4-Substituted benzaldehydes	Competitive	9-Hydroxy-4-methoxypsoraln	Noncompetitive
4-Substituted resorcinol	Competitive	5-Hydroxymethyl-2-furfural	Noncompetitive
p-Hydroxybenzaldehyde	Competitive		

*Adapted from (52).

However, competitive inhibitors of tyrosinase are the most commonly used skin-lightening agents. They include phenolic compounds, non-phenolic compounds and the combination formula (53). Phenolic compounds consist of compounds such as hydroquinone and its derivatives, for example, monobenzyl ether of hydroquinone, phenol and its compounds such as 4-methoxyphenol, 4-isopropylcatechol, 4-hydroxyanisol, and N-acetyl-4-S-cystaininylphenol. Kojic acid, azelaic acid, N-acetylcysteine, tretinoin, and L-ascorbic acid represent the non-phenolic compounds. The combination of hydroquinone and various other active lightening agents in different concentrations often describe the combination formula. The combination formulas often used are Kligman's formula (hydroquinone 5%, tretinoin 0.05–0.1%, dexamethasone or betamethasone valerate 0.1% in hydro-alcoholic base cream or ointment base), Pathak's formula (2% hydroquinone, tretinoin 0.05–0.1% in hydro-alcoholic base cream or ointment base) and Westerhofs formula (N-acetylcysteine 3%, hydroquinone 2%, hydrocortisone 1% in ointment base) (53). Other active compounds that have proven to have a strong inhibitory effect on melanin synthesis include resorcinol and its derivatives (4-butylresorcinol, 4-phenyl-ethylresorcinol), steroids and mercury (10, 50, 52, 54).

Furthermore, a myriad of plant extracts contain active compounds mainly flavonoids, gentisic acid, niacinamide, licorice, arbutin, aleosin and polyphenols, that have shown to inhibit melanogenesis, without melanocyte cytotoxicity or mutagenicity (55).

1.4 Skin-lightening agents

Currently, there are a plethora of skin depigmentation formulations available commercially which contain one or several active compounds. They may serve as prescription drugs and over-the-counter products. Others tend to be uncontrolled, traditional or prohibited products. Most skin-lightening formulations contain a combination of compounds in order to provide better potency via supplementary or synergistic actions. These include compounds like hydroquinone, mercury, corticosteroids, kojic acid, arbutin and many more. These agents expose users to certain additional health risks since the comparative clinical efficacy and safety regulations of the agents are not usually met (9). The following is a review of the most used and popular

SLAs reported in the literature, their known mechanism of action and potential side-effects. The structures of these compounds are shown in Figure 1.2.

1.4.1 Hydroquinone

Hydroquinone (structure shown in Figure 1.2) is known to be a dihydric phenol with two derivatives, viz. monobenzyl and monomethyl ether of hydroquinone (40). It is also known under different synonyms such as benzohydroquinone, *p*-benzenediol, 1,4-dihydroxybenzene, β-quinol, 1,4-diol/quinol, 1,4-benzenediol, *p*-hydroxyphenol, *p*-dioxobenzene, hydrochinone or tecquinol. Hydroquinone occurs naturally in tea, red wine, coffee beans, wheat and leaves of berries. Despite the inconsistent effects and safety concern of hydroquinone, it is still the most prescribed SLA worldwide and is the gold standard for skin-lightening (31, 56-59). Hydroquinone was initially believed to act mainly by inhibition of tyrosinase due to its structural analogy to melanin precursors (60). But, the inhibition of DNA and RNA synthesis, degradation of melanosomes and destruction of melanocytes, are other putative mechanisms of hydroquinone action (61).

The clinical efficacy of hydroquinone is gauged based on its concentration, nature of the vehicle and stability of the formulation. It is mostly used at a concentration of 1.5-5% (9). Several clinical studies have established the beneficial therapeutic effect of hydroquinone in the treatment of melasma and other pigmentary disorders. In a report, 2% of hydroquinone was found to produce a decrease in hyperpigmentation, which was as good to excellent in 14-17% of treated patients (62-64). Hydroquinone at high concentration showed more effectiveness but there were associated augmented side-effects, consisting mainly of irritation at the sites of application (53). The inclusion of antioxidants, in the form of sodium bisulfate or ascorbic acid, enhance the stability of hydroquinone by minimising its oxidation (53). Hydroquinone undergoes oxidation to benzoquinone. Both hydroquinone and benzoquinone are metabolites of each other, these makes the toxicity observed with one of the two also relevant for the other, although it may vary in potency (65). Hence, SLPs which contain hydroquinone may very likely contain benzoquinone as a result of auto-oxidation when exposed to air or dissolved in aqueous solution.

In Europe, most available commercial formulations contain 2% hydroquinone, which is considered safe and effective (53). High concentrations of hydroquinone are highly discouraged due to its side-effects (9). The US FDA proposed a maximum of 1.5-2.0% hydroquinone as an active ingredient in over-the-counter cosmetics, while the National Food and Drug Agency of Nigeria (NAFDAC) had initially allowed a maximum of 2% hydroquinone in lightening creams in Nigeria, but later banned hydroquinone due to the side-effects associated with prolonged usage and non-compliance with safety regulations (40). Likewise, the use of hydroquinone in over-the-counter SLPs is also completely banned in South Africa (66)

Prolonged and high concentrations of hydroquinone usage have proven to cause multiple cutaneous and systemic-side effects (67). Irritant contact dermatitis is the most common acute complication of hydroquinone, followed by post-inflammatory hyperpigmentation, hypopigmentation, and allergic contact dermatitis (67-69). Chronic exposure to hydroquinone results in nail discolouration (70-72), or pseudo yellow nail syndrome (40). Other side-effects include conjunctival pigmentation (73), corneal melanosis and degeneration (7, 73, 74), decreased skin elasticity (40), peripheral neuropathy (75), impaired wound healing, and wound dehiscence (40, 76), specifically after abdominal surgeries such as caesarian section or hysterectomy. Trimethylaminuria or fish odour syndrome is another unique side-effect of chronic hydroquinone use. It is characterised by a rotten fish body odour caused by excretion of trimethylamine in the saliva, sweat, urine, and vagina (77).

The most severe complication of chronic hydroquinone use is known as exogenous ochronosis (67, 78), with at least 789 reported cases, 756 of which occurred in Africa (79). Ochronosis can exist both in the form of endogenous and exogenous. Endogenous ochronosis is related to alkaptonuria, an autosomal recessive disorder which is characterised by the absence of homogentisic acid oxidase in 1 out of 25,000 of the population (80), while exogenous ochronosis is attributed typically to hydroquinone-containing compounds (67, 78). Pick (81) reported the first case of exogenous ochronosis in 1901, in a patient with prolonged exposure to phenols. The first cases of hydroquinone induced exogenous ochronosis were reported in 1975 by Findlay and colleagues (78), in 35 South African Bantu women with high hydroquinone exposure (3.5-7%) for many years. Exogenous ochronosis is characterised by grey-brown or blue-black macules

coalescing into patches, accompanied occasionally by pinpoint, dark brown, and caviar-like papules (78, 82, 83). Although, several attempts have been made experimentally to treat exogenous ochronosis, the results have not been satisfactory (84). Occasionally, hydroquinone discontinuation might undo hyperpigmentation, but this can take several years (78, 85).

Concern exists on the possible carcinogenicity of hydroquinone but studies need to be carried out to determine the risk of skin cancer in hydroquinone users (86, 87).

1.4.2 Benzoquinone

Benzoquinone is a poorly water soluble crystalline substance which can be produced in large amounts by oxidation of aniline or phenol (88). It can also occur naturally in small amounts in a variety of arthropods as it is excreted and synthesised by many insects (89). Other synonyms for benzoquinone include cyclohexa-2,5-diene-1,4-dione, 1,4-benzoquinone, *p*-benzoquinone, *p*-quinone or quinone. When present in aqueous solution, benzoquinone is susceptible to both redox and acid-based transformation, resulting in the formation of hydroquinone, semiquinone and reactive oxygen species (88, 90, 91).

Studies have shown benzoquinone to be readily absorbed from the gastrointestinal tract and subcutaneous tissue and excreted partly unchanged and partly as hydroquinone. Most of it is eliminated as its conjugates (88, 92). Results emanating from various animal studies described benzoquinone to be a skin sensitizer. It was reported to induce skin lesions and irritation when injected subcutaneously in guinea pigs (93). The acute toxicity carried out on animals indicates various neurological symptoms, including loss of reflexes, writhing and paralysis of the hind limbs (94, 95). In humans, high level exposure of benzoquinone in air may result in irritation of the eyes (94, 96) while dermal exposure may result in discolouration, dermatitis, severe irritation, erythema, swelling, and the formation of papules and vesicles (97, 98). The carcinogenicity studies of benzoquinone still remain unsatisfactory, although benzoquinone is classified in category 3B for carcinogenicity and cell germ mutagenicity in Germany (65). The America Conference of Governmental Industry Hygienists (ACGIH) established in 2001 a threshold limit

value (TLV) for exposure to benzoquinone of 0.44 mg m^{-3} as the 8 hours time weighted average (94).

1.4.3 Kojic acid

Kojic acid is a hydrophilic fungal derivative obtained from *aspergillus* and *penicillium* species, and is chemically known as 5-hydroxymethyl-4H-pyrane-4-one. It is used as an antioxidant by the cosmetics industry and is the second most effective over-the-counter SLA used as an alternative to hydroquinone in skin-lightening. It is also a popular agent used for the treatment of melasma (99). Despite being purported to have skin-lightening credentials, it is not currently approved by the US FDA for such use in over-the-counter pharmaceutical products (100).

Kojic acid is found to be a skin sensitizer and has proven to be mutagenic in cell culture studies (54). It is characterised by the ability to prevent tyrosinase activity by binding to copper (101). According to a survey conducted by the Personal Care Products Council of the United States, kojic acid is used at a concentration ranging from 0.1 to 2%, with the maximum concentration found in face and neck creams, lotions, and powders. In a report submitted by Health Canada's Cosmetics Notification System, 148 products were found to contain kojic acid, with uses in skin care products (100). It summarized the concentration ranges for kojic acid use in Canada to be 0.1% or less in 37 products, 0.1 to 0.3% in 11 products, 0.3 to 1% in 34 products, 1 to 3% in 45 products, 3 to 10% in 14 products, and 10 to 30% in 3 products. However, it is widely reported to be used in a concentration of 1 to 4% in Asia (102).

Based on a margin of safety calculation, the European Commission's Scientific Committee on Consumer Products (SCCP) concluded that the use of kojic acid at a maximum concentration of 1% in skin care formulations poses a risk to human health due to its potential systemic effects (100).

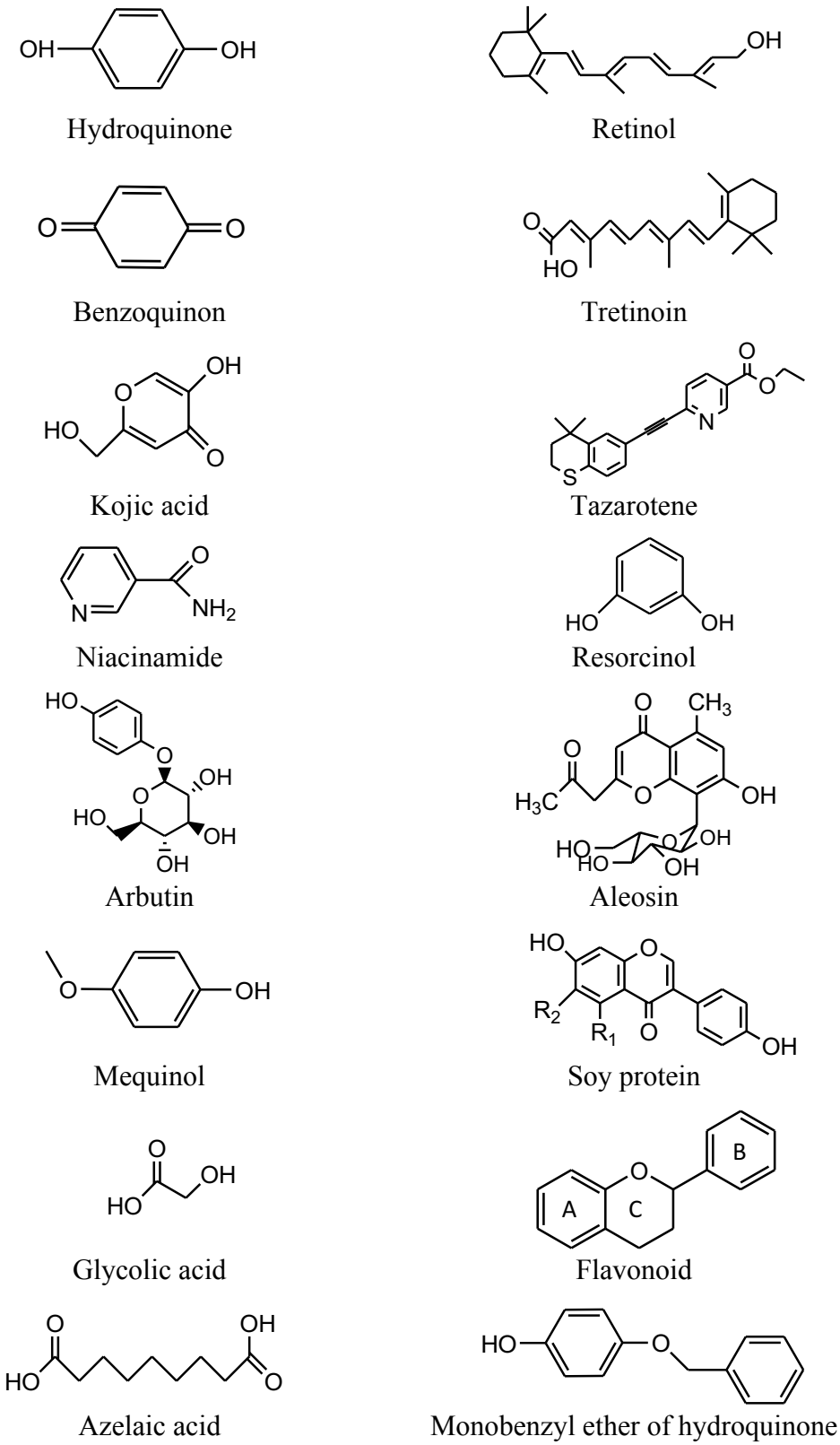


Figure 1.2: Chemical structures of common skin-lightening agents. Adapted from http://www.chemicalbook.com/ (Accessed on 28 June 2016).

1.4.4 Niacinamide

Niacinamide is the amide form of vitamin B_3. It acts by inhibiting the transfer of melanosomes to the epidermal keratinocytes. Reports have shown that niacinamide is of benefit in the treatment of melasma, and pustular acne (103). The efficacy of niacinamide was confirmed in one study where 3.5% niacinamide plus retinyl palmitate were used in the treatment of hyperpigmentation (104).

1.4.5 Arbutin

Arbutin (hydroquinone-B-D-glucopyranoside) and methyl arbutin are referred to as skin-lightening glucosides derived from bearberry fruit (*Arctostaphylos uva ursi*). Arbutin is also found in certain herbs and pear trees. Arbutin acts by inhibiting melanosomal tyrosinase activity. A 1% concentration of arbutin has been cited as effective for depigmentation by SLP manufacturers (102). However, 3% arbutin is also available, but a controlled study on the possible side-effects has not been carried out (102). Arbutin has been shown to be effective in treating solar lentigines and melasma, but a high concentration can bring about hyperpigmentation (103).

1.4.6 Glycolic acid

Glycolic acid is derived from sugarcane. It is an α-hydroxy-acid, which in low concentration produces rapid desquamation of pigmented keratinocytes, and in high concentration results in epidermolysis. In the removal of superficial layers of the epidermis with glycolic acid peels, a concentration of 30 to 70% is used and these can further enhance the penetration of other skin lighteners like hydroquinone (105). In the treatment of post-inflammatory hyperpigmentation, glycolic acid is usually used at a low concentration so as to avoid skin irritation (102).

1.4.7 Mequinol

Mequinol is chemically known as 4-hydroxyanisole, methoxyphenol, or monomethyl ether of hydroquinone. It is a substrate for tyrosinase; it acts as a competitive inhibitor in the formation of melanin precursors but the exact mechanism of action accounting for its skin-lightening attributes is unknown. It is the primary lightening alternative to hydroquinone approved for use in Europe and the United States. In the United States, mequinol is available at 2% concentration and can only be sold as a prescribed skin-lightener in combination with 0.01% tretinoin. Unlike hydroquinone, mequinol does not damage the melanocyte but has been noticed to cause longstanding depigmentation, though, this longstanding depigmentation generally repigments with time (54).

1.4.8 Azelaic acid

Azelaic acid is regarded as a naturally occurring non-phenolic, saturated, 9-carbon dicarboxylic acid. It is isolated from cultures of *pityrosporum ovale* and is associated with the hypomelanosis seen in *tinea versicolor* (53). *In vitro*, azelaic acid interferes with the function of tyrosinase and may also inhibit DNA synthesis and mitochondrial oxidoreductase (106), but does not seem to affect normal melanocytes (107). Therapeutically, azelaic acid has shown to have beneficial effects in acne and vulgaris and certain pigmentary disorders, such as melasma and lentigo maligna (53). In the treatment of melasma and post-inflammatory hyperpigmentation, azelaic acid is used at a concentration of 15 to 20% (108). At these concentrations, azelaic acid is tolerated in humans, but mild pruritus, transient erythema, irritation and scaling tend to be its adverse reactions (107, 102).

1.4.9 Retinoids (Topical)

Retinoids such as tretinoin and retinol are obtained from vitamin A. The efficacy of these products has been proven in the treatment of melasma, post-inflammatory hyperpigmentation, and other pigmentation disorders (102). Retinoids have been used as pigment lightening agents both directly and indirectly. For direct improvement in skin

pigmentation, the prescribed retinoids are tretinoin and tazarotene (109, 110). Retinol compared to tretinoin or tazarotene is less effective and less irritating. Retinol is mostly found in a variety of moisturisers and anti-aging products sold over the counter (102). Retinol has been shown to convert to retinoic acid in the skin in a two-step oxidation process (111). The main challenge to retinol formulations is its lack of stability at high concentration (54) .

Retinoids when use indirectly to lighten dyspigmentation serve as penetration enhancers. A side-effect of retinoids is irritant dermatitis which is characterised by erythema, dryness, and scaling (112, 113). These cutaneous changes damage the skin barrier, allowing easy penetration of other pigment-lightening agents such as hydroquinone and mequinol, to the melanocytes.

1.4.10 Resorcinol

Resorcinol and its derivates, such as rucinol (4-n-butylresorcinol), are known tyrosinase and TRP-1 inhibitors (50). They are considered as antibacterial, anti-acne and potent topical disinfectants in a concentration of 1 - 3%. In the treatment of moderate to severe facial dyschromia, acne, oily skin, texturally rough skin, fine wrinkle and pseudofolliculitis, 14% resorcinol is often combined with 14% salicylic acid and 14% lactic acid (114). A prolonged exposure to resorcinol can lead to thyroid dysfunction (115). Resorcinol is banned in over-the-counter SLPs in South Africa (66).

1.4.11 Aleosin

Aleosin, a low-molecular-weight glycoprotein, is derived from the aloe vera plant. It acts by inhibiting tyrosinase by competitive inhibition at the dihydroxyphenylalanine oxidation site (116, 117). Unlike hydroquinone, it is not characterised by cell cytotoxicity. Due to its hydrophilic nature, it has limited ability to penetrate the skin. Aleosin is commonly used in combination with arbutin or deoxyarbutin, and, these combinations have shown to decrease tyrosinase activity through several different mechanisms (54).

1.4.12 Soy proteins

Soy protein is a commonly used SLA in cosmeceutical moisturisers (54). It contains small serine proteases such as Bowman Brik inhibitor (BBI) and Soybean trypsin inhibitor (STI) which inhibit the protease activated receptor-2 (PAR-2) pathway expressed on keratinocytes (118). The PAR-2 pathway, can be inhibited after 3 weeks of raw soy milk application (54), resulting in diminished melanin transfer (119, 120). Thus far, soy extract has been shown to have minimal side-effects and has an excellent safety profile (54).

1.4.13 Flavonoids

Flavonoids belong to the group of plant polyphenols. They are classified into six major groups, which include flavanols, flavones, flavonols, flavanones, isoflavones, and anthocyanidins. Reports have shown flavonoids to inhibit enzymes due to their ability to chelate copper at the active site (121). In a recent fluorescence quenching study, the dihydroxy substitutions in both the A and B rings of flavonoids (see Figure 1.2) have been shown to be crucial in inhibiting tyrosinase activity (122).

1.4.14 Steroids

In dermatology, steroid preparations are pharmaceutical products usually prescribed for the treatment of inflammatory conditions. They are also called corticosteroids, glucocorticoids, or “cortisone”. Steroids are referred to as “systemic” if taken by mouth or given by intramuscular injection, or “topical” if absorbed through the skin. Topical steroids are categorised as mild, moderate, potent and very potent. Both mild and moderate topical steroids rarely cause side-effects, while potent and very potent topical steroids are very toxic as they are more absorbed through the skin and into the blood system. Betamethasone, clobetasol, fluocinonide, and triamcinolone acetonide are among the strongest topical steroids found in bleaching creams. They exert their depigmenting effects by inhibiting epidemic melanogenesis (123). Steroid formulations have been found to improve therapy for psoriasis conditions when prescribed in a concentration of

0.025 to 0.5% (m/m) (124). Sometimes, the efficacy of steroids is increased when combined with other SLAs. The chemical structures of commonly used topical steroids are shown in Figure 1.3.

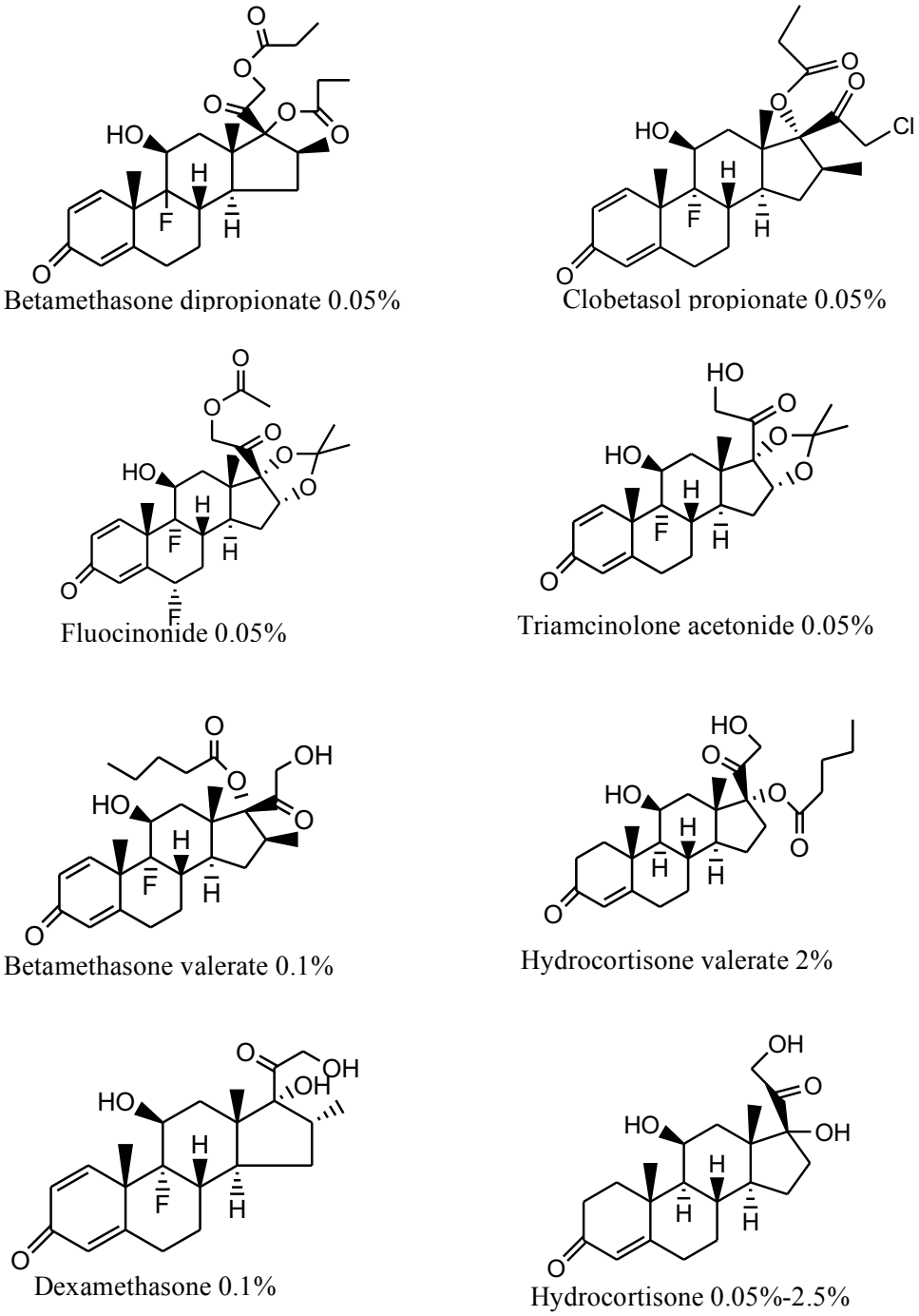


Figure 1.3: Chemical structures of topical steroids mostly present in skin-lightening products. Adapted from http://www.chemicalbook.com/ (Accessed on 28 June 2016).

The use of topical steroids is associated with multiple dermatological and systemic side-effects, especially when used incorrectly (123, 40, 76, 125, 126). Acne vulgaris (12, 76), allergic contact dermatitis (127, 128), hypertrichosis (76, 126), skin atrophy (129, 11, 130), perioral dermatitis (130), rosacea (40), striae atrophica (12, 11), and telangiectasias (126, 129), are among the common cutaneous complications associated with topical steroid application. Topical steroid use is also known to cause systemic complications such as hypothalamic-pituitary-adrenal (HPA) axis suppression, Cushing syndrome (76, 131, 132), diabetes mellitus (126), and hypertension (133).

Steroid addiction syndrome is the adverse effect from the chronic daily use of a potent topical steroid, which is associated with intense burning and potentially permanent erythema due to the withdrawal of vasodilatation. Ophthalmological conditions such as cataracts and glaucoma, also fall among complications associated with chronic topical steroid use (40).

1.4.15 Mercury

Mercury is a known melanotoxin. Mercury exists primarily in elemental, or in organic and inorganic forms. Elemental mercury is mostly used as a liquid metal in thermometers and is also still used in dental amalgams. Organic mercury compounds like thiomersal, methylmercury, and ethylmercury are used as preservatives. Mercury salts such as mercuric amidochloride or ammoniated mercury, mercuric chloride, and mercurous chloride or calomel are the kind of mercury commonly added to SLPs (134). Mercury is a highly volatile element with a long atmospheric half-life. As a result of these physical properties, it is present in every environment and exposure is not an isolated concern but rather a global concern to human health. In recent years, research has shown that even chronic exposure to very low concentrations has the ability to cause long-lasting neurological and kidney impairment (135).

Mercury compounds, when used to lighten skin pigment, have recorded varying success. The mercury ion is thought to act by inhibiting the synthesis of melanin (136). Inhibiting melanin production makes the skin more susceptible to skin cancer (137). When mercury is applied to the skin, it reacts with ultra-violet light and reoxidizes. This leads to more

pigmentation and premature ageing. However, further application of mercury in an attempt to correct the skin will lead to a darker blotchy appearance (40).

Chronic usage of mercury-containing skin-lightening preparations has led to the deposition of mercury in the body after absorption through the skin, especially in the tubular region of the kidney, giving rise to the occurrence of severe reactions (138-140). Studies have shown increased accumulation of mercury in the brain, kidney and liver tissues of both albino and pigmented mice when absorbed through the skin (141), and it was found to be highest in the kidney and lowest in the brain (142). Research has also shown that women using mercury-containing soaps and creams tend to attain mercury concentrations from 0.03 to 0.15 mg L^{-1} in their urine. At this concentration, the central nervous system, and kidneys are at major risk of negative effects, since such effects have been shown at a concentration of 0.02 to 0.5 mg L^{-1} (143).

The common cutaneous complications of mercury use are allergic contact dermatitis, flushing, erythroderma, purpura, nail discolouration, and gingivostomatitis (144, 145, 59). Paradoxic hyperpigmentation is a consequence of the chronic use of mercurial skin lighteners, which might be caused by dermal deposition of mercury-containing granules (123). The use of mercury cosmetics during pregnancy has also been shown to result in prenatal and postnatal intoxication, as mercury can cross the placenta (59). The amount of mercury applied to the skin is proportional to the amount excreted by the kidney as shown by Cole and colleagues (146).

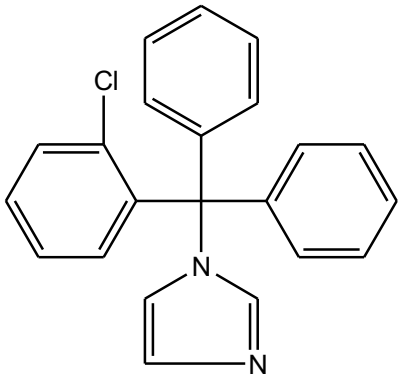
1.5 Clotrimazole

Clotrimazole is a broad-spectrum topical antifungal drug which belongs to the azole class. The azole class can be further subdivided into two classes, namely, imidazole and triazoles (147). Clotrimazole and other class members, econazole and miconazole (see Figure 1.4), fall into the imidazole sub-class and can be used for the topical treatment of tinea pedis (athlete's foot), tinea cruris and tinea corporis (148). It is also popularly used in the topical treatment of vulvovaginal and oropharyngeal candidiasis (147).

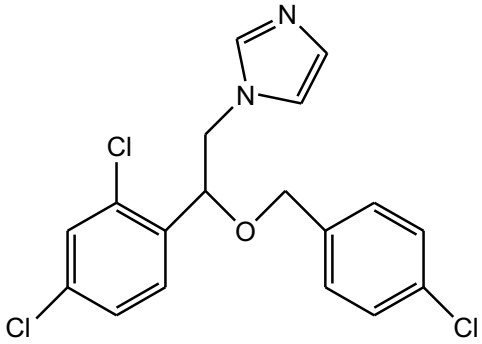
The mono-preparations of clotrimazole are generally available over-the-counter, but if present in a combined preparation will necessitate prescription. Clotrimazole is usually

at 1% (m/m) in a combined formulation (147). Treatment with a clotrimazole formulation does not usually exceed one week of application, especially if found in combination with topical steroids so as to avoid unwanted side-effects. The addition of clotrimazole to SLPs is very unconventional and should be a cause for concern since SLPs are used over a long period of time.

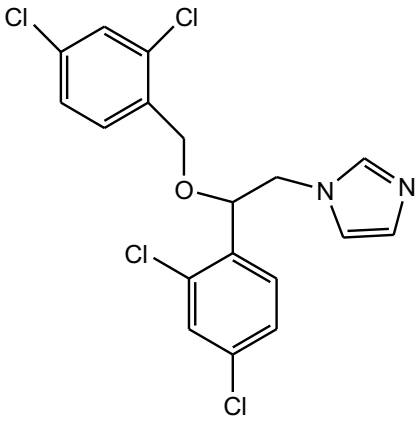
The side-effects resulting from clotrimazole application include burning, stinging, swelling, irritation, redness, pimple-like bumps, tenderness or flaking of the skin (149). Serious allergic reactions of clotrimazole-containing creams include rash, itching/swelling (especially of the face, tongue or throat), severe dizziness and troubled breathing (149).



Clotrimazole



Econazole



Miconazole

Figure 1.4: Chemical structures of some imidazole fungicides. Adopted from https://www.google.co.za. (Accessed on 9th December 2016).

1.6 Heavy metals in cosmetics and their health complications

Heavy metals are any metallic chemical elements that have a relatively higher density of at least five times greater than that of water (1 g cm^{-3}). They are naturally occurring in the environment, in rocks, soil, and water. They may also be found in pigments and other raw materials in all industries with no exception to the cosmetics industry. Examples of metals considered as heavy metals include antimony, arsenic, bismuth, cadmium, chromium, cobalt, copper, gallium, gold, iron, lead, manganese, mercury, nickel, platinum, selenium, silver, tellurium, thallium, tin, uranium, vanadium, and zinc (150).

Heavy metals are naturally occurring in the environment and make their way in trace quantities into raw materials and finally end up in the finished products we use and consume every day. These substances enter into the human body mainly through diet, inhalation, and skin exposure to soil and/or medication. Dermal exposure represents the most significant route for cosmetic products, as most of the cosmetics are applied directly on the skin (4, 151).

The intentional use of heavy metals and their compounds as active ingredients in cosmetic products has been banned by the US FDA unless they occur as impurities (150). Their presence in products is therefore a cause for concern if not well controlled. Annex 11 of Directive 76/7681/EEC of the European Union lists more than 1000 chemical substances that must not be included in the composition of cosmetic products due to their toxicity (152). In that list, heavy metals such as antimony (Sb), arsenic (As), cadmium (Cd), chromium (Cr), cobalt (Co), mercury (Hg), nickel (Ni) and lead (Pb) in cosmetics are banned as they are considered unsafe.

Long-term exposure to heavy metals can result in their accumulation in organisms as they are difficult to metabolise. From studies, metal ions, when bound to proteins, enzymes, and nucleic acids, are absorbed and form complexes with the carboxylic acid (-COOH), amine ($-NH_2$), and thiol (-SH) groups of proteins resulting in malfunctioning or death of cells (153, 154).

The use of cosmetic products often represents a possible source of long-term exposure to a myriad of chemicals (155). Some of these ingredients in cosmetic products can penetrate the skin and reach vital internal organs via the systemic circulation, as they are

applied to the skin (156). Unlike other cosmetic products that are applied to restricted parts of the body, the application of body creams is over the entire body surface, and hence, exposure to contaminants in these products is much greater (152).

Low-level exposure to Pb can result in disorders such as behavioural abnormalities, permanent neurological damage, decreased learning and hearing, and may also have diverse effects on the reproductive, hepatic and renal systems. Cadmium is a cell poison. It causes several damages, including cell death or an increase in cell proliferation. Due to the toxic nature of cadmium, it has been categorised as a group 2A carcinogen by the International Agency for Research in Cancer (IARC) (157).

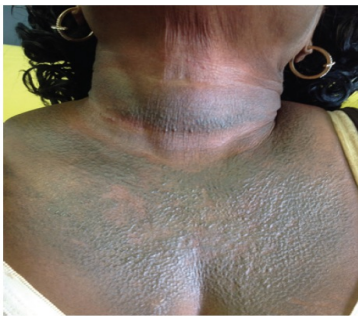
Arsenic has a pronounced affinity for skin and keratinizing structures including the hair and nail. Acute overexposure to arsenic can bring about skin eruptions, alopecia and characteristic striation of the nails (158). Overexposure to antimony and it compounds can also adversely affect the skin, liver, lungs and cardiovascular system (159).

Other heavy metals such as Cu, Cr, Fe, Ni, Mn and Zn are not completely harmful to humans as they are involved in many biological processes, even though Cr still has some controversies surrounding it. For instance, iron plays a vital role in oxygen transport and storage, and electron transport, in hydrogenase and other redox active enzymes. Manganese is present in superoxide dismutases and also in catalases. Cobalt, a vitamin B12 component, is essential in various rearrangement and methylation reactions. Copper is important in electron transfer (azurin and plastocyanin), oxygen atom transfer (oxygenases), and also respiration (cytochrome C oxidase). Nickel is required for the function of acetyl coenzyme synthesis, ureas, and various hydrogenases. Zinc plays an essential role in many enzymes including anhydrase and a group of proteases, such as carboxypeptidase A. Zinc is also involved in the transfer of genetic information via the zinc finger protein. Despite the essential nature of some of these heavy metals to humans and other organisms, they still can cause serious health challenges when present in cosmetics products, one of which is an allergy. Chromium, cobalt, and nickel are well-known allergens, while copper, manganese and zinc are referred to as weak allergens (152).

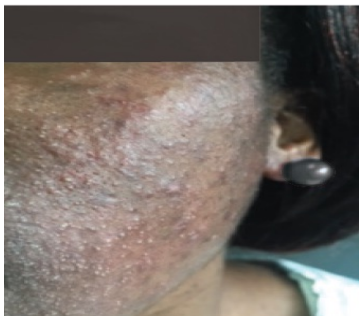
In the present study, 12 elements: As, Al, Cd, Co, Cr, Cu, Fe, Hg, Mn, Ni, Pb, and Zn were investigated for their presence in the studied SLPs.

1.7 Dermatological effects of skin-lightening products

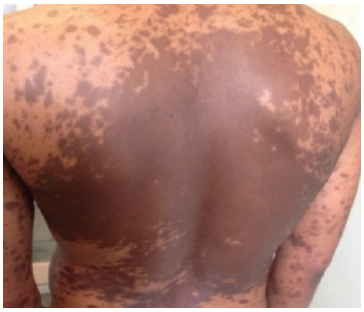
The occurring side-effects associated with skin bleaching depend on the nature and concentration of the SLPs used (15, 11). This is complicated when the actual concentrations of the active ingredients listed on the product labels are not consistent (160). Further contributing factors include the concurrent use of several depigmenting products, application of products over a wide spread of the body, and longtime use of the products (123). The most reported side-effects include exogenous ochronosis, allergic contact dermatitis, skin atrophy or thinning, acne, erythema, pigmented colloid milia, straie atrophicans, nail discoloration, skin peeling and redness, patchy hyperpigmentation and hypopigmentation, cushing syndrome, pityriasis, bleached panda effect, eczema, wrinkles, rosacea, and tinea (59). Thus, a knowledge of the concentration of SLAs in SLPs is essential. In Figure 1.5 are shown some photographs of patients diagnosed with various skin conditions by dermatologists, arising from the improper use of SLPs.



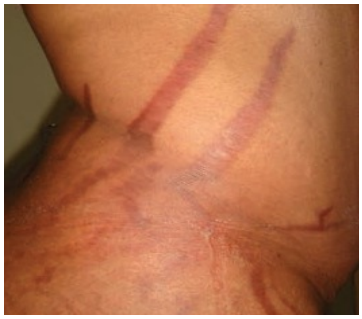
Permanent ochronosis



Acne vulgaris



Patchy pigmentation



Permanent stretch marks

Figure 1.5: Photographs of patients diagnosed with various skin conditions arising from the improper use of skin-lightening products. (Source: Prof N Dlova, Nelson Mandela School of Medicine).

1.8 Methods of analysis of skin-lightening agents

Before now, mercury and hydroquinone were the most frequently analysed SLAs found in lightening creams since their side-effects where recorded first. Currently, a myriad of SLPs containing newly introduced SLAs are available in the market. This development has led to the constant demand of new analytical procedures capable of quantifying such compounds. Methods that have been used over time to analyse SLAs include thin layer chromatography (TLC), capillary electrophoresis (CE), capillary electrochromatography (CEC), micellar electrokinetic chromatography (MEKC), electrochemistry, differential pulse voltammetry (DPV), gas chromatography (GC) and high performance liquid chromatography (HPLC) (161-163). Mass spectrometry coupled with either HPLC or GC is also used for the analysis of SLAs (162).

Among the methods listed above, the HPLC method was chosen in this study to analyse the selected SLAs present in the studied SLPs. HPLC represents one of the most reliable techniques regularly used in the analysis of SLAs since it can handle a wide range of target compounds. For instance, Gaudiano *et al*. reported an isocratic HPLC method for the determination of six glucocorticoids in whitening creams. The method employed a C_{18} column, a mobile phase of water/acetonitrile (50:50) and detection was done at a wavelength of 240 nm. The method was reported to be robust, linear, accurately sufficient and precise (161).

Huang *et al*. developed an HPLC method for the simultaneous determination of five whitening agents (magnesium ascorbyl phosphate, ascorbyl glucoside, kojic acid, arbutin and hydroquinone decomposed from arbutin in cosmetics). The method used a Cosmosil C_{18}-AR-II column, a mobile phase of 0.05 M KH_2PO_4 buffer solution (pH 2.5) and methanol (99:1), with detection wavelength set at 280 nm (164).

Balaquer *et al*. developed a HPLC method referred to as environmental friendly for the determination of ascorbic acid (vitamin C) and its derivatives (ascorbyl phosphate (as magnesium or sodium salts), ascorbyl palmitate, and ascorbyl glucoside) in skin-whitening cosmetics. The method made use of a C_{18} column and a mobile phase gradient of ethanol:50 mM phosphate buffer at different pHs (containing 0.1 M NaCl). Detection was carried out with a UV-visible detector set at different wavelengths. The method was reported to be robust (165).

Reepmeyer *et al*. developed a reversed-phase and a normal phase HPLC method for the detection of clobetasol propionate in some formulations. A NOVA-Pak C_{18} column and a mobile phase of acetonitrile-water (1:1) was used for the reversed phase chromatography, while a Chromegasphere SI 60 column and a mobile phase of isopropanol-heptane (1:4) was used for the normal phase chromatography. The chromatogram was monitored at 240 nm and the signal was scanned from 200 to 350 nm in order to record the UV spectra (166).

More studies have employed HPLC when investigating SLPs due to the complex matrices that require a variety of ingredients to be separated and quantified.

1.9 Methods of analysis of heavy metals

Typical examples of instrumentation methods used for heavy metal analysis include atomic absorption spectrometry (AAS), inductively coupled plasma-optical emission spectrometry (ICP-OES), inductively coupled plasma-mass spectrometry (ICP-MS), neutron activation analysis (NAA), X-ray fluorescence (XRF), and ion chromatography (IC) (167-171). However, the AAS, ICP-OES and ICP-MS are the most common instrumentation methods used when analysing the heavy metal content in cosmetic products.

For instance, Iwegbue *et al*. evaluated the levels of heavy metals present in commonly used moisturising and skin-lightening creams in Nigeria by means of AAS. The metals quantified included Al, Cd, Cr, Cu, Co, Fe, Mn, Pb and Zn (152). Similarly, Al-Salah *et al*. used the AAS method to investigate the presence of trace metals such as As, Cd, Cr, Hg, Ni, and Sb in lipsticks (172). Nnorom also used the AAS method to determine the presence of metals such as Pb, Cd, Co, and Cr in talcum powders (173). More studies have used the AAS technique when measuring the content of heavy metals in cosmetics products (4, 150). The AAS technique is easy to use, fast and cheap, but the technique is disadvantaged with moderate detection limits, element limitations and lack of screening ability.

The ICP-OES and ICP-MS are the most favoured techniques for heavy metal analysis. They have the advantage of multi-element analysis, high productivity, economical for

many samples and/or elements, few chemical interferences, excellent screening abilities, total dissolved solids and can handle both inorganic and organic samples (174).

Al-Qutob *et al*. reported the use of ICP-MS for the determination of heavy metals such as Ag, Al, Ba, Bi, Cd, Cr, Co, Cu, Mn, Mo, Ni, Pb and Zn in cosmetics products (168). Similarly, Bocca *et al*. used the ICP-MS to determine the presence of metals such as Cd, Co, Cr, Cu, Hg, Ir, Mn, Ni, Pb, Pd, Pt, Rh, and V in commercial body creams (175). Zeo *et al*. also used the ICP-MS for measurement of metals like Cr, Cd, Pb, Hg, As, Se, and Sb in cosmetic products such as lipsticks, nail polishes and skin creams (176).

Alquadami *et al*. used the ICP-OES to determine the presence of heavy metals such as As, Bi, Cd, Hg, Pb and Ti in some skin-whitening cosmetic creams (167). Likewise, Mayildurai *et al*. used the ICP-OES to investigate the presence of metals such as As, Cd, Cr, Hg, Ni, Pb, Co, Cu, in a deodorant, eyeshadows, a face cream, a hair dye, kohl, soap and talcum powder (177). Sabah *et al*. also used ICP-OES to quantify metals such as As, Cd, Pb, and Hg in face make-ups (178).

Furthermore, Emad *et al*. used both the ICP-MS and ICP-OES for the determination of elements in cosmetic products like eye-shadow, lipsticks, lip-gloss, eye-liners, mascaras, fairness products, kohl, hair dyes, foundations and hennas. Twenty three trace elements (Ag, As, Ba, Be, Cd, Co, Cr, Cu, Mn, Mo, Ni, Pb, Sn, Sr, Ti,Tl, U, Zn, Zr, Nb, Rb, Sb, and Se) were studied by using ICP-MS and twenty two elements (Al, Ba, Bi, Ca, Cd, Co, Cr, Cu, Fe, K, Mg, Mn, Na, Ni, P, Pb, Si, Sr, Th, Ti, U, and V) were studied by using ICP-OES (179).

Both the ICP-OES and ICP-MS are an excellent choice when analysing the heavy metal content of cosmetic products as they can measure a wide range of elements with good to excellent detection limits, but are associated with possible limitations of spectral interferences (174).

For mercury determination in cosmetic products, the cold vapour atomic absorption spectroscopy (CV-AAS) technique is one of the most widely used techniques and has been reported in a number of studies (137, 180).

In this study, the CV-AAS and ICP-OES methods were used to quantify the levels of mercury and other heavy metals respectively.

1.10 Quantitation methods

Basically, there are three different calibration methods that can be utilised in quantitative analysis; namely, external standard, internal standard and the standard addition method.

The external standard method is the basic quantification method in which both the calibration solutions and unknown samples are analysed under the same conditions. The accuracy of this method is affirmed when there is reproducibility of the injection volume. The external standard method is performed by preparing calibration standard solutions of known concentration of the analyte of interest, and making sure the concentration of unknown samples fall within the range bracket of the calibration solutions. A fixed amount of the calibration solutions and unknown samples are injected, and the peak heights or areas obtained for the calibration solutions are then plotted against the concentration of the analyte, giving a linear calibration plot passing through the origin. The resulting peak heights or areas from the unknown samples are then related to those of the calibration solutions, by using the calibration plot to estimate the amount of the unknown (181, 182).

The internal standard method is performed by adding a known amount of a compound different from the analyte of interest to the unknown sample and the calibration standards. The internal standard chosen must be chemically similar to the analyte of interest. The accuracy of this method is dependent on the stability of the internal standard and it must not in any way interfere with any of the sample components. With this method extraction efficiency can be evaluated if the internal standard is added before preparation of the sample. Quantification by using the internal standard method is achieved by using the ratio of peak height or area of the standard solution to the internal standard to calculate a response factor.(181). This response factor is then used to calculate the concentration of the unknown.

The standard addition method is most useful for samples with complex matrices and with potential to cause adverse effects to the signal (matrix effect), making it unreliable to relate the signal between the sample and standard by using the traditional calibration plot approach. In the standard addition method, known quantities are added directly to an unknown and the analyte concentration is determined from the increased signal (183).

In this study, the external standard method was employed to carry out quantitative analysis of the selected SLAs and heavy metals. When analysing samples with complex matrices like SLPs, which require a variety of ingredients to be quantified, the external standard approach is more suitable so as to avoid introduction of further interferences in the samples. The external standard method helps to calibrate the instrument and can be applied to a wide variety of methods.

1.11 Problem statement

The problem to be addressed in this research is the fact that some SLPs contain compounds which are illegal and harmful to the skin. Another problem area is that the active SLAs present in the products may be present at concentrations greater than allowed by regulatory control limits. All SLPs should have the active ingredients listed on the package labels and the actual contents should be consistent with those listed on the product labels. It is a concern that not all SLPs available on the market specify the composition and active ingredients in detail as would be informative to the end-user. Some products are labelled in a language that is confusing or difficult to understand. It is therefore of great importance for public health that the contents of commonly used SLPs be known.

1.12 Motivation

Considering the toxic effects of some SLAs, such as hydroquinone, mercury, and steroids, the practice of skin-lightening in an attempt to beautify and accomplish a light complexion, is worrisome. The NAFDAC had initially allowed a maximum of 2% hydroquinone in lightening creams in Nigeria, but due to the side-effects associated with prolonged use of hydroquinone and also the lack of compliance with content and labelling requirements, all forms of bleaching agents were prohibited in cosmetics and toiletries (40). In view of the increasing incidents of undesirable side-effects such as exogenous ochronosis, contact dermatitis, hyperpigmentation and hypopigmentation, nail discoloration, nephrotic syndrome and other health complications reported to be associated with the practice skin-lightening, it is necessary to investigate whether

prohibited additives such as hydroquinone and other toxic elements and compounds are still among the ingredients found in commercial SLPs on the Nigerian market. This work is of great importance for public awareness and safety.

1.13 Aim and objectives

The aim of this research work was to identify and quantify commonly used organic SLAs and heavy metals present in some selected SLPs sampled from Ilorin State in Nigeria.

This study had the following specific objectives:

I. To develop an extraction method for the selected SLAs.

II. To develop and validate a high-performance liquid chromatography (HPLC) method for the identification and quantification of the selected SLAs in the studied SLPs.

III. To extract trace amounts of heavy metals present from the SLPs by means of microwave-assisted digestion.

IV. To quantitatively determine the presence of mercury in the studied SLPs by means of cold vapour atomic absorption spectroscopy (CV-AAS).

V. To quantitatively determine the presence of other heavy metals in the studied SLPs by means of inductively coupled plasma-optical emission spectroscopy (ICP-OES).

VI. To evaluate the exposure risk derived from the heavy metal content in these products.

Chapter 2 of this dissertation provides the experimental procedures undertaken to achieve these objectives. The results of these experiments are presented and discussed in Chapter 3. The conclusions derived from this research are explicitly stated in Chapter 4.

Chapter 2

EXPERIMENTAL

This chapter describes the analytical techniques used for the analysis of some commonly used skin-lightening products (SLPs) in Nigeria. It also provides the materials and instruments used to carry out these analyses.

2.1 Materials

A total of 35 products were purchased from various stores in Ilorin, Nigeria. All the samples were stored at room temperature away from sunlight. The samples were stored in the refrigerator after extraction and before analysis. All samples were investigated for both the presence of organic active skin-lightening ingredients and heavy metals. A list of all the products investigated including their brand names, cost, ingredients listed on the label, company of production, and country of manufacture, can be found in Table 2.1.

The list of chemicals used are provided in Appendix A, together with their chemical grades and manufacturers. The reagents used were all of analytical grade. The solvents used, for example, methanol and acetonitrile, were of HPLC grade. The water used was purified with a Millipore Elix 5 UV water purification system.

2.2 Equipment

A list of all the equipment used to carry out this research can be found in Appendix B.

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

Code	**Price (Naira)**	**Brand name**	**Ingredients listed on packaging**	**Company of production**	**Country of manufacture**
SLP 1	4000	Anivat Lightening Lotion	Aqua (water), Glyceryl Stearate, Parafinum, Liquidum (mineral oil), Isopropyl Myristate, Cetostearyl Alcohol, Cetearch 20 & 25, Triethanolamine Puriss, Sodium Lauryl Sulfate, Butyrospermum parkii, *Glycyrrhiza Globra*, Murus Alba, Ascorbic Acid, Citric Acid, Methyl Paraben, Parfum (Fragrance), Psropyl Paraben, BHT.	Shinesarod Limited	USA
SLP 2	3500	Make Me White Fade Milk	Various Vitamins and Protein Herbal Extractions.	Shinesarod Limited	USA
SLP 3	3500	Nature White Whitening Milk	Water (Aqua), Cetearyl Alcohol, Cetearesth 20, Canola oil, Glycerin Glycol Stearate, PEG-4, Lactic Acid, Kojic Acid, Propylene Glycom, Mulberry (*Morou Bom Bycis*) Root Extract, Bearberry (Arctostaphylos Uvayrsil) Extract, Licorice 1 (*Glycyrrhiza Galabra*) Extract,		France

			Dimethicone, Phenoxyethanol Isobutylparaben, N-Butylparaben, Fragrance, Ethylhexyl Methoxycinnamate, Tocopheryl Acetate (Vitamin E).		
SLP 4	3500	Crystal White UV Lightening Milk	Aqua, Polysorbate 60, Glycerine, A.H.A, Glyceryl Monostearate, Cetyl Alcohol, Vitamin C, Stearic Acid, Paraben Complex, Carbomer, Triethanolamine, Vitamin E, Acetate, Fruity Fragrance, Eggyolk Mineral oil, Sodium EDTA.	Young and Beautiful Cosmetics	Phillipines
SLP 5	2500	Glutathione Whitening Lotion			
SLP 6	1800	Sure White Maxi Tona	Aqua (Water), ParaffinumLiquidum (Mineral oil), Glycerin, Cetearyl Alcohol and Ceteth-20 Phosphate and Dicetyl Phosphate, Di-PPG-2 Myreth-10 Adipate, Cetearyl Alcohol, Phenoxy-ethanol and methylparaben and ethylparaben, and propylparaben and butylparaben and Iso		Switzerland

			butylparaben, Lanolin Alcohol, Carbomer, Parfum (fragrance), Sodium Hydroxide, Caprylic/Capric Triglyceride Diacetyl Boldine, Disodium EDTA, CI 45430, Alpha-Hexyl Cinnamic Aldehyde (HCA), Benzl Benzoate, Citral, Limonene, Geraniol, Hydroxyisohexyl3-Cyclohexane Carboxaldehyde, Linalool.		
SLP 7	300	Clair Express	Aqua, Plant Extracts, Paraffinum Liquidum, Glycerin, Refined Palm, Stearin, Fragrances, Carbomer, Methylparaben, Propyl Paraben, stearic Acid, Lmidalidinyl Ure.	Hekin Inc.	Paris
SLP 8	900	Clearasil Lightening Lotion	Aqua (water), Glycerystearate, Parafinum Liquidum (mineral oil, Isopropyl Myristate, Cetosearyl Alcohol, Cetearch 20 & 25, Triethanolamine Puriss, Sodium Lauryl Sulfate, Butyrospermum Parkii, Glycyrthiza Globra, Murus Alba, Ascorbic Acid, Citric Acid, Methylparaben, Parfum (Fragrance), Propyl Paraben, BHT.	Xandas Ind. Limited	Nigeria

SLP 9	750	White Express	Eau Purfiee BP, Parafine Oil USP, Dimethicone BP, Isopropyl Myristate BP, Propylene Glycol BP, Methyl Paraben BP, Propyl Paraben BP, Clobetasol Propionate USP 0.05%w/w, Clotrimazole BP 1.00%w/w	Kremoint Pharma P. Limited	India
SLP 10	700	Hi White Exclusive Whitenizer Lotion	Deionised Water, Isopropyl Palmitate, Ceteareth-6 (and) Stearyl Alcohol, Stearic Acid, Glycerol, Cetostearyl Alcohol, Lanolin, Hydroquinone (2.0%), Hydrogenated Castor Oil, Fragrance, Silicon Fluid, Carboxy Methyl Cellulose, Octyl Methoxy Cinamate, Aloe Vera, Collagen, Allantoin, Vitamin c, Citric Acid, Triclosan, Vitamin E, Methyl Paraben, Propyl Paraben, Sodium Sulphite, Sodium Metabisulphite.	Skin Beauty Limited	Nigeria
SLP 11	650	Looking Good Skin Tone Lotion	Deionised Water, Cetostearyl Alcohol, Isopropyl Palmitate, Stearic Acid, Glycerol, Lanolin, Hydroquinone, Hydrogenated Castor Oil, Fragrance, Silicon Fluid, Carboxy Methyl Cellulose, Benzophenone-3, Sodium Lauryl Sulphate, Aloe Vera, Collegen, Allantoin, Citric Acid, Vitamin C,	Skin Beauty Limited	Nigeria

			Vitamin E, Irgasan DP300, Methyl Paraben, Propyl Paraben, Sodium Sulphite, Sodium Metabisuphite, FD &C Yellow No 6.		
SLP 12	650	Forever Young Plus Super-Maxitona	Isopropyl, Myristate, Deionised Water, Dimethicone, Lactic Acid (Alpha-Hydroxy Acids), Cetosteary Alcohol, P-Hydroxyphenol, Ceteareth-20, Carboxy Methyl Cellulose, Octyl Methoxycinnamate, Anhydrous Lanolin, Methyl Paraben, Propyl Paraben, Sodium Sulphite, Sodium Metabisulphite, Stearic-Acid, Vitamin A, Vitamin E, Citric Acid, Ascorbic Acid (Vitamin –C), Niacinamide (Vitamin B3), Fragrance.	Beauty Fair Laboratories Limited	Nigeria
SLP 13	650	Beauty Fair Supa Tona	Stabilized Aloe-Vera Gel, Deionized Water, Glycerin, Stearic Acid, Propylene Glycol, C12-C13 (Alky Benzoate), Hydrogenated Poly sobutene, Isopropyl Myrestate, Collegen, D-Penthanol (Pro Vitamin B5), Vitamin-C, Vitamin-E, Kojic Acid Dipalmitate (K.A.D), Hydrolyzed Elastin, Apricot	Beauty Fair Laboratories Limited	Nigeria

			Kernel Oil, Sodium Metabisulfite, Sodium Sulfite, Propyl Paraben, Methyl Paraben, Cetyl Alcohol, EDTA, Fragrance.		
SLP 14	600	Ken Perfect Action White	Deionized Water, Cosmowax, Cetyl Alcohol, Stearic Acid, Glycerin, Mineral Oil, Isopropyl Myristate, Alph Hydroxy Acid with Kolic Acid (AHA-K), Citric Acid, Propyl Paraben and Methyl Paraben.	Blueworld Cosmetics Nig. Limited	Nigeria
SLP 15	600	Diva Maxima Maxitone	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 Ssulfite, Citric Acid, Propylene Glycol, AHA (Alpha-Hydroxyacid), Allantoin, Lecithin Cholesterol, Ascorbic Acid, Fragrance.	N.P. Gandour	Côte d'Ivoire
SLP 16	600	Skin Magnet Whitening Lotion	Aqua (water), Glyceryl Stearate, Cetostearyl, Alcohol, Avocado Oil, Isopropyl Myristate, Glycerine, Allantoin, Ascorbic Acid, Sodium Metabisulphite,	Anochemical Cosmetic Industries Limited	Nigeria

			Sodium Sulphite, Fragrance, Sunscreen, Arbutin, Carrot Extract Alpha Hydroxyl Acid (AHA).		
SLP 17	600	Body White Paris	Water, Carbomer, Methylparaben, Propylparaben, Triethanolamine, Dimethicone, Peg-7 Glycerylcacoate, Bearberry Extracts, Mulberry Extracts, Licorice Extracts, Lemon Extracts, Kojic Acid, PPG-1-PEG-9 Lauryl Ether, Fragrance.	N.P. Gandour	Côte d'Ivoire
SLP 18	500	So White Skin Perfector Gel	Propylene Glycol, Cetearyl Alcohol, Hydroxy Phenol,Castor (Ricinus Communis) Oil, Carnauba Wax, Polyacrylamide/C13-14, Isoparaffin/Laureth-7, Parfum (Fragrance), Sodium Sulfite, Sodium Metabisulfite, CI14700.	Fair & White Paris	France
SLP 19	500	Perfect White Lightening Beauty Lotion	Vaseline, *Huile De Vaseline*, *Acide Stearique*, Glycerine, Acides De Fruits (AHA), *Acide Kojique*, Allantoine, *Huile De Silicone*, BHT, Lorol C16, Lorol C18, Parfum, EAU.	Dream Cosmetics	Côte d'Ivoire

SLP 20	450	Rapid Clair Gel Cream Plus	Eau Purfiee BP, Parrafine Oil USP, Dimethicone BP, Isopropyl Myristate BP, Propylene Glycol BP, Sorbic Acid BP, Clobetasol Propionate USP 0.05%w/w, Clotrimazole BP 1.00%w/w.	Laboratoires Biopharma S.A.	India
SLP 21	400	Swiss Collagen Toning Formula	Deionosed Water, Ceteareth-6 (and) Stearyl Alcohol, Cetostearyl Alcohol, Isopropyl Palmitate, Glycerol, Stearic Acid, Lanolin, Hydroquinone (2%), Hydrogenated Castor Oil, Fragrance, Sillicon Oil, Escalol 557, Aloe Vera, Collegen, D-Panthenol, Citric Acid, Vitamin E, Vitamin C, Methyl Paraben, Propyl Paraben, Sodium Sulphite, Sodium Metabisulphite, FD & C Red No.2.	Skin Beauty Limited	Nigeria
SLP 22	400	Caro White Lightening Beauty Lotion	Petrolatum, Mineral Oil, Stearic Acid, Cetearyl Alcohol, Isopropyl Myristate, Glycerin, Methyl & Propyl Paraben, *Huile De Carotte*, Vitamin E(0.2%), Hydroquinone (Max. 2%), Aqua (EAU), Fragrance.	Dream Cosmetics	Côte d'Ivoire

SLP 23	350	Carotone Brightening Body Lotion	Water, Cetyl Stearylic Alcohol, Stearic Acid, Petrolatum, Mineral Oil, Isopropyl Myristate, Propylparaben, BHT, UVA, UVB, Tocopheryl Acetate, Polysorbate 60, Methylparaben, Sodium Sulfite, Sodium Metabisulfite, Propylene Glycol, Ascorbic acid, Allantoin, Carrot Oil, Collagen, Hydroquinone 2%, Fragrance.	N.P. Gandour	Côte d'Ivoire
SLP 24	350	Venus Skin Toning Cream	Water, C12-15 Alkyl Benzoate, Mineral Oil, Octyl Stearate, Polyglyceryl-3 Methyl Glucose Distearate, Glycerin, Cetyl Alcohol, 4-Methylbenzylidene Camphor, Niacinamide, Benzophenone-3, Dimethicone, Glyceryl Stearate, Tocopheryl Acetate, Penthanol, Propylene Glycol, Cyclomethicone, Diazolidinyl Urea, Methylparaben, Propylparaben, Dimethiconol, BHT, Acrylates/C10-30 Alkyl Acrylate Crosspolymer, Disodium EDTA, Sodium Hydroxide, Fragrance. Contains Oxybenzone.	PZ Cussons Nig.	Nigeria

SLP 25	250	Euro White Cream	100g Contain Clobetasol Propionate 0.05 g Excipients q.s.	Pink Rose Cosmetics Limited	
SLP 26	250	Labidjanaise Carrot Cream	100 g Contiennet Clobetasol Propionate 0.05 g Excipients c.s.	H. Cosmetiques CI	Italy
SLP 27	250	Applevet Lightening Cream	Clobetasol Propionate B.P 0.05 % w/w Cream Base q.s.	Applefield Int'l Limited	Italy
SLP 28	250	Xtra White Gel	100 g Contiennet Betamethasone Dipropionate 0.065 g- Excipients c.s.	Stanelvis Pharmaceutic als Limited	Italy
SLP 29	250	Amos White Gel	Clobetasol Propionate 0.05 % w/w.	PT. Tempo Scan Pacific TBK	Indonesia
SLP 30	200	Janet Gel Plus	100 g Contain Clobetasol Propionate 0.05 g Excipient q.s.	H Cosmetiques CI	Italy
SLP 31	200	Tempovate Gel	Clobetasol Propionate 0.05 % w/w	PT. Tempo Scan Pacific TBK	Indonesia
SLP 32	200	Visible Difference Cream	Clobetasol Propionate 0.05 % w/w	Vinco Pharmaceutic als	Italy

SLP 33	200	Clear White Moon	Clobetasol Propionate U.S.P. 0.05 % w/w Excipient q.s.		India
SLP 34	150	Esapharma Movate Cream	100 g Contiennet: Clobetasol Propionate 0.05 g, Excipient: 1,2-Propandiol, Dyoxyde De Titane, Carbomer, *Eau purifiee q.s*.	Esapharma SRL Pharmaceutical Laboratory	Italy
SLP 35	150	Beneks' Fashion Fair Gel Plus	100 g Contient: Betamethasone Dipropionate 0.065 g, Excipient q.s.	Beltapharm S.P.A.	Italy

2.3 HPLC analysis of skin-lightening agents

Chromatography still represents the most commonly used technique for the determination of SLAs in cosmetic products including SLPs. It is categorised by the type of mobile phase used, as liquid, gas, or supercritical chromatography. A number of reports describe various HPLC methods used for the qualitative and quantitative determination of SLAs in SLPs. These were discussed in Section 1.8.

In this study, a reversed-phase HPLC technique was employed to separate, identify, and quantify the SLAs present in the studied SLPs. Reversed-phase HPLC is far superior to other HPLC modes in the variety of target compounds it can handle. The HPLC technique has a unique advantage in terms of the column and mobile phase interaction, or mechanism of separation, and the selectivity. It allows for the separation of high molecular mass and high polarity compounds and also gives one liberty to accomplish very difficult separations at low levels of detection, with high precision and accuracy.

2.3.1 Theory of HPLC

Chromatography is based on the partitioning of solutes between two phases and is, therefore, related to simple liquid–liquid extraction. In HPLC, however, one phase (the mobile phase) is in constant movement relative to the other one (the stationary phase). The sample molecules are partitioned between the phases; those in the stationary phase are retained, whereas those in the mobile phase move. The interaction between the solutes and the stationary phase is most often based on adsorption. During an HPLC separation, a solute normally partitions between the phases many thousand times. The basis of separation is that different kinds of molecules on average spend different amounts of time in the stationary phase. Due to a large number of partitioning steps, chromatography has enormous resolving power and can separate mixtures of components with very similar physical properties. In the common HPLC format, the stationary phase is a highly porous solid material packed inside a cylindrical column, whereas the mobile phase is a liquid. If a successful separation has been made of a binary sample, this will in the ideal case result in the elution of two Gaussian-shaped concentration peaks (184).

2.3.2 Components of an HPLC system

A typical HPLC instrument has five main units. These are the mobile phase reservoir, pump, column, injector system, and detector (Figure 2.1). A recorder and a waste unit are also required as they help complete a proper HPLC system.

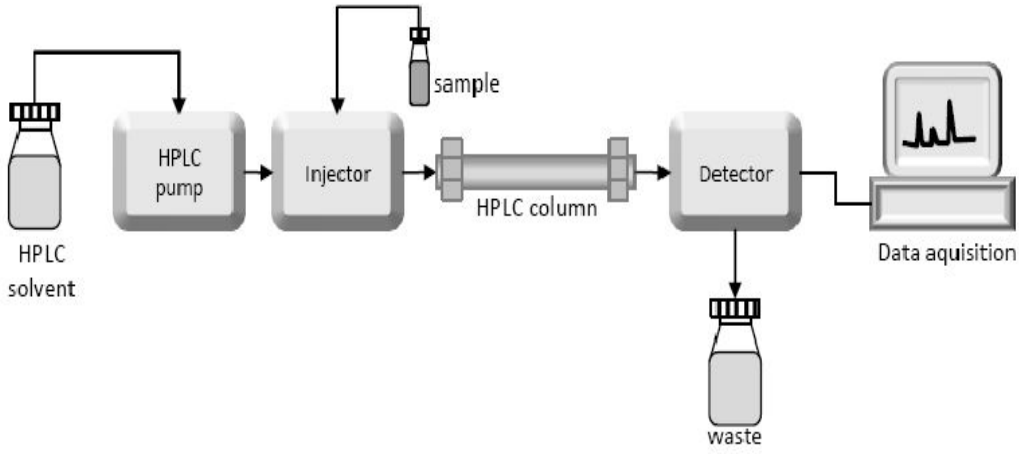


Figure 2.1: Components of an HPLC instrument (185).

The mobile phase reservoir is an inert container for mobile phase storage and transport. Generally, transparent glass bottles are used so as to facilitate visual inspection of the mobile phase level inside the container. Stainless steel particulate filters are placed inside the containers for removal of particulate impurities in the mobile phase, if any.

The column is a stainless steel tube packed with stationary phase, with the option of different lengths, bore sizes and packing materials, and is held in place by the column hardware. Columns are available in different dimensions including preparative, normal-, micro- and mini-bore, and capillary columns. The most widely used packing materials for HPLC separations are silica-based. The most popular material is octadecyl-silica (ODS-silica), which contains C18 coating, but materials with C1, C2, C4, C6, C8 and C22 coatings are also available. Other types of substances used as column packing materials include zirconia, polymer-based and monolithic columns. The right choice of column length combined with the correct packing material in correlation with the appropriate mobile phase is what determines an effective separation in HPLC. The two most commonly used modes of separation in HPLC are the reversed-phase and normal-phase. Reversed-phase columns contain a non-polar stationary phase (such as C_{18}) and

are mostly used for non- or mildly polar organic compounds, and are typically paired with a polar mobile phase. Normal-phase columns contain a polar stationary phase and are mostly used to separate polar molecules in combination with a non-polar mobile phase.

An injector or auto-sampler is used to provide a constant volume injection of the sample into the mobile phase stream. Inertness and reproducibility of injection are necessary to maintain a high level of accuracy.

The detector measures either the absorbance, fluorescence, conductivity or refractive index of the mobile phase. When a compound elutes from the column, a peak is observed. The retention time of the peak is used to identify the compound. The peak height (or area) is proportional to the amount of the compound in the sample. Some detectors provide the ability to obtain a spectrum of the eluting species. Detector choice depends on the properties of the compound being analysed. A UV detector or photodiode array (PDA), fluorescence detector and refractive index detector (RI) are the most widely available detectors for use in HPLC. When analysing a wide variety of substances that can absorb light within the ultraviolet to visible region (190-900 nm) a PDA detector is commonly used. The PDA detector allows simultaneous monitoring of more than one absorbing component at different wavelengths which is an advantage in terms of less solvent usage and time-saving. A mass spectrometer is the ultimate detector for liquid chromatography (LC-MS). It significantly enhances the application range for HPLC in terms of sensitivity and selectivity and its detection pattern is based on fragmentation of a molecule by an electric field and separation on the basis of mass to charge ratio of the fragmented molecule.

The pump or solvent delivery system serves to provide a pulse-free flow of the mobile phase through the column under constant pressure. Variations in the flow rates of the mobile phase affect the elution time of sample components and result in errors. The composition of the mobile phase can be kept constant during the separation (isocratic mode) or can be changed during the gradient separation mode. Gradient elution is employed when trying to resolve components which do not separate and elute under isocratic conditions. There are two options for gradient operation: high-pressure mixing (which requires a pump for each solvent) and low pressure mixing (which requires one pump). A control program is required for gradient operation.

The recorder reads out information from the detector. It interprets and records detected compounds by means of peaks and produces chromatograms as relevant. The analysed solutions flowing from the detector output make their way to the waste unit. Tubing lines and fittings serve to interconnect the pump, injector, column, and detector components in order to form an entire channel for the mobile phase, sample, and separated compounds. Modern HPLC systems are computer-based and software controls the operational parameters and also the acquisition and treatment of data.

2.3.3 Identification of skin-lightening agents

Identification of selected SLAs in the analysed products was done by comparing the UV spectra and retention times of pure analytes with those of the sample analytes. The HPLC detector used in this study was a photodiode array detector which has the ability to provide a UV spectrum for each individual peak in the chromatogram. With the help of UV-visible spectrophotometry, the wavelength of maximum absorption for each of the selected SLAs was obtained. By using these wavelengths that the selected SLAs absorb, one can differentiate the components separated based on their UV spectra and retention times with HPLC. The wavelengths of maximum absorption are also used to select appropriate detection wavelengths for collection of the chromatograms so that all analytes can be detected. Below is a brief introduction on UV-visible spectrophotometry and the spectral information of the selected SLAs detected in the analysed products.

2.3.3.1 Theory and instrumentation for UV-visible spectrophotometry

Ultraviolet and visible (UV-Vis) absorption spectroscopy is the measurement of the attenuation of a beam of light after it passes through a sample or after reflection from a sample surface. It measures the intensity of light as a function of its wavelength. Single-beam and double-beam are the two major classes of spectrophotometers. In single-beam spectrophotometers, all the light passes through the sample. To measure the intensity of the incident light the sample must be removed so that all the light can pass through. In a double-beam spectrophotometer, before the light reaches the sample, the light source is split into two separate beams. One beam passes through the sample and the second one

is used for reference. This gives an advantage because the reference reading and sample reading can take place at the same time.

Different analytes, such as transition metal ions, biological macromolecules, and highly conjugated organic compounds, can be analysed by UV-Vis spectroscopy. Organic compounds absorb light in the UV or visible region of the electromagnetic spectrum. Compounds that absorb visible light (400-780 nm) are coloured, whereas those that absorb UV light (190-400 nm) do not need to be coloured, which is in our case.

The wavelength at which an organic compound absorbs depends on how its electrons are tightly held in a molecule, but the wavelength of this light is inversely proportional to the energy of the radiation as explained by the equation below:

$$\mathbf{E = hc/\lambda.}$$

E is the energy of electromagnetic radiation being absorbed, h is Plank's constant, c is the speed of light and λ is the wavelength of absorption.

As energy is absorbed in the UV region, there occur changes in the electronic energy of the molecule. Once a molecule absorbs energy, an electron is promoted from an occupied molecular orbital (usually a non-bonding n or bonding π orbital) to an unoccupied molecular orbital (an antibonding π^* or σ^* orbital) of greater potential energy. The lowest-energy occupied molecular orbitals for most molecule are σ orbitals (see Figure 2.2), which correspond to σ bonds and the π orbitals lie at relatively higher energy levels than σ orbitals. The non-bonding orbitals that hold unshared pairs of electrons lie at even higher energies. The antibonding orbitals (π^* and σ^*) are orbitals of highest energy.

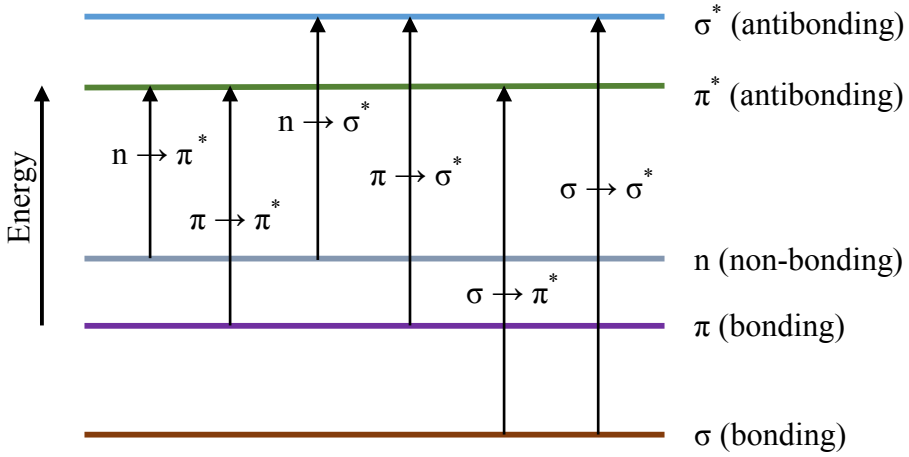


Figure 2.2: Relative energies of orbitals and the types of electronic transitions.

To determine the concentration of an absorbing species in a solution with a fixed path length by UV-visible spectroscopy, the Beer-Lambert law comes into play. It states that the absorbance of a solution is directly proportional to the concentration of the absorbing substances in the solution and the path length, which is written symbolically as

$$\mathbf{A = \log I_o / I = \log 100 / T = \varepsilon\, c\, b}$$

where A is the absorbance (no unit of measurement), ε is the molar absorptivity ($dm^3\ mol^{-1}\ cm^{-1}$), c is the molar concentration (mol dm^{-3}), and b is the path length (cm). The concentration of the samples must be low in order to obey the Beer-Lambert law.

The minimum requirements of an instrument to study absorption spectra (a spectrophotometer) include a source, a monochromator, sample compartment and a detector (see Figure 2.3). The commonly used light sources, such as a tungsten halogen lamp (> 350 nm) and a deuterium lamp (< 350 nm), are required to be stable during the measurement period, i.e. the intensity of the emitted radiation should not fluctuate, and that there should be adequate intensity over as large a wavelength region as possible.

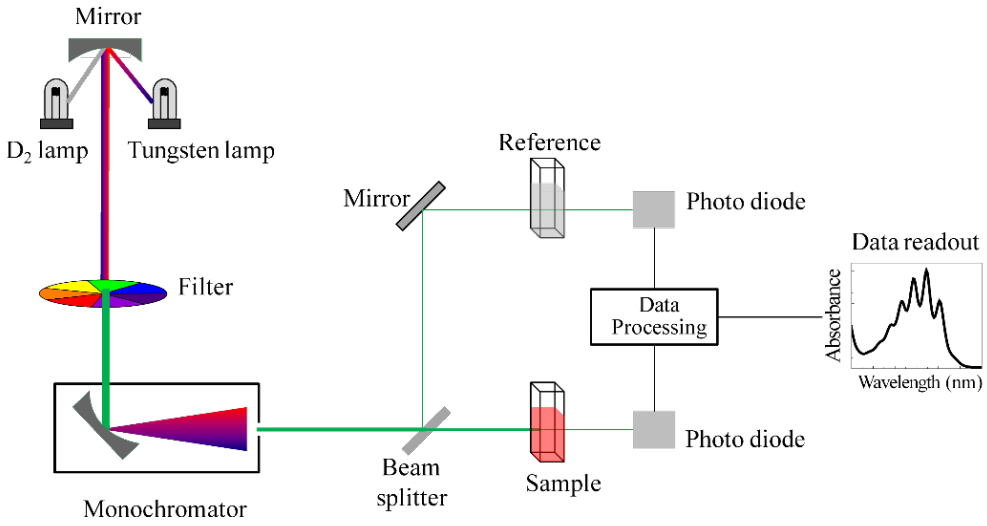


Figure 2.3: Schematic of a double-beam UV-visible spectrophotometer (186).

The function of a monochromator is to produce a beam of monochromatic (single wavelength) radiation that can be selected from a wide range of wavelengths. The essential components are the entrance slit, collimating device (to produce parallel light), a wavelength selection or dispersing system, a focus lens or mirror, and an exit slit. The

sample compartment serves to accommodate the cuvette that carries the sample solution. The detector, which servers to measures the absorbance of samples, is usually a photomultiplier, silicon diode, or diode array.

In this study, a PerkinElmer Lambda 25 double-beam UV-visible spectrophotometer (Figure 2.4) was used to measure the maximum absorbance of the selected SLAs.

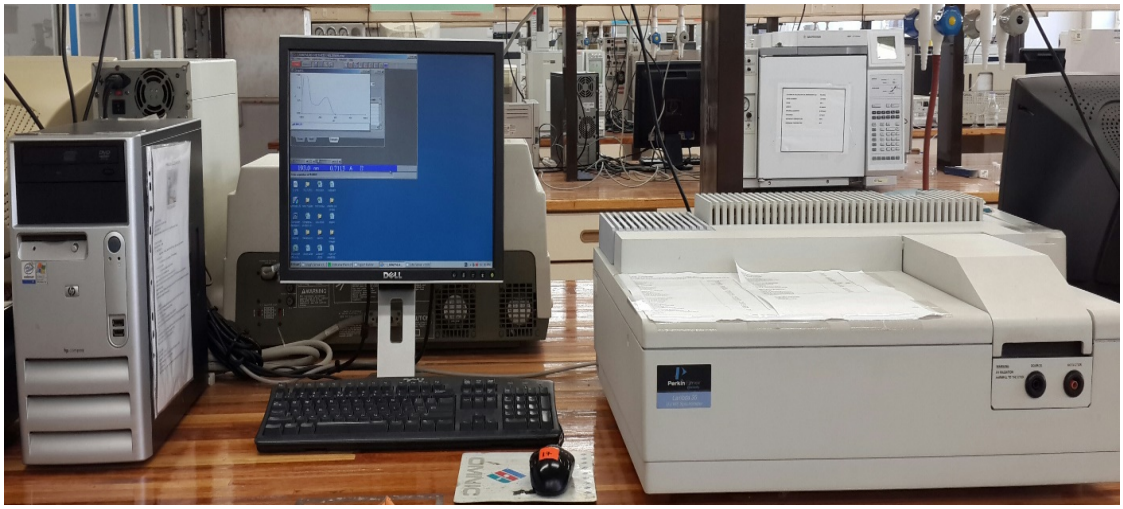


Figure 2.4: A photograph of the PerkinElmer Lambda 25 double-beam UV-visible spectrophotometer used for measuring the UV spectra of the selected SLAs.

2.3.3.2 Determination of UV absorbance of skin-lightening agents

In order to carry out the UV analysis, pure standards of selected SLAs, which included clobetasol propionate, betamethasone dipropionate, clotrimazole, hydroquinone, benzoquinone, kojic acid, niacinamide and arbutin, were dissolved in methanol. The UV analysis of the SLAs was done by using 1 cm path length quartz cuvettes and methanol as the blank in the reference beam. Their UV absorption bands were obtained over the wavelength range of 190 nm to 400 nm. The spectra of the SLAs are shown in Figures 2.5 to 2.12 and their wavelengths of maximum absorption are listed in Table 2.2.

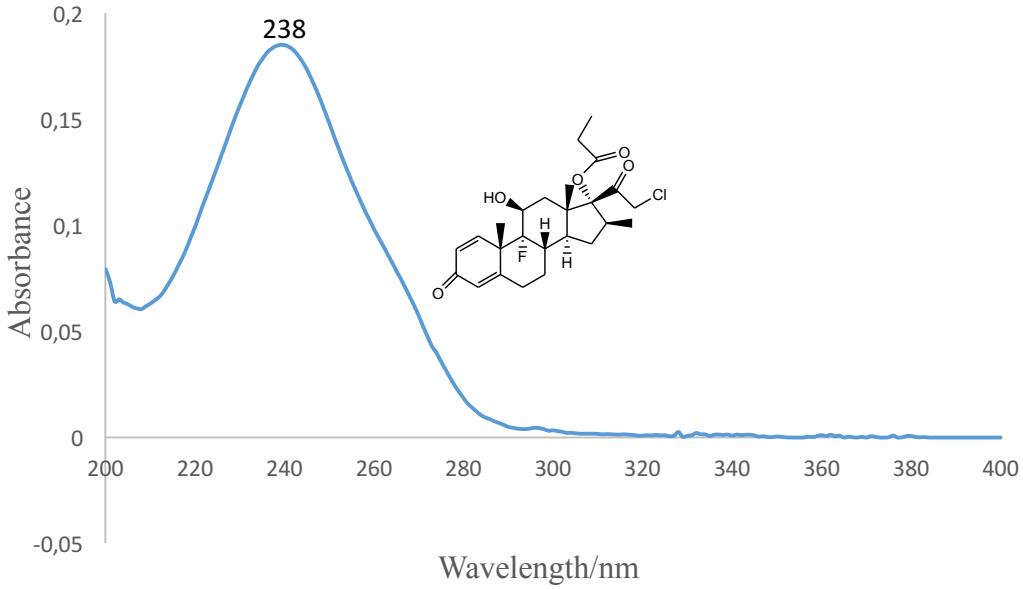


Figure 2.5: UV spectrum of 5 mg L^{-1} clobetasol propionate (CP) dissolved in methanol.

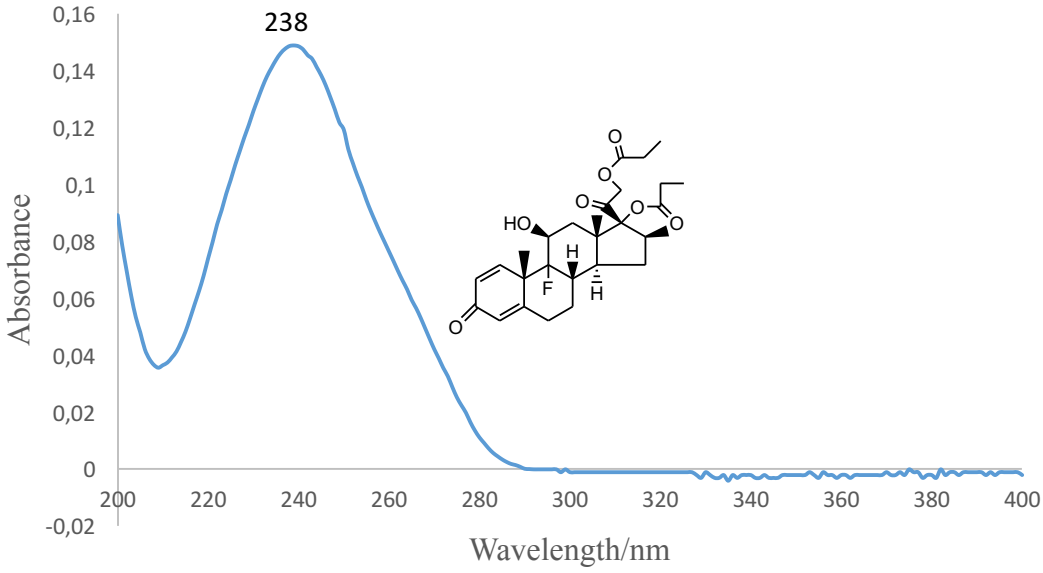


Figure 2.6: UV spectrum of 5 mg L^{-1} betamethasone dipropionate (BD) dissolved in methanol.

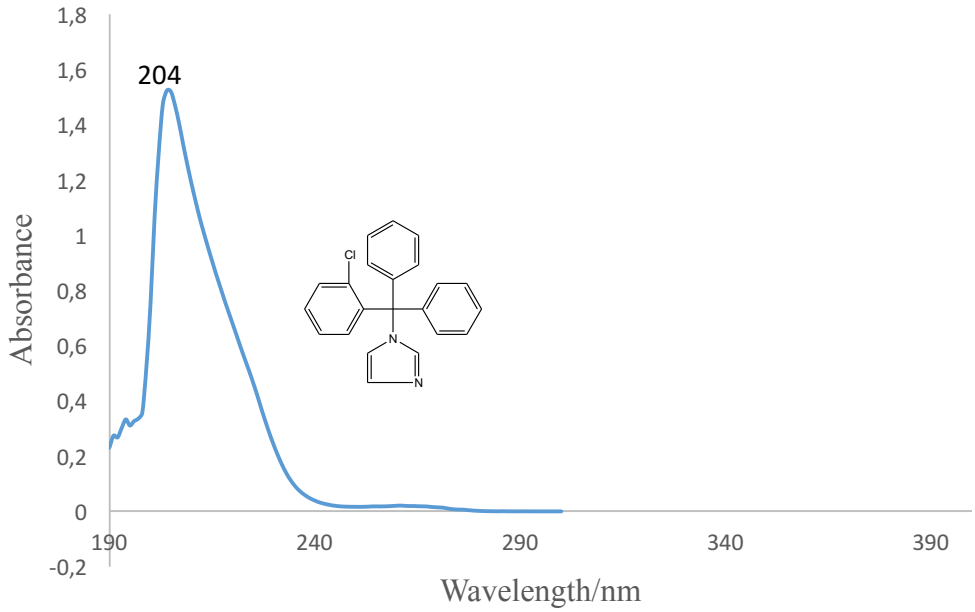


Figure 2.7: UV spectrum of 10 mg L^{-1} clotrimazole (CT) dissolved in methanol.

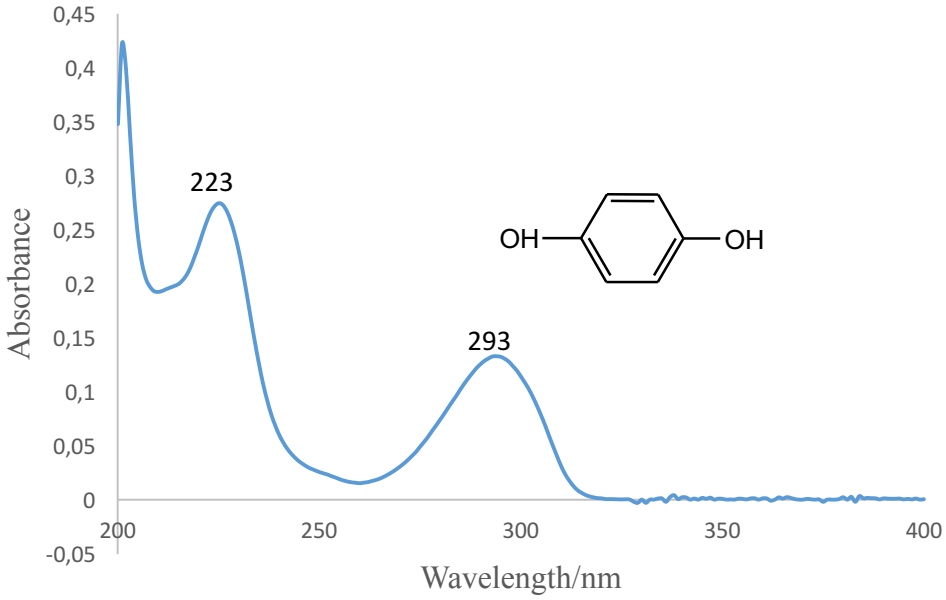


Figure 2.8: UV spectrum of 5 mg L^{-1} hydroquinone (HQ) dissolved in methanol.

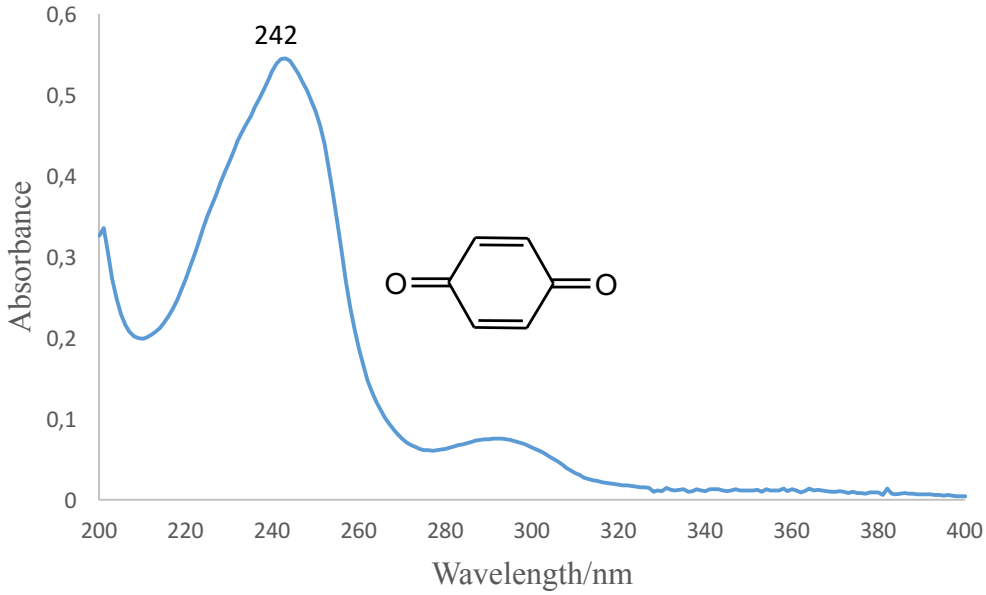


Figure 2.9: UV spectrum of 5 mg L^{-1} benzoquinone (BQ) dissolved in methanol.

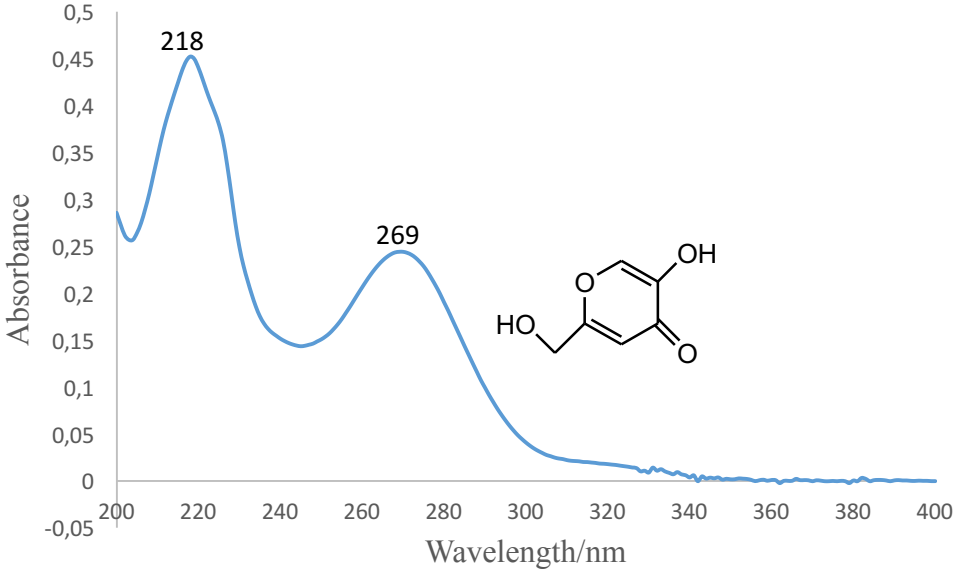


Figure 2.10: UV spectrum of 5 mg L^{-1} kojic acid (KA) dissolved in methanol.

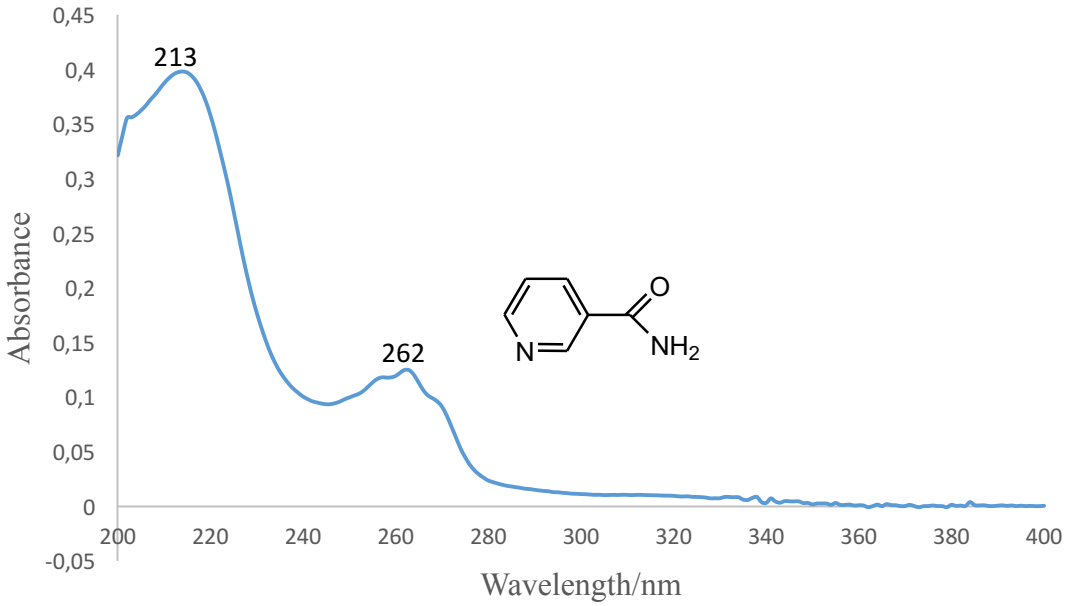


Figure 2.11: UV spectrum of 5 mg L^{-1} niacinamide (NC) dissolved in methanol.

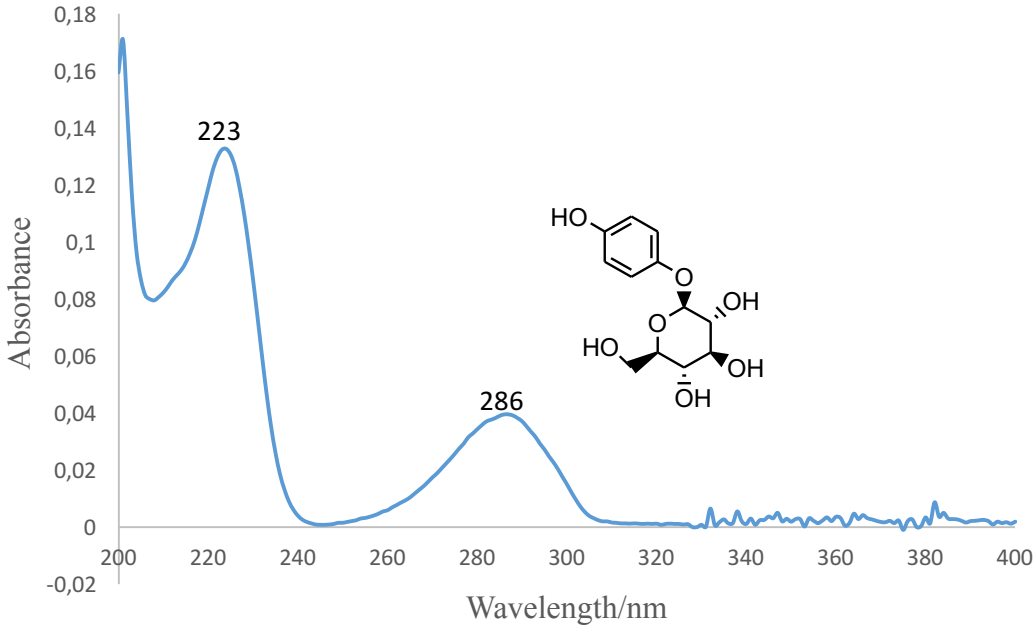


Figure 2.12: UV spectrum of 5 mg L^{-1} arbutin (ARB) dissolved in methanol.

Table 2.2: The wavelength of maximum absorption for each skin-lightening agent obtained by UV-visible spectrophotometry.

Active ingredients	Wavelength of maximum absorption/nm
Clobetasol propionate (CP)	238
Betamethasone dipropionate (BD)	238
Clotrimazole (CT)	204
Hydroquinone (HQ)	293
Benzoquinone (BQ)	242
Kojic acid (KA)	269
Niacinamide (NC)	262
Arbutin (ARB)	286

2.3.4 HPLC method development

The selection of the appropriate HPLC column and mobile phase, and optimisation of other operational parameters are what defines the method development. The process is typically influenced by the nature of the sample and analytes involved.

However, certain precautions need to be taken during the HPLC method development so as to consistently obtain reproducible results. This includes the use of HPLC-grade solvents and ultrapure water obtained from a Milli-Q unit for preparation of all solutions including the mobile phase. The mobile phase is one of the most important factors in HPLC method development and if it contains water it should be prepared fresh daily. It must be filtered through Millipore 0.45 μm HV organic aqueous filters for removal of any particulates before degassing with high purity helium at a rate of 100 mL min^{-1} for 5 minutes prior to use. The process of degassing serves to prevent a change in PDA signal to noise ratio caused by light scattering which can be due to air bubbles in the eluent. When using acidic or basic mobile phases, the column must be confirmed to be compatible with the pH range and should be immediately flushed out thoroughly after use with the appropriate solvent so as to avoid precipitation of salts that could damage and score the moving parts of the pump.

In order to clear the entire system lines of air bubbles and previously used solvent, the HPLC pumps should be left over a long time to pump at a rate of 0.2 ml min^{-1} with the mobile phase of interest. This is referred to as purging and is done without the column so as to avoid its contamination. Before use, the column should be equilibrated by allowing at least ten column volumes of mobile phase to pass through the column with the pressure of the pumps not exceeding the maximum pressure allowed for the instrument. This reduces the problems of pressure fluctuations and noisy baseline which can result in inconsistent retention times. The lamp of the PDA detector should be warmed-up for a period of 30 minutes before use.

In order to avoid the introduction of any particulate matter onto the column all samples must be filtered through 0.45 μm Millex HV solvent resistant syringe filters. Carry-over of samples between injections is checked with blank injections in between samples.

2.3.4.1 Determination of optimum separation method

Separation in HPLC is dependent upon the physical and chemical interactions between the analyte and the mobile and stationary phases. Each of these factors may be altered in order to change or optimise the selectivity of an HPLC separation.

To achieve optimum separation by isocratic elution, the studied SLAs were grouped into two so as to avoid co-elution. The first group consisted mainly of steroid compounds such as clobetasol propionate, betamethasone dipropoinate and clotrimazole. The test separations were performed on the mixed standard solutions on a Brownlee analytical C18 (100 × 4.6 mm, 3 μm) column. Several mobile phases were tested in order to find the best separation. The mobile phases included a mixture of methanol and water (50:50, v/v), methanol and water (60:40 v/v), methanol and 0.2% acetic acid in water (70:30, v/v), and methanol and water (80:20, v/v). The mobile phase flow rate was altered between 0.5, 0.8, and 1.0 mL min^{-1} while the injection volume was 10 μL. The column temperature was kept at ambient throughout.

The second group of SLAs, which included hydroquinone, benzoquinone, kojic acid, niacinamide and arbutin, were separated under different liquid chromatographic conditions since the method chosen for the first group of SLAs proved unsuccessful for

these analytes. Just like for the first group of SLAs, trial separation experiments were done by using pure standard solutions. Here the stationary phase used was an SGE C18 (250 × 4.6 mm, 5 µm) column, as it is known that a change in column is one of the most popular ways to alter the selectivity of a separation. The mobile phases which were tried included a mixture of methanol and 0.05 M KH_2PO_4 at a pH of 2.5 (70:30, v/v), methanol and 0.05 M $K_2HPO_4.3H_2O$ at a pH of 3.0 (80:20, v/v), methanol and 0.2% acetic acid in water (90:10, v/v), and 0.5% orthophosphoric acid in methanol and 0.5% orthophosphoric acid in water (5:95, v/v). The mobile phase flow rate was varied between 0.3, 0.5, 0.8, 1.0, and 1.2 mL min^{-1} and the injection volume of the studied analytes was 10 µL. The column temperature was varied between 25 °C and 35 °C. The result of these various trials are presented and discussed in Section 3.1.3.

2.3.4.2 Determination of optimum extraction method

The extraction of SLAs in the studied SLPs is an important factor for their quantitative analysis. The extraction method must be good enough to extract the maximum amount of SLAs in the SLPs. This required a preliminary extraction test so as to determine the best solvent to be used. Four solvents were tested as the extractant, namely, methanol, acetonitrile, 50% methanol in water, and a mixture of 50% acetonitrile and 50% methanol. The extraction method for the sample preparation was studied with eight SLA standards and the analysis was done by HPLC with the columns described in Section 2.3.4.1 for the two groups of SLAs.

In order to optimise extraction of SLAs from the cream formulations, 0.05 g of a SLP containing none of the studied SLAs was weighed into a glass flask, after which a known amount of a mixture containing the same concentration of each of the SLAs was immediately added and then diluted with 4 mL of the extraction solvent. The solution was stirred for a few minutes to allow for proper mixing before being placed in an ultrasonic water bath at 40 °C for 20 minutes. After ultrasonication, the solution was allowed to cool down and was then quantitatively transferred into a 10 mL volumetric flask and made up to the mark with the extractant. This method was repeated for each of the four extractants on both groups of SLAs. The resulting solutions were analysed by HPLC with the folowing chromatographic conditions: a Brownlee analytical C18 (100 ×

4.6 mm, 3 μm) column, mobile phase of methanol and water (80:20, v/v), injection volume of 10 μL, flow rate of 0.8 mL min^{-1}, and ambient column temperature for the first group of SLAs and an SGE C18 (250 × 4.6mm, 5 μm) column, mobile phase of 0.5% orthophosphoric acid in methanol and 0.5% orthophosphoric acid in water (5:95, v/v), injection volume of 10 μL, flow rate of 1.0 mL min^{-1} and a column temperature of 35 °C for the second group of SLAs. The results of the extraction test are presented and discussed in Section 3.1.4.

2.3.4.3 Standard solution preparation and analysis

A 1000 mg L^{-1} standard stock solution was prepared for each of the SLAs by dissolving 0.025 g of the pure materials with methanol in 25 mL volumetric flasks. The solutions were then placed in an ultrasonic bath for 10 minutes so as to completely solubilise the solutes present in them. The solutions were subsequently made up to the mark with methanol. For each SLA, the standard stock solutions were prepared in triplicate, from which a 100 mg L^{-1} multi-standard working stock solution was prepared for clobetasol propionate, betamethasone dipropionate, clotrimazole, and a 400 mg L^{-1} multi-standard working stock solution for hydroquinone, benzoquinone, kojic acid and niacinamide. In the case of arbutin, a 100 mg L^{-1} standard working stock solution was prepared separately as it always co-elutes with kojic acid.

From these working stock solutions of standards, another set of dilute working standard solutions was prepared by serial dilution with methanol. For clobetasol propionate, betamethasone propionate and clotrimazole, eight different concentrations of working standard solutions were prepared in the range of 0.2 – 60.0 mg L^{-1}. In the case of hydroquinone, benzoquinone, kojic acid, niacinamide and arbutin seven different concentrations of working standard solutions were prepared in the range of 0.5 – 400 mg L^{-1}, 0.2 – 20 mg L^{-1}, 0.5 – 90 mg L^{-1}, 0.8 – 110 mg L^{-1}, and 1.0 – 20 mg L^{-1} respectively. Aliquots of each of the standard solutions were subjected to HPLC analysis so as to generate calibration curves for quantification of the SLAs.

Certain chromatographic conditions were set during the analysis. The autosampler was set to perform three injections per standard solution, with a blank injection of methanol

in between each injection so as to avoid any carry-over from the previous injection into the column. The injection volume was 10 µL throughout and the flow rate was set at 0.8 mL min^{-1} for the first group of SLAs and 1.0 mL min^{-1} for the second group of SLAs. The column temperature was room temperature for the first group of SLAs and 35 °C for the second group of SLAs. Chromatograms were collected at detection wavelengths of 225 nm, 240 nm, 270 nm and 289 nm so as to observe all SLAs optimally and the maximum run time was 20 minutes.

2.3.4.4 Sample preparation and analysis

A mass of 0.05 g of each SLP was accurately weighed into a glass flask in which 4 ml of methanol was added and properly shaken for 5 minutes. The flask was then placed in an ultrasonic water bath set at 40 °C for 20 minutes. After ultrasonication, the sample was allowed to cool to room temperature and then quantitatively transferred into a 10 mL volumetric flask and made up to volume with methanol. The sample was filtered through a 0.45 µm Millipore syringe filter to remove all particulate matter that could clog the column before being transferred into the HPLC injection vials. Three replicates were prepared for each of the SLPs for HPLC analysis. The analysis was carried out by using the same precautions and chromatographic conditions as described in Section 2.3.4.3.

Both samples and standard solutions were analysed on a Brownlee analytical C18 (100 × 4.6 mm, 3 µm) column for the first group of SLAs and a SGE C18 (250 × 4.6 mm, 5 µm) column for the second group of SLAs with a mobile phase composed of methanol : water (80:20, v/v) and 0.5% orthophosphoric acid in methanol : 0.5% orthophosphoric acid in water (5:95, v/v) for the first and second groups of SLAs respectively.

All HPLC analyses were carried out on a Shimadzu Prominence $UFLC_{XR}$ instrument (see Figure 2.13) (Shimadzu Corporation, Japan) with the following components: a DGU-$20A_3$ degasser, LC-20AD XR binary pumps, SIL-20A XR autosampler, SPD-M20A diode array detector, CTO-20A column oven, CBM-20A communicator bus module, FRC-10A fraction collector and LC/Lab solution system software.

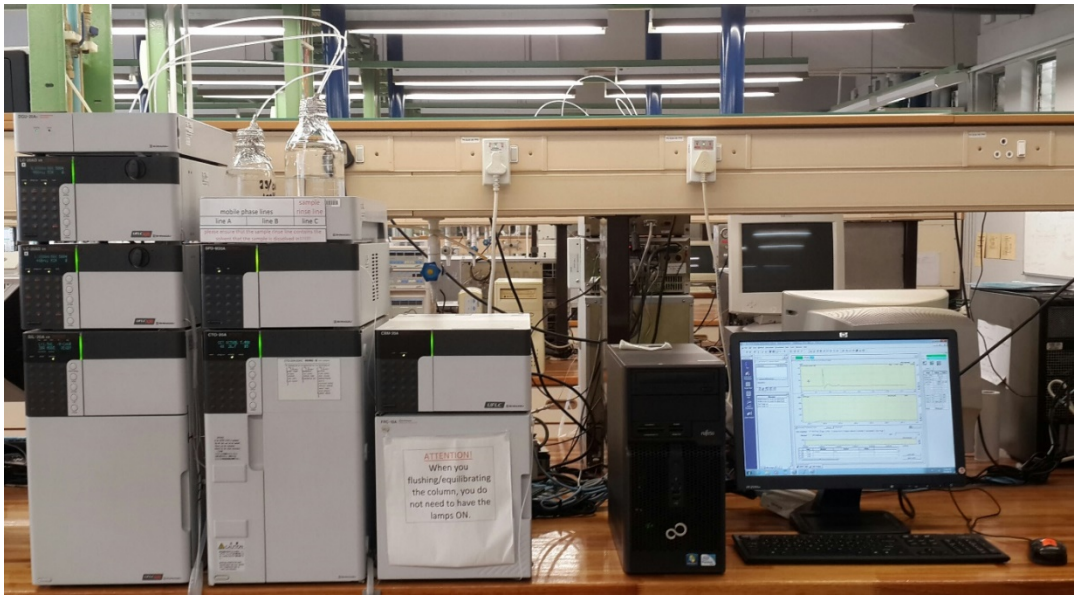


Figure 2.13: A photograph of the Shimadzu Prominence UFLC$_{XR}$ instrument used for the determination skin-lightening agents in this study.

2.3.4.5 Recovery analysis

Recoveries were performed in order to determine the accuracy of the methods developed. This was done by spiking samples with a known amount of the SLA standards. The samples used were previously analysed to ensure that they did not contain any of the SLAs under investigation. Two spiked solutions of different concentration were prepared for each of the studied SLAs. This was done by pipetting 1 mL and 5 mL of clobetasol propionate, 1 mL and 5 mL of betamethasone propionate, 1 mL and 5 mL of clotrimazole, 1 mL and 2 mL of hydroquinone, 1.5 mL and 2 mL of benzoquinone, 0.8 mL and 2 mL of kojic acid, 1 mL and 2 mL of arbutin, and 1.5 mL and 2 mL of niacinamide from a 100 mg L^{-1} of their respective standard stock solutions to a mass of 0.05 g of the SLA-free sample and made up to volume with methanol in a 10 mL volumetric flask. The spiked sample solutions were then shaken vigorously for 5 minutes and placed in an ultrasonic water bath at 40°C for 20 minutes. After this, the spiked sample solutions were allowed to cool and filtered through a 0.45 µm Millipore syringe filter into an HPLC injection vial for analysis. The entire procedure was performed in triplicate and analyses were carried out following the methods described in Sections 2.3.4.3 and 2.3.4.4. The results obtained from the recovery analysis are presented and discussed in Section 3.2.2.

2.3.4.6 Intra-day and inter-day analysis

Intra-day and inter-day analyses were carried out in order to determine the precision of the method. This was done by a repeated analysis of the studied SLA standards. Three different standard solutions were prepared for each of the SLAs, which included 2, 10, and 20 mg L^{-1} for hydroquinone; 2, 8, and 20 mg L^{-1} for benzoquinone; 1, 10, and 20 mg L^{-1} for kojic acid; 5, 10, and 20 mg L^{-1} for niacinamide; 2, 12, and 20 mg L^{-1} for arbutin and the same concentrations of 1, 4, and 8 mg L^{-1} for clobetasol propionate, betamethasone dipropionate, and clotrimazole. The entire procedure was completed in triplicate. The HPLC methods described in Sections 2.3.4.3 and 2.3.4.4 were used to analyse the standards.

The intra-day analysis was done by repeated analysis of the three standards three times a day over a time interval of three hours, while the inter-day analysis was done by repeated analysis of the three standards over a period of 24 hours for three consecutive days. The results of the intra-day and inter-day analyses are given and discussed in Section 3.2.3.

2.4 Analysis of heavy metal content

The qualitative and quantitative analysis of heavy metals in various types of samples has been carried out by using different analytical techniques. These were discussed in Section 1.9. Usually, the choice of technique is dependent on cost, run-time, sensitivity (LOD), physical state of the matrix and instrument availability.

In the present study, ICP-OES was employed as it was the best available option at our disposal. This technique allows multi-element determination and also permits the scanning of liquid samples in order to obtain overall information on the inorganic composition of the sample under study. The technique involves short-run times, has high sensitivity and is relatively free from complex matrices. For mercury determination, cold vapour atomic absorption spectroscopy (CV-AAS) was used, since mercury is volatile.

2.4.1 Extraction of heavy metals

An acid digestion procedure was employed in this study for the extraction of heavy metals in the studied SLPs. This was achieved by dissolving the samples in a mixture of acids and heating until complete decomposition of the matrix, while avoiding loss or contamination of the analytes before introduction into the ICP-OES or CV-AAS in liquid form for analysis.

Open acid digestion and closed acid digestion are the two types of acid digestion procedure. Both are carried out with the aid of a wide variety of reagents. Aside from mineral acids, such as nitric acid, hydrochloric acid, nitro-hydrochloric acid, hydrofluoric acid, or sulfuric acid, other reagents, such as hydrogen peroxide, potassium peroxide sulfate, boric acid and many more, are also employed. However, the selection of specific reagents or the preparation of the reagent mixture depends on the sample to be digested.

The open acid digestion method is carried out either with a reflux system or in a beaker on a laboratory hot plate. This method is challenged with temperature limitations as a consequence of the solution boiling point and the risk of contamination from the air. A volatile element like mercury may be lost during the digestion process, leading to lower results. Generally, the quality of digestion is mostly unsatisfactory despite the long digestion times of 2 – 15 hours.

For the present study, the closed acid digestion method was used. With this method, the temperature limitation can be overcome by working with a closed pressure vessel since it allows temperatures in the range of 200 – 260 °C to be reached. This results in a dramatic increase in the reaction kinetics, allowing acid digestion to be carried out in a matter of 20 – 40 minutes if microwave heating is employed. However, one can conclude that temperature represents what is actually the most important reaction parameter. It is the ultimate determinant of digestion quality, but also results in a pressure increase in the vessel as the reaction between the acid and sample takes place, making it a potential safety hazard. Thus, the pressure must ultimately be closely monitored. Section 2.4.1.1 describes the closed acid digestion method used in this study with the help of a microwave digester.

2.4.1.1 Microwave-assisted digestion

Microwave-assisted digestion involves irradiation of the sample with microwave electromagnetic radiation of a high-frequency electric field to achieve fast heating of the sample through the direct absorption of microwave radiation. This allows for an extremely rapid, simultaneous heating of about 8-12 sample solutions which represents another advantage of microwave heating. Once the setpoint temperature is reached, the decomposition reaction of the sample solutions proceeds at the same rate.

As mentioned earlier, the combination of acids during digestion depends on the matrix of the sample. A combination of nitric acid, hydrochloric acid, and/or hydrofluoric acid has been reported to provide better recoveries for digestion of some cosmetics such as lipsticks, eye shadows and eyeliners in a closed system (187). Such a reagent mixture was not suitable for use for SLP digestion as it would explode (188). Thus, nitric acid alone was chosen for the digestion of SLPs in this study as it is known to be a strong oxidising agent.

A sample of each SLP of a mass of a 0.250 g was placed into a high-pressure resistant TFM-vessel to which 10 mL of conc. nitric acid was added. The samples were allowed to pre-digest for 30 minutes in the fume hood uncovered, after which the vessels were sealed and subjected to microwave irradiation in a Mars 6 microwave digestion system (see Figure 2.14). The temperature was gradually increased from room temperature to 200 °C in a time frame of 15 minutes before the vessels were held at this temperature for a period 20 minutes and then cooled down to ± 65 °C over a period of 15 minutes. After cooling to room temperature, the vessel modules were disassembled and both the lids and vessels were rinsed with double distilled water and the rinse solutions collected with the digest. The samples were subsequently filtered through a 11 μm grade one Whatman filter paper into 50 mL volumetric flasks and then made up to mark with double distilled water. Filtration of samples was necessary in order to avoid any residue that could clog the instrument lines or result in data fluctuation. Both the samples and blanks were digested in triplicate and the resulting solutions were stored in plastic bottles.

Figure 2.14: A photograph of the Mars 6 microwave digestion device and the XP-1500 TFM reaction vessels used for digestion of skin-lightening samples in this study.

2.4.2 Inductively coupled plasma-optical emission spectrometry

Inductively coupled plasma-optical emission spectrometry (ICP-OES) is one of the most powerful and popular analytical tools for the determination of metals in a variety of different sample matrices. The technique is based on the spontaneous emission of photons from atoms and ions that have been excited in a radio-frequency discharge. Thus, the wavelength of the photons emitted is used to identify the elements from which they originated. The total number of photons is directly proportional to the concentration of the originating element in the sample.

The ICP-OES instrument has two main parts: the torch, and the optical spectrometer (Figure 2.15). The ICP torch is made up of three concentric quartz glass tubes. Part of this quartz torch is surrounded by the work coil of the radio-frequency generator. Argon gas typically serves in creating the plasma. 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. This radio-frequency signal is created by the radio-frequency generator which is, effectively, a high power radio-transmitter driving the work coil the same way as a typical radio transmitter drives a transmitting antenna. Typical ICP-OES instruments run at either 27 or 40 MHz. 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 ionisation process. Once the plasma is "ignited", the Tesla unit is turned off. The argon gas is ionised in the intense electromagnetic field and flows in a particular rotationally symmetrical pattern towards the magnetic field of the radio-frequency coil. A stable, high-temperature plasma of about 7000 K is then generated as the result of the inelastic collisions created between the neutral argon atoms and the charged particles.

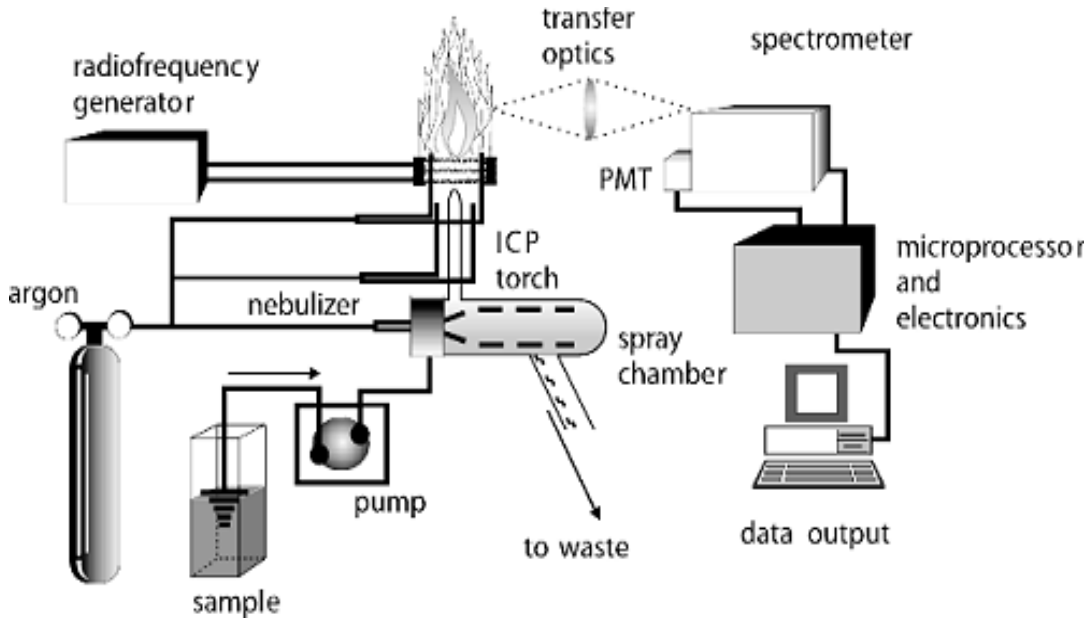


Figure 2.15: Schematic of an ICP-OES instrument (189).

A peristaltic pump delivers an aqueous or organic sample into the analytical nebuliser where the sample is changed into mist and introduced directly inside the plasma discharge. The sample components immediately collide with the electrons and charged ions in the plasma and are broken down into charged ions. The various molecules break up into their respective atoms, which then lose electrons and recombine repeatedly in the plasma, giving off radiation at the characteristic wavelengths of the elements involved.

In this work a PerkinElmer Optima 5300 DV simultaneous ICP-OES spectrometer (see Figure 2.16) was used for the determination of heavy metals in SLP samples.

2.4.2.1 Preparation of standard solutions of heavy metals

All standard solutions were prepared from 1000 mg L^{-1} analytical grade stock solutions of the respective metals. From the stock solutions, a multi-element working standard solution of 5 mg L^{-1} for each component was prepared for Al, Cr, Cu, Fe, Mn, Ni, Pb, and Zn in a 100 mL volumetric flask. This was done by first placing 10 mL of concentrated nitric acid in the volumetric flask and then transferring an aliquot of 0.5 mL of each metal ion into the flask before making it up to the mark with double distilled water. The essence of the nitric acid was to minimise matrix effects. From here, further dilution was made with double distilled water to prepare nine different concentrations for each of the metals and these more dilute solutions were used for the construction of calibration curves. The concentration range was 0.01 – 1.6 mg L^{-1} for all the metals.

Standard solutions were not prepared for As, Cd and Co since the rough scan run on all the samples did not indicate their presence.

2.4.2.2 Determination of heavy metals

All samples were analysed for As, Al, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, and Zn. Certain operational conditions were set on the ICP-OES instrument before the analysis began, which included generator power, 1000 W; plasma gas flow rate, 12 L min^{-1}; nebuliser pressure, 3 bars; and sample flow rate, 1 mL min^{-1}.

A scan run was initially performed on all the sample solutions to confirm the presence or absence of the targeted elements. This was helpful in knowing what metal standards to prepare and their concentration ranges. Also, three different analytical wavelengths were preselected for each element out of which one of these wavelengths with high intensity, low detection limits and minimal impact on the background of an emission line were chosen for each element for further quantitative determinations. The wavelengths chosen for use in this study for each metal ion analysed are listed in Table 2.3. To begin the analysis, standard solutions and samples were allowed to aspirate for 60 seconds before sampling and the instrument was set for sampling each solution five times and the average was recorded.

Figure 2.16: A photograph of the PerkinElmer Optical 5300 DV simultaneous ICP-OES device used for the determination of heavy metals in this study.

Table 2.3: Emission wavelengths used for analysis of heavy metals by ICP-OES.

Element	**Wavelength/nm**
Arsenic (As)	188.979
Aluminium (Al)	396.153
Cadmium (Cd)	214.440
Cobalt (Co)	228.6116
Chromium (Cr)	267.716
Copper (Cu)	324.752
Iron (Fe)	259.939
Manganese (Mn)	257.610
Nickel (Ni)	231.604
Lead (Pb)	217.000
Zinc (Zn)	213.857

2.4.3 Cold-vapour atomic absorption spectrometry

Atomic absorption spectrometry is an analytical technique used to determine the presence and concentration of metals in liquid samples. Samples are atomised by a flame or graphite furnace and light of a specific wavelength, from a hollow cathode lamp or an electrodeless discharge lamp, is passed through the atomic vapour of the element of interest, and measurement is made of the attenuation of the intensity of the light as a result of absorption by free ground-state atoms of the element in the sample. In the case of mercury, free mercury atoms can exist at room temperature and consequently this metal can be analysed by atomic absorption spectrometry without employing a flame or graphite furnace. The technique used for the analysis of mercury is what is referred to as cold-vapour atomic absorption spectrometry (CV-AAS).

In the cold vapour technique, mercury is reduced chemically to the free atomic state with a strong reducing agent solution like stannous chloride ($SnCl_2$) or sodium borohydride ($NaBH_4$) in a closed reaction system. The reaction quantitatively releases mercury from the sample solution and the mercury atoms are carried by a stream of air, nitrogen or argon through a quartz absorption cell placed in the light path of the spectrometer. A manually operated accessory, such as the MHS 15 mercury hydride system, is required for vapour generation when carrying out mercury analysis with an atomic absorption spectrometer. The pneumatic system of the MHS 15 accessory is described by Figure 2.17. The MHS 15 unit is composed of a reaction assembly (a reaction flask, reservoir bottle and carrier gas pipelines) and a quartz tube atomiser (QTA) assembly. Hence, the efficacy of this technique is dependent upon the proper connection of the MHS 15 unit to the gas supply and the spectrometer.

When $SnCl_2$ is used as the reductant during the analysis of mercury, the inert gas from the pressure reducer comes via restrictor F_2 and line *b* to the changeover valve, and then travels via line *e* to the reaction flask. The gas pressure applied by means of ports *1* to *4* and then through line *g*, forces the transfer of $SnCl_2$ reductant solution through line *h* into the reaction flask. The mercury vapour is then transported by the combined gas streams through the transfer tube *f* to the QTA, where its atomic absorption is measured.

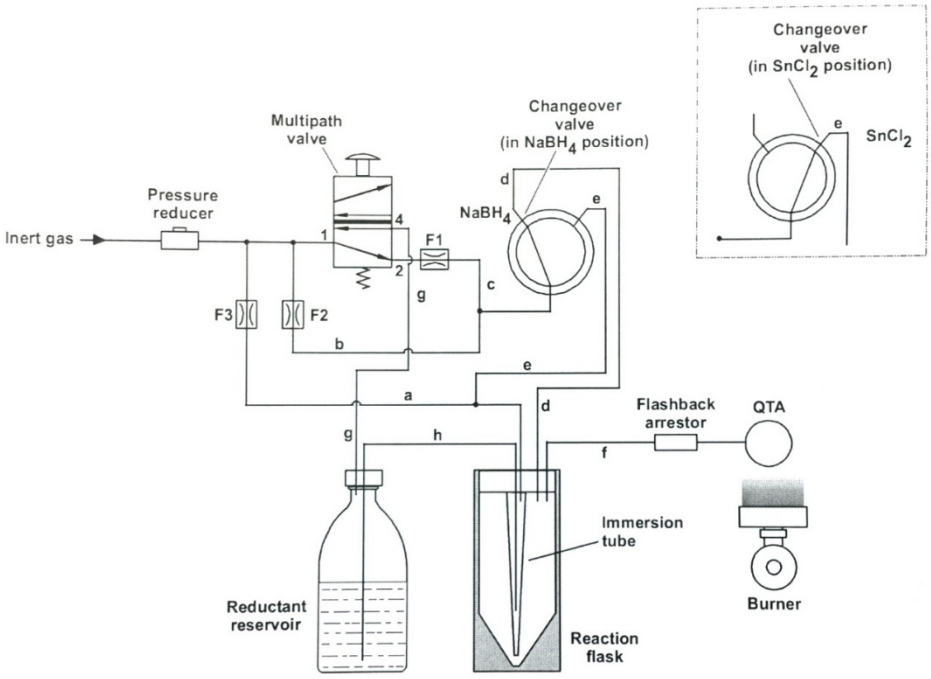


Figure 2.17: Schematic of the PerkinElmer MHS 15 mercury hydride pneumatic system (190).

2.4.3.1 Preparation of reagents and standard solutions

A 5% (m/v) $SnCl_2 \cdot 2H_2O$ (reductant solution) solution was prepared by dissolving 50 g of the salt in 100 mL of hot conc. HCl and then diluted to 1 L with double distilled water. A dilute nitric acid solution of 1.5% was also prepared which served as the diluent throughout the analysis. 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 double distilled water and the solution was diluted to 100 mL in a volumetric flask with double distilled water. After this a 1 mg L^{-1} mercury working standard solution was prepared. This was done by pipetting a volume of 100 µL of the 100 mg L^{-1} stock solution into a 100 mL volumetric flask and then diluted to the mark with double distilled water. From here, lower concentrations of mercury working standards were prepared for construction of calibration curves as described in Section 2.4.3.2.

2.4.3.2 Determination of mercury

Mercury determination was carried out with a PerkinElmer Analyst 200 atomic absorption spectrometer fitted with a mercury (Hg) lamp at a wavelength of 253.7 nm and a PerkinElmer MHS 15 system was also connected to the instrument for vapour generation. The instrument set-up is shown in Figure 2.18.

Figure 2.18: A photograph of the PerkinElmer MHS 15 mercury hydride system connected to the PerkinElmer Analyst 200 atomic absorption spectrometer instrument and gas supply during mercury determination in this study.

During analysis, 10 mL of the diluent (1.5% HNO_3) was transferred into the reaction flask, to which aliquots of 100, 200, 300, 400, 500, 600, 700, and 800 µL of a 1 mg L^{-1} mercury working stock solution were added sequentially to generate mercury calibration curves by mass. These aliquots correspond to 100, 200, 300, 400, 500, 600, 700, and 800 ng of mercury respectively. Once the reagent reservoir and reaction flask were attached to the reactor assembly, the extractor was then switched on and the argon gas was allowed to flow through the immersion tube placed into the reaction flask. While the plunger (multipath valve) is manually depressed and held between 20 to 30 seconds, an argon stream transports the reducing agent ($SnCl_2$) from the reagent reservoir into the reaction

flask to reduce mercury either in the standard or sample resulting in mercury vapour which is driven out through the immersion tube to the QTA. The special conical form of the immersion tube and reaction flask provides excellent mixing of the standard or sample and $SnCl_2$, guaranteeing a spontaneous reaction. As the mercury vapour passes into the QTA, the measured absorbance gradually increases and then decreases as the mercury diminishes. It was ensured that the reaction flask and the outside of the immersion tube were rinsed with the diluent after every single measurement. The maximum absorbance detected for each measurement was recorded and the absorbance for the standard solutions was used to plot the calibration curve. Both standard solutions and digested samples were analysed in triplicate.

2.4.4 Validation of heavy metal and mercury analyses

The accuracy of both the analytical techniques, namely, ICP-OES and CV-AAS, was verified by using spike recovery methods. A known amount of each element under study was introduced into an already analysed sample and the sample reanalyzed. Two spiked solutions were prepared for each of the metals by spiking a sample mass of 0.250 g with aliquots from two different standard solutions as indicated in Table 2.4. Thereafter, 10 mL of concentrated nitric acid was added to the solution and the mixture allowed to predigest. The digest was completely solubilized by the microwave procedure described in Section 2.4.1.1. After microwave-assisted digestion, the digest was filtered, diluted into a 50 mL volumetric flask with double distilled water and analysed according to the analysis conditions of the parent sample.

Table 2.4: Details of the solution preparation in 50 mL volumetric flasks for validation of heavy metal and mercury analyses.

Metal	**Standard solution/mg L^{-1}**	**Aliquot pipetted from standard solution/mL**	**Spiked concentration in sample solution/mg L^{-1}**
Al	10	0.25	0.05
	20	3.0	1.20
Cr	10	0.1	0.02
	20	0.5	0.20
Cu	10	0.1	0.02
	20	0.175	0.07
Fe	10	0.4	0.08
	20	2.75	1.10
Mn	10	0.1	0.02
	20	0.1	0.04
Ni	10	0.1	0.02
	20	0.75	0.30
Pb	10	0.25	0.05
	20	2.5	1.00
Zn	10	0.4	0.08
	20	3.75	1.50
Hg	1.0	1.25	250 ng
	1.0	2.25	450 ng

2.5 Data analysis

The data obtained in this study were evaluated by using the Microsoft Excel Analysis Tool Pak. Calibration curves were determined by using the linear least-squares regression equation given as:

$$y = m\varkappa + c \qquad (2.1)$$

where y is the HPLC peak area or corrected intensity or absorbance, $\varkappa$ is the concentration of analyte, m is the slope, and c is the intercept. By assuming that c = 0 at the 95% confidence level, only m needs to be determined to establish the calibration line taking into account the error in each y_o c and m, and thereby to produce an estimated standard deviation $SD\varkappa_o$ for the point estimate $\varkappa_o = (y_o - c)/m$. The correlation coefficient (R^2) measures the degree of linear association between the peak area, or corrected intensity or absorbance, and the concentration of each analyte.

The slope and standard error (SE) of the calibration line (for each analyte) was used to calculate the limit of detection (LOD) and the limit of quantitation (LOQ). This is given by the equation:

$$\text{LOD} = {}^{3SE}/_{m} \qquad (2.2)$$

(*SE* is the standard error of the slope and m is the slope of the calibration line) and the limit of quantitation was calculated as:

$$\text{LOQ} = {}^{10SE}/_{m} \qquad (2.3)$$

The accuracy of the method developed is expressed in terms of the percent recovery and estimated by using equation (2.4):

$$\% \text{ Recovery} = \frac{\text{mean of found concentration}}{\text{spiked concentration}} \times 100 \qquad (2.4)$$

The precision of the method developed is expressed in terms of percent relative standard deviation and estimated by using the following equations:

$$\% \text{ R.S.D.} = \frac{\text{standard deviation}}{\text{mean concentration}} \times 100 \qquad (2.5)$$

$$\% \text{ Accuracy} = \frac{\text{mean of found concentration}}{\text{theoretical amount}} \times 100 \qquad (2.6)$$

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

$$\% = \frac{C_s \times V_s \times Df}{W_s} \times 100 \qquad (2.7)$$

where *Cs* is the concentration of an analyte of interest from the standard curve (mg L^{-1}), *Vs* is the volume of sample extract, *Df* is the dilution factor (where applicable), and *Ws* is the mass of the cream sample.

The risk of human exposure to heavy metals contained in the SLPs was estimated by using the uncertainty factor called the Margin of Safety (MoS) in accordance with the Scientific Committee on Consumer Safety (SCCS) (191). This is given by the equation:

$$\text{MoS} = \frac{NO(A)EL}{SED} \qquad (2.8)$$

NO(A)EL is the no-observed-adverse-effect-level of the contaminant and can be estimated by using the formula:

$$\text{NO(A)EL} = \text{RFD} \times \text{UF} \times \text{MF} \qquad (2.9)$$

UF and MF are the uncertainty factor and the modifying factor with default values of 100 and 1 respectively. RFD is the oral reference dose, which is an estimate of daily exposure of the metals to the human population, including sensitive sub-groups. The RFD values (in mg g^{-1} day^{-1}) for each metal are as follows: Al (1.0×10^{-1}) (192), Cr (3.0×10^{-3}), Cu (4.0×10^{-2}), Fe (7.0×10^{-1}), Mn (1.4×10^{-1}), Ni (2.0×10^{-2}), Pb (4.0×10^{-3}), Zn (3.0×10^{-1}) (193), and Hg (5.0×10^{-4}) (194).

A minimum MoS value of 100 has been proposed by the World Health Organization (WHO) as acceptably safe for the product to be used on the human body (191).

SED is the systemic exposure dosage. Certain factors need to be considered in order to obtain information on the SED for cosmetics. This includes the amount of cosmetic applied to the body per day, the applied surface area, the concentration of the metal in the cosmetic under investigation, the dermal absorption of the particular contaminant and a human body weight value (191). Thus, SED can be estimated by using equation (2.10):

$$\text{SED (mg kg}^{-1}\text{ bw day}^{-1}) = \frac{DA_a \times SSA \times F}{BW} \times 10^{-3} \qquad (2.10)$$

DA_a is the dermal absorption reported as μg/cm^2. It is the amount of ingredient absorbed per square centimeter, resulting from an assay under an in-use mimicking condition. In case no dermal absorption data is available, 100% dermal absorption is used (191). DA_a can be estimated by using the equation:

$$DA_a = \frac{C_S \times AA}{SSA} \qquad (2.11)$$

Cs is the concentration of the particular metal in the product (mg kg^{-1}); AA is the amount of cosmetic product applied per day (7.82 g for body cream); SSA is the surface area of the skin onto which the cosmetic is applied (15670 cm^2 for body cream); F is the frequency of application (2.28/day for body cream); BW is the body weight (60 kg for default body weight) and 10^{-3} is the unit conversion factor (191).

The results obtained following the procedures described in this chapter are presented and discussed in Chapter 3.

Chapter 3

RESULTS AND DISCUSSION

The results of the experiments described in Chapter 2 are presented and discussed in this chapter. This chapter is divided into two main parts as described below.

The first part discusses the results obtained for the identification and quantification of the organic SLAs such as clobetasol propionate (CP), betamethasone propionate (BD), clotrimazole (CT), hydroquinone (HQ), benzoquinone (BQ), kojic acid (KA), niacinamide (NC), and arbutin (ARB) in the 35 Nigerian commercially available SLPs. The second part of the chapter present the results obtained for the heavy metal content of these same products and also provides an assessment of the possible exposure risk through the use of these products from the heavy metal content.

3.1 HPLC method development for the determination of organic skin-lightening agents

In order to determine the active organic ingredients in the studied samples, a suitable HPLC method was developed for the separation, identification, and quantification of the selected SLAs. The HPLC instrument (Shimadzu Prominence) used for the analysis consisted of a binary solvent manager, autosampler, and a PDA detector. Separation was performed in an isocratic mode with a Brownlee analytical C18 column (100 × 4.6 mm, 3 μm) and a SGE C18 column (250 × 4.6mm, 5 μm) for the two groups of SLAs as described in Section 2.3.4.1.

3.1.1 Optimum detection wavelengths

For skin-lightening active ingredients to be detected by an HPLC instrument equipped with a PDA detector, optimum detection wavelengths must be set. The UV spectra and the wavelength of maximum absorption for each of the SLAs were first obtained by means

of UV-visible spectrophotometry. The optimum wavelength for detection was selected based on the wavelength that gives the highest sensitivity. The wavelength of maximum absorption identified for each SLA was provided in Table 2.2. The UV spectrum for each SLA obtained from a UV-visible spectrophotometer (provided in Section 2.3.3.2) was used to confirm the UV spectra generated by the PDA detector in HPLC.

The PDA detector was set to acquire chromatograms at 225 nm for CT, 240 nm for CP, BD and BQ, 270 nm for KA and NC, and 289 nm for HQ and ARB, as their highest sensitivity was observed at or close to these wavelengths and these would enhance the quantitation of the SLAs. For CT its wavelength of maximum absorption of 204 nm could not be used since many other analytes as well as the solvent absorb close to this wavelength. Hence, four UV absorption wavelengths (225, 240, 270 and 289 nm) were selected for the SLA analysis in this study and the HPLC chromatograms were acquired simultaneously at these wavelengths with the PDA detector.

3.1.2 Identification of skin-lightening agents

Since each component has its own distinct UV-visible spectrum and retention time at which it elutes, identification of the SLAs in all samples was achieved by comparing the HPLC-UV-visible spectra and retention times of the pure analytes (standards) with those of the sample analytes. It must be borne in mind that there is a slight variability in the retention time for each of the SLAs as indicated in Table 3.1 but an acceptable range is 0.1 – 0.3 minutes.

Table 3.1: Retention times and detection wavelengths for the skin-lightening agents

Active ingredient	Retention time/min	Detection wavelength/nm
Clobetasol propionate	2.80-3.00	240
Betamethasone dipropionate	3.50-3.70	240
Clotrimazole	4.50-4.80	225
Hydroquinone	7.90-8.10	289
Benzoquinone	15.45-15.70	240
Kojic acid	6.15-6.30	270
Niacinamide	2.60-2.70	270
Arbutin	6.0-6.15	289

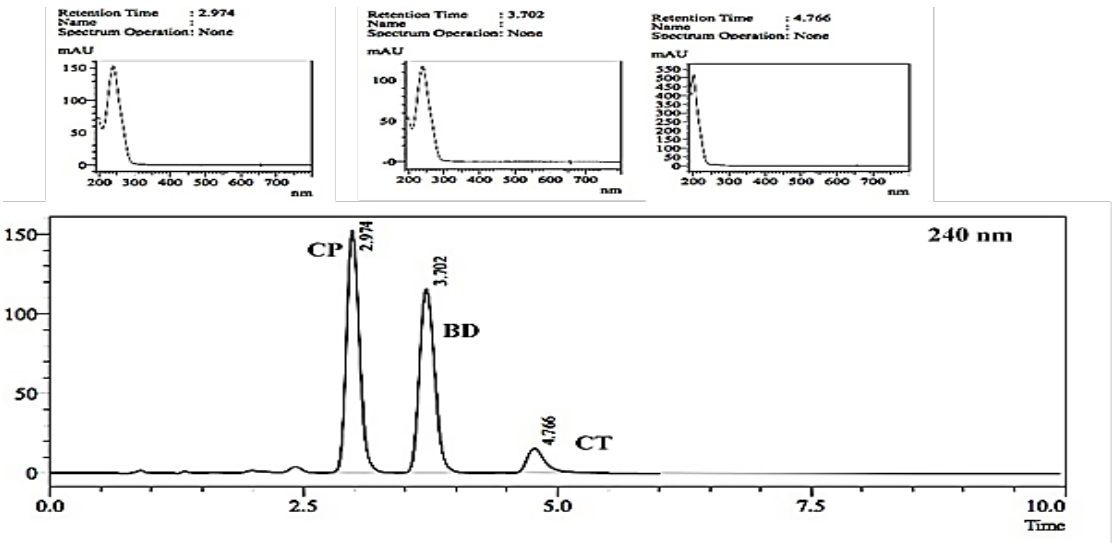
3.1.3 Optimum separation conditions

Obtaining optimum resolution and separation in a minimum time is the most important aspect in HPLC . This is achieved by first knowing the chemistry of the analytes in question, and also the use of the best mobile phase combination and stationary phase. Since most of the studied SLAs are relatively polar molecules, the use of polar solvents such as methanol and acetonitrile as constituents of the mobile phase and a non-polar stationary phase in a reversed phase HPLC separation seems appropriate. Therefore, in order to obtain a good separation for the simultaneous analysis of the studied SLAs, mobile phases with different compositions of methanol, acids, buffers and water, with flow rates varying between 0.3-1.2 mL min^{-1} on a Brownlee analytical C18 column (100 × 4.6 mm, 3 μm) and a SGE C18 column (250 × 4.6 mm, 5 μm) were tried in an isocratic mode.

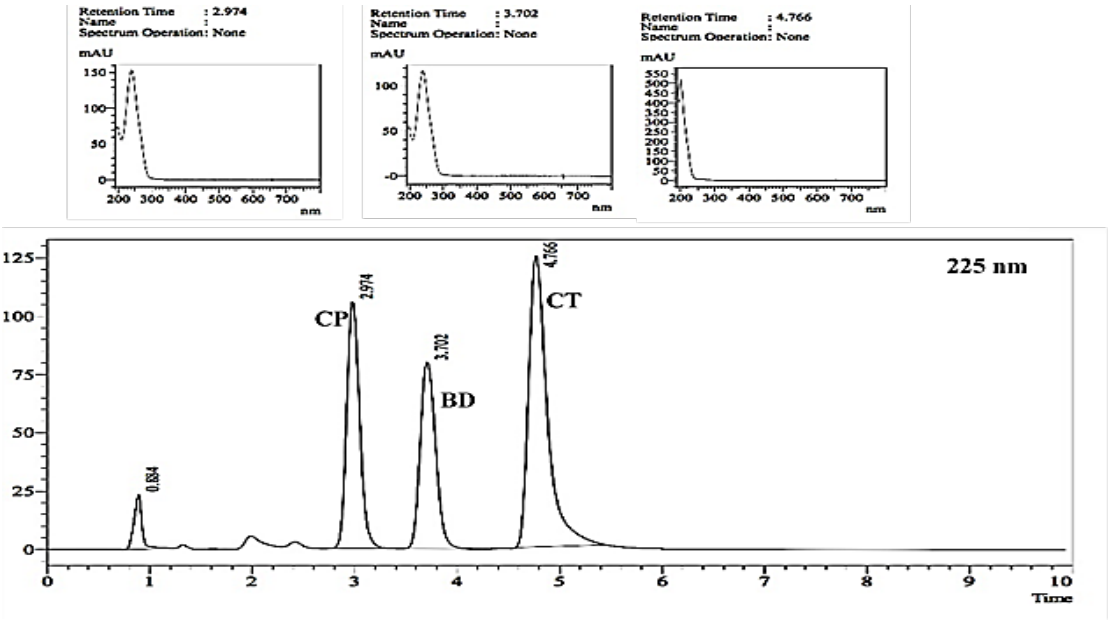
The pure standards of each SLA were first analysed individually to obtain the retention times. These were then mixed to prepare a multicomponent standard solution that could be used to check for proper resolution and separation of the compounds. Two multicomponent standard solutions were prepared. The first included steroid compounds, such as CP, BD and CT, and their separation was effected on a Brownlee analytical C18 column (100 × 4.6 mm, 3 μm). When a mobile phase consisting of either 50 or 60% (v/v)

methanol in water was used at flow rates of 0.5, 0.8 and 1.0 mL min^{-1}, all three SLAs were separated but the peak for CT was very broad. In an attempt to correct the CT broadened peak, 70% methanol in water containing 0.2% acetic acid at flow rates of 0.5, 0.8 and 1.0 mL min^{-1} was used. The introduction of acetic acid was meant to change the pH of the mobile phase which in turn would alter the degree of ionisation of some analytes by affecting their hydrophobicity. Nevertheless, it was observed that all three SLA peaks clustered together at flow rates of 0.8 and 1.0 mL min^{-1}. However, with a lower flow rate of 0.5 mL min^{-1}, all three SLA peaks were separated but the CT peak still remained broad. With the first group of SLA, it was generally observed that an increase in the percentage of methanol in the mobile phase caused the analytes to elute faster with well resolved peaks but the peaks co-eluted or broadened when the wrong flow rate was chosen. The three SLAs were finally well resolved and separated when 80% (v/v) methanol in water was used at a flow rate of 0.8 mL min^{-1}. The total run time was 10 minutes and the column temperature was kept at ambient. Figure 3.1 shows the optimum chromatogram for the separation of the first group of SLAs which includes CP, BD and CT. The first two chromatograms (A and B) shown in Figure 3.1 clearly show the effect of the detection wavelength, particularly for CT. The peak for CT is much more prominent when detected at 225 nm which is close to its wavelength of maximum absorption, namely 204 nm, than in the case of detection at 240 nm. The third chromatogram (C) shows an example of one of the steroid SLAs separated in one of the samples.

(A)



(B)



(C)

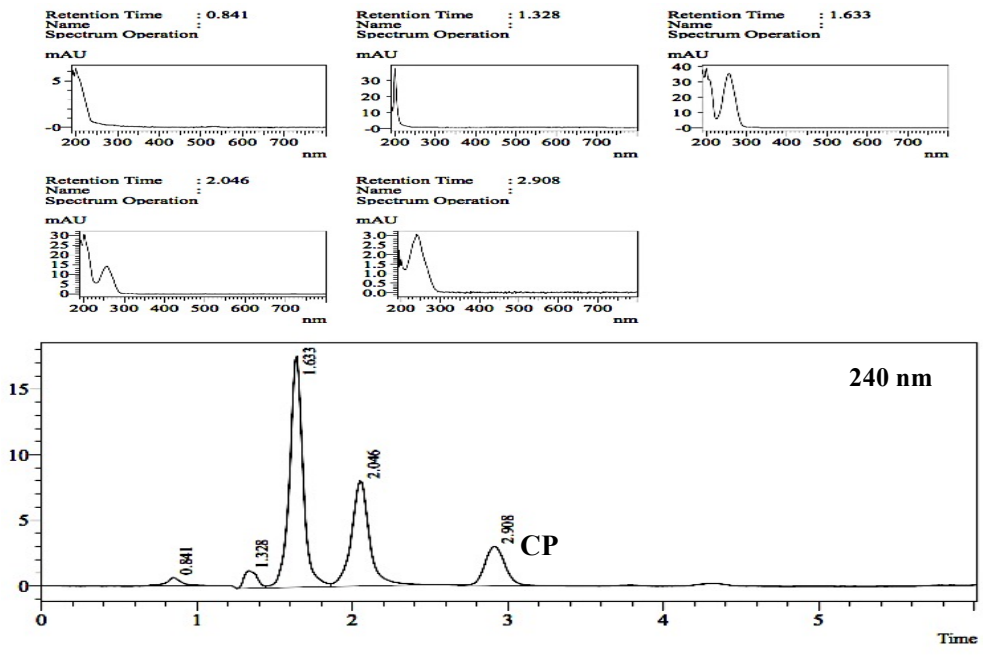
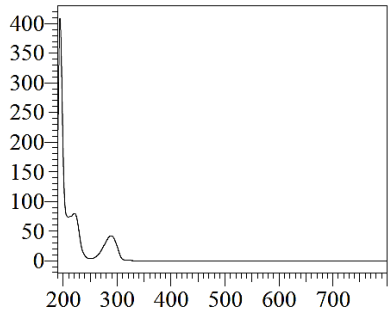
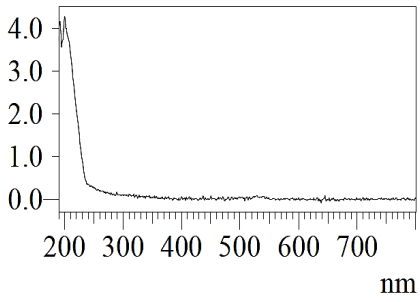


Figure 3.1: Chromatogram for the separation of active ingredients CP, BD and CT, obtained at detection wavelengths of (A) 240 nm, (B) 225 nm and (C) 240 nm. The chromatographic separation was achieved on a Brownlee analytical C18 column (100 × 4.6 mm, 3 μm), with a mobile phase of MeOH-H_2O 80:20 (v/v), an injection volume of 10 μL, a flow rate of 0.8 mL min^{-1}, and ambient column temperature.

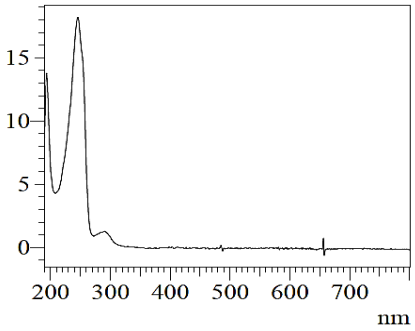
As seen in chromatogram (C) of Figure 3.1, there is good resolution and separation of the peak of the steroid compound (CP) at a retention time of 2.908 minutes when the optimum conditions used for separating the steroid standard mixture were applied on the sample. It can also be observed that there are peaks of other analytes on the chromatogram which are not of interest. These peaks can be found, if not all, but at least some, in all the steroid containing samples analysed. They have consistent retention times of approximately 0.8, 1.3, 1.6 and 2.0 minutes as shown in Appendix E, Figures E25 – E37. The peaks of these undesired analytes were confirmed not to belong to any of the other SLAs investigated in this study when comparison of their UV–visible spectra was made (see Figure 3.2). However, these peaks are suspected to be those of paraben preservatives (methyl, propyl, ethyl and butyl paraben) judging from their UV–visible spectra. Just like most of the SLAs investigated in this study, paraben preservatives are also polar compounds and could easily have been extracted from the samples during the extraction process of the SLAs with 100% methanol. Moreover, separation of parabens have been reported to be carried out in a reversed-phase HPLC with a similar mobile phase combination (methanol : water) and column (C18) to this study (195, 196).



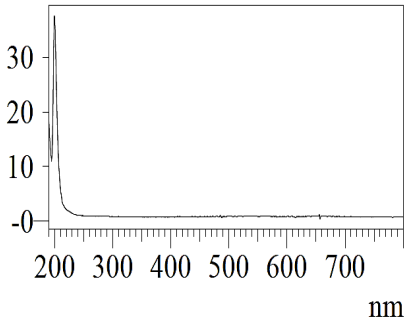
Hydroquinone



Unknown



Benzoquinone



Unkown

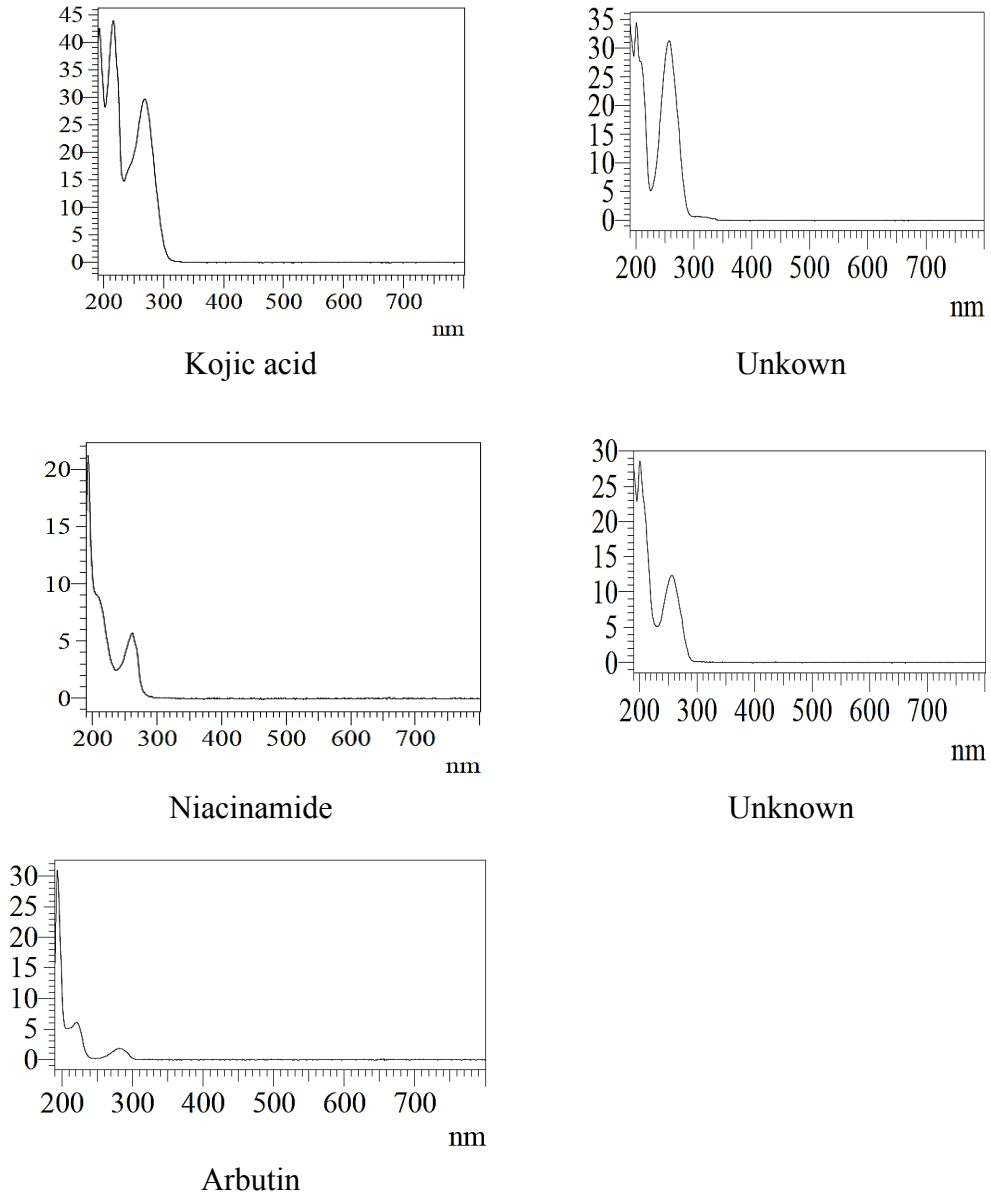
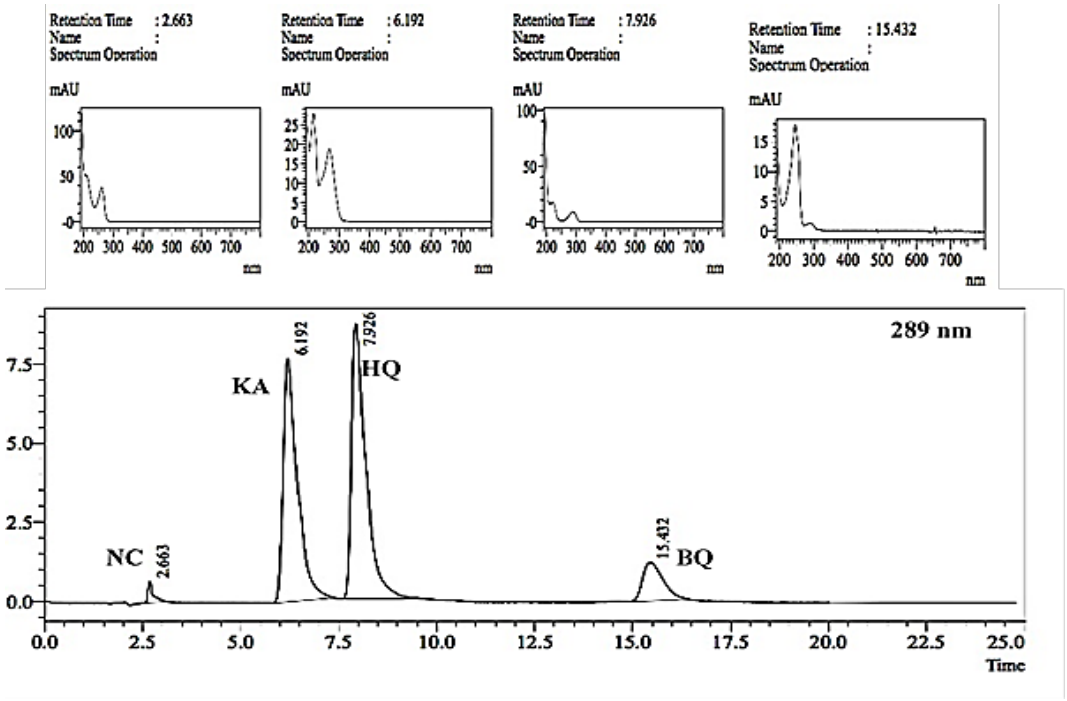


Figure 3.2: Comparison of the UV–visible spectra of other SLAs investigated in this study with those of the unknown analytes.

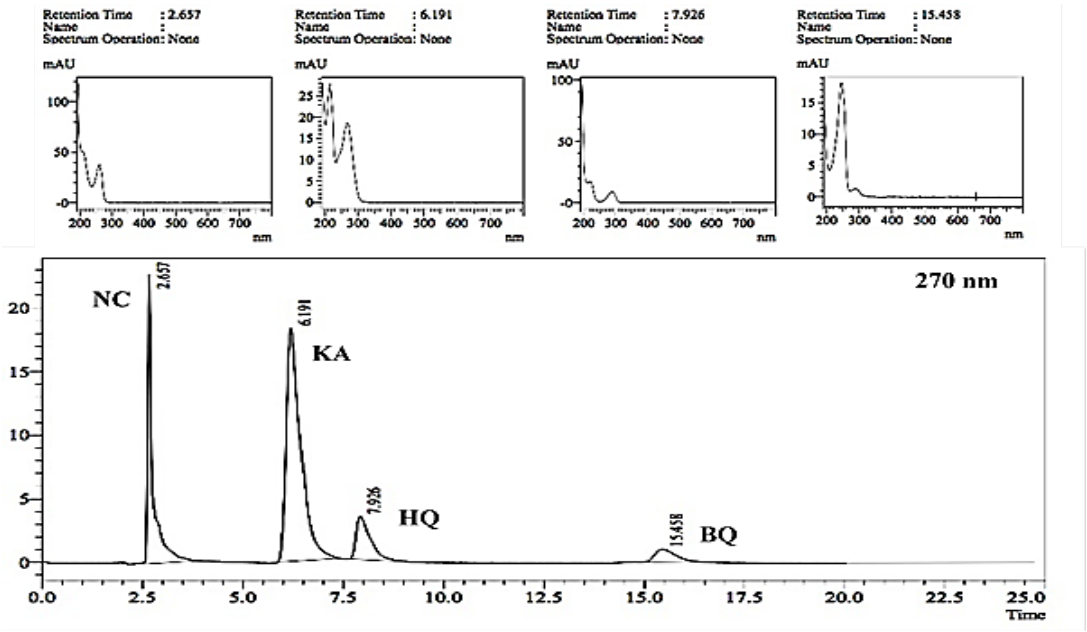
The second group of SLAs, which included HQ, BQ, KA, NC and ARB were separated on a SGE C18 column (250 × 4.6 mm, 5 μm). When the column and conditions used to separate the first group of SLAs was used for these analytes, a poor separation resulted. This then led to a change in the stationary phase, which is one of the effective ways of altering the selectivity of the separation in HPLC. When a mobile phase consisting of either 70% methanol with 0.05 M KH_2PO_4 at a pH of 2.5, or 80% methanol with 0.05 M K_2HPO_4 at a pH of 3.0, was used at flow rates of 0.3, 0.5, 0.8, 1.0 and 1.2 mL min^{-1}, the peak for NC was separated, but the peaks for HQ and BQ co-eluted and likewise for KA and ARB. By using 90% methanol in water containing 0.2% acetic acid as the mobile phase, at different flow rates, all the SLAs were found to elute earlier than before but the peaks were clustered at very close retention times. With the second group of SLAs, it was generally observed that an increase in the percentage of methanol in the mobile phase led to early elution of all the analytes at flow rates above 0.8 mL min^{-1}, causing the analytes to co-elute. Whereas with flow rates ranging between 0.3 to 0.8 mL min^{-1}, the elution times of all analytes are increased and the separation improved but the peaks were broadened. This indicated that a mobile phase containing far less methanol was needed for optimal separation.

Separation of the second group of SLAs was best achieved when 5% methanol containing 0.5% orthophosphoric acid and 95% water containing 0.5% orthophosphoric acid was used as a mobile phase. The flow rate was set at 1.0 mL min^{-1} and the total run time was 20 minutes. Figure 3.3 shows the chromatograms for the separation of the second group of SLAs, which included NC, KA, HQ, BQ and ARB. ARB was removed from the standard mixture and was analysed separately with the same optimum conditions used for these SLAs as it co-eluted with KA. Moreover, the column temperature was set at 35 °C. An increase in temperature was observed to reduce tailing in some of the analyte peaks. Again the effect of detection wavelength is particularly evident in the case of NC and BQ in their chromatograms acquired at different detection wavelengths of 270 and 240 nm respectively in Figure 3.3. Chromatogram (E) of the same figure shows the separation of some of these SLAs effected on a sample.

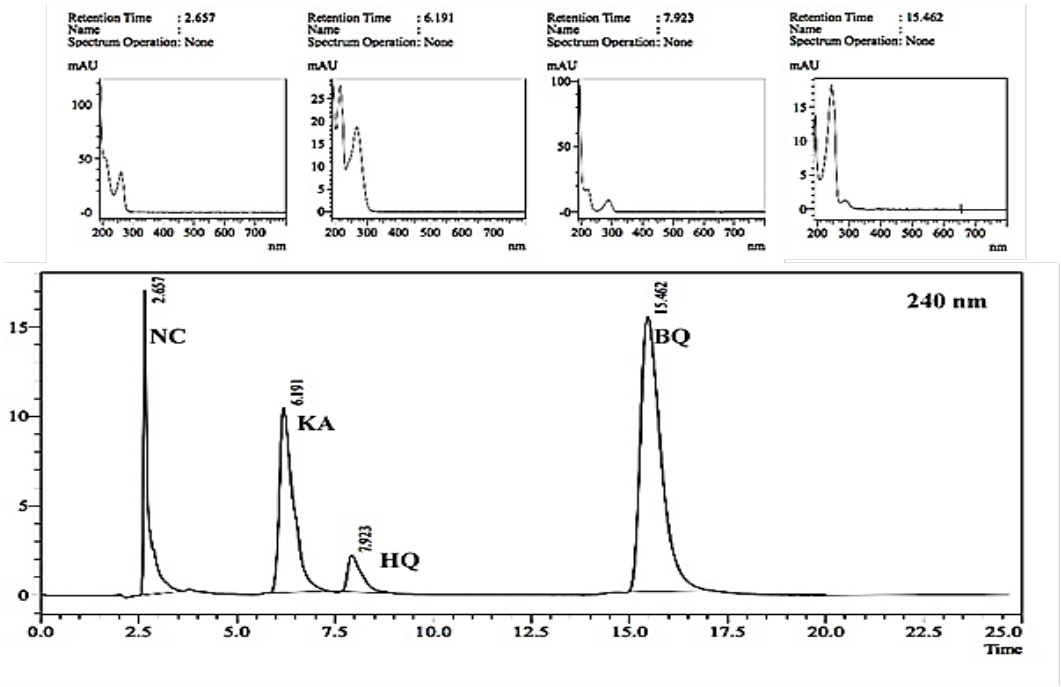
(A)


Retention Time : 2.663
Name :
Spectrum Operation
Retention Time : 6.192
Name :
Spectrum Operation
Retention Time : 7.926
Name :
Spectrum Operation
Retention Time : 15.432
Name :
Spectrum Operation
mAU
nm
289 nm
NC
2.663
KA
6.192
HQ
7.926
BQ
15.432
Time

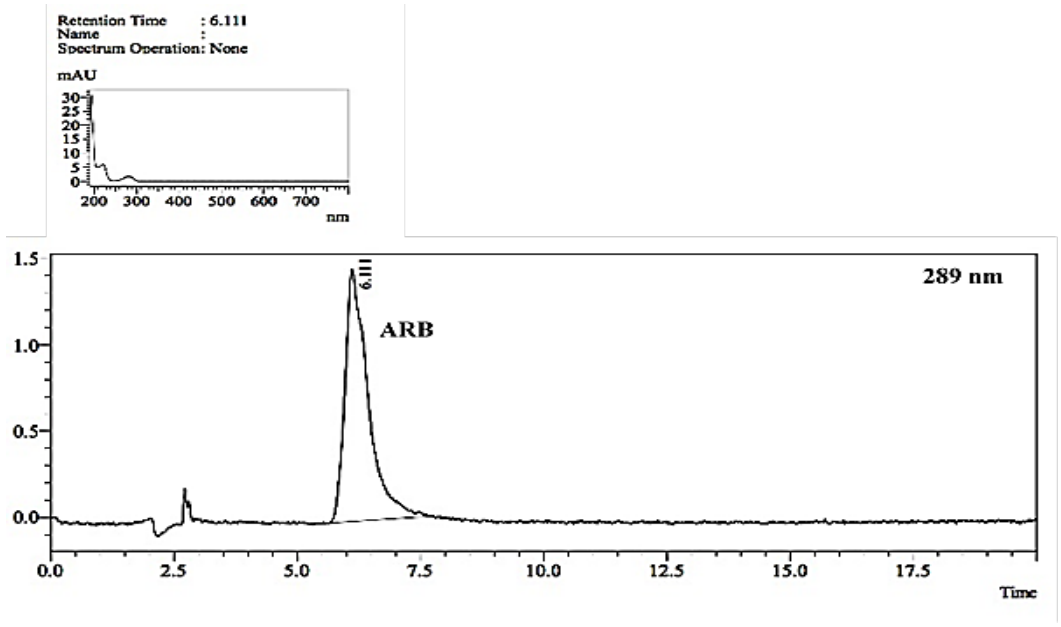
(B)


Retention Time : 2.657
Name :
Spectrum Operation: None
Retention Time : 6.191
Name :
Spectrum Operation: None
Retention Time : 7.926
Name :
Spectrum Operation: None
Retention Time : 15.458
Name :
Spectrum Operation: None
mAU
nm
270 nm
NC
2.657
KA
6.191
HQ
7.926
BQ
15.458
Time

(C)


Retention Time : 2.657
Name :
Spectrum Operation: None
Retention Time : 6.191
Name :
Spectrum Operation: None
Retention Time : 7.923
Name :
Spectrum Operation: None
Retention Time : 15.462
Name :
Spectrum Operation: None
mAU
nm
240 nm
NC
KA
HQ
BQ
2.657
6.191
7.923
15.462
Time

(D)


Retention Time : 6.111
Name :
Spectrum Operation: None
mAU
nm
289 nm
ARB
6.111
Time

(E)

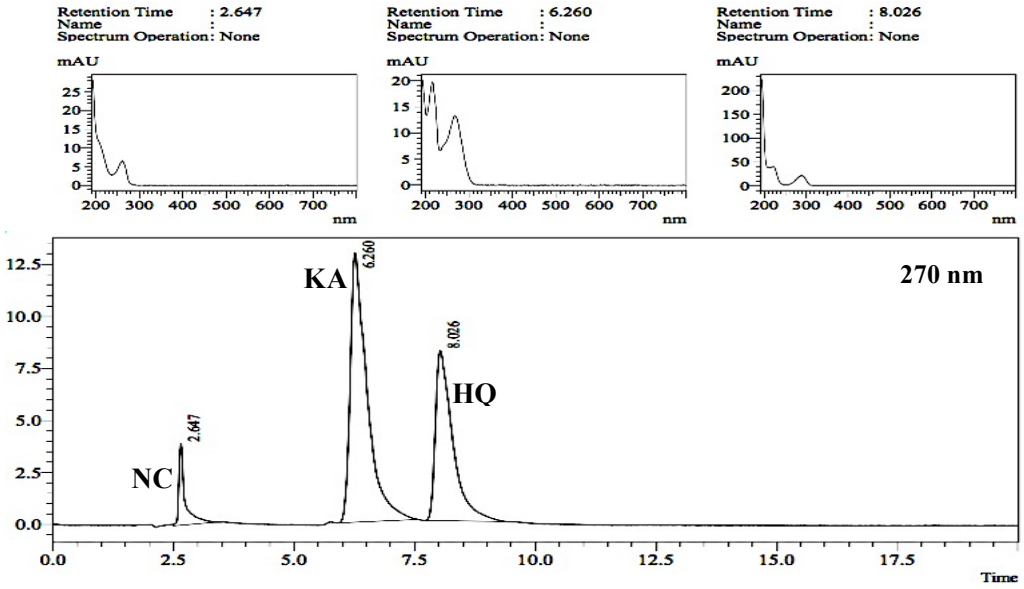


Figure 3.3: Chromatograms for the separation of active ingredients NC, KA, HQ, BQ and ARB obtained at detection wavelengths of (A) 289 nm, (B) 270 nm, (C) 240 nm (D) 289 nm and (E) 270 nm. The chromatographic separation was achieved on a SGE C18 column (250 × 4.6 mm, 5 µm) with a mobile phase of 0.5% orthophosphoric acid in methanol and 0.5% orthophosphoric acid in water 5:95 (v/v), an injection volume of 10 µL, a flow rate of 1.0 mL min^{-1} and a column temperature of 35 °C.

3.1.4 Optimum extraction solvent for sample preparation

In order to determine the optimum extraction method for the studied SLAs, four different solvents were examined. This was done following the experimental method described in Section 2.3.2.4. The peak areas of the SLAs obtained were plotted and served as a direct reflection of the solvent extraction strength.

When solutions of the SLAs were ultra-sonicated in a water bath at 40 °C for 20 minutes, the best extraction solvent was found to be 100% methanol. When 100% acetonitrile and a 1:1 (v/v) mixture of methanol and acetonitrile were used, a similar extraction result was obtained for both solvents but with less efficacy than 100% methanol. A 1:1 (v/v) mixture of methanol and water was found to be the solvent with the least extraction strength among those examined as shown in Figure 3.4. Thus, 100% methanol was used in this study for the extraction of the studied SLAs in our sample products

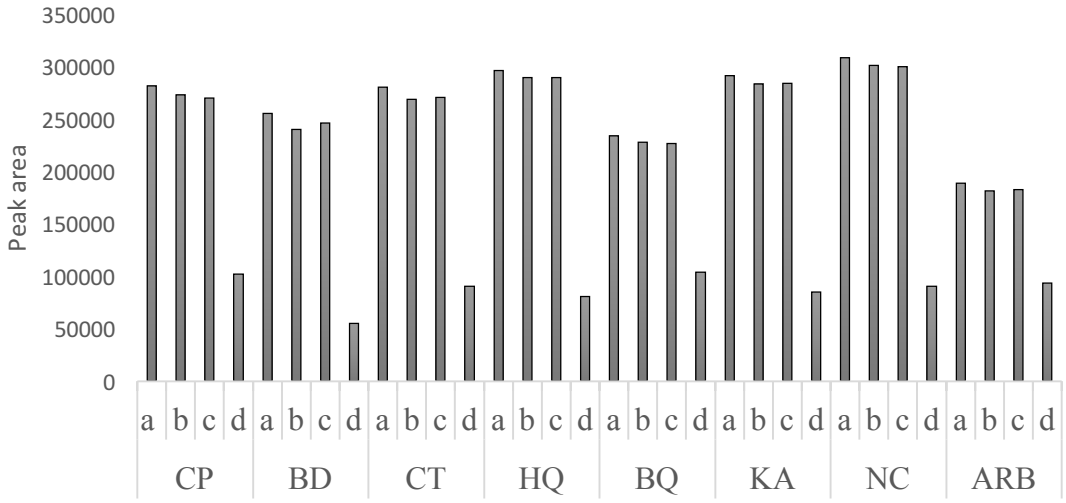


Figure 3.4: Optimisation of four different solvents for the extraction of CP, BD, CT, HQ, BQ, KA, NC, and ARB: a) methanol (100%), b) acetonitrile (100%), c) methanol:acetonitrile (1:1, v/v), and d) methanol:water (1:1, v/v). The optimum HPLC conditions described in Section 3.1.3 were used to obtain the peak areas.

3.2 Validation of HPLC analytical method

Analytical method validation is an important requirement in any HPLC analysis. The validation of an analytical method must demonstrate the fulfilment of all the analytical conditions required to ensure the reliability of the results. For this reason, the adequateness of the HPLC method developed in this study was tested for linearity, accuracy, precision, and limits of detection and quantitation.

3.2.1 Linearity of calibration curves and limits of detection and quantitation

An external standard calibration method was used to assess the linearity of the calibration curves used for quantitation of the SLAs identified in the sample products. A minimum of seven different concentrations in three replicates were prepared for each of the active ingredients and the calibration curves were determined in triplicate. Linear regression analysis (at the 95% confidence level) of the calibration data for each SLA was performed by using Microsoft Excel 2013 Analysis Tool Pak. The calibration curves were

constructed by plotting a graph of the HPLC peak areas against concentration. Moreover, equation (2.1) given in Section 2.5 was used for the quantitation of the studied SLAs in the sample products. The calibration data used to construct the linear regression curves are given in Appendix C while the corresponding calibration curves and residual plots for each of the studied SLAs are shown in Appendix D.

Good linearity was observed for all the SLA standards over the concentration range tested and was confirmed from the correlation coefficient R^2 values which were greater than 0.990. Table 3.2 gives the results of the linear regression analysis, and the limits of detection and limits of quantitation. The lowest correlation coefficient value for the studied SLAs was found to be 0.9942 and the highest value was 0.9995. The LOD values were found to be in the range of 0.007 – 0.029 mg L^{-1} and the LOQ values ranged from 0.025 – 0.097 mg L^{-1}.

Table 3.2: Results of the linear regression analysis of the calibration curves for the skin-lightening active ingredients analysed by HPLC.

Parameters	**CP**	**BD**	**CT**	**HQ**	**BQ**	**KA**	**NC**	**ARB**
Maximum absorption/nm	239	238	205	293	242	269	262	286
Detection wavelength/nm	240	240	225	289	240	270	270	289
Calibration range/mg L^{-1}	0.2-60	0.2-60	0.2-60	0.5-400	0.2-20	0.5-90	0.8-110	1.0-20
Slope/L mg^{-1}	22293	20003	27456	11708	38293	27397	11327	2310.5
Standard error of slope	63.099	52.915	69.235	113.883	326.682	124.798	58.988	9.047
Correlation Coefficient (R^2)	0.9994	0.9995	0.9995	0.9942	0.9955	0.9987	0.9983	0.9991
LOD/mg L^{-1}	0.008	0.007	0.007	0.029	0.026	0.014	0.016	0.012
LOQ/mg L^{-1}	0.028	0.026	0.025	0.097	0.085	0.046	0.052	0.039

Limit of Detection (LOD)/mg L^{-1} = (3 × standard error of slope)/slope.
Limit of quantitation (LOQ)/mg L^{-1} = (10 × standard error of slope)/slope.

3.2.2 Accuracy

The accuracy of the quantitation method was examined by performing a recovery test. By using a sample free of the active ingredients, two solutions of different concentration were prepared with known amounts of the pure active ingredients added. These were extracted and the extracts injected in triplicate on the HPLC. The percent recoveries of the spiked active ingredient were then calculated. No interferences were observed throughout the test. The results of the accuracy studies are summarised in Table 3.3 and it is evident that the method is accurate within the acceptable recovery range of 80 to 120% (197). The percent recoveries obtained ranged from 84.23 to 110.33% and the percent relative standard deviation (%R.S.D.) ranged from 0.11 to 3.93%.

Table 3.3: Percentage recoveries of skin-lightening active ingredients (n = 3).

Active ingredients	Spiked amount/mg L^{-1}	Found/mg L^{-1}	%Recovery	%R.S.D.
CP	10	8.42±0.03	84.23	0.41
	50	49.58±0.21	99.17	0.44
BD	10	9.33±0.04	93.36	0.44
	50	47.09±0.08	94.19	0.18
CT	10	9.35±0.07	93.49	0.80
	50	49.03±0.14	98.07	0.28
HQ	10	11.03±0.20	110.33	1.84
	20	20.15±0.48	100.73	2.39
BQ	15	14.86±0.58	99.09	3.93
	20	19.63±0.02	98.15	0.11
KA	8.0	7.27±0.02	90.83	0.21
	20	19.16±0.07	95.80	0.37
NC	15	14.33±0.36	95.56	2.49
	20	19.13±0.12	95.67	0.62
ARB	10	10.03±0.28	100.29	2.84
	20	19.61±0.26	98.07	1.37

Recovery (%) = (mean of found concentration/spiked concentration) × 100.
R.S.D. (%) = (S.D./mean concentration) × 100.

3.2.3 Precision

The precision of analysis measures the degree of repeatability of an analytical method and is normally expressed as the percent relative standard deviation for a significant number of samples with different ranges of concentration. The precision of the method was determined from the intra-day (same day) and inter-day (over a period of three days) analysis. Three different standard solutions were prepared for each of the active ingredients and analysed following the method described in Section 2.3.4.6.

The results obtained from the intra-day and inter-day precision analysis are summarised in Table 3.4. The percent relative standard deviation (%R.S.D.) for the intra-day precision ranged from 0.17 to 8.16% while that of the inter-day precision ranged from 0.14 to 4.78%. Likewise, the % accuracies obtained from the precision analysis fell within an acceptable range. The intra-day analysis % accuracy ranged from 83.1 to 114.2% and the inter-day analysis % accuracy ranged from 84.2 to 114.4%.

Table 3.4: Determination of precision for intra-day and inter-day variability.

Active ingredients	Retention time/min	Concentration/ mg L^{-1}	Intra-day (n = 9)			Inter-day (n = 9)		
			Found/mg L^{-1}	R.S.D. /%	Accuracy /%	Found/mg L^{-1}	R.S.D. /%	Accuracy/%
CP	2.80-3.00	1.0	0.94±0.004	0.39	94.1	0.94±0.007	0.79	94.3
		4.0	3.74±0.025	0.67	93.5	3.76±0.023	0.61	93.9
		8.0	7.82±0.042	0.54	97.7	7.95±0.033	0.41	99.4
BD	3.50-3.70	1.0	0.92±0.005	0.59	91.9	0.92±0.012	1.27	91.7
		4.0	3.72±0.006	0.17	93.1	3.73±0.019	0.52	93.2
		8.0	7.83±0.098	1.25	97.9	7.92±0.038	0.48	99.0
CT	4.50-4.80	1.0	0.83±0.029	3.55	83.1	0.84±0.024	2.81	84.2
		4.0	3.65±0.137	3.76	91.1	3.74±0.026	0.69	93.5
		8.0	7.96±0.024	0.29	99.5	7.98±0.011	0.14	99.7
HQ	7.90-8.10	2.0	2.18±0.112	5.15	109.2	2.14±0.100	4.69	106.9
		10	11.48±0.087	0.76	114.2	11.4±0.087	0.76	114.4
		20	18.71±0.191	1.02	93.5	18.83±0.227	1.21	94.2
BQ	15.45-15.70	2.0	1.91±0.066	3.46	95.7	1.96±0.026	1.35	97.8
		8.0	7.74±0.064	0.83	96.8	7.72±0.091	1.18	96.5

		20	19.17±0.067	0.35	95.8	19.19±0.069	0.36	95.9
KA	6.15-6.30	1.0	0.94±0.077	8.16	94.4	0.94±0.045	4.78	93.9
		10	10.13±0.054	0.53	101.3	11.15±0.111	0.99	111.5
		20	18.08±0.126	0.69	90.4	18.16±0.064	0.35	90.8
NC	2.60-2.70	5.0	4.57±0.078	1.71	91.5	4.59±0.042	0.93	91.9
		10	9.66±0.129	1.34	96.6	11.0±0.093	0.85	110.1
		20	19.81±0.066	0.33	99.1	19.9±0.063	0.32	99.6
ARB	6.0-6.15	2.0	1.93±0.116	5.99	96.4	1.96±0.091	4.63	97.9
		12	12.05±0.245	2.03	100.4	12.19±0.216	1.77	101.6
		20	19.32±0.147	0.76	96.6	19.42±0.322	1.66	97.1

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

3.3 Amount of skin-lightening agents detected in skin-lightening products

A total of 35 cream samples were prepared and quantitatively analysed for the presence of SLAs, such as CP, BD, CT, HQ, BQ, KA, NC, and ARB, following the HPLC method developed. It was found that out of the 35 cream samples analysed, 32 contained at least one of these eight agents (see Table 3.5). The HPLC chromatograms of these cream samples are shown in Appendix E and the peak areas obtained for the detected SLAs are given in Appendix F.

Fifteen out of the thirty-five cream samples analysed contained HQ in the range of 0.017 – 7.096% (m/m). Of these, only five cream samples listed HQ in the ingredients with four stating the content percentage to be 2%. Two more cream samples (SLP 12 and 18) were found to indicate HQ in the list of ingredients but as *p*-hydroxyphenol. This can be perceived as deliberately misleading since this is an unpopular name for HQ among users of skin bleaching creams. As seen in Table 3.5, two cream samples contain HQ below 1.0% (m/m) (0.017 – 0.924% m/m), nine cream samples have HQ above 1% (m/m) (1.024 – 1.916% m/m) and four cream samples have HQ above 2% (m/m) (2.429 – 7.096% m/m) which counters the proposed US FDA ruling of 2% HQ in marketing over-the-counter skin-lightening creams. Six cream samples containing HQ were found to also contain BQ. This can be attributed to the hydrolysis of HQ into BQ as it is usually unstable in some formulations. The BQ concentration in our cream samples was found to be in the range of 0.005 – 0.015% (m/m).

For the purpose of dermatological therapy, HQ was one of the most prescribed agents for treating hyperpigmentation until 2006 when the US FDA placed a ban on over-the-counter HQ products due to its toxicity (16). Currently, products containing HQ above 2% are only sold by prescription in the US. Reports have revealed the health risks associated with the use of high concentrations of HQ and its long-term exposure. They include irritant contact dermatitis, allergic contact dermatitis, nail discolouration, decreased skin elasticity, trimethylaminuria, exogenous ochronosis and much more (59). In contrast to the US FDA, the Nigerian Food and Drug Agency (NAFDAC) and the South Africa government have placed a total ban on any amount of HQ in over-the-counter products (40, 198). This is also the case in the UK and EU (16). Of great concern

is that the cream sample (SLP 18) with the highest amount of HQ (7.096% m/m) was found to be manufactured in one of the EU countries which could imply that the inclusion of HQ as an active ingredient in over-the-counter SLPs is permitted for export only in some EU countries. From the results obtained, it shows that despite the bans, products containing high concentrations of HQ are still available and sold illegally in the Nigerian market. Regulations on the quality of import products is weak or not there at all.

KA was found in nine cream samples ranging from 0.017 – 1.412% (m/m), with only three of the creams indicating KA among their ingredients. Only one of the nine cream samples (SLP 15) was found to contain KA above 1% (m/m). The allowed amount of KA is still not unanimously agreed on but according to reports from the Personal Care Product Council (100), KA is commonly used at a concentration of 0.1 – 2.0 %. However, based on the margin of safety calculation, KA at a maximum concentration of 1% is considered unsafe (100). Nevertheless, KA is still to be approved by the US FDA in over-the-counter products (100) and has been reported to be banned in Japan due to its mutagenicity concerns (9). Thus, going by the afore-mentioned reports, it is safer to avoid over-the-counter SLPs with KA as the active ingredient.

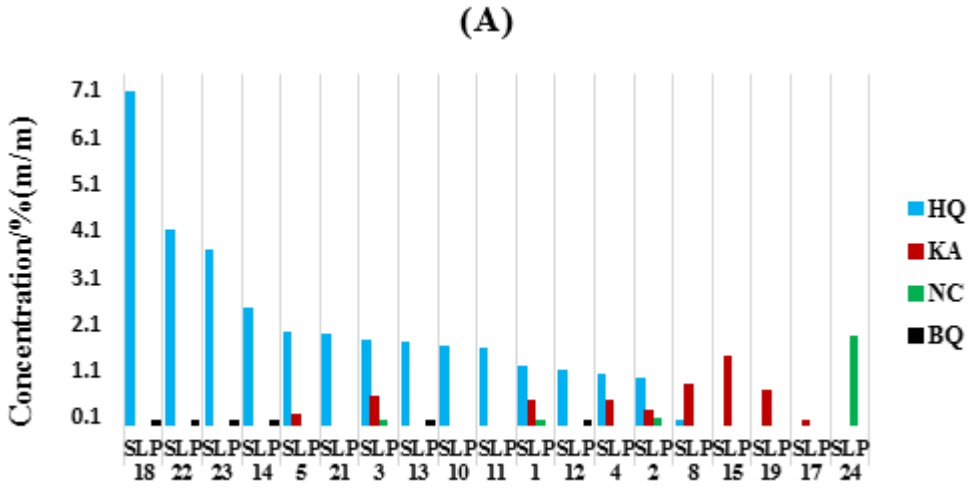
NC was also identified in four cream samples ranging from 0.029 – 1.827% (m/m). Only one of the four cream samples (SPL 24) indicated NC in the list of ingredients. NC was also listed in another of the cream samples (SLP 12) but was not detected from the analysis. The amount of NC allowed in SLPs is still to be established and this could be the reason why some manufacturers introduce the use of NC and some other bleaching agents with unknown safety profiles in over-the-counter SLPs.

One cream sample (SLP 16) was indicated to contain ARB in the list of ingredients but upon analysis, this agent could not be detected. However, ARB has been shown to be a safer SLA in comparison with HQ (102).

Steroid compounds, such as CP, BD and CT, were detected in 13 cream samples with two of the samples (SLP 9 and 20) containing both CP and CT. The steroid compounds were all indicated in the list of ingredients, stating their content percentage (see Table 2.1). Although one of the cream samples (SLP 25) claimed to contain CP, it was found to contain BD instead. Ten of the cream samples contain CP in the range of 0.007 – 0.035% (m/m), three contained BD in the range of 0.019 – 0.027% (m/m) and two contained CT

in the range of 0.007 – 0.012% (m/m). Hence, all the steroid compounds contained in the cream samples do not exceed the allowed limits of 0.05% (m/m) CP, 0.065% (m/m) BD and 1.0% (m/m) CT for medical use. However, these cream samples are all sold as over-the-counter products for skin bleaching purposes. Ideally, any cream containing steroid compounds should be considered a pharmaceutical product and should only be sold as a prescription drug or when sold as an over-the-counter product should only be used within seven days under medical supervision. Failure to observe this can be very detrimental to the users' health. The addition of steroid compounds in skin bleaching creams has been seriously warned against by the US FDA irrespective of the potency or amount due to their severe side-effects (16). Steroids can be absorbed systematically and result in several detrimental health effects such as acne vulgaris, allergic contact dermatitis, Cushing syndrome, diabetes mellitus, steroid addiction syndrome, ophthalmological conditions such as cataracts and glaucoma, and many more (59).

Samples 6, 7 and 16 were found to contain none of the studied SLAs in this study. Thus, in this selection of Nigerian SLPs, the most prevalent SLAs were HQ, BQ, KA, NC, CP, BD and CT as shown in Figure 3.5. As shown in bar graph (A) it can be observed that HQ, the gold standard for skin-bleaching, is the most detected SLA in the studied SLPs followed by KA, BQ and then NC. Whereas in SLPs containing steroid compounds (see bar graph B), CP is the most detected SLA followed by BD and then CT.



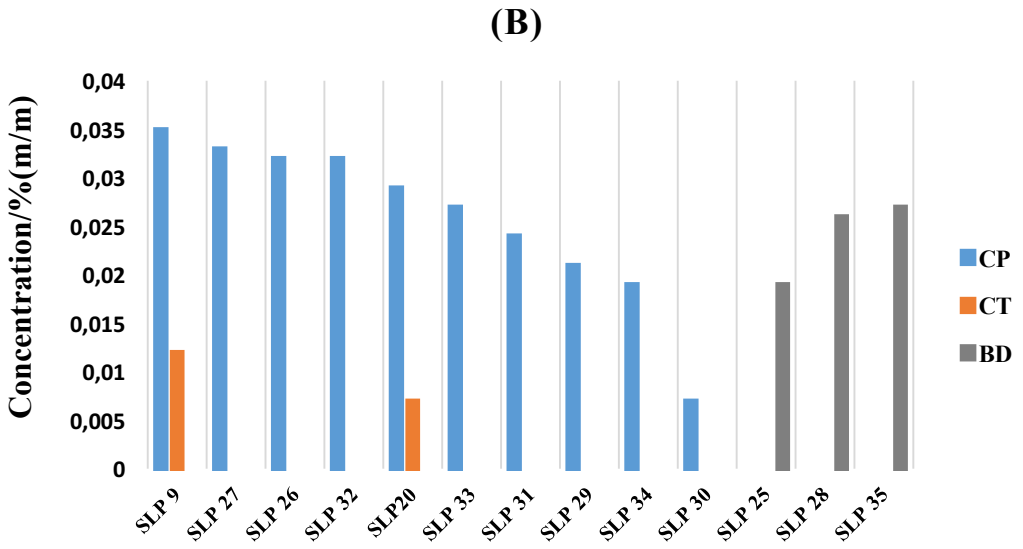
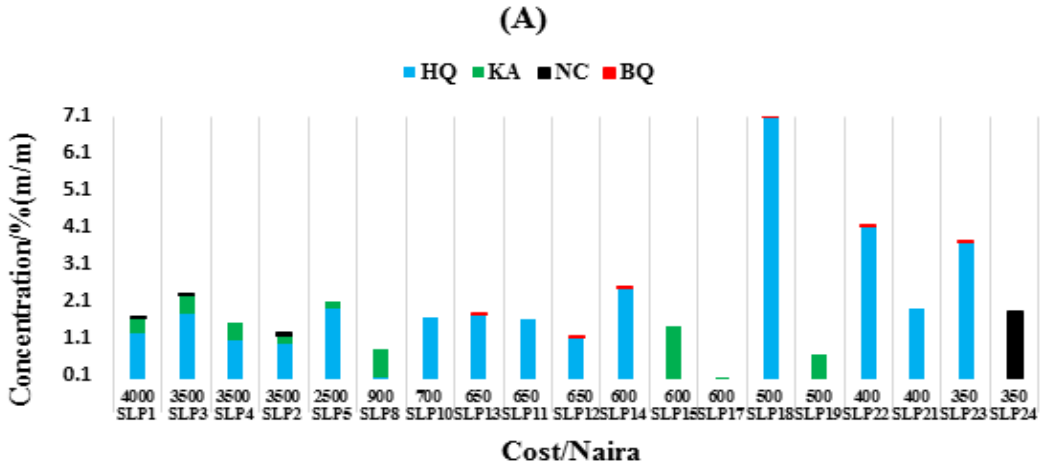


Figure 3.5: Prevalence of skin-lightening agents detected in the investigated products.

Each of the SLPs analysed in this study has its own purchase price (see Table 2.1) and these can actually be a deciding factor on their sales. Looking at the price range (4000-150 Naira) of the studied SLPs, it is only ideal for one to assume that the more expensive products might be the safer products to use in terms of the type or level of SLAs they contained. Figure 3.6 relates the cost of each of the studied SLPs to the level of their detected SLAs in order to confirm if the most expensive products are actually safer in comparison with the cheaper products.



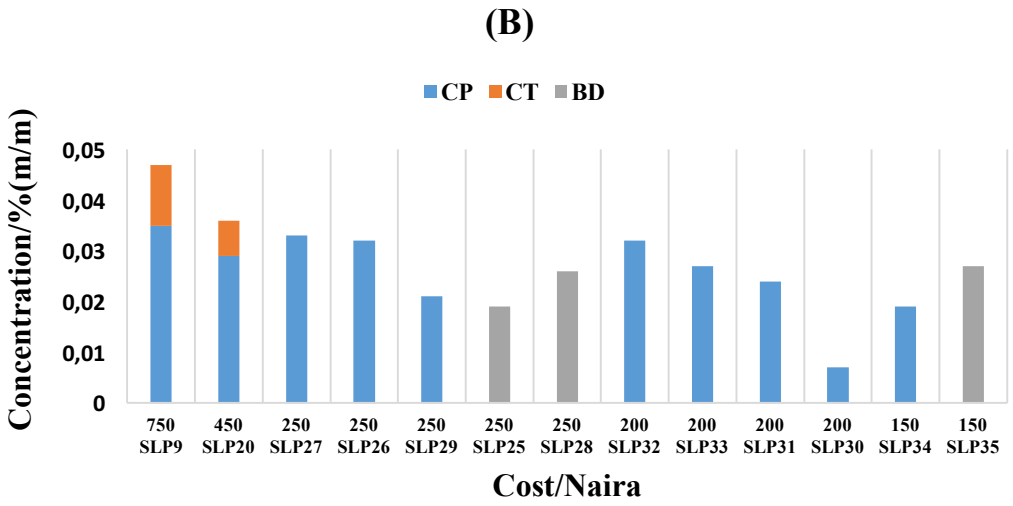


Figure 3.6: Levels of SLAs detected in the studied SLPs compared to their cost.

For the purpose of this study, SLPs above 1000 Naira are considered expensive, those above 500 Naira are considered moderately cheap and those below 500 Naira are considered cheap since most of the population can afford SLPs at this cost.

As shown in bar graph (A) of Figure 3.6, the cost of the SLPs ranged from the most expensive sample (4000 Naira) to the cheapest sample (350 Naira). It can be observed that all the expensive SLPs have HQ below the 2% proposed limit by the US FDA. Nevertheless, the expensive SLPs were observed to contain more than one SLA. This can be HQ combined with either KA or NC or all the three SLAs together. There are no established guide-lines for KA and NC, but KA is still to be approved by the US FDA for use in over-the-counter products due to its mutagenic concerns (100). In assessing other SLPs that fall in the range of moderately cheap to cheap it can be observed that most of these samples have HQ in line with the proposed US FDA regulations too, except in the case of some samples (SLP 14, 18, 22, and 23) containing HQ above 2%. Another notable observation with SLPs in this range is the unstable nature of HQ in them. The presence of BQ in some of these cheaper products proves that HQ has been oxidised. This means HQ is unstable in the formulation.

As observed in bar graph (B) of Figure 3.6, only SLPs containing steroid compounds were compared for their cost relative to the level of SLAs detected. The price ranged from

750-150 Naira. It can be seen that the sample that cost most contained the highest amount of CP. Here, there is no significant difference in relation to the levels of SLAs detected and the purchase price of the SLPs except in the case of SLP 30 with a lower level of CP.

Judging from the results of this study, there is no convincing proof to show that the more expensive SLPs are actually safer than the cheaper SLPs. One can only make reference to some of the moderately cheap and cheap SLPs (SLP 14, 18, 22, and 23) as the most unsafe products in this study since many other cheaper SLPs have similar levels of detected SLAs as the expensive ones. It is also important to note that most of the so called 'expensive SLPs' analysed in this study declared none of the SLAs detected or false ingredients on their labels. However, some of the investigated SLAs were positively detected. This is a clear tactic of luring users to buy the expensive products.

Table 3.5: Levels of active ingredients detected (n = 3).

Sample code	**Sample label claim**	**Active ingredient detected**	**Mean concentration /%(m/m) ± confidence interval**
SLP 1	——	HQ	1.205±0.228
	——	KA	0.442±0.025
	——	NC	0.029±0.035
SLP 2	——	HQ	0.924±0.227
	——	KA	0.245±0.025
	——	NC	0.063±0.035
SLP 3	KA	KA	0.530±0.025
	——	HQ	1.741±0.229
	——	NC	0.049±0.035
SLP 4	——	HQ	1.024±0.227
	——	KA	0.476±0.025
SLP5	——	HQ	1.916±0.229
	——	KA	0.155±0.025
SLP 8	——	HQ	0.017±0.226
	——	KA	0.786±0.026
SLP 9	CP	CP	0.035±0.008
	CT	CT	0.012±0.007

SLP 10	HQ	HQ	1.635±0.229
SLP 11	HQ	HQ	1.592±0.228
SLP 12	HQ	HQ	1.111±0.227
	——	BQ	0.015±0.011
SLP 13	——	HQ	1.729±0.229
	——	BQ	0.007±0.011
SLP 14	——	HQ	2.429±0.231
	——	BQ	0.009±0.011
SLP 15	——	KA	1.412±0.028
SLP 17	KA	KA	0.017±0.025
SLP 18	HQ	HQ	7.096±0.265
	——	BQ	0.006±0.011
SLP 19	KA	KA	0.663±0.025
SLP 20	CP	CP	0.029±0.008
	CT	CT	0.007±0.007
SLP 21	HQ	HQ	1.889±0.229
SLP 22	HQ	HQ	4.129±0.240
	——	BQ	0.005±0.011
SLP 23	HQ	HQ	3.700±0.238
	——	BQ	0.009±0.011
SLP 24	NC	NC	1.827±0.039
SLP 25	CP	BD	0.019±0.007
SLP 26	CP	CP	0.032±0.008
SLP 27	CP	CP	0.033±0.008
SLP 28	BD	BD	0.026±0.007
SLP 29	CP	CP	0.021±0.008
SLP 30	CP	CP	0.007±0.008
SLP 31	CP	CP	0.024±0.008
SLP 32	CP	CP	0.032±0.008
SLP 33	CP	CP	0.027±0.008
SLP 34	CP	CP	0.019±0.008
SLP 35	BD	BD	0.027±0.007

3.4 Validation of ICP-OES and CV-AAS methods

The MHS 15 mercury hydride system together with a PerkinElmer Analyst 200 AAS and a PerkinElmer Optical 5300 DV ICP-OES were used to determine the levels of mercury and other heavy metals in the studied SLPs respectively. The linearity and accuracy of the techniques used were determined by using the external standard calibration method and recovery studies. The results of these validations are provided in Sections 3.4.1 and 3.4.2 respectively. The problem of spectral interference which usually occurs for elemental analysis with ICP-OES was well minimised by choosing detection wavelengths where minimal interference occurred.

3.4.1 Linearity of calibration plots for heavy metal analysis

An external standard method was used to obtain nine-point calibration curves for each of the metals. The curves were plotted by using the data generated from the standard solutions. The standard solutions were prepared in three replicates for each of the metals following the procedure described in Section 2.4.2.1 and were measured in the same way to obtain three data points. The calibration data for the metals is given in Appendix G and the calibration curves with their corresponding residual plots are shown in Appendix H.

Linear regression analysis (at the 95% confidence level) of the calibration data for the metals was performed by using Microsoft Excel 2013 Analysis Tool Pak. The LOD and LOQ of each metal was estimated from the slope and standard error of the calibration curves by using equation (2.2) and (2.3) given in Section 2.5 respectively. As seen in Table 3.6, the method had LODs in the range of 0.007 – 0.011 mg L^{-1} and LOQs in the range of 0.025 – 0.088 mg L^{-1}. Likewise, acceptable correlation coefficient (R^2) values greater than 0.995 were obtained for the calibration curves of all the metals.

Table 3.6: Results from the linear regression analysis of the calibration data for heavy metals.

Parameters	**Al**	**Cr**	**Cu**	**Fe**	**Mn**	**Ni**	**Pb**	**Zn**
Calibration range/mg L^{-1}	0.01-1.6	0.01-1.6	0.01-1.6	0.01-1.6	0.01-1.6	0.01-1.6	0.01-1.6	0.01-1.6
Slope/L mg^{-1}	59756	38901	219185	59962	285242	19683	1165.3	36679
Standard error of slope	215.7	91.6	1170.7	526.1	1154.5	62.9	4.2	92.1
Correlation Coefficient (R^2)	0.9997	0.9999	0.9993	0.9980	0.9996	0.9997	0.9997	0.9998
LOD/mg L^{-1}	0.011	0.007	0.016	0.026	0.012	0.009	0.011	0.008
LOQ/mg L^{-1}	0.036	0.024	0.053	0.088	0.040	0.032	0.036	0.025

The results of the linear regression analysis for mercury are given in Table 3.7. An acceptable correlation coefficient, R^2, value of 0.9989 was obtained from the calibration curve of mercury and the LOD and LOQ values were less than 0.025 and 0.08 ng respectively.

Table 3.7: Results from the linear regression analysis of the calibration data for the determination of mercury.

Parameters	**Value**
Calibration range/ng	100-800
Slope/ng	7.334×10^{-4}
Standard error of slope	5.184×10^{-6}
Correlation Coefficient (R^2)	0.9989
LOD/ng	0.022
LOQ/ng	0.074

3.4.2 Accuracy

Recovery tests were carried out so as to check the accuracy of the methods used for quantitation of metals. This was done by spiking cream samples with known amounts of the element of interest as described in Section 2.4.4. The results obtained from the recovery tests are summarised in Table 3.8. The mean percentage recovery for all the metals analysed ranged from 95.6 – 112.7% and the percent relative standard deviation (%R.S.D.) ranged from 0.12 to 5.64%.

Table 3.8: Recovery data for heavy metal quantification in skin-lightening products (n = 3).

Element	Spiked amount /µg g^{-1}	Found/µg g^{-1}	Recovery %	Standard deviation	%R.S.D.
Al	0.05	0.049	98.14	0.0004	0.89
	1.20	1.181	98.39	0.0092	0.78
Cr	0.02	0.020	100.24	0.0002	0.99
	0.20	0.219	109.51	0.0091	4.17
Cu	0.02	0.019	99.3	0.0009	4.89
	0.07	0.068	98.28	0.0018	2.62
Fe	0.08	0.079	99.07	0.0007	0.88
	1.10	1.083	98.43	0.0013	0.12
Hg	0.25	0.282	112.76	2.974	1.05
	0.45	0.496	110.26	2.182	0.44
Mn	0.02	0.019	99.17	0.0011	5.64
	0.04	0.038	95.63	0.0005	1.23
Ni	0.02	0.019	98.07	0.0002	1.22
	0.30	0.299	99.87	0.0023	0.76
Pb	0.05	0.049	97.43	0.0008	1.59
	1.0	0.978	97.83	0.0023	0.23
Zn	0.08	0.078	97.75	0.0008	1.08
	1.50	1.485	98.99	0.0119	0.80

3.5 Levels of mercury and heavy metals detected in skin-lightening products

A total of 35 SLPs were investigated for the presence of 12 heavy metals (As, Al, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, Zn and Hg) in this study. As seen in Table 3.9, only nine of the metals (Al, Cr, Cu, Fe, Mn, Ni, Pb, Zn and Hg) were detected in the studied SLPs. Arsenic, cadmium and cobalt could not be detected.

Aluminium was detected in all the cream samples investigated at concentrations ranging from 0.039 to 0.354 μg g^{-1}. Among the cream samples analysed, SLP 12 was found to contain the highest level of Al but this value is much lower than the level found in Brazilian make-up products (199). Although, no guideline has yet been established for Al in cosmetic products, studies have shown it to cause changes in some biological processes. It is suggested that Al at high concentration can result in a decreased rate of DNA synthesis, an increase in DNA replication errors and affinity of linker histones for DNA (200).

Chromium was detected in 21 cream samples. The concentration of Cr found ranged from 0.010 to 0.217 μg g^{-1}. The levels of Cr found in the cream samples were similar to some of those found in body creams from Dubai, India, and Pakistan (201), and some personal care products in Nigeria (4). The level of Cr in the cream samples was found to be lower than the reported value capable of inducing immediate side-effects. This could imply that continued or long-term exposure to cosmetic products with a low level of Cr is unsafe too. Side-effects, such as skin rashes, kidney and liver damage, lung cancer, and respiratory problems, have been reported to be associated with chromium exposure (4). However, complications associated with chromium exposure are dependent upon its oxidation state. In accordance with the IARC classification, Cr(VI) compounds are considered carcinogenic to humans while metallic Cr and Cr(III) compounds were not classifiable as human carcinogens due to lack of reassuring evidence in humans (202). Reports have also shown that Cr(VI) can diffuse through the skin more easily than Cr(III) due to its higher solubility (203, 204), with the contact time, sweat at low pH and the application of a cleanser being contributing factors (205).

Lead was detected in all the studied cream samples, however, at low concentration. The concentration of Pb ranged from 0.012 to 0.026 μg g^{-1}. The concentrations of Pb in the cream samples are lower than the concentrations found in some cosmetic products in Nigeria (150). Pb concentrations in the cream samples were below the 10 μg g^{-1} limit that was established for Pb in specified cosmetics by Health Canada (206) and the 20 μg g^{-1} limit established as an impurity in colour additives in cosmetics for external use (207). However, even at low concentration, Pb has been indicated as a cause for concern since it can be absorbed through the skin especially after long-term exposure (208). Hence, the concentrations of Pb in the studied cream samples do not indicate an immediate health concern.

Nickel was detected in 26 cream samples with concentrations ranging from 0.010 to 0.433 μg g^{-1}. The levels of Ni in the studied cream samples were similar to those found in moisturising and skin-lightening creams in Nigeria (152). Ni with other metals, such as Cr and Co, are known inducers of allergic contact dermatitis both at occupational and environmental levels (175). However, the levels of Ni in our cream samples were below the 5 μg g^{-1} limit proposed by scientific literature as a good manufacturing practice and an ultimate target level of 1 μg g^{-1} in order to minimise the risk of sensitization for very sensitive individuals (209).

The copper concentrations ranged from 0.017 to 0.050 μg g^{-1} and copper was detected in all the studied cream samples. The level of Cu in the cream samples is very low to cause any immediate health concerns. However, cases of immune reactions, as a result of Cu exposure from intra-uterine devices or handling of euro coins, have been reported (152).

Iron and manganese are two essential elements involved in a number of biological processes. They are both reported to have relatively low toxicity. Neurological disorders, such as anger, aggression, and criminality, are said to be associated with Mn exposure (210) and the cumulative effect of long-term exposure to low levels of Fe may result in cellular death or colorectal cancer (211, 212). The concentration of Fe in the studied cream samples ranged from 0.112 to 1.252 μg g^{-1} which is lower than those reported in moisturising and skin-lightening creams in Nigeria (152). Fe was detected in all the cream samples. Low levels of Mn were detected in 22 of the studied cream samples. The concentrations of Mn in the cream samples ranged from 0.012 to 0.053 μg g^{-1}.

Zinc was detected in all the studied cream samples, with SLP 14 (1.535 μg g^{-1}) and SLP 33 (76.33 μg g^{-1}) having the highest concentrations of Zn. The concentrations of Zn in the cream samples ranged from 0.018 to 76.33 μg g^{-1}. High levels of Zn have been reported in body creams in Dubai, India, and Pakistan (201) and in personal care products in Nigeria (4). Cream samples found to have a high concentration of Zn may serve in the form of ZnO as a broad spectrum inorganic ultraviolet (UV) radiation filter in creams. Despite the importance of Zn to the body, cumulative effects of high concentration dosages can cause brittle nails, neurological abnormalities, as well as gastrointestinal disorders and convulsions (152).

Mercury was detected in only one of the studied cream samples (SLP 6) at a concentration of 69.86 μg g^{-1}. Under conditions of good manufacturing practice, trace amounts of mercury in cosmetics are unavoidable and it has been established by the US FDA that mercury in cosmetics should be less than 1 μg g^{-1} (213). The concentration of Hg in cream sample SLP 6 is far above the allowed limit of 1 μg g^{-1} by the World Health Organization (WHO) (214) and the 3.0 μg g^{-1} limit allowed by Health Canada (206). High concentrations of Hg have been reported in skin-lightening creams in Syria and extremely high concentrations of Hg in Mexican facial whitening creams (215). Hg can be absorbed through the skin and can accumulate in the brain, kidney, and liver tissues (141). This may result in several health issues such as psychological, neurological, and immunological conditions and much more (137). Cream SLP 6 was not found to contain any other SLA (see Section 3.3). Thus, the high amount of Hg found here is indicative that it was not an impurity from the manufacturing process but that it was added as the active SLA. It is well known that mercury salts bleach the skin (16). The use of Hg as a SLA is banned and should not be found present in SLPs.

3.6 Systemic exposure dosage and margin of safety for metals

The systemic exposure dosage (SED) and margin of safety (MoS) in this study were estimated by using equations 2.10 and 2.8, respectively, as given in Section 2.5. The values of SED and MoS obtained from the use of the studied SLPs are displayed in Tables 3.10 and 3.11 for 100% dermal absorption respectively. A minimum MoS value of 100

has been proposed by the World Health Organization (WHO) as acceptably safe for a product to be used on the human body (191).

The estimated SED for Al in this study ranged from 1.16×10^{-5} to 1.05×10^{-4} mg kg^{-1} bw day^{-1}. This does not exceed the provisional tolerable daily intake (PTDI) of 1.43×10^{-1} mg kg^{-1} bw day^{-1} set by the WHO (216). The estimated SED of Pb from the use of the studied SLPs ranged from 3.57×10^{-6} to 1.93×10^{-5} mg kg^{-1} bw day^{-1}. A PTDI of 3.6×10^{-3} mg kg^{-1} bw day^{-1} was used as the indicative value for comparing the results, even though the existing PTDI has been withdrawn by the combined FOA/WHO in 2011 as it could not be considered health protective (217). The estimated SED values for Ni, Cr and Cu of the investigated SLPs ranged from 2.97×10^{-6} to 1.29×10^{-4}, 3.86×10^{-6} to 6.45×10^{-5} and 5.05×10^{-6} to 2.59×10^{-5} mg kg^{-1} bw day^{-1}, respectively. They are all below their respective recommended daily intake/tolerable intake values of 1.2×10^{-2}, 3.3×10^{-3} and 8.33×10^{-2} mg kg^{-1} bw day^{-1} (217). The recommended daily intake (RDI) for Mn, Zn and Fe is set at 1.5×10^{-1} (218), 12.0 and 12.5 mg kg^{-1} bw day^{-1}, respectively (217). Their respective SEDs in the current study ranged from 3.57×10^{-6} to 1.57×10^{-5}, 5.65×10^{-6} to 2.27×10^{-2} and 1.93×10^{-6} to 3.72×10^{-4} mg kg^{-1} bw day^{-1}. The SED of Hg in sample SLP 6 was 2.07×10^{-2} mg kg^{-1} bw day^{-1}. This is above the established WHO PTDI value of 7.14×10^{-4} mg kg^{-1} bw day^{-1} (194, 219), making the product unsafe for use. The estimated SED for other metals studied were within their respective recommended PTDI or RDI values.

As shown in Table 3.11, the MoS value of Hg (2.4085) in sample SLP 6 is far less than the minimum value of 100 proposed by WHO to guarantee product safety. This should be a cause for concern since the safety of the product cannot be guaranteed. All other metals examined in the studied SLPs have MoS values greater than 100. This makes the level of the metal contents detected in other SLPs safe.

Table 3.9: Levels of heavy metals detected in different samples of skin-lightening products analysed in this study (n = 3).

Sample codes	Mean concentration ± SD/µg g^{-1}								
	Al	**Cr**	**Cu**	**Fe**	**Hg**	**Mn**	**Ni**	**Pb**	**Zn**
SLP 1	0.137±0.0005	0.010±0.0003	0.022±0.0003	0.209±0.0059	-	0.013±0.0001	0.017±0.0011	0.022±0.0099	0.030±0.0006
SLP 2	0.132±0.0008	0.019±0.0006	0.043±0.0001	0.243±0.0021	-	0.012±0.0001	0.064±0.0005	0.014±0.0024	0.023±0.0001
SLP 3	0.122±0.0031	0.217±0.0016	0.051±0.0010	1.252±0.0108	-	0.053±0.0001	0.433±0.0018	0.020±0.0120	0.023±0.0003
SLP 4	0.137±0.0009	0.032±0.0001	0.056±0.0005	0.304±0.0010	-	0.015±0.0001	0.069±0.0005	0.020±0.0073	0.018±0.0004
SLP 5	0.117±0.0014	0.013±0.0002	0.025±0.0003	0.169±0.0003	-	<LOD	0.032±0.0001	0.016±0.0016	0.021±0.0005
SLP 6	0.089±0.0005	<LOD	0.022±0..0001	0.149±0.0001	69.86±0.8921	<LOD	0.018±0.0007	0.020±0.0113	0.021±0.0003
SLP 7	0.196±0.0010	0.171±0.0009	0.046±0.0004	0.458±0.0016	-	0.026±0.0001	0.166±0.0016	0.022±0.0033	0.145±0.0005
SLP 8	0.091±0.0047	0.013±0.0007	0.034±0.0034	0.160±0.0060	-	0.012±0.0004	0.061±0.0031	0.017±0.0011	0.070±0.0005
SLP 9	0.173±0.0025	0.064±0.0003	0.051±0.0003	0.298±0.0009	-	0.017±0.0001	0.059±0.0004	0.024±0.0058	0.113±0.0003
SLP 10	0.050±0.0001	0.043±0.00004	0.034±0.0001	0.280±0.0003	-	00.023±0.0001	0.145±0.0013	0.025±0.0108	0.019±0.0003
SLP 11	0.074±0.0003	0.013±0.0005	0.053±0.0002	0.207±0.0007	-	0.017±0.0002	0.035±0.0007	0.024±0.0092	0.032±0.0003
SLP 12	0.354±0.0019	<LOD	0.035±0.0003	0.150±0.0006	-	0.022±0.0001	0.010±0.0006	0.025±0.0016	0.042±0.0003
SLP 13	0.118±0.0002	0.033±0.0005	0.018±0.0003	0.252±0.0002	-	0.021±0.0001	0.097±0.0013	0.018±0.0069	0.061±0.0007
SLP 14	0.243±0.0022	0.021±0.0006	0.037±0.0004	0.135±0.0005	-	0.013±0.0001	0.041±0.0009	0.025±0.0100	1.535±0.0140
SLP 15	0.073±0.0024	0.032±0.0006	0.024±0.0012	0.147±0.0021	-	<LOD	0.010±0.0004	0.018±0.0146	0.059±0.0007
SLP 16	0.049±0.0010	0.036±0.0002	0.033±0.0002	0.175±0.0007	-	0.015±0.0002	0.059±0.0008	0.012±0.0070	0.030±0.0006
SLP 17	0.043±0.0003	<LOD	0.026±0.0002	0.127±0.0002	-	<LOD	<LOD	0.017±0.0088	0.022±0.0002
SLP 18	0.046±0.0010	<LOD	0.031±0.0001	0.122±0.0006	-	<LOD	<LOD	0.022±0.0027	0.026±0.0001
SLP 19	0.061±0.0019	0.030±0.0009	0.035±0.0026	0.315±0.0136	-	0.020±0.0004	0.080±0.0014	0.019±0.0020	0.028±0.0003
SLP 20	0.075±0.0005	0.115±0.0013	0.047±0.0005	0.218±0.0029	-	0.013±0.0002	0.099±0.0012	0.024±0.0055	0.052±0.0001
SLP 21	0.104±0.0005	0.017±0.0003	0.050±0.0003	0.225±0.0010	-	0.020±0.0001	0.073±0.0014	0.021±0.0038	0.038±0.0001

SLP 22	0.039±0.0008	0.041±0.0004	0.022±0.0002	0.151±0.0008	-	0.012±0.0001	0.030±0.0008	0.023±0.0060	0.019±0.0003
SLP 23	0.051±0.0007	<LOD	0.019±0.0002	0.120±0.0002	-	<LOD	0.032±0.0004	0.023±0.0088	0.026±0.0001
SLP 24	0.062±0.0008	<LOD	0.018±0.0002	0.124±0.0009	-	<LOD	<LOD	0.014±0.0037	0.032±0.0004
SLP 25	0.258±0.0045	0.121±0.0004	0.017±0.0001	0.373±0.0026	-	0.012±0.0001	0.050 ±0.0006	0.015±0.0149	0.041±0.0003
SLP 26	0.141±0.0081	<LOD	0.023±0.0026	0.122±0.0066	-	<LOD	<LOD	0.019±0.0018	0.063±0.0003
SLP 27	0.106±0.0250	<LOD	0.020±0.0017	0.112±0.0051	-	<LOD	0.010±0.0004	0.017±0.0026	0.020±0.0002
SLP 28	0.068±0.0009	<LOD	0.048±0.0005	0.065±0.0004	-	<LOD	<LOD	0.022±0.0082	0.239±0.0002
SLP 29	0.304±0.0028	<LOD	0.024±0.0006	0.122±0.0003	-	0.012±0.0001	<LOD	0.019±0.0031	0.074±0.0004
SLP 30	0.095±0.0027	<LOD	0.035±0.0013	0.139±0.0035	-	0.013±0.0003	0.013±0.0006	0.026±0.0081	0.052±0.0003
SLP 31	0.302±0.0053	0.090±0.0006	0.051±0.0003	0.285 ±0.0013	-	0.012±0.0001	0.025±0.0012	0.019±0.0062	0.020±0.0001
SLP 32	0.162±0.0001	<LOD	0.031±0.0002	0.114±0.0002	-	<LOD	<LOD	0.021±0.0054	0.067±0.0002
SLP 33	0.050±0.0013	0.014±0.0004	0.024±0.0013	0.136±0.0003	-	0.012±0.0001	0.014±0.0005	0.065±0.0029	76.33±0.3055
SLP 34	0.060±0.0007	<LOD	0.087±0.0007	0.120±0.0002	-	<LOD	<LOD	0.013±0.0016	0.035±0.0003
SLP 35	0.093±0.0066	<LOD	0.023±0.0032	0.122±0.0062	-	<LOD	<LOD	0.017±0.0054	0.083±0.0008

Table 3.10: Systemic exposure dosage values of metals in skin-lightening products for 100% dermal absorption.

Sample codes	Systemic Exposure Dosage/mg kg^{-1} bw day^{-1}								
	Al	**Cr**	**Cu**	**Fe**	**Hg**	**Mn**	**Ni**	**Pb**	**Zn**
SLP 1	4.07×10^{-5}	2.97×10^{-6}	6.54×10^{-6}	6.21×10^{-5}	0.00	3.86×10^{-6}	5.05×10^{-6}	6.54×10^{-6}	8.91×10^{-6}
SLP 2	3.92×10^{-5}	5.65×10^{-6}	1.28×10^{-5}	7.22×10^{-5}	0.00	3.57×10^{-6}	1.90×10^{-5}	4.16×10^{-6}	6.83×10^{-6}
SLP 3	3.63×10^{-5}	6.45×10^{-5}	1.52×10^{-5}	3.72×10^{-4}	0.00	1.57×10^{-5}	1.29×10^{-4}	5.94×10^{-6}	6.83×10^{-6}
SLP 4	4.07×10^{-5}	9.51×10^{-6}	1.66×10^{-5}	9.03×10^{-5}	0.00	4.46×10^{-6}	2.05×10^{-5}	5.94×10^{-6}	5.35×10^{-6}
SLP 5	3.48×10^{-5}	3.86×10^{-6}	7.43×10^{-6}	5.02×10^{-5}	0.00	0.00	9.51×10^{-6}	4.75×10^{-6}	6.24×10^{-6}
SLP 6	2.64×10^{-5}	0.00	6.54×10^{-6}	4.43×10^{-5}	2.08×10^{-2}	0.00	5.35×10^{-6}	5.94×10^{-6}	6.24×10^{-6}
SLP 7	5.82×10^{-5}	5.08×10^{-5}	1.37×10^{-5}	1.36×10^{-4}	0.00	7.73×10^{-6}	4.93×10^{-5}	6.54×10^{-6}	4.31×10^{-5}
SLP 8	2.70×10^{-5}	3.86×10^{-6}	1.01×10^{-5}	4.75×10^{-5}	0.00	3.57×10^{-6}	1.81×10^{-5}	5.05×10^{-6}	2.08×10^{-5}
SLP 9	5.14×10^{-5}	1.90×10^{-5}	1.52×10^{-5}	8.86×10^{-5}	0.00	5.05×10^{-6}	1.75×10^{-5}	7.13×10^{-6}	3.36×10^{-5}
SLP 10	1.49×10^{-5}	1.28×10^{-5}	1.01×10^{-5}	8.32×10^{-5}	0.00	6.83×10^{-6}	4.31×10^{-5}	7.43×10^{-6}	5.65×10^{-6}
SLP 11	2.19×10^{-5}	3.86×10^{-6}	1.57×10^{-5}	6.15×10^{-5}	0.00	5.05×10^{-6}	1.04×10^{-5}	7.13×10^{-6}	9.51×10^{-6}
SLP 12	1.05×10^{-4}	0.00	1.04×10^{-5}	4.46×10^{-5}	0.00	6.54×10^{-6}	2.97×10^{-6}	7.43×10^{-6}	1.25×10^{-5}
SLP 13	3.51×10^{-5}	9.81×10^{-6}	5.35×10^{-6}	7.49×10^{-5}	0.00	6.24×10^{-6}	2.88×10^{-5}	5.35×10^{-6}	1.81×10^{-5}
SLP 14	7.22×10^{-5}	6.24×10^{-6}	1.09×10^{-5}	4.01×10^{-5}	0.00	3.86×10^{-6}	1.22×10^{-5}	7.43×10^{-6}	4.56×10^{-4}
SLP 15	2.17×10^{-5}	9.51×10^{-6}	7.13×10^{-6}	4.37×10^{-5}	0.00	0.00	2.97×10^{-6}	5.35×10^{-6}	1.75×10^{-5}
SLP 16	1.46×10^{-5}	1.07×10^{-5}	9.81×10^{-6}	5.20×10^{-5}	0.00	4.46×10^{-6}	1.75×10^{-5}	3.57×10^{-6}	8.91×10^{-6}
SLP 17	1.28×10^{-5}	0.00	7.73×10^{-6}	3.77×10^{-5}	0.00	0.00	0.00	5.05×10^{-6}	6.54×10^{-6}
SLP 18	1.37×10^{-5}	0.00	9.21×10^{-6}	3.63×10^{-5}	0.00	0.00	0.00	6.54×10^{-6}	7.73×10^{-6}
SLP 19	1.81×10^{-5}	8.91×10^{-6}	1.04×10^{-5}	9.36×10^{-5}	0.00	5.94×10^{-6}	2.38×10^{-5}	5.65×10^{-6}	8.32×10^{-6}

SLP 20	2.23×10^{-5}	3.42×10^{-5}	1.39×10^{-5}	6.48×10^{-5}	0.00	3.86×10^{-6}	2.94×10^{-5}	7.13×10^{-6}	1.55×10^{-5}
SLP 21	3.09×10^{-5}	5.05×10^{-6}	1.49×10^{-5}	6.69×10^{-5}	0.00	5.94×10^{-6}	2.17×10^{-5}	6.24×10^{-6}	1.13×10^{-5}
SLP 22	1.16×10^{-5}	1.22×10^{-5}	6.54×10^{-6}	4.49×10^{-5}	0.00	3.57×10^{-6}	8.91×10^{-6}	6.83×10^{-6}	5.65×10^{-6}
SLP 23	1.52×10^{-5}	0.00	5.65×10^{-6}	3.57×10^{-5}	0.00	0.00	9.51×10^{-6}	6.83×10^{-6}	7.73×10^{-6}
SLP 24	1.84×10^{-5}	0.00	5.35×10^{-6}	3.68×10^{-5}	0.00	0.00	0.00	4.16×10^{-6}	9.51×10^{-6}
SLP 25	7.67×10^{-5}	3.59×10^{-5}	5.05×10^{-6}	1.11×10^{-4}	0.00	3.57×10^{-6}	1.49×10^{-5}	4.46×10^{-6}	1.22×10^{-6}
SLP 26	4.19×10^{-5}	0.00	6.83×10^{-6}	3.63×10^{-5}	0.00	0.00	0.00	5.65×10^{-6}	1.87×10^{-5}
SLP 27	3.15×10^{-5}	0.00	5.94×10^{-6}	3.33×10^{-5}	0.00	0.00	2.97×10^{-6}	5.05×10^{-6}	5.94×10^{-6}
SLP 28	2.02×10^{-5}	0.00	1.43×10^{-5}	1.93×10^{-5}	0.00	0.00	0.00	6.54×10^{-6}	7.10×10^{-5}
SLP 29	9.03×10^{-5}	0.00	7.13×10^{-6}	3.63×10^{-5}	0.00	3.57×10^{-6}	0.00	5.65×10^{-6}	2.19×10^{-5}
SLP 30	2.82×10^{-5}	0.00	1.04×10^{-5}	4.13×10^{-5}	0.00	3.86×10^{-6}	3.86×10^{-6}	7.73×10^{-6}	1.55×10^{-5}
SLP 31	8.97×10^{-5}	2.67×10^{-5}	1.52×10^{-5}	8.47×10^{-5}	0.00	3.57×10^{-6}	7.43×10^{-6}	5.65×10^{-6}	5.94×10^{-6}
SLP 32	4.81×10^{-5}	0.00	9.21×10^{-6}	3.39×10^{-5}	0.00	0.00	0.00	6.24×10^{-6}	1.99×10^{-5}
SLP 33	1.49×10^{-5}	4.16×10^{-6}	7.13×10^{-6}	4.04×10^{-5}	0.00	3.57×10^{-6}	4.16×10^{-6}	1.93×10^{-5}	2.27×10^{-2}
SLP 34	1.78×10^{-5}	0.00	2.59×10^{-5}	3.57×10^{-5}	0.00	0.00	0.00	3.86×10^{-6}	1.04×10^{-5}
SLP 35	2.76×10^{-5}	0.00	6.83×10^{-6}	3.63×10^{-5}	0.00	0.00	0.00	5.05×10^{-6}	2.47×10^{-5}

Table 3.11: Margin of safety values of metals in skin-lightening products for 100% dermal absorption.

Sample codes	Margin of Safety								
	Al	**Cr**	**Cu**	**Fe**	**Hg**	**Mn**	**Ni**	**Pb**	**Zn**
SLP 1	245634.3	100955.7	611852.8	1127097.1	0.00	3624051.3	395904.8	61185.3	3365190.5
SLP 2	254938.7	53134.6	313040.9	969396.4	0.00	3926055.5	105162.2	96148.3	4389378.9
SLP 3	275835.3	4652.3	263936.5	188149.6	0.00	888918.2	15543.6	67303.8	4389378.9
SLP 4	245634.3	31548.7	240370.7	774879.4	0.00	3140844.4	97541.8	67303.8	5608650.8
SLP 5	287623.1	77658.2	538430.5	1393865.9	0.00	0.00	210324.4	84129.8	4807414.9
SLP 6	378111.3	0.00	611852.8	1580961.9	2.4085	0.00	373910.1	67303.8	4807414.9
SLP 7	171693.4	5903.8	292625.3	514330.4	0.00	1812025.6	40544.5	61185.3	696246.3
SLP 8	369801.2	77658.2	395904.8	1472270.8	0.00	3926055.5	110334.1	79180.9	1442224.5
SLP 9	194519.7	15774.3	263936.5	790480.9	0.00	2771333.3	114074.3	56086.5	893413.4
SLP 10	673038.1	23478.1	395904.8	841297.6	0.00	2048376.8	46416.4	53843.0	5313458.6
SLP 11	454755.5	77658.2	253976.6	1137987.1	0.00	2771333.3	192296.6	56086.5	3154866.1
SLP 12	95061.9	0.00	384593.2	1570422.2	0.00	2141484.8	673038.1	53843.0	2403707.5
SLP 13	285185.6	30592.6	747820.1	934775.1	0.00	2243460.3	69385.4	74782.0	1655011.7
SLP 14	138485.2	48074.1	363804.4	1744913.6	0.00	3624051.3	164155.6	53843.0	65769.2
SLP 15	460984.9	31548.7	560865.1	1602471.7	0.00	0.00	673038.1	74782.0	1711113.8
SLP 16	686773.6	28043.3	407901.9	1346076.2	0.00	3140844.4	114074.3	112173.0	3365190.5
SLP 17	782602.4	0.00	517721.6	1854829.4	0.00	0.00	0.00	79180.9	4588896.1
SLP 18	731563.1	0.00	434218.1	1930846.9	0.00	0.00	0.00	61185.3	3882912.1
SLP 19	551670.6	33651.9	384593.2	747820.1	0.00	2355633.3	84129.8	70846.1	3605561.2

SLP 20	448692.1	8778.8	286399.2	1080565.7	0.00	3624051.3	67983.6	56086.5	1941456.0
SLP 21	323576.0	59385.7	269215.2	1046948.1	0.00	2355633.3	92196.9	64098.9	2656729.3
SLP 22	862869.4	24623.3	611852.8	1560022.1	0.00	3926055.5	224346.0	58525.1	5313458.6
SLP 23	659841.3	0.00	708461.2	1963027.8	0.00	0.00	210324.4	58525.1	3882912.1
SLP 24	542772.7	0.00	747820.1	1899704.3	0.00	0.00	0.00	96148.3	3154866.1
SLP 25	130433.7	8343.4	791809.5	631537.1	0.00	3926055.5	134607.6	89738.4	2462334.5
SLP 26	238665.9	0.00	585250.5	1930846.9	0.00	0.00	0.00	70846.1	1602471.7
SLP 27	317470.8	0.00	673038.1	2103244.0	0.00	0.00	673038.1	79180.9	5047785.7
SLP 28	494880.9	0.00	280432.5	3624051.1	0.00	0.00	0.00	61185.3	422408.8
SLP 29	110697.1	0.00	560865.1	1930846.9	0.00	3926055.5	0.00	70846.1	1364266.4
SLP 30	354230.6	0.00	384593.2	1694700.2	0.00	3624051.3	517721.6	51772.2	1941456.0
SLP 31	111430.1	11217.3	263936.5	826538.0	0.00	3926055.5	269215.2	70846.1	5047785.7
SLP 32	207727.8	0.00	434218.1	2066345.0	0.00	0.00	0.00	64098.9	1506801.7
SLP 33	673038.1	72111.2	560865.	1732083.3	0.00	3926055.5	480741.5	20708.9	1322.6
SLP 34	560865.1	0.00	154721.1	1963027.8	0.00	0.00	0.00	103544.3	2884448.9
SLP 35	361848.4	0.00	585250.5	1930846.9	0.00	0.00	0.00	79180.9	1216333.9

Chapter 4

CONCLUSIONS AND RECOMMENDATIONS

A number of skin-lightening formulations commonly available in the Nigerian market were investigated in this study. Only 28% of the sample products were manufactured in Nigeria. The remaining 72% of the sample products were either manufactured in America, Europe, Asia or other African Countries.

A total of 35 skin-lightening formulations were analysed for the quantitative determination of some selected organic active ingredients and heavy metals. The organic active ingredients were quantified by means of HPLC and the heavy metals were quantified by means of ICP-OES and/or CV-AAS. All analytical methods employed in this study were fully validated in terms of precision, detection and quantification limits, linearity and recovery.

Determination of skin-lightening agents by means of HPLC

The active ingredients investigated in our sample products by means of HPLC included clobetasol propionate, betamethasone dipropionate, clotrimazole, hydroquinone, benzoquinone, kojic acid, niacinamide and arbutin.

An external standard method was used to prepare a calibration curve for each active ingredient. The correlation coefficients of all the calibration curves were in the acceptable range of a value greater than 0.95. The hydroquinone standard had the lowest correlation coefficient of 0.9942, while both betamethasone dipropionate and clotrimazole had the highest correlation coefficient values of 0.9995. The limits of detection obtained for hydroquinone, benzoquinone, kojic acid, niacinamide, arbutin, clobetasol propionate, betamethasone dipropionate and clotrimazole were 0.029, 0.026, 0.014, 0.016, 0.012, 0.008, 0.007 and 0.007 mg L^{-1} respectively and the limits of quantitation were 0.097, 0.085, 0.046, 0.052, 0.039, 0.028, 0.026 and 0.025 respectively.

Intra-day and inter-analyses were used to test the precision of the method. The percent relative standard deviation (%R.S.D.) for the intra-day precision ranged from 0.17 to 8.16% while that of the inter-day precision ranged from 0.14 to 4.78%. A recovery

analysis was used to test the accuracy of the method. The calculated recoveries for all the active ingredient were within an acceptable range. The obtained percent recoveries ranged from 84.23 to 110.33%.

Arbutin was not detected in any of the products. A total number of 32 samples (92%) were found to contain at least one or more of the other active ingredients. Hydroquinone was detected in 15 samples with only five of the samples properly labelled. The concentration of hydroquinone in the samples ranged from 0.017 to 7.096%. Some of the samples containing hydroquinone were found to contain benzoquinone. Hydroquinone is usually unstable in some formulations and hydrolyses to benzoquinone. The six samples found to contain benzoquinone ranged from 0.005 to 0.015%. Kojic acid was detected in nine samples in the range of 0.017 to 1.412%. However, only three samples specified kojic acid as an ingredient in the formulations. Four samples were found to contain niacinamide, with only one listing niacinamide on the label. Although, one more sample listed niacinamide among its ingredients, it was not detected after analysis. The concentration of niacinamide in the samples ranged from 0.029 to 1.827%. Arbutin was indicated to be present in one of the sample products but, upon analysis, it was not detected. Steroid compounds were detected in 13 of the studied samples with all the samples properly labelled. Although one sample claimed to contain clobetasol propionate, after analysis it was found to contain betamethasone dipropionate. Eight samples were found to contain clobetasol propionate alone, two samples were found to contain both clobetasol propionate and clotrimazole, and three samples were found to contain betamethasone dipropionate only. The concentrations of clobetasol propionate, betamethasone dipropionate and clotrimazole were found in the range of 0.007 to 0.035, 0.019 to 0.027 and 0.007 to 0.012% (m/m) respectively.

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

All skin-lightening formulations were screened for the presence of arsenic, aluminium, cadmium, cobalt, chromium, copper, iron, manganese, nickel, lead, and zinc by means of ICP-OES and mercury was screened for by means of CV-AAS.

The correlation coefficients for all the different metal calibration curves ranged from 0.9980 to 0.9999. The limits of detection and quantitation of the different metals varied

from 0.007 to 0.026 and 0.024 to 0.088 mg L^{-1} respectively. The methods were found to have good recovery values ranging from 95.6 to 112.7%.

Arsenic, cadmium and cobalt were not detected in any of the sample products. Aluminium was detected in all the samples and the maximum concentration detected was 0.354 μg g^{-1}. There is no established guideline for aluminium, but certain health concerns have been reported from its high dosage. Iron, manganese, and copper were detected in most of the samples at levels not exceeding 1.252, 0.053, and 0.056 μg g^{-1}, respectively. This should not be a threat to the health of users of the products since these are essential elements required in a number of biological processes at low concentration. However, cumulative effects of long-term exposure to these elements even at low concentration is unsafe. Zinc was detected in all the samples but was relatively high in one of the samples (76.33 μg g^{-1}). It may have been present in this sample as a sunscreen filter to protect the user against the harmful effects derived from solar ultraviolet exposure. This is particularly important in SLPs where the formation of the natural UV filter, melanin, is being prevented. Zinc is a vital element to the body but not required at high concentration. Chromium was found in twenty-one samples at a maximum concentration (0.217 μg g^{-1}) that cannot induce allergenic contact dermatitis.

Lead was detected in all samples with concentrations not exceeding 0.026 μg g^{-1} and nickel was detected in 26 samples at a maximum concentration of 0.433 μg g^{-1}. The levels detected for both metals do not exceed the maximum limits allowed as impurities in colour additives in cosmetics for external use. One sample (SLP 6) was found to have a relatively high level of mercury (69.86 μg g^{-1}). This level of mercury in a skin-lightening cream is alarming due to its high toxicity. The severe health implications of mercury are why it is completely banned in cosmetics.

The values of SED and MoS obtained for the studied SLPs show that the level of the metal contents detected in these products are safe for use except in the case of the sample SLP 6 containing a relatively high level of Hg, where it's SED and MoS values are outside the WHO limits.

As gathered from the results of this study, the initial concern of some skin-lightening formulations commonly available in the Nigerian market containing either banned ingredients or exceeding imposed limits of allowed ingredients and improper labelling of

these products was justified. The findings in this study further expose the continued inclusion of banned and toxic ingredients, such as hydroquinone, mercury and very potent steroids like clobetasol propionate and betamethasone dipropionate, in SLPs. These ingredients are known to be detrimental and pose a serious health risk if the frequency of application, the surface area of the body applied, and the duration of practice are considered. Severe and permanent side-effects such as exogenous ochronosis, decreased skin elasticity, trimethylaminuria, Cushing syndrome, diabetes mellitus, steroid addiction syndrome, gingivostomatitis, paradoxical hyperpigmentation and many more have been reported from using SLPs containing these toxic ingredients (59).

Often, users are advised to double check the labelling of any skin-care product or any other cosmetics products before purchase. However, it is worrisome to know that even when users double check the labelling, one cannot be certain of the actual contents of these products as evident from some of the findings in this study. We, therefore, discourage the use of these products for skin-lightening purposes. For skin conditions that do require treatment with such products, appropriate consultation from a medical practitioner is recommended.

Furthermore, regulating agencies, especially in a country like Nigeria where skin-bleaching is fashionable, must establish and enforce more stringent regulations restricting the use of banned and harmful ingredients in commercial skin-lightening products. Such regulations should extend to importation or smuggling of skin-lightening or any other cosmetic products containing banned or toxic ingredients since 72% of the cream samples investigated in this study were found to be manufactured in other countries but are in circulation in the study area. Efforts must also be made by appropriate authorities to enlighten users and the general public on the possible dangers associated with skin-bleaching practices.

Future work can investigate some newly used plant extracts as active ingredients in skin-lightening formulations. Manufacturers have now opted for the inclusion of some plant extracts with unknown safety profiles in skin-lightening products not minding the amount added as far as the desired effects of skin-lightening are achieved. Examples of such extracts include mulberry root extracts, licorice extracts, lemon extracts, and more. Moreover, parabens used as preservatives in skin-lightening products also need to be

investigated. Most of the studied cream samples in this study were found to contain one or more of these parabens (methyl, ethyl, propyl, and butyl parabens). Toxicity concerns of preservatives at high concentration have been reported (220, 221).

REFERENCES

1. Brenner, M. and V. J. Hearing (2008) Modifying skin pigmentation–approaches through intrinsic biochemistry and exogenous agents. *Drug Discovery Today: Disease Mechanisms* **5,** e189-e199.

2. Carsberg, C., H. Warenius and P. Friedmann (1994) Ultraviolet radiation-induced melanogenesis in human melanocytes. Effects of modulating protein kinase C. *Journal of Cell Science* **107,** 2591-2597.

3. Al-Saleh, I., S. Al-Enazi and N. Shinwari (2009) Assessment of lead in cosmetic products. *Regulatory Toxicology and Pharmacology* **54,** 105-113.

4. Ayenimo, J., A. Yusuf, A. Adekunle and O. Makinde (2010) Heavy metal exposure from personal care products. *Bulletin of Environmental Contamination and Toxicology* **84,** 8-14.

5. Nnorom, I., J. Igwe and C. Oji-Nnorom (2005) Trace metal contents of facial (make-up) cosmetics commonly used in Nigeria. *African Journal of Biotechnology* **4,** 1133-1138.

6. Nourmoradi, H., M. Foroghi, M. Farhadkhani and M. Vahid Dastjerdi (2013) Assessment of lead and cadmium levels in frequently used cosmetic products in Iran. *Journal of Environmental and Public Health* **2013,** 1-5.

7. DeCaprio, A. P. (1999) The toxicology of hydroquinone—relevance to occupational and environmental exposure. *Critical reviews in toxicology* **29,** 283-330.

8. Findlay, G. and H. De Beer (1980) Chronic hydroquinone poisoning of the skin from skin-lightening cosmetics. *South Africa Medical Journal* **57,** 187-190.

9. Petit, L. and G. Pierard (2003) Skin-lightening products revisited. *International journal of cosmetic science* **25,** 169-181.

10. Gillbro, J. and M. Olsson (2011) The melanogenesis and mechanisms of skin-lightening agents–existing and new approaches. *International Journal of Cosmetic Science* **33,** 210-221.

11. Ly, F., A. S. Soko, D. A. Dione, S. O. Niang, A. Kane, T. I. Bocoum, M. T. Dieng and B. Ndiaye (2007) Aesthetic problems associated with the cosmetic use of bleaching products. *International Journal of Dermatology* **46,** 15-17.

12. Mahe, A., F. Ly, G. Aymard and J. M. Dangou (2003) Skin diseases associated with the cosmetic use of bleaching products in women from Dakar, Senegal. *British Journal of Dermatology* **148,** 493-500.

13. Panusa, A., M. Ottaviani, M. Picardo, E. Camera, L. Gagliardi, P. Chimenti, A. Granese and D. Tonelli (2004) Analysis of corticosteroids by high performance liquid chromatography–electrospray mass spectrometry. *Analyst* **129,** 719-723.

14. Halder, R. and G. Richards (2006) In Photoaging in pigmented skins. *Dermatology and dermatological therapy of pigmented skins, Boca Raton***,** 211-220.

15. Mahé, A., F. Ly and J. L. Perret (2005) Systemic complications of the cosmetic use of skin-bleaching products. *International Journal of Dermatology* **44,** 37-38.

16. Al-Saleh, I., R. Elkhatib, R. Al-Rouqi, S. Al-Enazi and N. Shinwari (2012) The dangers of skin-lightening creams. *Toxicological & Environmental Chemistry* **94,** 195-219.

17. Blay, Y. A. (2011) Skin bleaching and global white supremacy: By way of introduction. *Journal of Pan African Studies* **4,** 4-46.

18. Glenn, E. N. (2008) Yearning for lightness: Transnational circuits in the marketing and consumption of skin lighteners. *Gender & Society***,** 1-22.

19. Adebajo, S. (2002) An epidemiological survey of the use of cosmetic skin lightening cosmetics among traders in Lagos, Nigeria. *Mercury* **5,** 43-48.

20. Del Giudice, P. and P. Yves (2002) The widespread use of skin lightening creams in Senegal: a persistent public health problem in West Africa. *International Journal of Dermatology* **41,** 69-72.

21. Malangu, N. and G. Ogunbanjo (2006) Predictors of topical steroid misuse among patrons of pharmacies in Pretoria. *South African Family Practice* **48,** 14-14e.

22. Kandarova, H. (2006) Evaluation and validation of reconstructed human skin models as alternatives to animal tests in regulatory toxicology. PhD thesis, Freie Universität Berlin.

23. Wagner, H., N. Zghoul, C.-M. Lehr and U. F. Schäfer (2002) Human skin and skin equivalents to study dermal penetration and permeation. *Cell culture models of biological barriers: in vitro test systems for drug absorption and delivery***,** 289-309.

24. Snell, R. (1967) An electron microscopic study of the human epidermal keratinocyte. *Zeitschrift für Zellforschung und Mikroskopische Anatomie* **79,** 492-506.

25. Eckert, R. L. (1989) Structure, function, and differentiation of the keratinocyte. *Physiological Reviews* **69,** 1316-1346.

26. Schaefer, H. and T. Redelmeier (1996) Composition and structure of the stratum corneum. *Principles of Percutaneous Absorption***,** 43-86.

27. Muehlenbein, M. P. (2010) *Human Evolutionary Biology*. Cambridge University Press, New York.

28. Webb, A. R. (2006) Who, what, where and when—influences on cutaneous vitamin D synthesis. *Progress in Biophysics and Molecular Biology* **92,** 17-25.

29. Mosher, D., T. Fitzpatrick, J. Ortonne and Y. Hori (1999) Normal skin color and general considerations of pigmentary disorders. *Dermatology in General Medicine. New York, Mcgraw-Hill***,** pp 936-944.

30. Jablonski, N. G. (2012) *Living color: the biological and social meaning of skin color.* University of California Press.

31. Fitzpatrick, T. B. (1988) The validity and practicality of sun-reactive skin types I through VI. *Archives of Dermatology* **124,** 869-871.

32. Mercurio, D., J. Segura, M. Demets and P. Maia Campos (2013) Clinical scoring and instrumental analysis to evaluate skin types. *Clinical and Experimental Dermatology* **38,** 302-309.

33. Meredith, P. and T. Sarna (2006) The physical and chemical properties of eumelanin. *Pigment Cell Research* **19,** 572-594.

34. Riley, P. A. (1997) Melanin. *The International Journal of Biochemistry & Cell Biology* **29,** 1235-1239.

35. Slominski, A., D. J. Tobin, S. Shibahara and J. Wortsman (2004) Melanin pigmentation in mammalian skin and its hormonal regulation. *Physiological Reviews* **84,** 1155-1228.

36. Ito, S., K. Wakamatsu and H. Ozeki (2000) Chemical analysis of melanins and its application to the study of the regulation of melanogenesis. *Pigment Cell Research* **13,** 103-109.

37. Vincensi, M., A. Napolitano, E. Procaccini, G. Riccio, G. Monfrecola, P. Santoianni and G. Prota (1998) Phaeomelanin versus eumelanin as a chemical indicator of ultraviolet sensitivity in fair-skinned subjects at high risk for melanoma: a pilot study. *Melanoma Research* **8,** 53-58.

38. D'Orazio, J., S. Jarrett, A. Amaro-Ortiz and T. Scott (2013) UV radiation and the skin. *International Journal of Molecular Sciences* **14,** 12222-12248.

39. Dyall-Smith, D. J. and J. P. Scurry (1990) Mercury pigmentation and high mercury levels from the use of a cosmetic cream. *The Medical Journal of Australia* **153,** 409-410, 414-405.

40. Olumide, Y. M., A. O. Akinkugbe, D. Altraide, T. Mohammed, N. Ahamefule, S. Ayanlowo, C. Onyekonwu and N. Essen (2008) Complications of chronic use of skin lightening cosmetics. *International Journal Of Dermatology* **47,** 344-353.

41. Plensdorf, S. and J. Martinez (2009) Common pigmentation disorders. *American Family Physician* **79,** 109-116.

42. Al-Saleh, I. and I. Al-Doush (1997) Mercury content in skin-lightening creams and potential hazards to the health of Saudi women. *Journal of Toxicology and Environmental Health* **51,** 123-130.

43. Prota, G. (1995) The chemistry of melanins and melanogenesis. In *Fortschritte der Chemie organischer Naturstoffe/Progress in the Chemistry of Organic Natural Products*. pp. 93-148. Springer.

44. Ito, S. (2003) A chemist's view of melanogenesis. *Pigment Cell Research* **16,** 230-236.

45. Pawelek, J. M. (1991) After dopachrome? *Pigment Cell Research* **4,** 53-62.

46. Carstam, R., C. Brinck, A. Hindemith-Augustsson, H. Rorsman and E. Rosengren (1991) The neuromelanin of the human substantia nigra. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease* **1097,** 152-160.

47. Double, K., L. Zecca, P. Costi, M. Mauer, C. Griesinger, S. Ito, D. Ben-Shachar, G. Bringmann, R. Fariello and P. Riederer (2000) Structural characteristics of human substantia nigra neuromelanin and synthetic dopamine melanins. *Journal of Neurochemistry* **75,** 2583-2589.

48. Zecca, L., D. Tampellini, M. Gerlach, P. Riederer, R. Fariello and D. Sulzer (2001) Substantia nigra neuromelanin: structure, synthesis, and molecular behaviour. *Molecular Pathology* **54,** 414.

49. Kolbe, L., T. Mann, W. Gerwat, J. Batzer, S. Ahlheit, C. Scherner, H. Wenck and F. Stäb (2013) 4-n-butylresorcinol, a highly effective tyrosinase inhibitor for the topical treatment of hyperpigmentation. *Journal of the European Academy of Dermatology and Venereology* **27,** 19-23.

50. Chang, T.-S. (2012) Natural melanogenesis inhibitors acting through the down-regulation of tyrosinase activity. *Materials* **5,** 1661-1685.

51. Tsong-Min, C. (2012) Tyrosinase and tyrosinase inhibitors. *Journal of Biocatalysis & Biotransformation* **1,** 1-2.

52. Parvez, S., M. Kang, H. S. Chung, C. Cho, M. C. Hong, M. K. Shin and H. Bae (2006) Survey and mechanism of skin depigmenting and lightening agents. *Phytotherapy Research* **20,** 921-934.

53. Katsambas, A. D. and A. J. Stratigos (2001) Depigmenting and bleaching agents: coping with hyperpigmentation. *Clinics in Dermatology* **19,** 483-488.

54. Draelos, Z. D. (2007) Skin lightening preparations and the hydroquinone controversy. *Dermatologic Therapy* **20,** 308-313.

55. Kamagaju, L., R. Morandini, E. Bizuru, P. Nyetera, J. B. Nduwayezu, C. Stévigny, G. Ghanem and P. Duez (2013) Tyrosinase modulation by five Rwandese herbal medicines traditionally used for skin treatment. *Journal of Ethnopharmacology* **146,** 824-834.

56. Dooley, T. (1997) Topical skin depigmentation agents: current products and discovery of novel inhibitors of melanogenesis. *Journal of Dermatological Treatment* **8,** 275-283.

57. Jimbow, K. and Y. Minamitsuji (2001) Topical therapies for melasma and disorders of hyperpigmentation. *Dermatologic Therapy* **14,** 35-45.

58. Xiaoying, R. C. W. J. M., H. R. C. S. S. Hongbo and Z. D. Q. H. I. Maibach (1998) Human in vivo and in vitro hydroquinone topical bioavailability, metabolism, and disposition. *Journal of Toxicology and Environmental Health Part A* **54,** 301-317.

59. Ladizinski, B., N. Mistry and R. V. Kundu (2011) Widespread use of toxic skin lightening compounds: medical and psychosocial aspects. *Dermatologic Clinics* **29,** 111-123.

60. Smith, C. J., K. B. O'hare and J. C. Allen (1988) Selective cytotoxicity of hydroquinone for melanocyte-derived cells is mediated by tyrosinase activity but independent of melanin content. *Pigment Cell Research* **1,** 386-389.

61. Palumbo, A., M. d'Ischia, G. Misuraca and G. Prota (1991) Mechanism of inhibition of melanogenesis by hydroquinone. *Biochimica et Biophysica Acta-General Subjects* **1073,** 85-90.

62. Pathak, M. A., T. B. Fitzpatrick and E. W. Kraus (1986) Usefulness of retinoic acid in the treatment of melasma. *Journal of the American Academy of Dermatology* **15,** 894-899.

63. Glenn, M., P. Grimes, M. Chalet, E. Pitts and A. Kelly (1991) Evaluation of clinical and light microscopic effects of various concentrations of hydroquinone. *Clinical Research* **39,** A83-A83.

64. Garcia-Lopez, M. (1989) Double-blind comparison of azelaic acid and hydroquinone in the treatment of melasma. *Acta Derm Venereol (Stockh)* **143,** 58-61.

65. Health-Council-of-the-Netherlands (2012) Hydroquinone and benzoquinone. *Healthbased recommended occupational exposure limit. The Hague: Health Council of the Netherlands*.

66. Government Gazette no. R2892; (1988) 'Ban of Hydroquinone and Other Harmful Ingredients in Skin Lighteners' in South Africa.

67. Nordlund, J., P. Grimes and J. P. Ortonne (2006) The safety of hydroquinone. *Journal of the European Academy of Dermatology and Venereology* **20,** 781-787.

68. Baliña, L. M. and K. Graupe (1991) The treatment of melasma 20% azelaic acid versus 4% hydroquinone cream. *International Journal of Dermatology* **30,** 893-895.

69. Grimes, P. E. (2004) A microsponge formulation of hydroquinone 4% and retinol 0.15% in the treatment of melasma and postinflammatory hyperpigmentation. *Cutis* **74,** 362-368.

70. Garcia, R. L., J. W. White Jr and W. F. Willis (1978) Hydroquinone nail pigmentation. *Archives of Dermatology* **114,** 1402.

71. Arndt, K. A. and T. B. Fitzpatrick (1965) Topical use of hydroquinone as a depigmenting agent. *Jama* **194,** 965-967.

72. Mann, R. and R. Harman (1983) Nail staining due to hydroquinone skin-lightening creams. *British Journal of Dermatology* **108,** 363-365.

73. Anderson, B. (1947) Corneal and conjunctival pigmentation among workers engaged in manufacture of hydroquinone. *Archives of Ophthalmology* **38,** 812-826.

74. Naumann, G. (1966) Corneal damage in hydroquinone workers: a clinicopathologic study. *Archives of Ophthalmology* **76,** 189-194.

75. Karamagi, C., E. Owino and E. Katabira (2001) Hydroquinone neuropathy following use of skin bleaching creams: case report. *East African Medical Journal* **78,** 223-224.

76. Ajose, F. O. (2005) Consequences of skin bleaching in Nigerian men and women. *International Journal of Dermatology* **44,** 41-43.

77. Ruocco, V. and M. Florio (1995) Fish-odor syndrome: An olfactory diagnosis. *International Journal of Dermatology* **34,** 92-93.

78. Findlay, G. H., J. Morrison and I. Simson (1975) Exogenous ochronosis and pigmented colloid milium from hydroquinone bleaching creams. *British Journal of Dermatology* **93,** 613-622.

79. Levitt, J. (2007) The safety of hydroquinone: a dermatologist's response to the 2006 Federal Register. *Journal of the American Academy of Dermatology* **57,** 854-872.

80. Phornphutkul, C., W. J. Introne, M. B. Perry, I. Bernardini, M. D. Murphey, D. L. Fitzpatrick, P. D. Anderson, M. Huizing, Y. Anikster and L. H. Gerber (2002) Natural history of alkaptonuria. *New England Journal of Medicine* **347,** 2111-2121.

81. Pick, L. (1906) Uber die ochronosis klin. *Wochenschr* **43,** 478-480.

82. Dogliotti, M. and M. Leibowitz (1979) Granulomatous ochronosis--a cosmetic-induced skin disorder in Blacks. *South African Medical Journal* **56,** 757-760.

83. Charlín, R., C. B. Barcaui, B. K. Kac, D. B. Soares, R. Rabello-Fonseca and L. Azulay-Abulafia (2008) Hydroquinone-induced exogenous ochronosis: a report of four cases and usefulness of dermoscopy. *International Journal of Dermatology* **47,** 19-23.

84. Levin, C. Y. and H. Maibach (2001) Exogenous ochronosis. *American Journal of Clinical Dermatology* **2,** 213-217.

85. Hoshaw, R. A., K. G. Zimmerman and A. Menter (1985) Ochronosislike pigmentation from hydroquinone bleaching creams in American blacks. *Archives of Dermatology* **121,** 105-108.

86. Ly, F., A. Kane, A. Déme, N. Ngom, S. Niang, R. Bello, L. Rethers, J. Dangou, M. Dieng and P. Diousse (2010) First cases of squamous cell carcinoma associated with cosmetic use of bleaching compounds. *Annales de Dermatologie et de Venereologie* **137,** 128-131.

87. Addo, H. (2000) Squamous cell carcinoma associated with prolonged bleaching. *Ghana Medical Journal* **34**.

88. IARC (1999) 1,4-Benzoquinone (para-quinone). In: Re-evaluation of some organic chemicals, hydrazine and hydrogen peroxide. *Proceedings of the IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. International Agency for Research on Cancer, Lyon, France*.

89. Omaye, S., R. Wirtz and J. Fruin (1980) Toxicity of substituted p-benzoquinones found in the secretion of tenebrionid flour beetles. *Proceedings of the Western Pharmacology Society* **24,** 169-171.

90. Iskander, K. and A. K. Jaiswal (2005) Quinone oxidoreductases in protection against myelogenous hyperplasia and benzene toxicity. *Chemico-Biological Interactions* **153,** 147-157.

91. Joseph, P. and A. Jaiswal (2000) A unique cytosolic activity related but distinct from NQO1 catalyses metabolic activation of mitomycin C. *British Journal of Cancer* **82,** 1305-1311.

92. IARC (1977) Hydroquinone and benzoquinone. In: IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, Some fumigants, the herbicides 2,4-D and 2,4,5-T, chlorinated dibenzodioxins and miscellaneous industrial chemicals. *International Agency for Research on Cancer, Lyon, France* **15**.

93. Rajka, G. and S. Blohm (1970) The allergenicity of paraphenylendiamine. II. *Acta Dermato-Venereologica* **50,** 51-54.

94. ACGIH (2001) Quinone. In: Documentation of the Threshold Limit Values and Biological Exposure Indices. *American Conference of Governmental Industrial Hygienists, Cincinnati, Ohio, USA*.

95. Serif, G. S. and L. Seymour (1963) Catabolism and excretion of the antithyroid substance, diacetyl-2, 6-diiodohydroquinone. *Biochemical Pharmacology* **12,** 885-889.

96. ACGIH (2008) In: Documentation of the Threshold Limit Values and Biological Exposure Indices. *American Conference of Governmental Industrial Hygienists, Cincinnati, Ohio, USA*.

97. US EPA (2008) Quinone (p-benzoquinone) (CAS 106-51-4) US Environmental Protection Agency,Washington DC, USA; 2000. http://www.epa.gov/ttn/atw/hlthef/quinone.html (Accessed 25th October 2016).

98. NIOSH (2005) Hydroquinone US Department of Health and Human Services, National Institute for Occupational Safety and Health, Washington DC, USA. http://www.cdc.gov/niosh/npg/npgd0338.html (Accessed 25th October 2016).

99. Lim, J. T. E. (1999) Treatment of melasma using kojic acid in a gel containing hydroquinone and glycolic acid. *Dermatologic Surgery* **25,** 282-284.

100. Burnett, C. L., W. F. Bergfeld, D. V. Belsito, R. A. Hill, C. D. Klaassen, D. C. Liebler, J. G. Marks, R. C. Shank, T. J. Slaga and P. W. Snyder (2010) Final report of the safety assessment of kojic acid as used in cosmetics. *International journal of toxicology* **29,** 244S-273S.

101. Garcia, A. and J. E. Fulton (1996) The combination of glycolic acid and hydroquinone or kojic acid for the treatment of melasma and related conditions. *Dermatologic surgery* **22,** 443-447.

102. Rendon, M. I. and J. I. Gaviria (2005) Review of skin-lightening agents. *Dermatologic Durgery* **31,** 886-890.

103. Dlova, N. C., N. E. Hendricks and B. S. Martincgh (2012) Skin-lightening creams used in Durban, South Africa. *International journal of dermatology* **51,** 51-53.

104. Hakozaki, T., L. Minwalla, J. Zhuang, M. Chhoa, A. Matsubara, K. Miyamoto, A. Greatens, G. Hillebrand, D. Bissett and R. Boissy (2002) The effect of niacinamide on reducing cutaneous pigmentation and suppression of melanosome transfer. *British Journal of Dermatology* **147,** 20-31.

105. Sarkar, R., M. Bhalla and A. J. Kanwar (2002) A comparative study of 20% azelaic acid cream monotherapy versus a sequential therapy in the treatment of melasma in dark-skinned patients. *Dermatology* **205,** 249-254.

106. Schallreuter, K. U. and J. Wood (1987) Azelaic acid as a competitive inhibitor of thioredoxin reductase in human melanoma cells. *Cancer letters* **36,** 297-305.

107. Robins, E., A. Breathnach, D. Bennett, B. Ward, Y. Bhasin, L. Ethridge, M. Nazzaro-porro, S. Passi and M. Picardo (1985) Ultrastructural observations on the effect of azelaic acid on normal human melanocytes and a human melanoma cell line in tissue culture. *British Journal of Dermatology* **113,** 687-697.

108. Breathnach, A. C., M. Nazzaro-Porro, S. Passi and G. Zina (1989) Azelaic acid therapy in disorders of pigmentation. *Clinics in dermatology* **7,** 106-119.

109. Kligman, A. M., G. L. Grove, R. Hirose and J. J. Leyden (1986) Topical tretinoin for photoaged skin. *Journal of the American Academy of Dermatology* **15,** 836-859.

110. Kligman, A. M. (1989) The treatment of photoaged human skin by topical tretinoin. *Drugs* **38,** 1-8.

111. Duell, E. A., S. Kang and J. J. Voorhess (1997) Unoccluded retinol penetrates human skin in vivo more effectively than unoccluded retinyl palmitate or retinoic acid. *Journal of investigative dermatology* **109,** 301-305.

112. Weinstein, G. D., T. P. Nigra, P. E. Pochi, R. C. Savin, A. Allan, K. Benik, E. Jeffes, L. Lufrano and E. G. Thorne (1991) Topical tretinoin for treatment of photodamaged skin: a multicenter study. *Archives of dermatology* **127,** 659-665.

113. Kimbrough-Green, C. K., C. E. Griffiths, L. J. Finkel, T. A. Hamilton, S. M. Bulengo-Ransby, C. N. Ellis and J. J. Voorhees (1994) Topical retinoic acid (tretinoin) for melasma in black patients: a vehicle-controlled clinical trial. *Archives of dermatology* **130,** 727-733.

114. Kim, Y.-J. and H. Uyama (2005) Tyrosinase inhibitors from natural and synthetic sources: structure, inhibition mechanism and perspective for the future. *Cellular and Molecular Life Sciences* **62,** 1707-1723.

115. Bennett, M. L. and R. L. Henderson (2003) Introduction to cosmetic dermatology. *Current Problems in Dermatology* **15,** 43-83.

116. Choi, S., Y. I. Park, S. K. Lee, J. E. Kim and M. H. Chung (2002) Aloesin inhibits hyperpigmentation induced by UV radiation. *Clinical and experimental dermatology* **27,** 513-515.

117. Jones, K., J. Hughes, M. Hong, Q. Jia and S. Orndorff (2002) Modulation of melanogenesis by aloesin: a competitive inhibitor of tyrosinase. *Pigment cell research* **15,** 335-340.

118. Lin, J.-W., H.-M. Chiang, Y.-C. Lin and K.-c. Wen (2008) Natural products with skin-whitening effects. *Journal of Food and Drug Analysis* **16,** 1-10.

119. Seiberg, M., C. Paine, E. Sharlow, P. Andrade-Gordon, M. Costanzo, M. Eisinger and S. S. Shapiro (2000) Inhibition of melanosome transfer results in skin lightening. *Journal of Investigative Dermatology* **115,** 162-167.

120. Minwalla, L., Y. Zhao, J. Cornelius, G. F. Babcock, R. R. Wickett, I. C. Le Poole and R. E. Boissy (2001) Inhibition of melanosome transfer from melanocytes to keratinocytes by lectins and neoglycoproteins in an in vitro model system. *Pigment Cell Research* **14,** 185-194.

121. Kubo, I., I. Kinst-Hori, S. K. Chaudhuri, Y. Kubo, Y. Sánchez and T. Ogura (2000) Flavonols from Heterotheca inuloides: tyrosinase inhibitory activity and structural criteria. *Bioorganic & medicinal chemistry* **8,** 1749-1755.

122. Kim, D., J. Park, J. Kim, C. Han, J. Yoon, N. Kim, J. Seo and C. Lee (2006) Flavonoids as mushroom tyrosinase inhibitors: a fluorescence quenching study. *Journal of agricultural and food chemistry* **54,** 935-941.

123. Dadzie, O. and A. Petit (2009) Skin bleaching: highlighting the misuse of cutaneous depigmenting agents. *Journal of the European Academy of Dermatology and Venereology* **23,** 741-750.

124. Guillot, B. (2013) Glucocorticoid-induced cutaneous adverse events. *La Revue de medecine interne/fondee... par la Societe nationale francaise de medecine interne* **34,** 310-314.

125. Ly, F. (2006) Skin diseases associated with the use of skin-bleaching products in Africa. *Annales de dermatologie et de venereologie* **133,** 899-906.

126. Nnoruka, E. and O. Okoye (2006) Topical steroid abuse: its use as a depigmenting agent. *Journal of the National Medical Association* **98,** 934.

127. Boyle, J. and R. Peachey (1984) Allergic contact dermatitis to Dermovate® and Eumovate®. *Contact dermatitis* **11,** 50-50.

128. Lutz, M. E. and R. A. El-Azhary (1997) Allergic contact dermatitis due to topical application of corticosteroids: review and clinical implications. *Mayo Clinic Proceedings* **72,** 1141-1144.

129. Keane, F., S. Munn, N. Taylor and A. Du Vivier (2001) Unregulated use of clobetasol propionate. *British journal of dermatology* **144,** 1095-1096.

130. Otley, C. C. and A. Sober (1994) Over-the-counter clobetasol propionate. *Archives of dermatology* **130,** 121-121.

131. Staughton, R. and P. August (1975) Cushing's syndrome and pituitary-adrenal suppression due to clobetasol propionate. *British Medical Journal* **2,** 419-421.

132. May, P., E. J. Stein, R. J. Ryter, F. S. Hirsh, B. Michel and R. P. Levy (1976) Cushing syndrome from percutaneous absorption of triamcinolone cream. *Archives of internal medicine* **136,** 612-613.

133. Bwomda, P., E. Sermijn, P. Lacor and B. Velkeniers (2014) Glucocorticoid hypertension due to the use of bleaching skin cream, a case report. *Acta Clinica Belgica>International Journal of Clinical and Laboratory Medicine* **60,** 146-149.

134. Hamann, C. R., W. Boonchai, L. Wen, E. N. Sakanashi, C.-Y. Chu, K. Hamann, C. P. Hamann, K. Sinniah and D. Hamann (2014) Spectrometric analysis of mercury content in 549 skin-lightening products: Is mercury toxicity a hidden global health hazard? *Journal of the American Academy of Dermatology* **70,** 281-287.e283.

135. Hutson, D., B. Dean, T. Brooks and G. Hudson-Walker (1999) Genetic Toxicology testing of 41 Industrial Chemicals. *Research* **153,** 57-77.

136. Giunta, F., D. Di Landro, M. Chiaranda, L. Zanardi, A. Dal Palu, G. Giron, G. Bressa and L. Cima (1983) Severe acute poisoning from the ingestion of a permanent wave solution of mercuric chloride. *Human toxicology* **2,** 243-246.

137. Amponsah, D. (2010) Levels of mercury and hydroquinone in some skin-lightening creams and their potential risk to the health of consumers in Ghana. Department of Chemistry, Kwame Nkrumah University of Science and Technology.

138. Marzulli, F. and W. Brown (1972) Potential systemic hazards of topically applied mercurials. *Journal of the Society of Cosmetic Chemists* **23,** 875-886.

139. Barr, R., B. Woodger and P. Rees (1973) Levels of mercury in urine correlated with the use of skin lightening creams. *American journal of clinical pathology* **59,** 36-40.

140. Bourgeois, M., A. Dooms-Goossens, D. Knockaert, D. Sprengers, M. Van Boven and T. Van Tittelboom (1986) Mercury intoxication after topical application of a metallic mercury ointment. *Dermatology* **172,** 48-51.

141. Al-Saleh, I., N. Shinwari, I. El-Doush, G. Billedo, M. Al-Amodi and F. Khogali (2004) Comparison of mercury levels in various tissues of albino and pigmented mice treated with two different brands of mercury skin-lightening creams. *Biometals* **17,** 167-175.

142. Al-Saleh, I., I. El-Doush, N. Shinwari, R. Al-Baradei, F. Khogali and M. Al-Amodi (2005) Does low mercury containing skin-lightening cream (fair & lovely) affect the kidney, liver, and brain of female mice? *Cutaneous and ocular toxicology* **24,** 11-29.

143. Glahder, C. M., P. W. Appel and G. Asmund (1999) Mercury in soap in Tanzania. *National Environmental Research Institute* **306,** 1-19.

144. de la Cuadra, J. (1992) Cutaneous sensitivity to mercury and its compounds. *Annales de Dermatologie et de Venereologie* **120,** 37-42.

145. Böckers, M., R. Wagner and O. Oster (1985) Nail dyschromia as the leading symptom in chronic mercury poisoning caused by a cosmetic bleaching preparation. *Zeitschrift fur Hautkrankheiten* **60,** 821-829.

146. Cole, H., N. Schreiber and T. Sollmann (1930) Mercurial ointments in the treatment of syphilis: their absorption as measured by studies on excretion. *Archives of Dermatology and Syphilology* **21,** 372-393.

147. Crowley, P. and H. Gallagher (2014) Clotrimazole as a pharmaceutical: past, present and future. *Journal of applied microbiology* **117,** 611-617.

148. Remington, J. P., D. B. Troy and P. Beringer (2006) *Remington: The science and practice of pharmacy.* Lippincott Williams & Wilkins.

149. http://www.webmd.com/drugs/2/drug-4316/clotrimazole-topical/details (Accessed on 7th December 2016).

150. Umar, M. and H. Caleb (2013) Analysis of metals in some cosmetic products in FCT-Abuja, Nigeria. *International Journal of Research in Cosmetic Science* **3,** 14-18.

151. Polefka, T., R. Bianchini and S. Shapiro (2012) Interaction of mineral salts with the skin: a literature survey. *International journal of cosmetic science* **34,** 416-423.

152. Iwegbue, C. M., F. I. Bassey, G. O. Tesi, S. O. Onyeloni, G. Obi and B. S. Martincigh (2015) Safety evaluation of metal exposure from commonly used moisturizing and skin-lightening creams in Nigeria. *Regulatory Toxicology and Pharmacology* **71,** 484-490.

153. Sainio, E. L., R. Jolanki, E. Hakala and L. Kanerva (2000) Metals and arsenic in eye shadows. *Contact Dermatitis* **42,** 5-10.

154. Caito, S., L. G. Costa and M. Aschner Toxicology of Metals. Proceedings of the Elsevier Inc.2014.

155. Piccinini, P., M. Piecha and S. F. Torrent (2013) European survey on the content of lead in lip products. *Journal of pharmaceutical and biomedical analysis* **76,** 225-233.

156. Gondal, M., Z. Seddigi, M. Nasr and B. Gondal (2010) Spectroscopic detection of health hazardous contaminants in lipstick using laser induced breakdown spectroscopy. *Journal of Hazardous Materials* **175,** 726-732.

157. WHO (2004) Water Sanitation:Guidelines for drinking-water quality. Vol. 1, Recommendations.

158. Guy, R. H. (1999) *Metals and the skin: topical effects and systemic absorption.* CRC Press.

159. Sundar, S. and J. Chakravarty (2010) Antimony toxicity. *International journal of environmental research and public health* **7,** 4267-4277.

160. Boyle, J. and C. Kennedy (1986) Hydroquinone concentrations in skin lightening creams. *British Journal of Dermatology* **114,** 501-504.

161. Gaudiano, M. C., D. Lucente, E. Antoniella, P. Bertocchi, N. Muleri, L. Manna, M. Bartolomei, S. Alimonti, L. Valvo and A. L. Rodomonte (2010) "For export only" medicines come back to Europe: A RP-LC method for the screening of six glucocorticoids in illegal and counterfeit anti-inflammatory and lightening creams. *Journal of Pharmaceutical and Biomedical Analysis* **53,** 158-164.

162. Görög, S. (2004) Recent advances in the analysis of steroid hormones and related drugs. *Analytical sciences* **20,** 767-782.

163. Wang, L.-H. (1995) Simultaneous determination of hydroquinone ethers in cosmetics after preconcentration at a carbon paste electrode. *Analyst* **120,** 2241-2244.

164. Huang, S.-C., C.-C. Lin, M.-C. Huang and K.-C. Wen (2004) Simultaneous determination of magnesium ascorbyl phosphate, ascorbyl glucoside, kojic acid, arbutin and hydroquinone in skin whitening cosmetics by HPLC. *Journal of Food and Drug Analysis* **12**.

165. Balaguer, A., A. Chisvert and A. Salvador (2008) Environmentally friendly LC for the simultaneous determination of ascorbic acid and its derivatives in skin-whitening cosmetics. *Journal of separation science* **31,** 229-236.

166. Reepmeyer, J. C., L. K. Revelle and I. Vidavsky (1998) Detection of clobetasol propionate as an undeclared steroid in zinc pyrithione formulations by high-performance liquid chromatography with rapid-scanning ultraviolet spectroscopy and mass spectrometry. *Journal of Chromatography A* **828,** 239-246.

167. ALqadami, A. A., M. A. Abdalla, Z. A. ALOthman and K. Omer (2013) Application of solid phase extraction on multiwalled carbon nanotubes of some heavy metal ions to analysis of skin whitening cosmetics using ICP-AES. *International journal of environmental research and public health* **10,** 361-374.

168. Al-Qutob, M. A., H. M. Alatrash and S. Abol-Ola (2013) Determination of different heavy metals concentrations in cosmetics purchased from the Palestinian markets by ICP/MS. *Advances in Environmental Sciences* **5,** 287-293.

169. Lee, S.-M., H.-J. Jeong and H.-K. Kim (2008) Simultaneous Determination of Heavy Metals in Cosmetic Products by Ion Chromatography. *Journal of the Society of Cosmetic Scientists of Korea* **34,** 57-62.

170. Somervaille, L. J., D. R. Chettle and M. C. Scott (1985) In vivo measurement of lead in bone using x-ray fluorescence. *Physics in medicine and biology* **30,** 929.

171. Steinnes, E. (1980) Atmospheric deposition of heavy metals in Norway studied by the analysis of moss samples using neutron activation analysis and atomic absorption spectrometry. *Journal of Radioanalytical Chemistry* **58,** 387-391.

172. Al-Saleh, I. and S. Al-Enazi (2011) Trace metals in lipsticks. *Toxicological & Environmental Chemistry* **93,** 1149-1165.

173. Nnorom, I. (2011) Trace metals in cosmetic facial talcum powders marketed in Nigeria. *Toxicological & Environmental Chemistry* **93,** 1135-1148.

174. Elemental, T. (2001) ICP or ICP-MS? Which technique should I use? An elementary overview of elemental analysis.

175. Bocca, B., G. Forte, F. Petrucci and A. Cristaudo (2007) Levels of nickel and other potentially allergenic metals in Ni-tested commercial body creams. *Journal of pharmaceutical and biomedical analysis* **44,** 1197-1202.

176. Zoe Grosser, Lee Davidowski and L. Thompson (2012) The Determination of Metals in Cosmetics. (Edited by I. PerkinElmer).

177. Rajagopal Mayildurai, A. R., Nanjan Velmani (2015) ICP – OES investigations of heavy metal contents in cosmetic products. *Journal of Pharmacy Research* **9,** 27-30.

178. Eltegani, S. E., H. M. Ali and A. Y. Hammad (2013) The Hazards of Hidden Heavy Metals in Face Make-ups. *British Journal of Pharmacology and Toxicology* **4,** 188-193.

179. Farrag, E. A., A. Sei'leek, H. Mohammed, A. Al-Sayyed and I. Mohammed (2015) Study of heavy metals concentration in cosmetics purchased from Jordan markets by ICP-MS and ICP-OES. *Advances in Environmental Sciences* **7**.

180. Peregrino, C. P., M. V. Moreno, S. V. Miranda, A. D. Rubio and L. O. Leal (2011) Mercury levels in locally manufactured Mexican skin-lightening creams. *International Journal of Environmental Research and Public Health* **8,** 2516-2523.

181. Kupiec, T. (2004) Quality-Control Analytical Methods:High-Performance Liquid Chromatography. *International Journal of Pharmaceutical Compounding* **8,** 223-227.

182. Chromacademy (2016) Quantitative & Qualitative HPLC. http://www.chromacademy.com/ (Accessed on 14th October 2016).

183. Daniel, H. C. (2003) *Qantitative chemical analysis. 6th Edition.* New York: W.H. Freeman.

184. Fornstedt, T., P. Forssén and D. Westerlund (2015) Basic HPLC Theory and Definitions: Retention, Thermodynamics, Selectivity, Zone Spreading, Kinetics, and Resolution. pp. 1-24.

185. https://www.google.co.za/search?q=component+of+an+hplc+system (Accessed 22nd July, 2016).

186. https://www.google.co.za/search?q=component+of+uv-vis+spectrophotometer (Accessed 22nd July, 2016).

187. Sukender, K., S. Jaspreet, D. Sneha and G. Munish (2012) AAS estimation of heavy metals and trace elements in Indian herbal cosmetic preparations. *Research Journal of Chemical Sciences* **2,** 46-51.

188. Niyibizi, C. (2014) An investigation of commonly used skin-ligthening creams. *M.Sc. Dissertation, University of KwaZulu-Natal, South Africa*.

189. https://www.google.co.za/search?q=THEORY+OF+ICP-OES (Accessed 25th August 2016).

190. PerkinElmer (2000) *Mercury hydride system (MHS) 15 user guide.*

191. SCCS (2012) Scientific Committee on Consumer Safety (SCCS/1501/12). The sccs's notes of guidance for the testing of cosmetic substances and their safety evaluation. 8th revision. The SCCS adopted this opinion at its 17th plenary meeting of 11 December 2012.

192. RAIS. The Risk Assessment Information System. Toxicity Profiles. RAGs A Format for Aluminum - CAS Number 7429905. https://rais.ornl.gov/tox/profiles/Aluminum_ragsa.html. (Accessed 17th November 2106).

193. Iwegbue, C. M., O. S. Emakunu, G. E. Nwajei, F. I. Bassey and B. S. Martincigh (2016) Evaluation of human exposure to metals from some commonly used bathing soaps and shower gels in Nigeria. *Regulatory Toxicology and Pharmacology* **83,** 38-45.

194. Storelli, M. (2008) Potential human health risks from metals (Hg, Cd, and Pb) and polychlorinated biphenyls (PCBs) via seafood consumption: estimation of target hazard quotients (THQs) and toxic equivalents (TEQs). *Food and Chemical Toxicology* **46,** 2782-2788.

195. Barbas, C., J. Rupérez, A. Dams and R. Majors (2005) Separation of Paraben Preservatives by Reversed-Phase HPLC. *Agilent Technologies, Inc***,** 1-3.

196. Kumar, S., S. Mathkar, C. Romero and A. Rustum (2011) Development and validation of a single RP-HPLC assay method for analysis of bulk raw material batches of four parabens that are widely used as preservatives in pharmaceutical and cosmetic products. *Journal of chromatographic science* **49,** 405-411.

197. Shabib, G. A. (2004) A Practical Approach to Validation of HPLC Methods Under Current Good Manufacturing Practices. *Journal of Validation Technology***,** 29-37.

198. Thomas, L. M. Feature: race, bodies and beauty Skin Lighteners, Black Consumers and Jewish Entrepreneurs in South Africa. pp. 259-283. Oxford University Press, Proceedings of the History Workshop Journal 2012.

199. Dalmázio, I. and M. Â. de BC Menezes (2011) Multi-elemental Profile of some Brazilian Make-up Products by Instrumental Neutron Activation Analysis. In *International Nuclear Atlantic Conference*. pp. 24-28, Brazil.

200. Laussac, J.-P. and G. Commenges (1983) 1H, 13C, 31P and 27Al NMR study of aluminium-ATP complexes. A possible relation with its biological application. *Nouveau journal de chimie* **7,** 579-585.

201. Ullah, H., S. Noreen, A. Rehman, A. Waseem, S. Zubair, M. Adnan and I. Ahmad (2013) Comparative study of heavy metals content in cosmetic products of different countries marketed in Khyber Pakhtunkhwa, Pakistan. *Arabian Journal of Chemistry* http://dx.doi.org/10.1016/j.arabjc.2013.09.021**,** 1-9.

202. IARC (1990) Chromium, nickel and welding. *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans*.

203. Larese, F., A. Gianpietro, M. Venier, G. Maina and N. Renzi (2007) In vitro percutaneous absorption of metal compounds. *Toxicology letters* **170,** 49-56.

204. Van Lierde, V., C. C. Chéry, N. Roche, S. Monstrey, L. Moens and F. Vanhaecke (2006) In vitro permeation of chromium species through porcine and human skin as determined by capillary electrophoresis–inductively coupled plasma–sector field mass spectrometry. *Analytical and bioanalytical chemistry* **384,** 378-384.

205. Filon, F. L., F. D'Agostin, M. Crosera, G. Adami, M. Bovenzi and G. Maina (2008) In vitro percutaneous absorption of chromium powder and the effect of skin cleanser. *Toxicology in Vitro* **22,** 1562-1567.

206. Health Canada (2011) Consumer product safety: Draft guidance on heavy metal impurities in cosmetics. http://www.hcsc.gc.ca/cpsspc/legislation/consultation/_cosmt./metalmtaux-consultengphp (Accessed 23rd September 2016).

207. US FDA (2007) Summary of color additives listed for use in the United State in foods, drugs/cosmetics and medical devices, color additives approved for use in cosmetic Part 73, subpart C: color additive exempt from batch certification. Available from: http://www.cfsan.fda.gov/dms/opa_col2.html (Accessed 23rd September 2016).

208. Filon, F. L., M. Boeniger, G. Maina, G. Adami, P. Spinelli and A. Damian (2006) Skin absorption of inorganic lead (PbO) and the effect of skin cleansers. *Journal of occupational and environmental medicine* **48,** 692-699.

209. Basketter, D. A., G. Angelini, A. Ingber, P. S. Kern and T. Menné (2003) Nickel, chromium and cobalt in consumer products: revisiting safe levels in the new millennium. *Contact Dermatitis* **49,** 1-7.

210. Ababneh, F. A., K. A. Abu-Sbeih and I. F. Al-Momani (2013) Evaluation of allergenic metals and other trace elements in personal care products. *Jordan Journal of Chemistry* **8,** 179-190.

211. Miyajima, H., S. Kono, Y. Takahashi and M. Sugimoto (2002) Increased lipid peroxidation and mitochondrial dysfunction in aceruloplasminemia brains. *Blood Cells, Molecules, and Diseases* **29,** 433-438.

212. Senesse, P., S. Méance, V. Cottet, J. Faivre and M.-C. Boutron-Ruault (2004) High dietary iron and copper and risk of colorectal cancer: a case-control study in Burgundy, France. *Nutrition and cancer* **49,** 66-71.

213. US FDA (1992) Cosmetics Hand-book. U.S. Department of Health and Human Services. Public Health Service, Food and Drug Administration. .

214. WHO (1991) Environmental Health Criteria 118: Inorganic mercury, Geneva.

215. Al-Saleh I and E.-D. I (1997) Mercury content in skin lightening creams and potential hazards to the health of Saudi women. *Toxicol Environ Health* **51,** 131–148.

216. WHO (2007) Joint FAO/WHO Expert Committee on Food Additives. Meeting (67th : 2006 : Rome, Italy). Evaluation of certain food additives and contaminants : sixty-seventh report of the Joint FAO/WHO Expert Committee on Food Additives.

217. Iwegbue, C. M., O. S. Emakunu, G. Obi, G. E. Nwajei and B. S. Martincigh (2016) Evaluation of human exposure to metals from some commonly used hair care products in Nigeria. *Toxicology Reports* **3,** 796-803.

218. http://www.popstoolkit.com/tools/HHRA/TDI_HealthCanada.aspx (Accessed 2nd December 2016).

219. US EPA (1997) Mercury study report to Congress Health Effects of Mercury and Mercury Compounds, volume. V. Environmental Protection agency, Washington (DC), United States, EPA-452/R-97-007.

220. Cashman, A. L. and E. M. Warshaw (2005) Parabens: a review of epidemiology, structure, allergenicity, and hormonal properties. *Dermatitis* **16,** 57-66.

221. Darbre, P., A. Aljarrah, W. Miller, N. Coldham, M. Sauer and G. Pope (2004) Concentrations of parabens in human breast tumours. *Journal of applied toxicology* **24,** 5-13.

Appendix A

MATERIALS

This appendix contains the list of chemicals used during the course of this study.

A1 Chemicals used during the analysis of skin-lightening agents by HPLC

Chemical	Supplier
Acetonitrile (HPLC grade)	Sigma-Aldrich
Methanol (HPLC grade)	Sigma-Aldrich
Millipore water	Millipore Elix 5 UV water purification unit
Betamethasone dipropionate	Sigma Life Science
Clobetasol propionate (98%)	Sigma-Aldrich
Clotrimazole	Sigma-Aldrich
Hydroquinone (99.09%)	Fluka
Benzoquinone (99.5%)	Fluka
Kojic acid (98%)	Fluka
Niacinamide (99.9%)	Supelco Analytical
Arbutin (96%)	Sigma-Aldrich
Acetic acid (99.9%)	BDH Chemicals Limited
Orthophosphoric acid (98%)	Rochelle Chemicals
Dipotassium hydrogen phosphate	BDH Laboratory Supplies
Potassium dihydrogen phosphate	BDH Laboratory Supplies

A2 Chemicals used during the analysis of heavy metals by ICP-OES and CV-AAS

Chemical	Supplier
Tin chloride dihydrate	BDH Laboratory Supplies
Mercury chloride ($HgCl_2$)	Sigma-Aldrich
Analytical grade nitric acid (69%)	Emparta ACS
Analytical grade hydrochloric acid (32%)	B & M Scientific
Analytical grade, aluminum (Al) 1000 ppm	Fluka Chemicals
Analytical grade, chromium (Cr) 1000 ppm	Fluka Chemicals
Analytical grade, copper (Cu) 1000 ppm	Fluka Chemicals
Analytical grade, iron (Fe) 1000 ppm	Fluka Chemicals
Analytical grade, manganese (Mn) 1000 ppm	Fluka Chemicals
Analytical grade, nickel (Ni) 1000 ppm	Fluka Chemicals
Analytical grade, lead (Pb) 1000 ppm	Fluka Chemicals
Analytical grade, zinc (Zn) 1000 ppm	Fluka Chemicals

Appendix B

EQUIPMENT

This appendix contains the list of instruments and equipment used during the course of this study.

B1 Instruments

Shimadzu Prominence HPLC equipped with:

DGU-20A_3 Prominence degasser,
LC-20AD XR Prominence binary pumps,
SIL-20A XR Prominence autosampler,
SPD-M20A Prominence diode array detector,
CTO-20A Prominence column oven,
CBM-20A Prominence communicator bus module,
FRC-10A Prominence fraction collector and
LC/Lab solution system software.

PerkinElmer Lambda 25 UV-VIS dual-beam spectrophotometer

PerkinElmer Optima 5300 DV ICP-OES

PerkinElmer Analyst 200 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)

Brownlee HPLC column; C18 (100 × 4.6 mm, 3 μm)

SGE HPLC column; C18 (250 × 4.6mm, 5 μm)

Ultrasonic bath (Ultra Manufacturing Company)

Helium degasser (Sp8700 XR)

Millipore Elix 5 UV water purification system

Ministart RC 25 hydrophilic 0.45 μm single use syringe filters

Millipore Durapore 0.45 μm membrane solvent filters

Appendix C

CALIBRATION DATA FOR SKIN-LIGHTENING AGENTS

The calibration data for all skin-lightening agents analysed is given in this section.

Table C1: Calibration data for the determination of clobetasol propionate (CP).

Concentration/ mg L^{-1}	0.2	0.5	2.0	6.0	10	15	25	60
1	4536 4747 4436	10789 10505 10490	41035 41166 41072	120086 120274 121111	231943 229865 225699	355800 355766 357276	563897 560181 556997	1359590 1347034 1334156
2	4613 4682 4577	10754 10447 11304	41098 41006 41553	120469 121076 121399	229100 235115 228312	359494 358454 361845	561059 564270 556528	1352021 1335777 1325635
3	4516 4521 4476	10647 10686 10603	41631 41994 41740	123159 123089 121582	220243 220730 221664	363496 367185 367366	553125 545843 547637	1316128 1308212 1309439
Mean	4567.1	10691.7	41366.1	121360.6	226963.4	360742.4	556615.2	1331999
Std Dev	99.3	258.3	366.9	1118.9	5241.1	4502.5	6643.3	18622.18
RSD%	2.2	2.4	0.9	0.9	2.3	1.2	1.2	1.4

Table C2: Calibration data for the determination of betamethasone dipropionate (BD).

Concentration/ mg L^{-1}	0.2	0.5	2.0	6.0	10	15	25	60
1	4317 4195 4060	9795 9577 9510	36901 36714 37316	107677 107699 108173	205433 203793 201815	317332 318832 317993	504338 500463 499561	1220964 1206853 1200922
2	3963 4158 4097	9535 9393 9415	36800 37014 37017	107449 107798 110488	202658 204690 200788	319932 319869 320734	502447 501796 497112	1214803 1201043 1191210
3	4250 4269 4207	9453 9613 9393	37609 37726 37721	108370 109425 109460	196911 197499 198290	326104 327538 326811	494210 489803 489855	1182946 1175806 1176594
Mean	4168.4	9520.4	37202	108504.3	201319.7	321682.8	497731.7	1196793
Std Dev	111.9	130.2	400.4	1046.9	3160.1	4000.9	5372.3	16264.5
RSD%	2.7	1.4	1.1	1.0	1.6	1.2	1.1	1.4

Table C3: Calibration data for the determination of clotrimazole (CT).

Concentration/ mg L^{-1}	0.2	0.5	2.0	6.0	10	15	25	60
1	4955 5259 4912	12512 13075 12442	48404 47451 49601	143404 142451 142909	280668 280618 278063	434332 436414 436607	692762 687259 685055	1661478 1646585 1641680
2	5440 5277 5362	12977 12886 12817	48296 48887 48534	141709 142341 138152	280697 280121 277909	429958 439054 438071	687877 683043 683256	1653851 1641229 1641101
3	5162 5110 5071	12933 12812 12885	49303 51968 48545	141156 142472 141581	278482 278926 277773	445679 443579 445960	667432 664049 663059	1639308 1640503 1642042
Mean	5172	12815.4	48998.8	141797.2	279250.8	438850.4	679310.2	1645309
Std Dev	178.7	208.8	1272.3	1530.9	1265.8	5369.4	11281.2	7527.8
RSD%	3.5	1.6	2.6	1.1	0.5	1.2	1.7	0.5

Table C4: Calibration data for the determination of hydroquinone (HQ).

Concentration/ mg L^{-1}	0.5	20	80	150	250	300	400
1	5058 5078 4317	196307 198875 194085	841082 839943 834169	1572541 1608575 1557722	2744875 2757385 2728820	3310542 3306661 3304408	5074334 5084454 5070686
2	4598 4543 4450	192351 193345 192112	832839 834164 836750	1565425 1563204 1599316	2719200 2718679 2714615	3292200 3292619 3277468	5055476 5060818 5057442
3	4268 4151 4201	191216 191767 192843	839432 836431 831855	1597967 1617219 1591295	2712364 2693866 2716994	3289059 3275839 3285176	5061405 5052795 5036873
Mean	4518.2	193655.7	836296.1	1585918	2722978	3292664	5061587
Std Dev	345.4	2470.2	3297.9	21669.5	18695.3	12433.5	13745.0
RSD%	7.6	1.3	0.4	1.4	0.7	0.4	0.3

Table C5: Calibration data for the determination of benzoquinone (BQ).

Concentration/ mg L^{-1}	0.2	1.0	5.0	10	15	18	20
1	1221 1280 1265	12092 12448 12726	135317 138276 138475	334185 336403 334037	582125 581318 584290	724154 726876 725326	767714 767977 764435
2	1135 1184 1197	13883 13320 13445	139823 140297 140919	339660 330229 336633	568514 565736 566205	719540 719691 719667	759702 760894 758319
3	1483 1459 1439	14022 14657 14682	145498 145415 145007	339933 339371 334056	581400 580335 583895	739302 740081 740762	770865 773210 771128
Mean	1295.9	13475	141003	336056.3	577090.9	728377.7	766027.1
Std Dev	130.9	926.9	3601.4	3259.5	7837.6	9141.4	5431.1
RSD%	10.1	6.9	2.6	0.9	1.4	1.3	0.7

Table C6: Calibration data for the determination of kojic acid (KA).

Concentration/ mg L^{-1}	0.5	10	25	40	55	70	90
1	13070 13052 12954	241327 242670 240741	697749 695263 701177	1045285 1054650 1039749	1458728 1447199 1451643	1900416 1904541 1895457	2584462 2549813 2560902
2	12538 12544 12324	238300 237199 236378	690050 691463 691740	1031024 1040369 1037203	1433120 1443531 1435473	1890323 1886321 1903344	2547224 2554898 2535975
3	13468 12916 13425	236934 236439 235818	696373 695980 690306	1035302 1041636 1034138	1428666 1434019 1428066	1879521 1885786 1878179	2546847 2540858 2558017
Mean	12921.2	238422.9	694455.7	1039928	1440049	1891543	2553222
Std Dev	393.5	2511.9	3801.9	6983.9	10753.2	9920.2	14138.9
RSD%	3.0	1.1	0.5	0.7	0.7	0.5	0.6

Table C7: Calibration data for the determination of niacinamide (NC).

Concentration/ mg L^{-1}	0.8	10	30	50	70	90	110
1	11399 11026 10995	95559 96529 98104	308690 307957 303646	540514 540930 540344	768367 770116 769845	1037663 1065898 1035897	1284557 1281038 1274572
2	10753 11476 11425	97278 95634 95470	300992 300275 300797	537865 537295 533058	759042 760110 761316	1018334 1019420 1018560	1324506 1321825 1327996
3	11314 11466 11543	99668 97816 98154	306871 303240 305645	537193 540850 537330	756003 758707 759439	1001649 1004059 1002936	1255602 1258282 1250562
Mean	11266.3	97134.7	304234.8	538375.4	762549.4	1022713	1286549
Std Dev	273.9	1447.1	3201.4	2580.9	5378.1	20893.6	30888.1
RSD%	2.4	1.5	1.1	0.5	0.7	2.0	2.4

Table C8: Calibration data for the determination of arbutin (ARB).

Concentration/ mg L^{-1}	1.0	4.0	6.0	10	15	18	20
1	1946 1942 1954	9716 9179 9506	13205 13586 13295	23441 23199 23468	33852 33828 33923	41278 41443 41882	48830 48686 48181
2	1944 1956 1964	9268 9768 9803	13705 13331 13045	21646 21847 22169	33173 33588 32446	42426 42014 42910	47046 46905 46471
3	1922 1940 1981	9417 9217 9809	13710 13951 13774	22458 22511 22402	33987 33085 32747	41094 42453 42492	46323 46110 45514
Mean	1949.9	9520.3	13511.3	22571.2	33403.2	41999.1	47118.4
Std Dev	16.6	261.2	303.2	665.4	562.1	624.8	1183.7
RSD%	0.9	2.7	2.2	2.9	1.7	1.5	2.5

Appendix D

CALIBRATION GRAPHS AND RESIDUAL PLOTS FOR SKIN-LIGHTENING AGENTS

The calibration curves and residual plots of all skin-lightening agents analysed by HPLC are presented here.

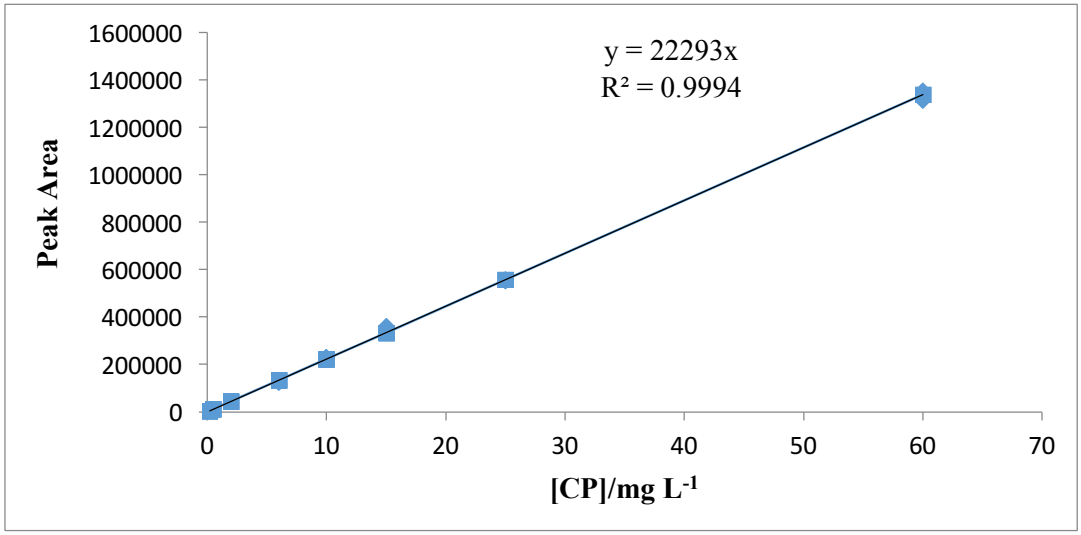


Figure D1: Calibration graph for the determination of clobetasol propionate at 240 nm.

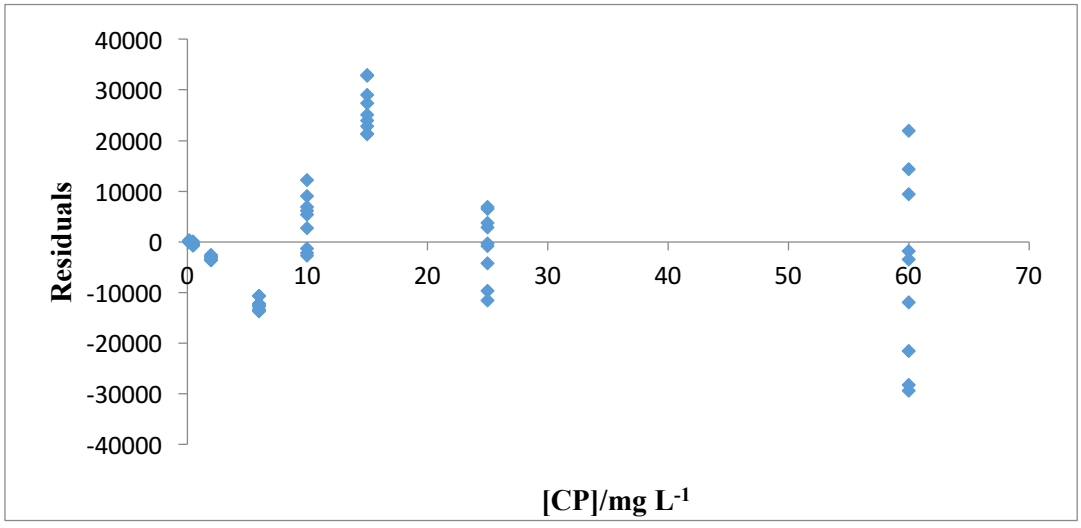


Figure D2: Residual plot for the calibration graph of clobetasol propionate.

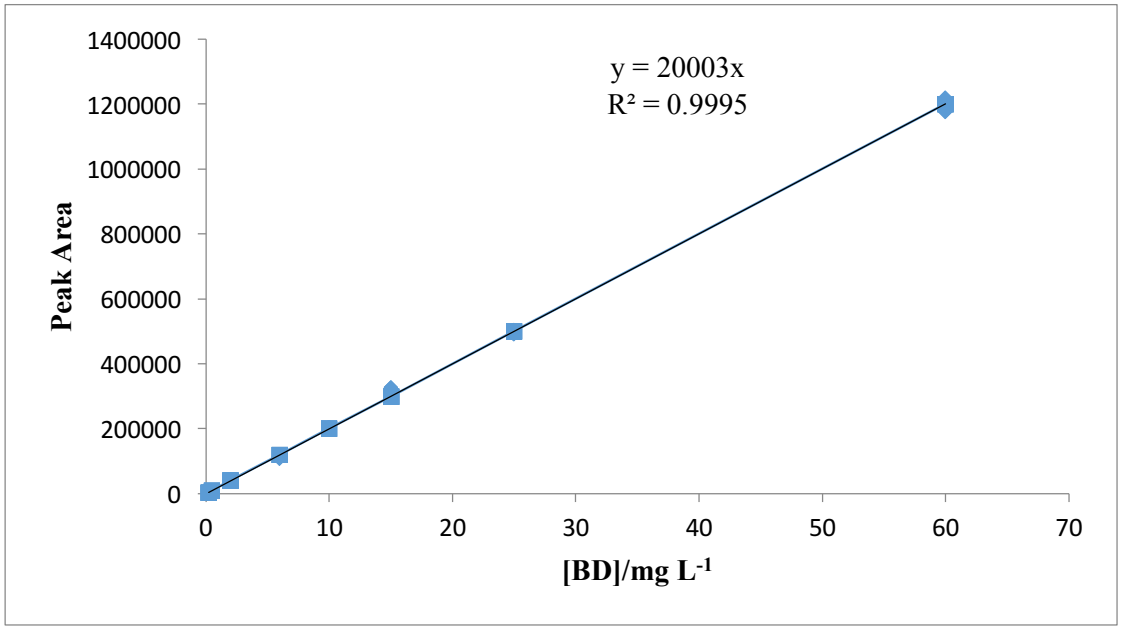


Figure D3: Calibration graph for the determination of betamethasone dipropionate at 240 nm.

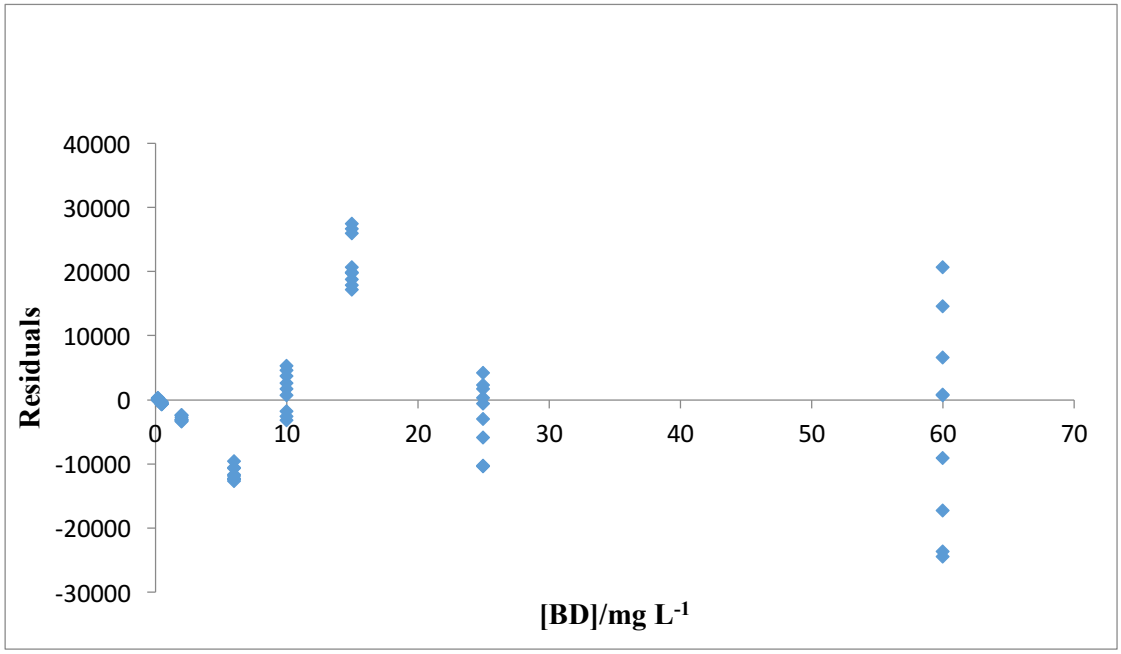


Figure D4: Residual plot for the calibration graph of betamethasone dipropionate.

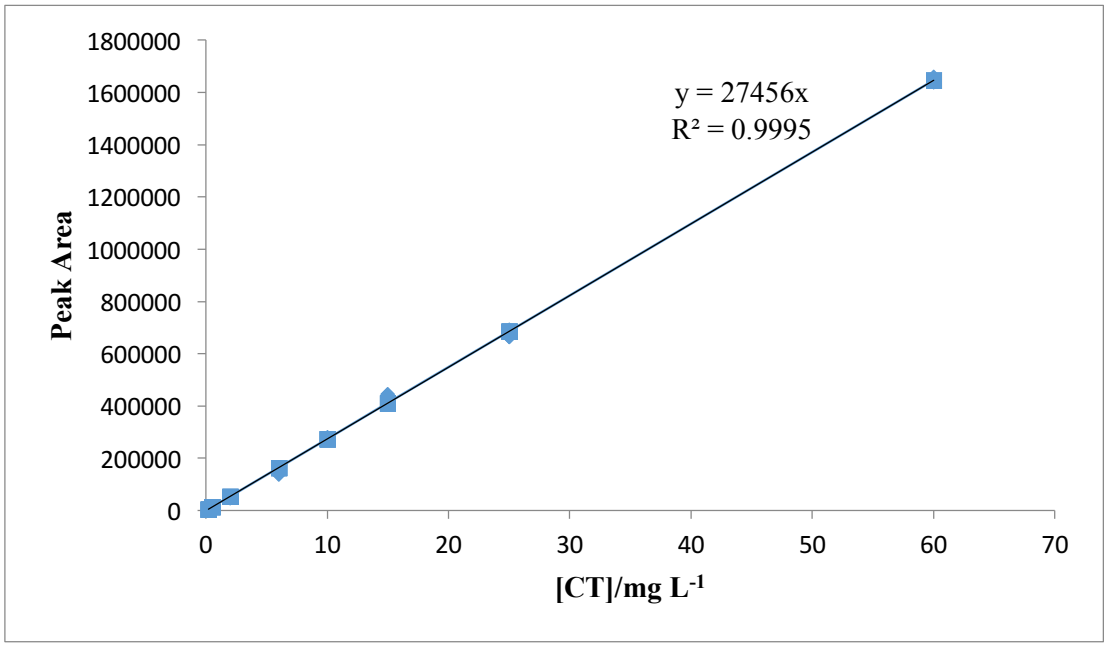


Figure D5: Calibration graph for the determination of clotrimazole at 225 nm.

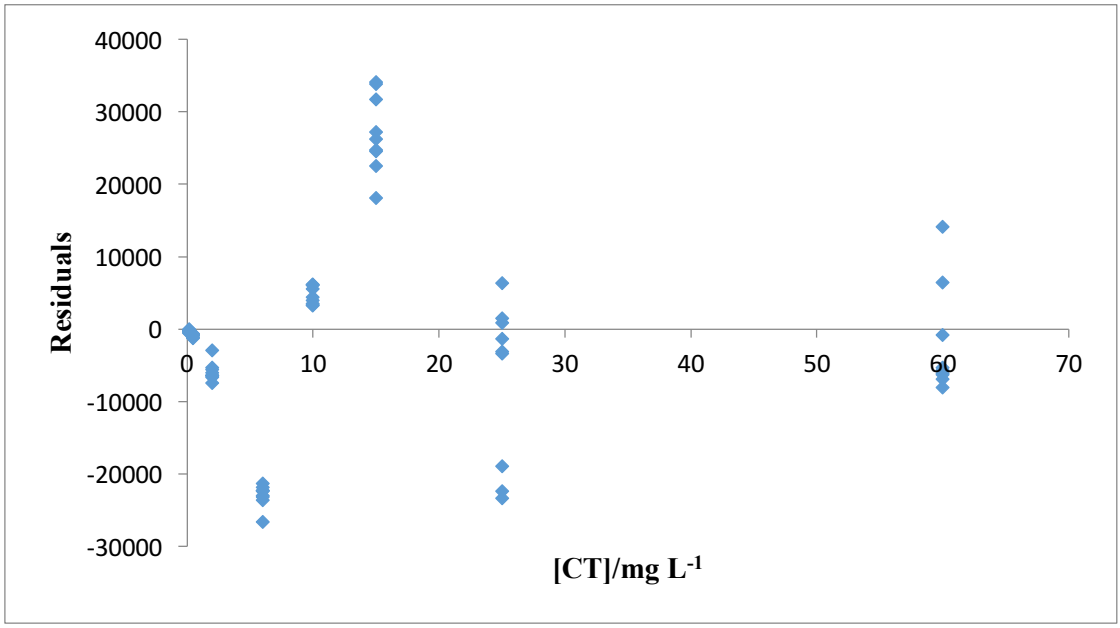


Figure D6: Residual plot for the calibration graph of clotrimazole.

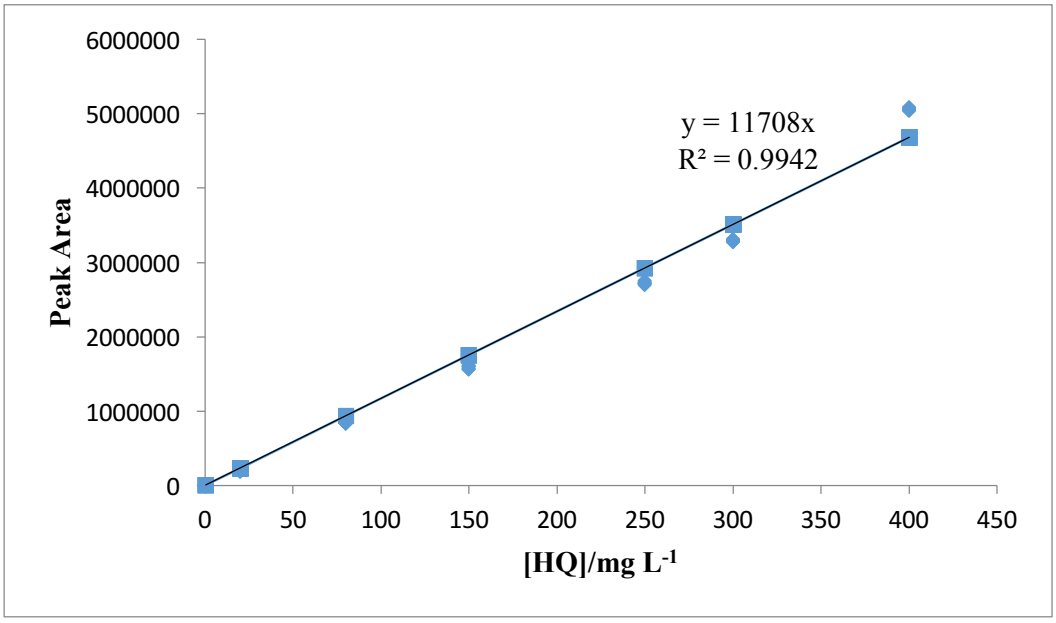


Figure D7: Calibration graph for the determination of hydroquinone at 289 nm.

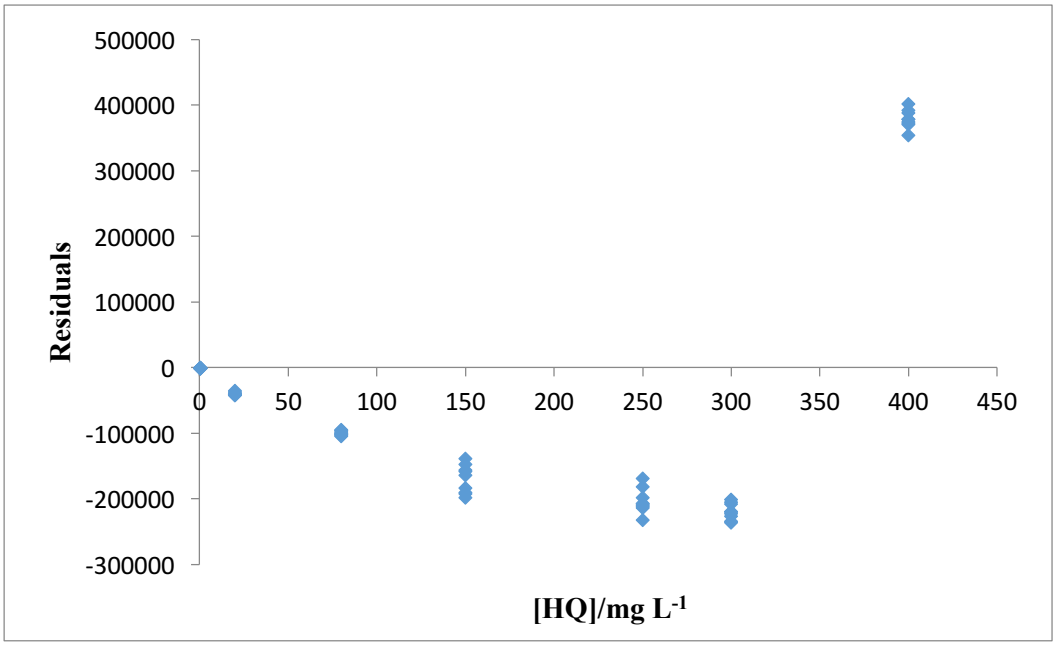


Figure D8: Residual plot for the calibration graph of hydroquinone.

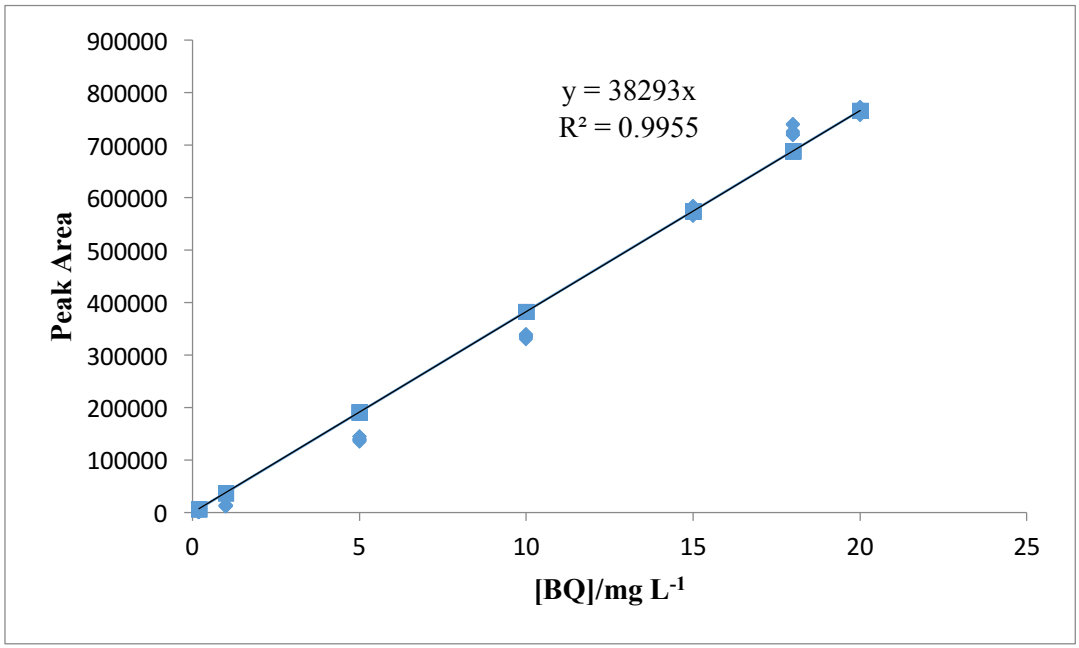


Figure D9: Calibration graph for the determination of benzoquinone at 240 nm.

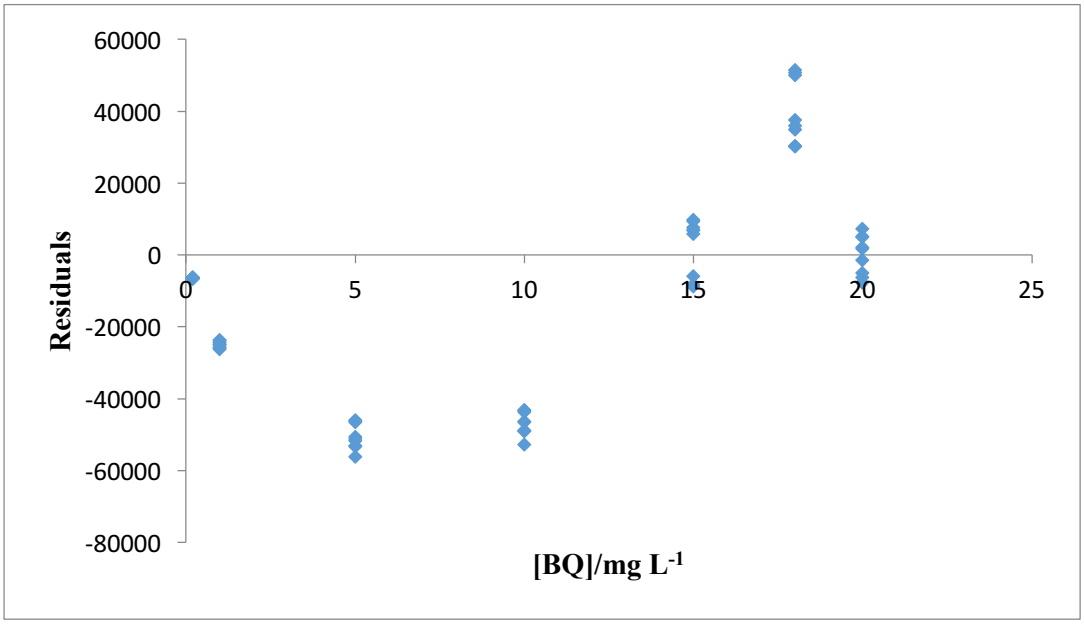


Figure D10: Residual plot for the calibration graph of benzoquinone.

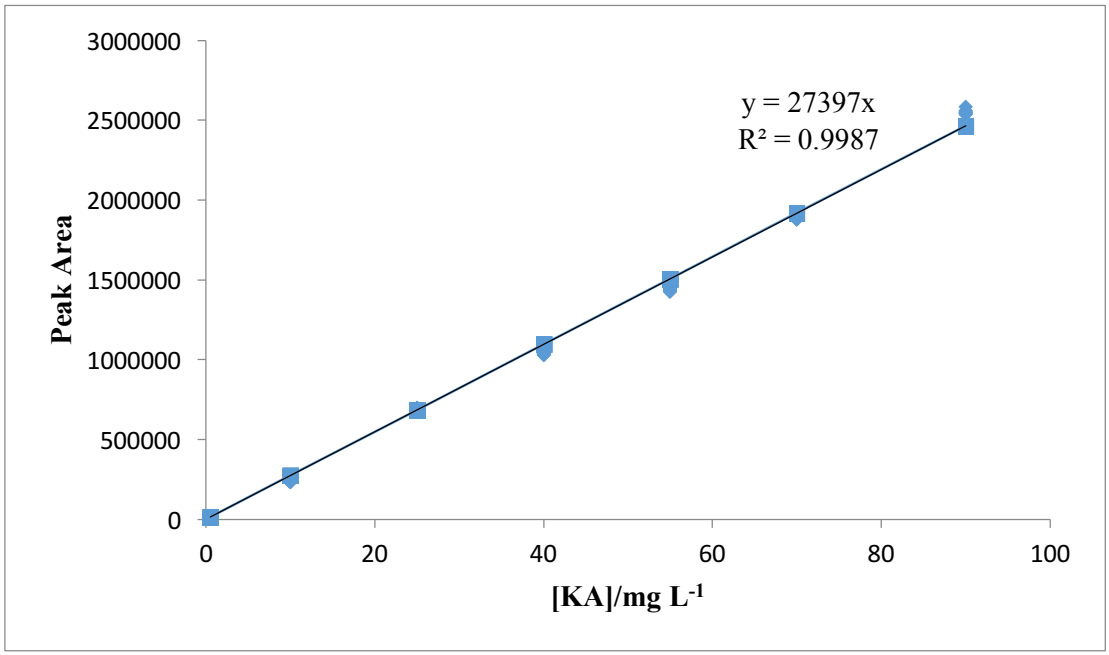


Figure D11: Calibration graph for the determination of kojic acid at 270 nm.

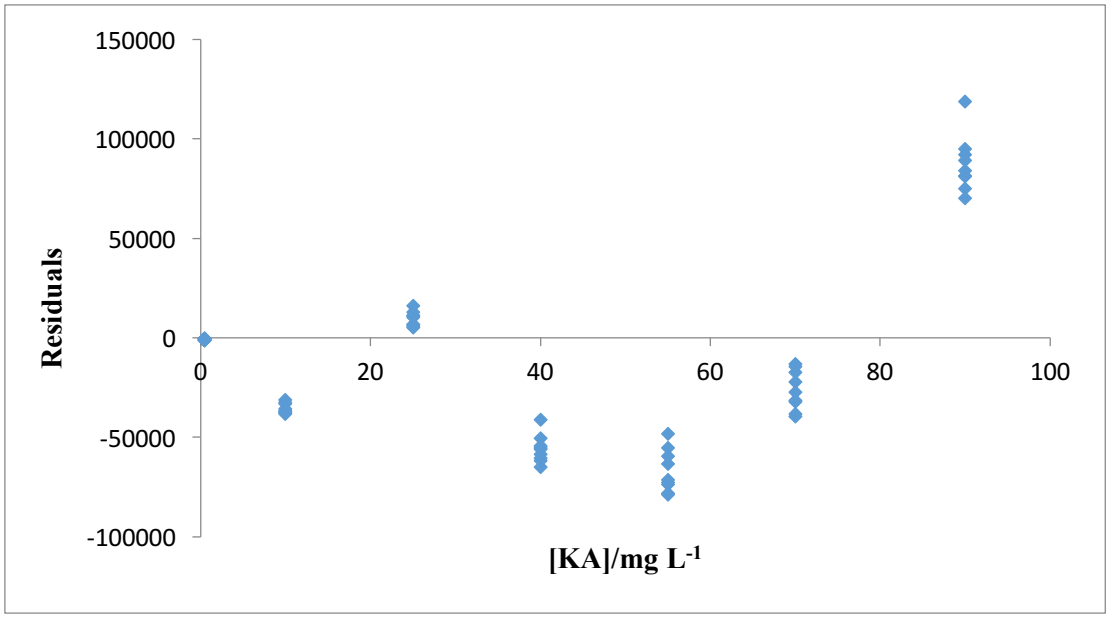


Figure D12: Residual plot for the calibration graph of kojic acid.

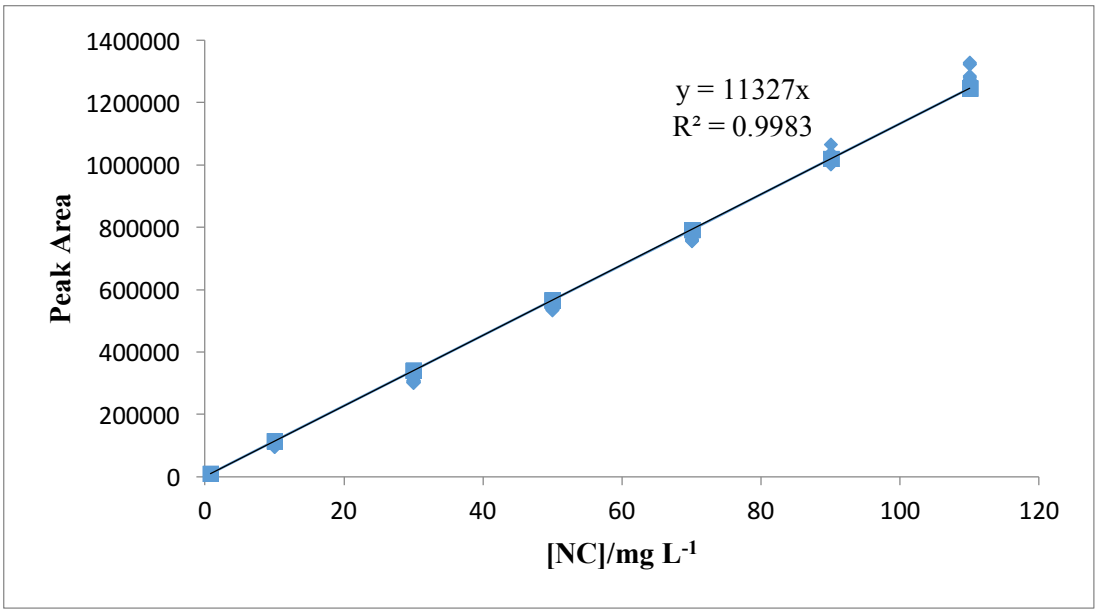


Figure D13: Calibration graph for the determination of niacinamide at 270 nm.

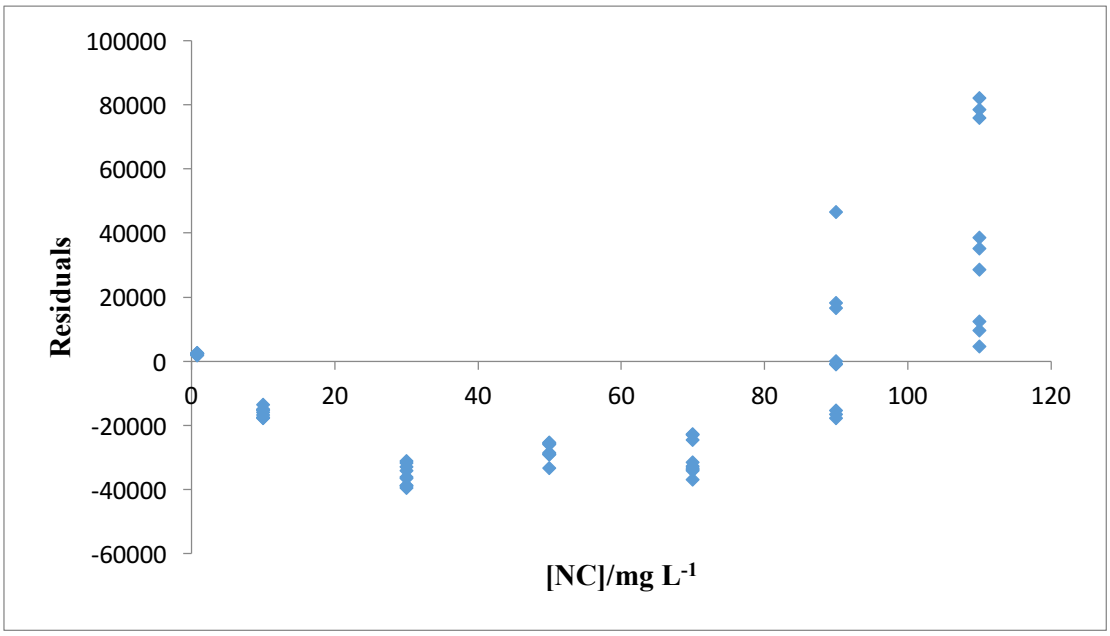


Figure D14: Residual plot for the calibration graph of niacinamide.

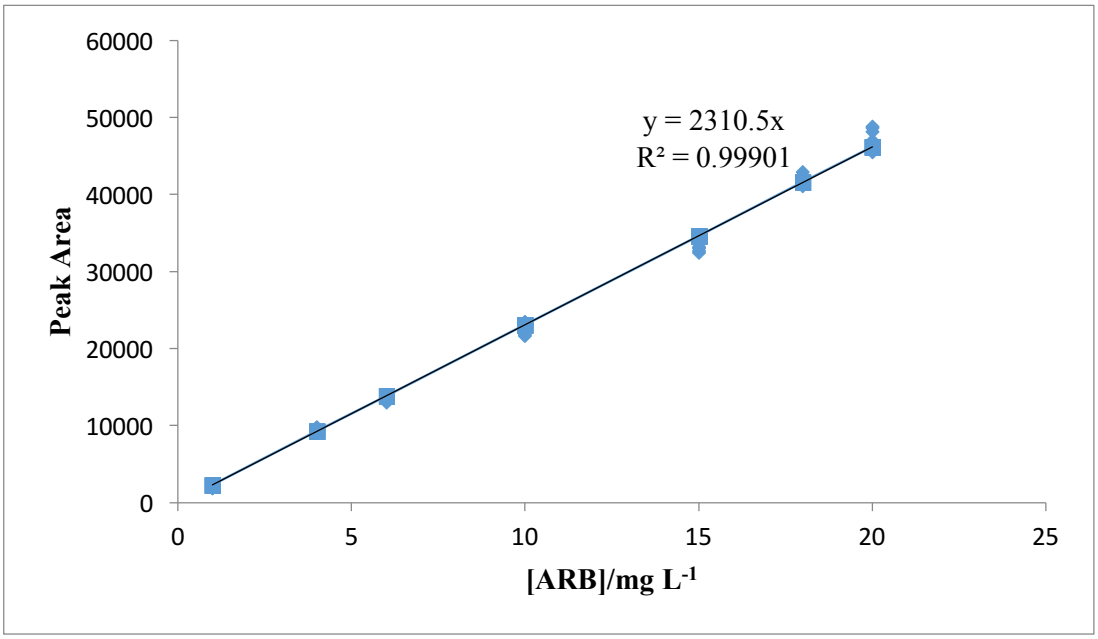


Figure D15: Calibration graph for the determination of arbutin at 289 nm.

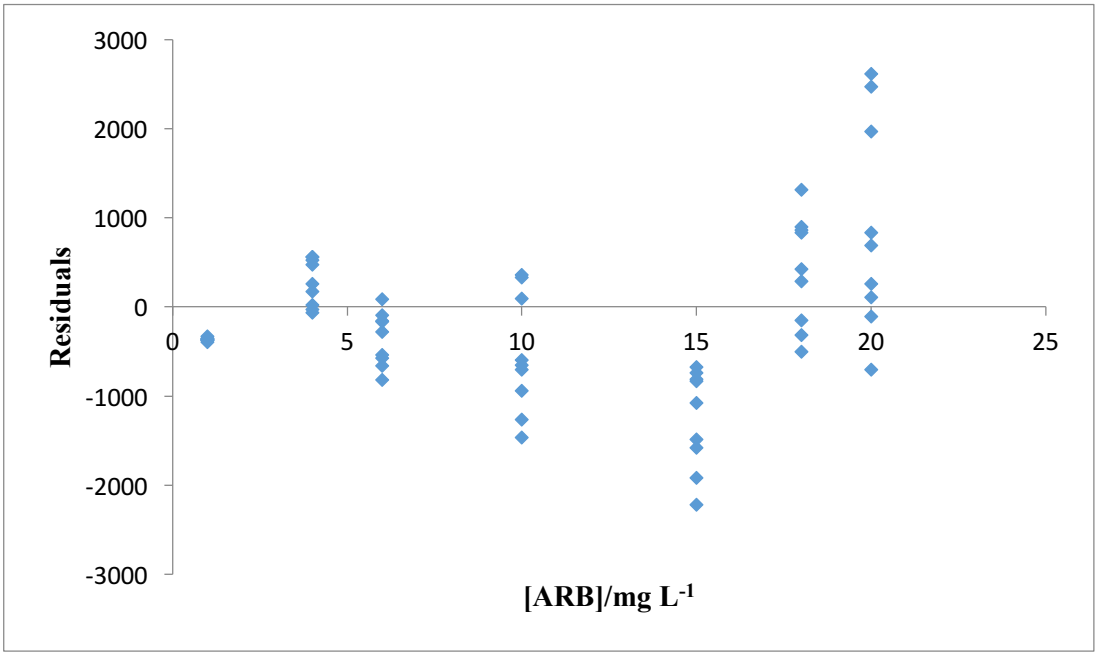


Figure D16: Residual plot for the calibration graph of arbutin.

Appendix E

HPLC CHROMATOGRAMS OF STANDARD MIXTURES OF SKIN-LIGHTENING AGENTS AND SKIN-LIGHTENING PRODUCTS

I. Chromatograms for the separation of standard mixtures of skin-lightening agents

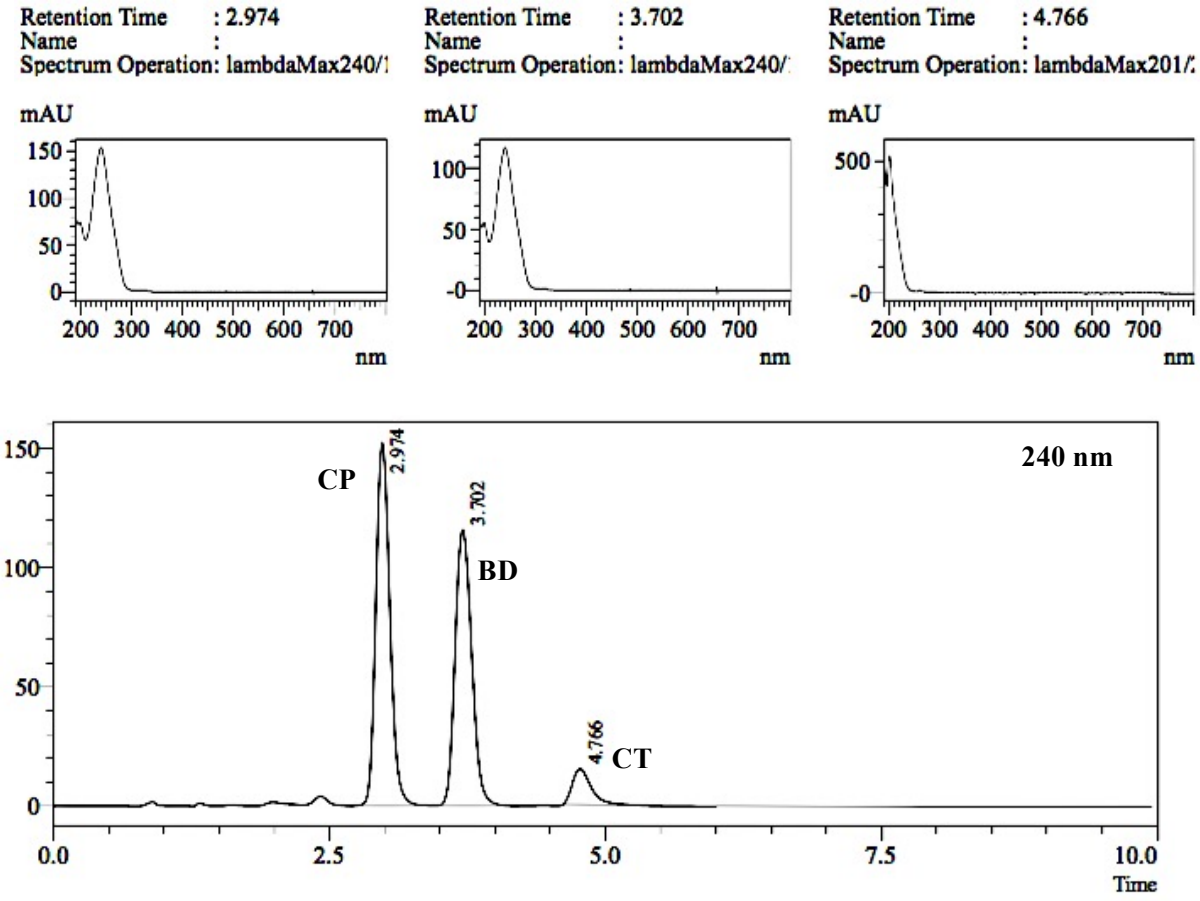


Figure E1: Chromatogram for the separation of CP, BD and CT at 240 nm. The retention times were 2.974, 3.702, and 4.766 minutes respectively. The chromatographic conditions used were: Brownlee analytical C18 (100 × 4.6 mm, 3 µm) column; mobile phase: MeOH-H_2O 80:20 (v/v); injection volume: 10 µL; flow rate: 0.8 mL min^{-1}; and column temperature: ambient.

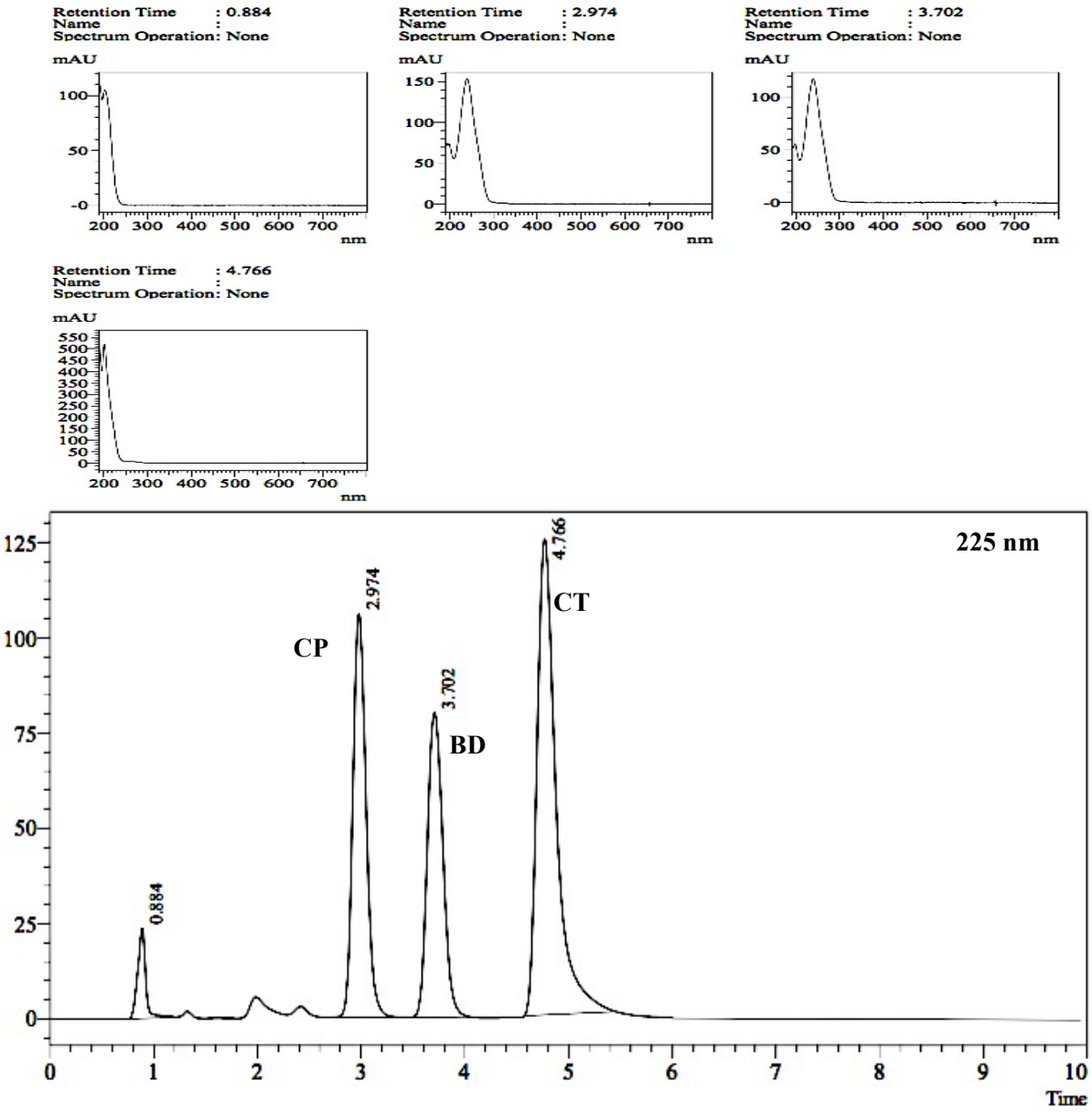


Figure E2: Chromatogram for the separation of CP, BD and CT at 225 nm. The retention times were 2.974, 3.702, and 4.766 minutes respectively. The chromatographic conditions used were: Brownlee analytical C18 (100 × 4.6 mm, 3 µm) column; mobile phase: MeOH-H_2O 80:20 (v/v); injection volume: 10 µL; flow rate: 0.8 mL min^{-1}; and column temperature: ambient.

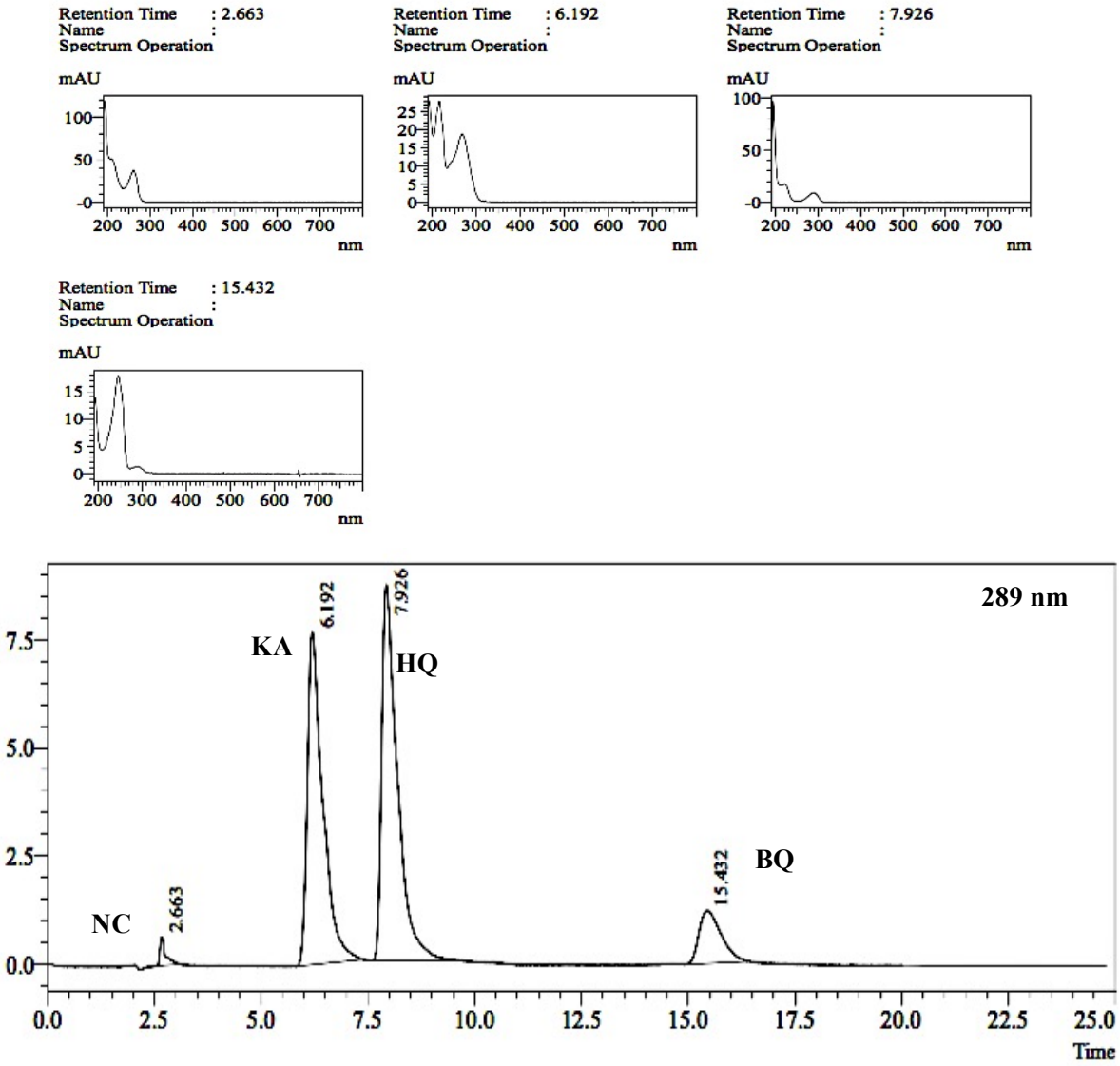


Figure E3: Chromatogram for the separation of NC, KA, HQ, and BQ at 289 nm. The retention times were 2.663, 6.192, 7.926 and 15.432 minutes respectively. The chromatographic conditions used were: SGE C18 (250 × 4.6mm, 5 µm) column; mobile phase: 0.5% orthophosphoric acid in methanol and 0.5% orthophosphoric acid in water 5:95 (v/v); injection volume: 10 µL; flow rate: 1.0 mL min^{-1}; and column temperature: 35 °C.

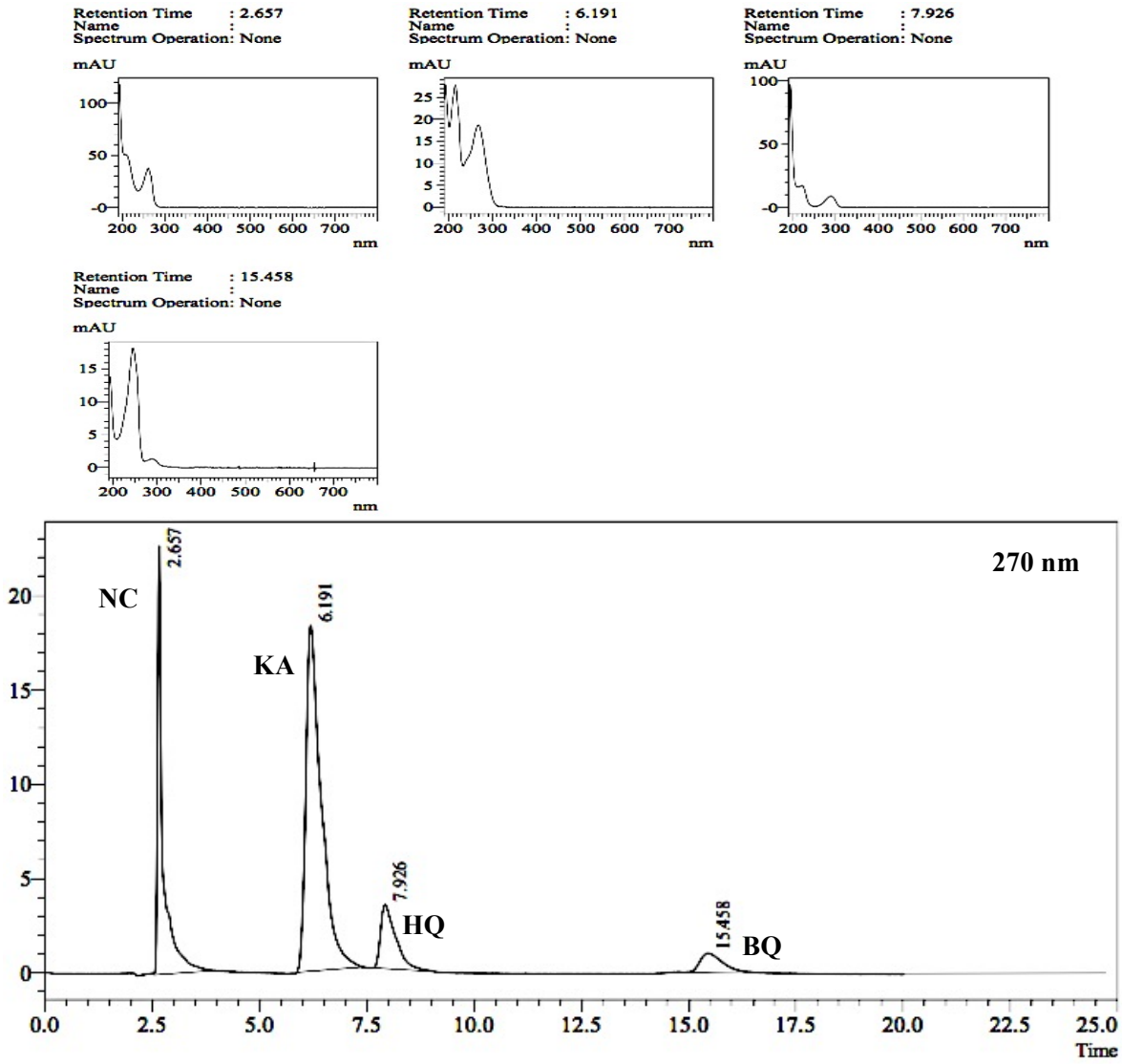


Figure E4: Chromatogram for the separation of NC, KA, HQ, and BQ at 270 nm. The retention times were 2.657, 6.191, 7.926 and 15.458 minutes respectively. The chromatographic conditions used were: SGE C18 (250 × 4.6mm, 5 µm) column; mobile phase: 0.5% orthophosphoric acid in methanol and 0.5% orthophosphoric acid in water 5:95 (v/v); injection volume: 10 µL; flow rate: 1.0 mL min^{-1}; and column temperature: 35 °C.

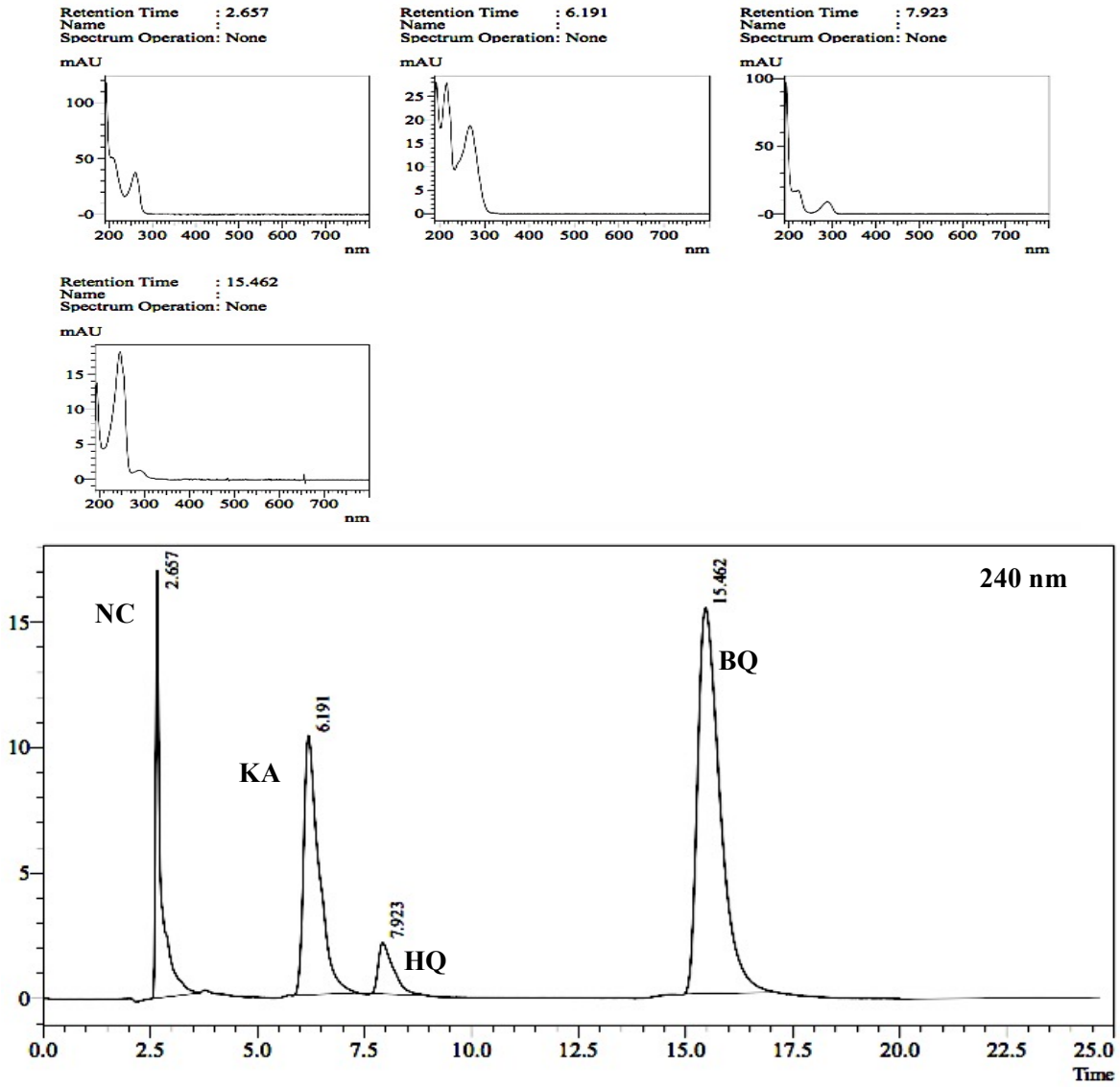


Figure E5: Chromatogram for the separation of NC, KA, HQ, and BQ at 240 nm. The retention times were 2.657, 6.191, 7.923 and 15.462 minutes respectively. The chromatographic conditions used were: SGE C18 (250 × 4.6mm, 5 µm) column; mobile phase: 0.5% orthophosphoric acid in methanol and 0.5% orthophosphoric acid in water 5:95 (v/v); injection volume: 10 µL; flow rate: 1.0 mL min^{-1}; and column temperature: 35 °C.

II. Chromatograms for the determination of HQ, KA, NC, and BQ in skin-lightening products at detection wavelengths of 289, 270, 270 and 240 nm respectively. The chromatographic conditions used were: SGE C18 (250 × 4.6mm, 5 µm) column; mobile phase: 0.5% orthophosphoric acid in methanol and 0.5% orthophosphoric acid in water 5:95 (v/v); injection volume: 10 µL; flow rate: 1.0 mL min^{-1}; and column temperature: 35 °C.

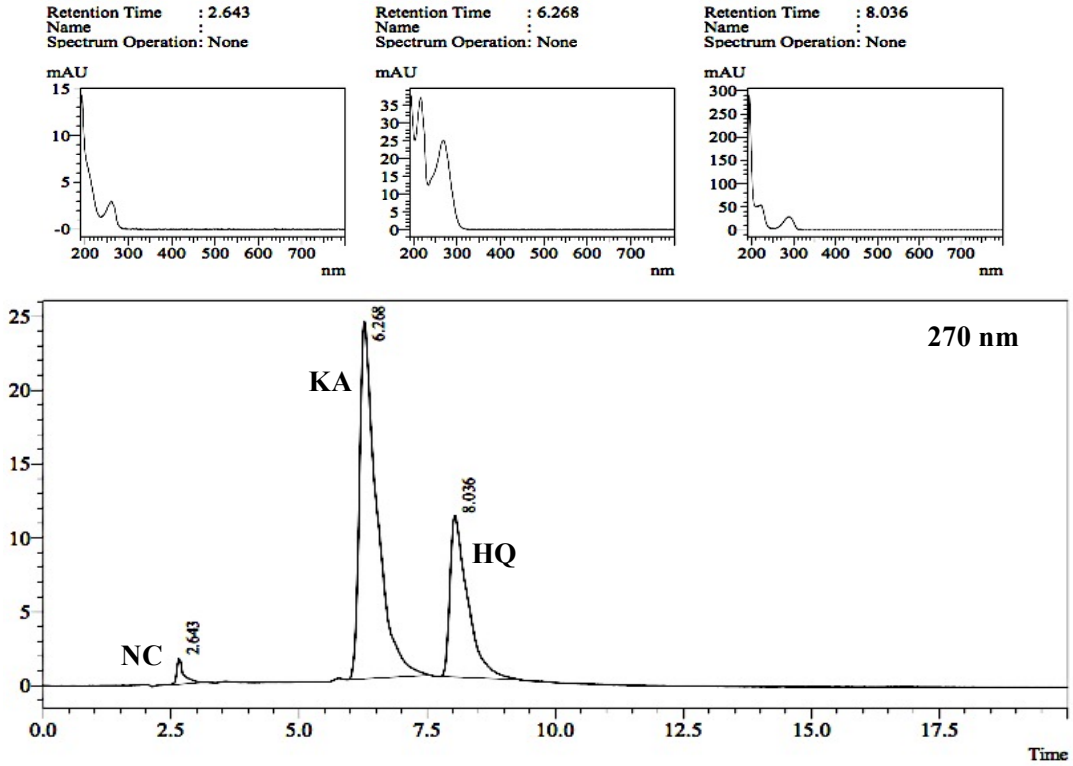


Figure E6: Chromatogram of SLP 1. The active ingredients detected are NC, KA, and HQ. The retention times were 2.643, 6.268 and 8.036 minutes respectively.

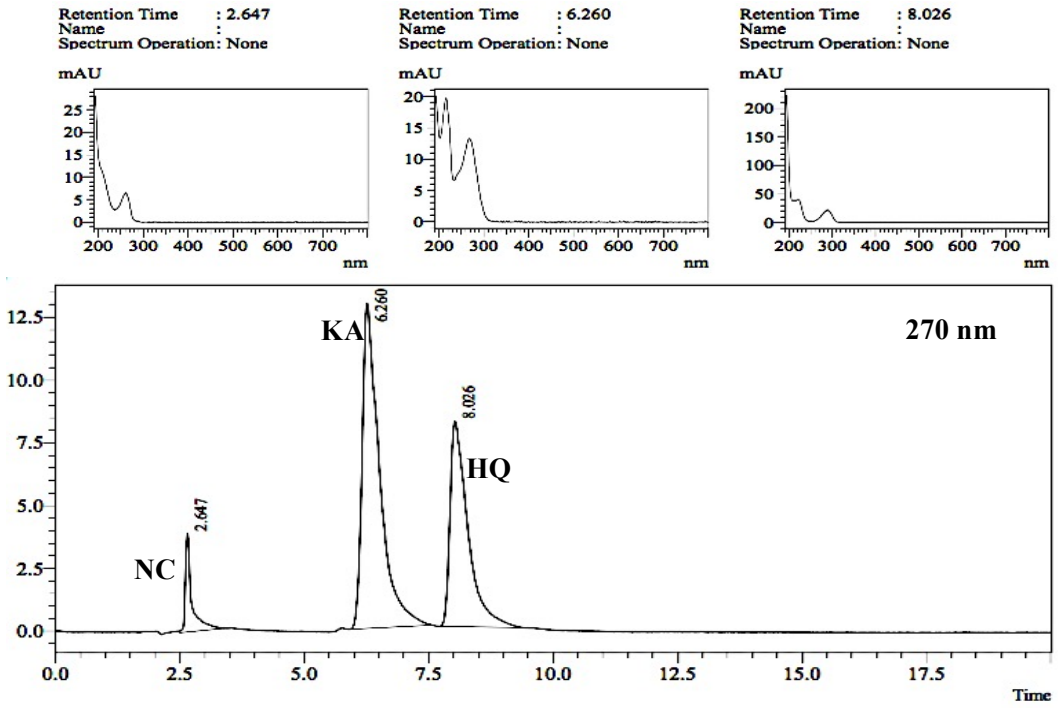


Figure E7: Chromatogram of SLP 2. The active ingredients detected are NC, KA, and HQ. The retention times were 2.647, 6.260 and 8.026 minutes respectively.

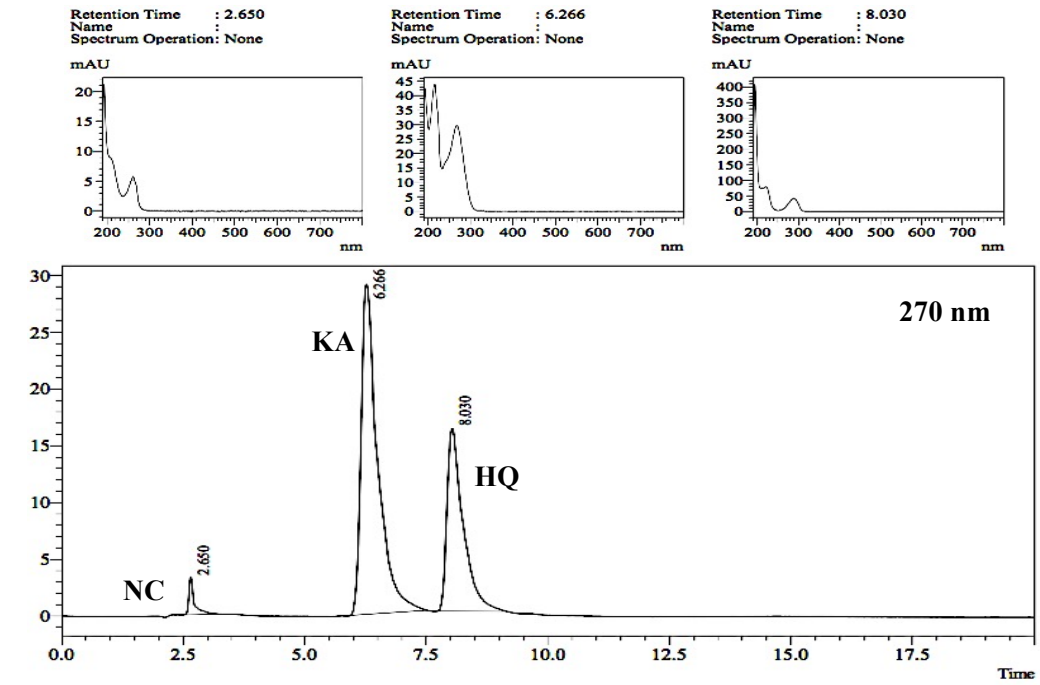


Figure E8: Chromatogram of SLP 3. The active ingredients detected are NC, KA, and HQ. The retention times were 2.650, 6.266 and 8.030 minutes respectively.

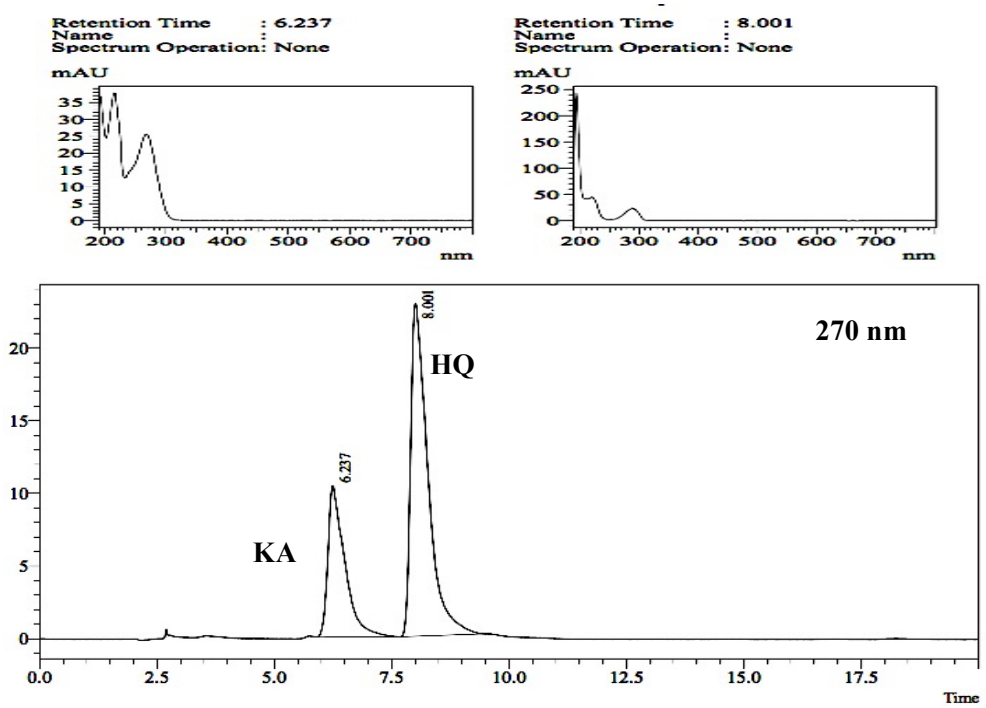


Figure E9: Chromatogram of SLP 4. The active ingredients detected are KA and HQ. The retention times were 6.2376 and 8.001 minutes respectively.

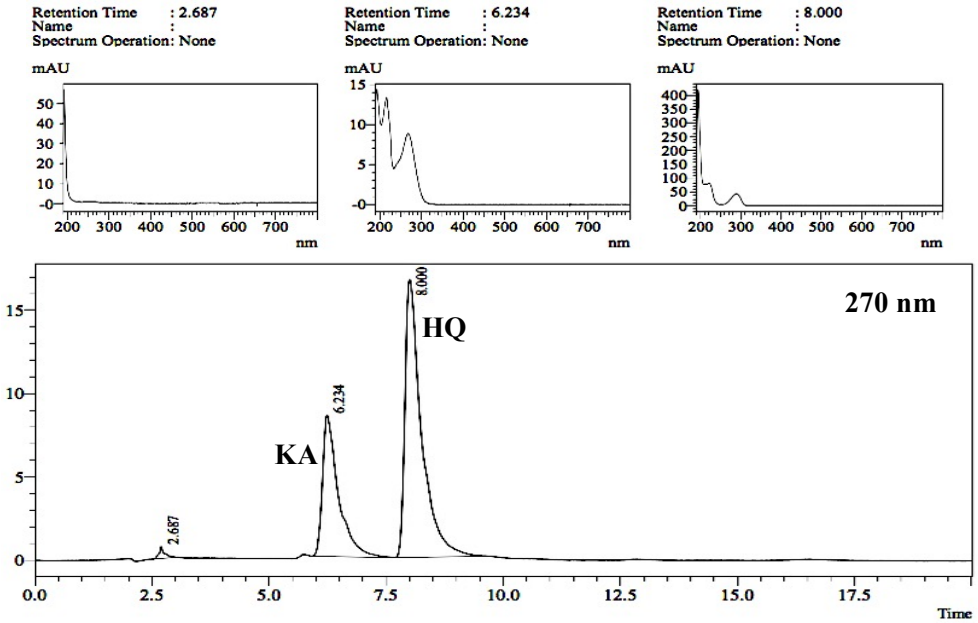


Figure E10: Chromatogram of SLP 5. The active ingredients detected are KA and HQ. The retention times were 6.234 and 8.000 minutes respectively.

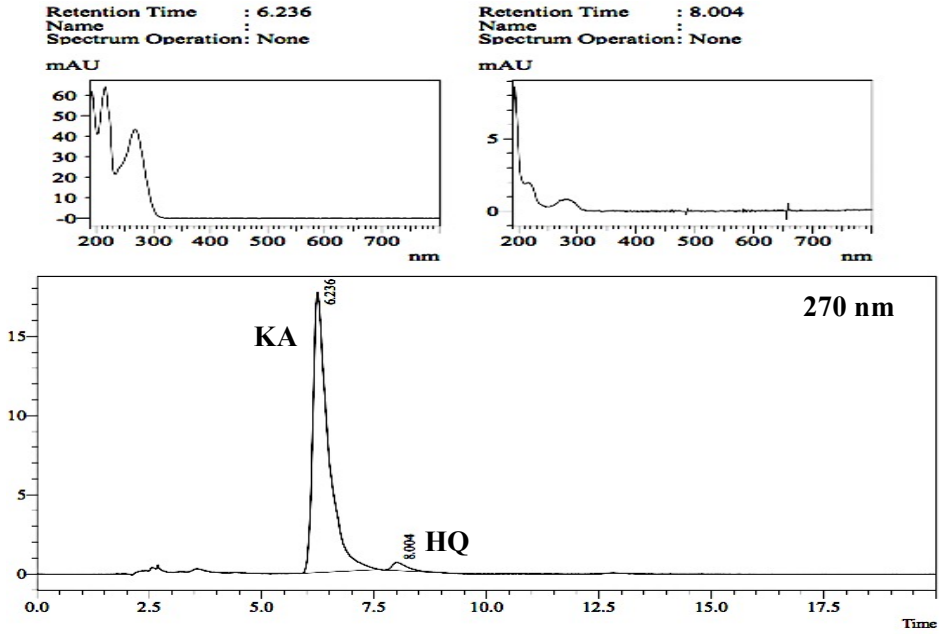


Figure E11: Chromatogram of SLP 8. The active ingredients detected are KA and HQ. The retention times were 6.236 and 8.004 minutes respectively.

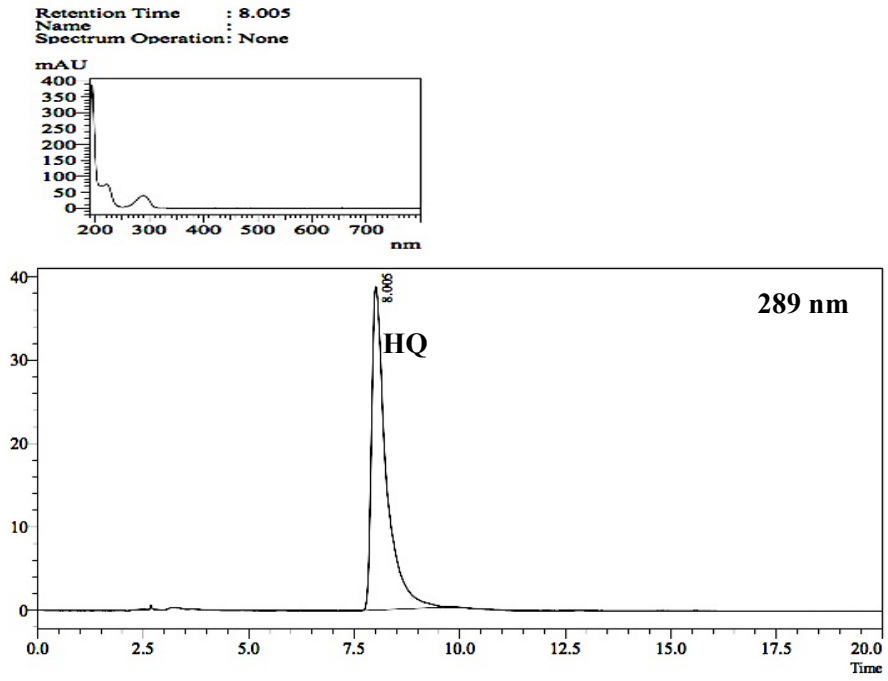


Figure E12: Chromatogram of SLP 10. The active ingredient detected is HQ. The retention time is 8.005 minutes.

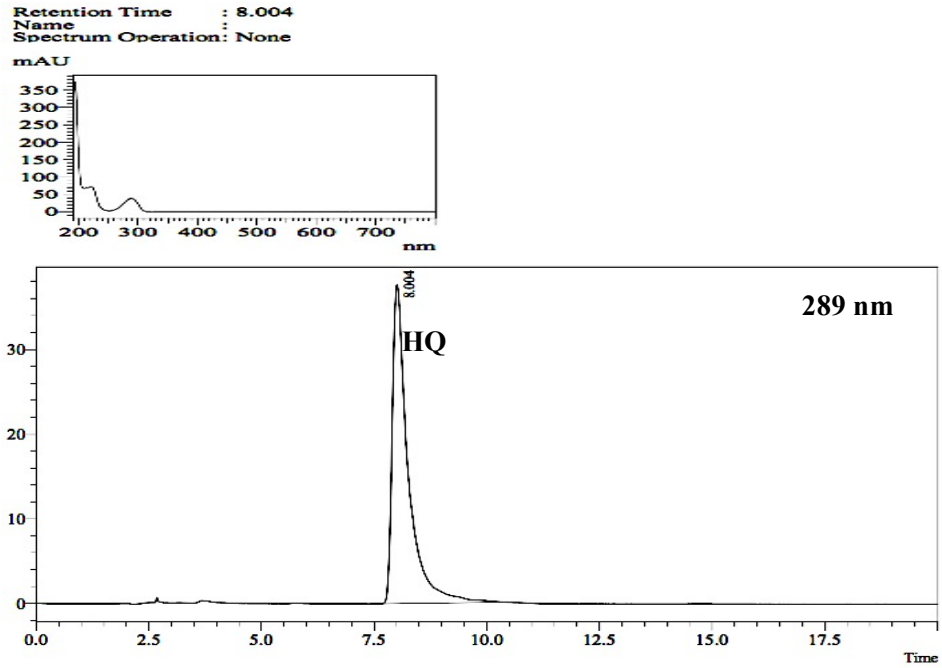


Figure E13: Chromatogram of SLP 11. The active ingredient detected is HQ. The retention time is 8.004 minutes.

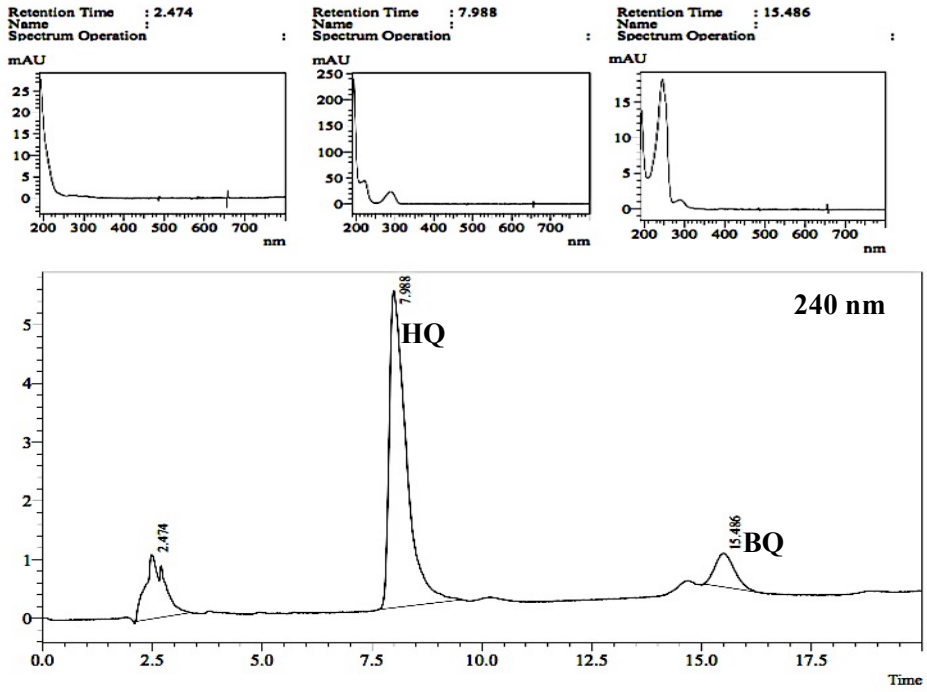


Figure E14: Chromatogram of SLP 12. The active ingredients detected are HQ and BQ. The retention times were 7.988 and 15.486 minutes respectively.

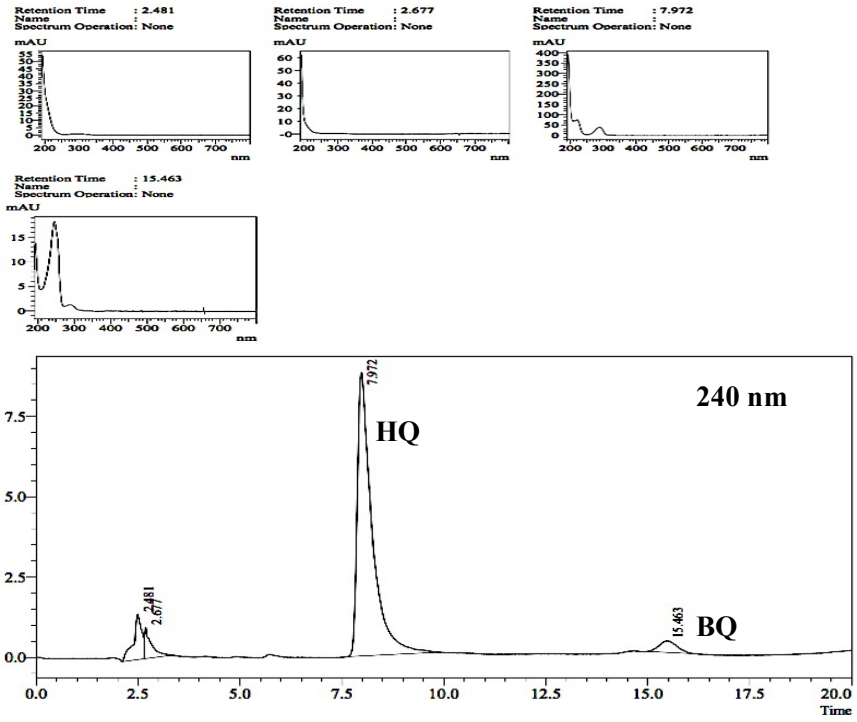


Figure E15: Chromatogram of SLP 13. The active ingredients detected are HQ and BQ. The retention times were 7.972 and 15.463 minutes respectively.

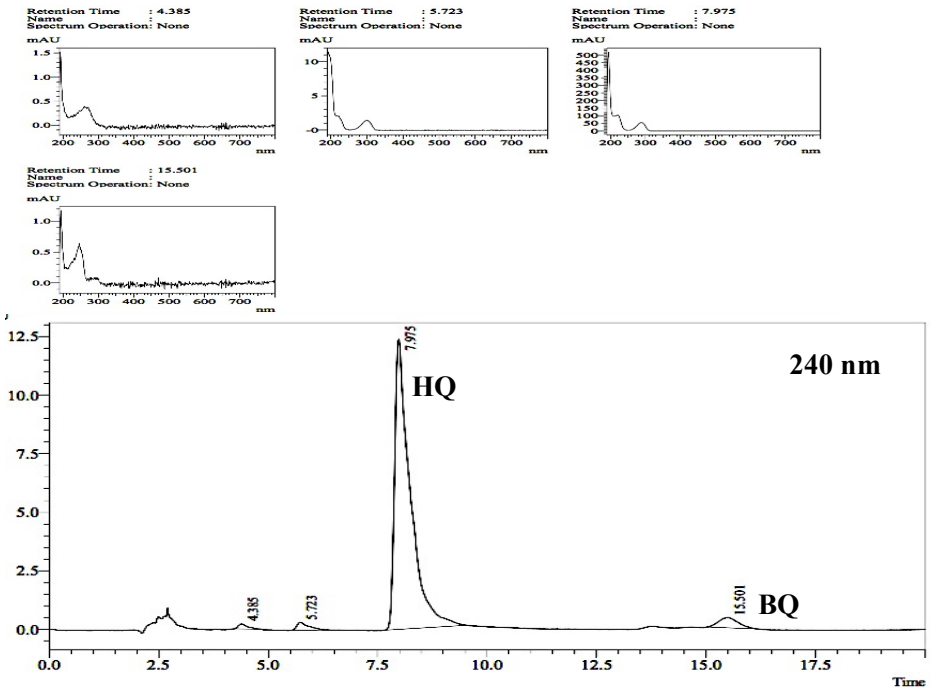


Figure E16: Chromatogram of SLP 14. The active ingredients detected are HQ and BQ. The retention times were 7.975 and 15.501 minutes respectively.

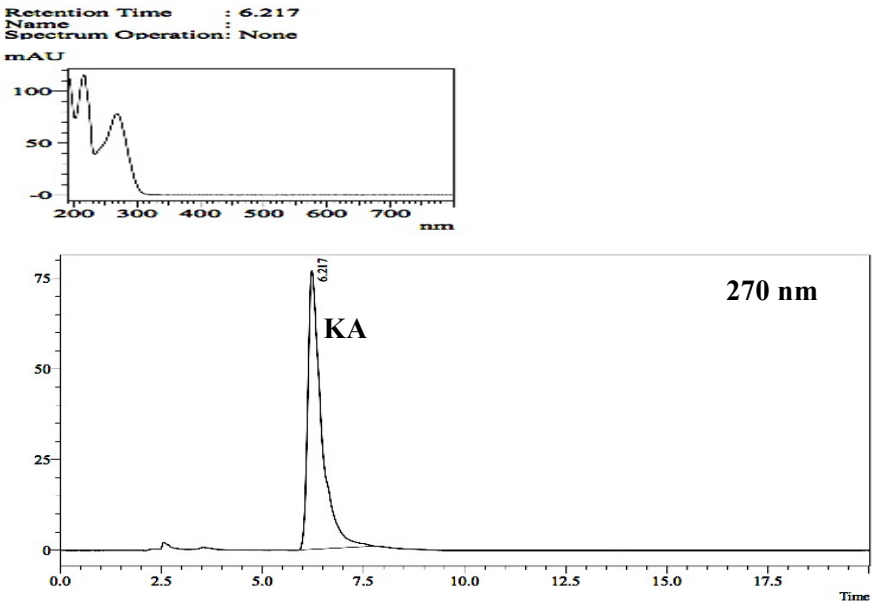


Figure E17: Chromatogram of SLP 15. The active ingredient detected is KA. The retention time is 6.217 minutes.

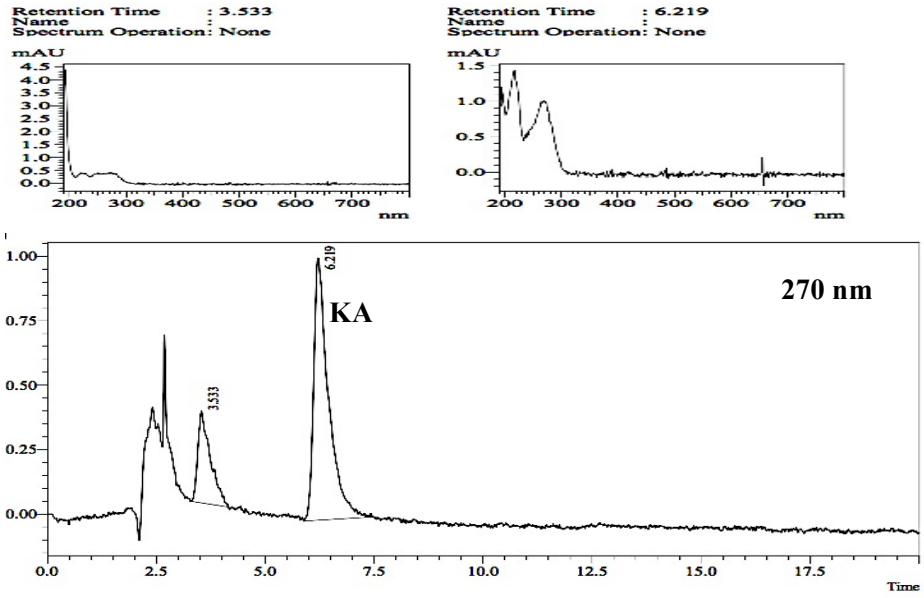


Figure E18: Chromatogram of SLP 17. The active ingredient detected is KA. The retention time is 6.219 minutes.

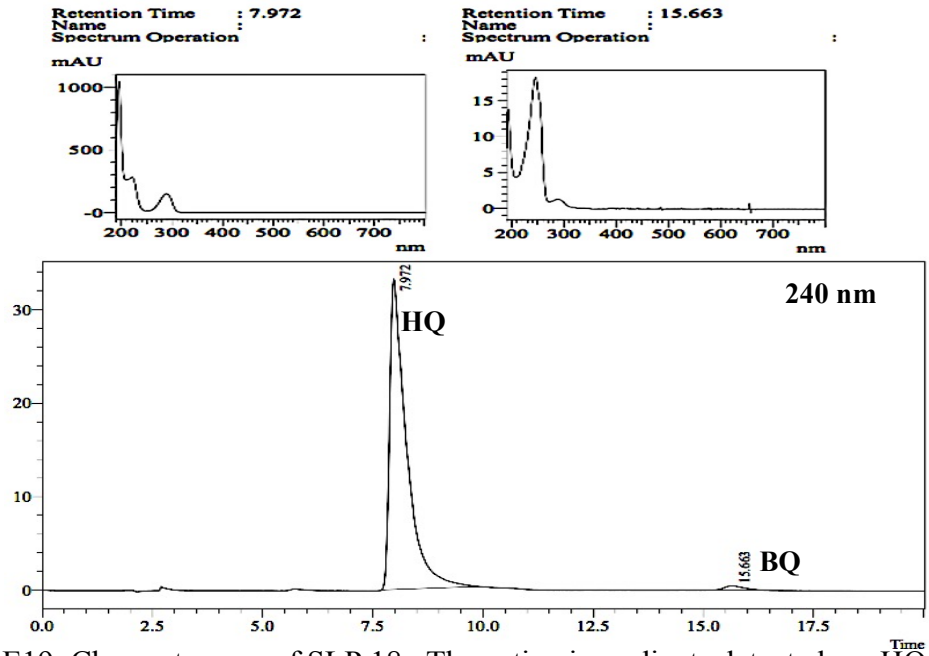


Figure E19: Chromatogram of SLP 18. The active ingredients detected are HQ and BQ. The retention times were 7.927 and 15.663 minutes respectively.

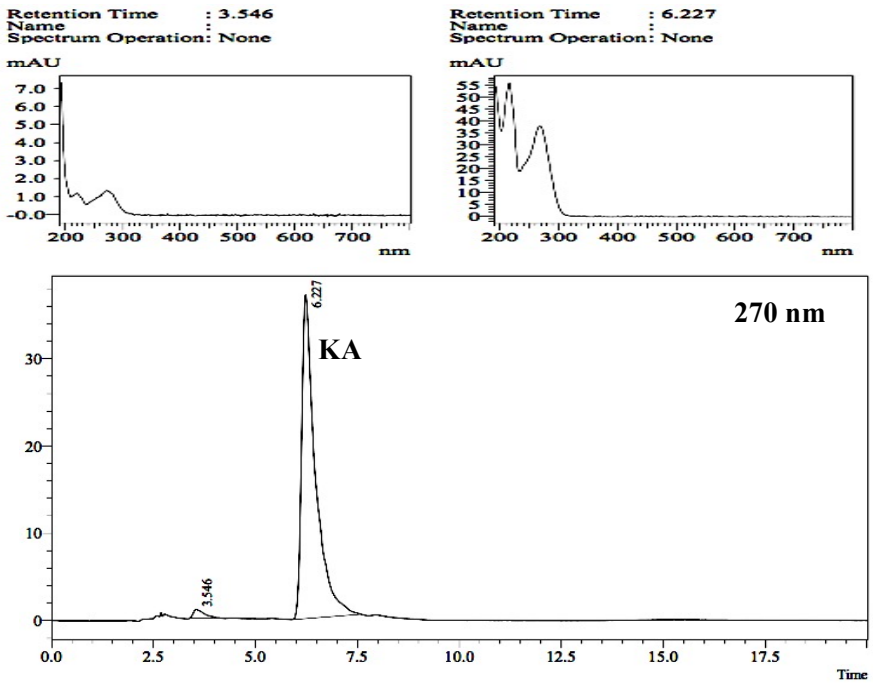


Figure E20: Chromatogram of SLP 19. The active ingredient detected is KA. The retention time is 6.227 minutes.

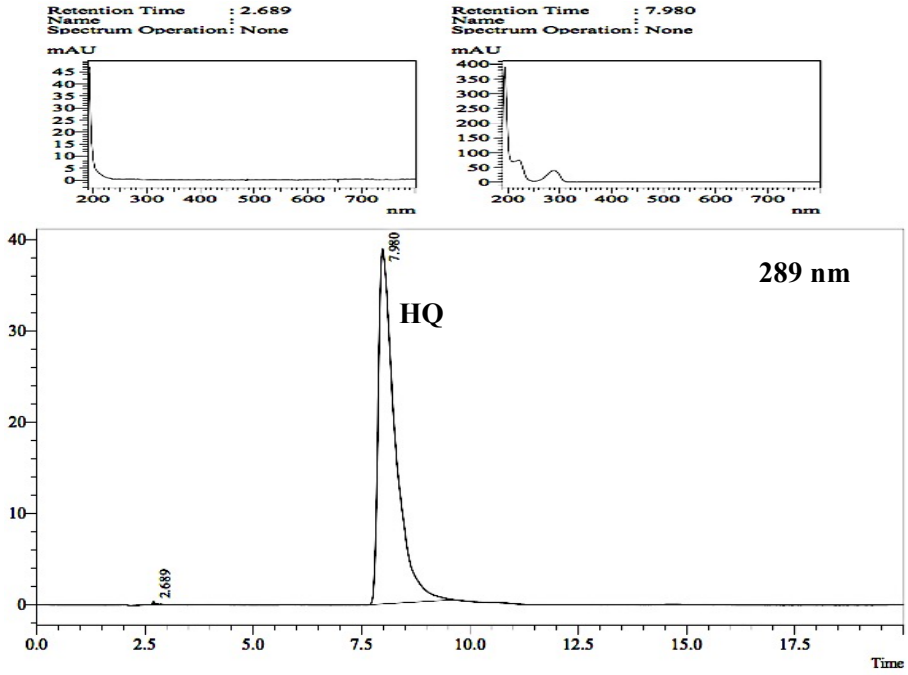


Figure E21: Chromatogram of SLP 21. The active ingredient detected is HQ. The retention time is 7.980 minutes.

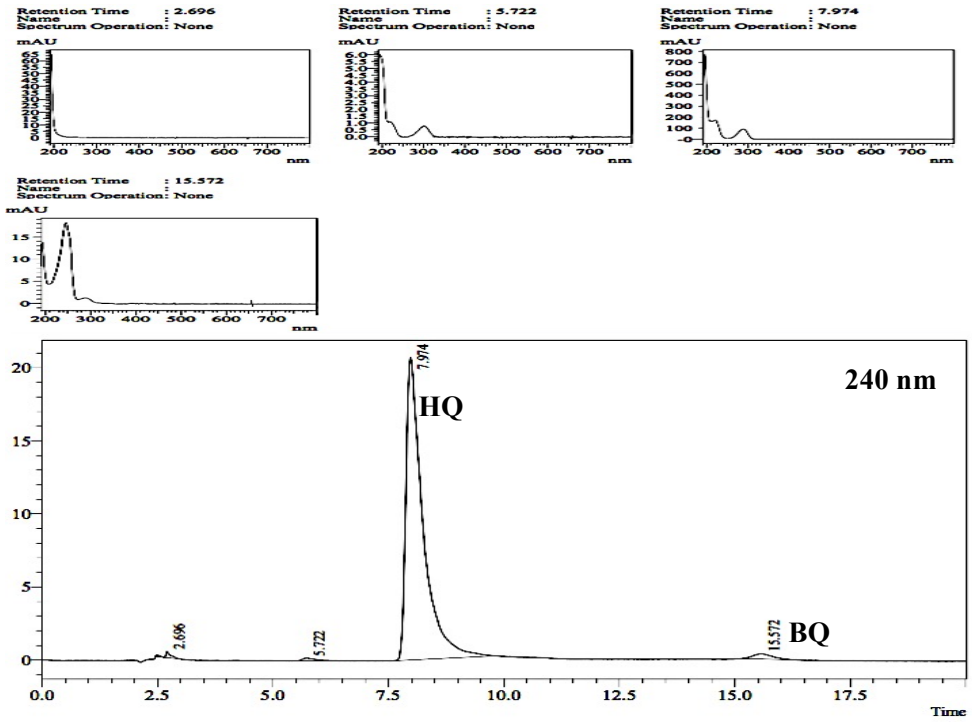


Figure E22: Chromatogram of SLP 22. The active ingredients detected are HQ and BQ. The retention times were 7.974 and 15.572 minutes respectively.

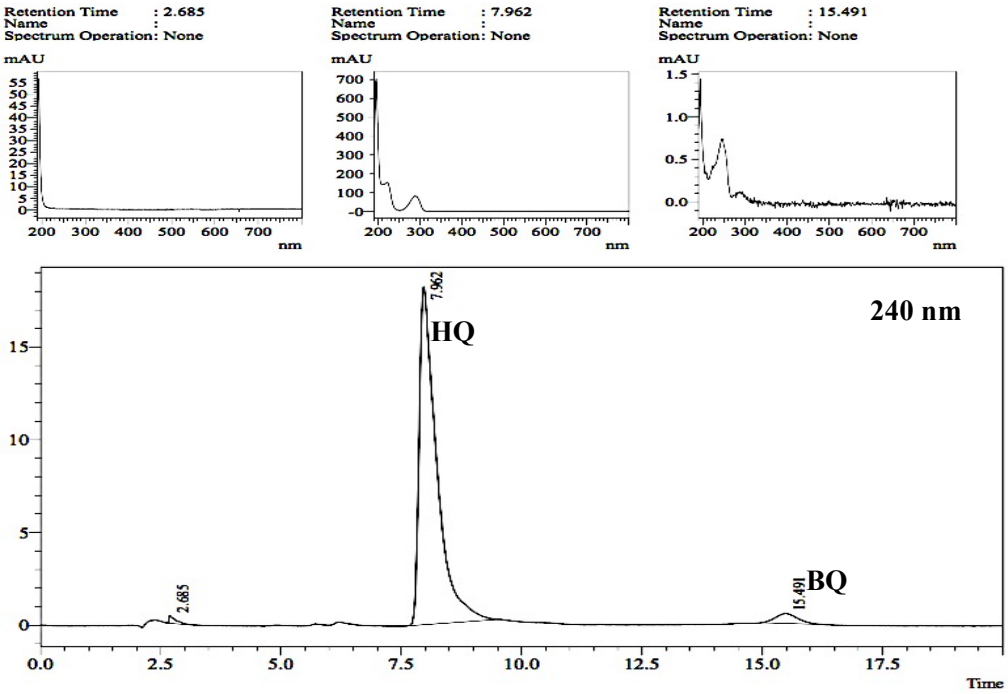


Figure E23: Chromatogram of SLP 23. The active ingredients detected are HQ and BQ. The retention times were 7.962 and 15.491 minutes respectively.

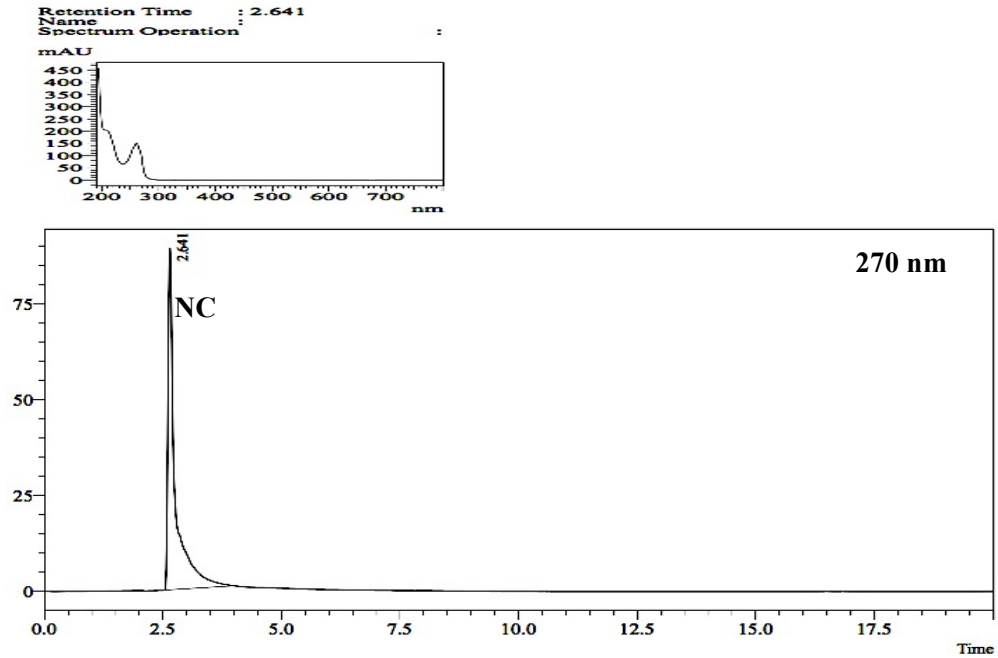


Figure E24: Chromatogram of SLP 24. The active ingredient detected is NC. The retention time is 2.641 minutes.

III. Chromatograms for the determination of CP, BD, and CT in skin-lightening products at detection wavelengths of 240, 240, and 225 nm respectively. The chromatographic conditions used were: Brownlee analytical C18 (100 × 4.6 mm, 3 µm) column; mobile phase: MeOH-H_2O 80:20 (v/v); injection volume: 10 µL; flow rate: 0.8 mL min^{-1}; and column temperature: ambient.

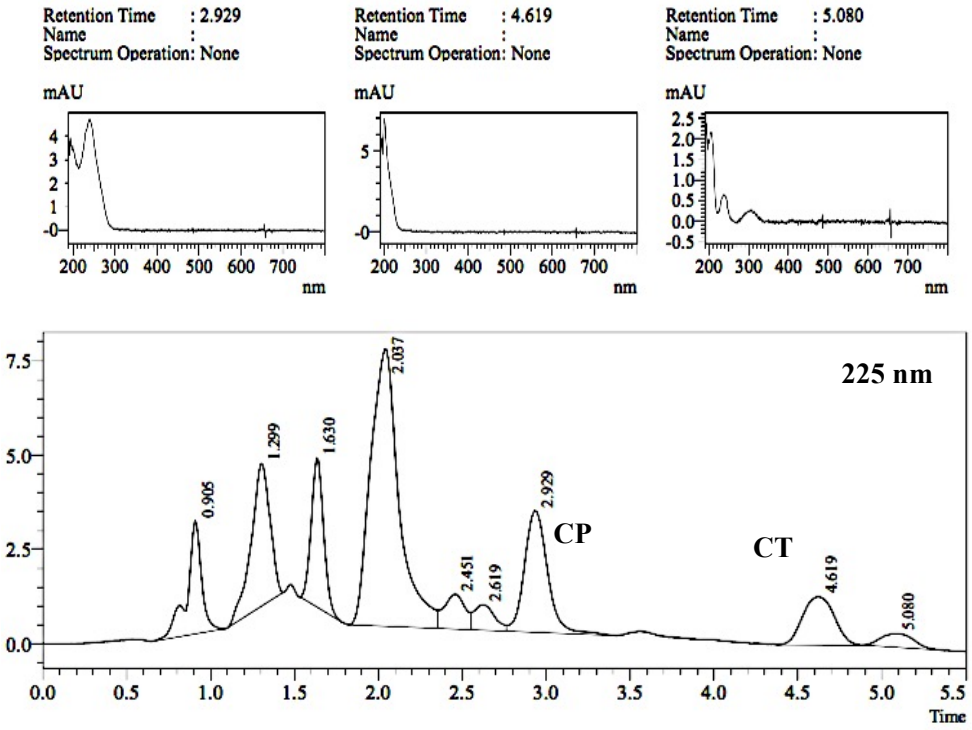


Figure E25: Chromatogram of SLP 9. The active ingredients detected are CP and CT. The retention times were 2.929 and 4.619 minutes respectively.

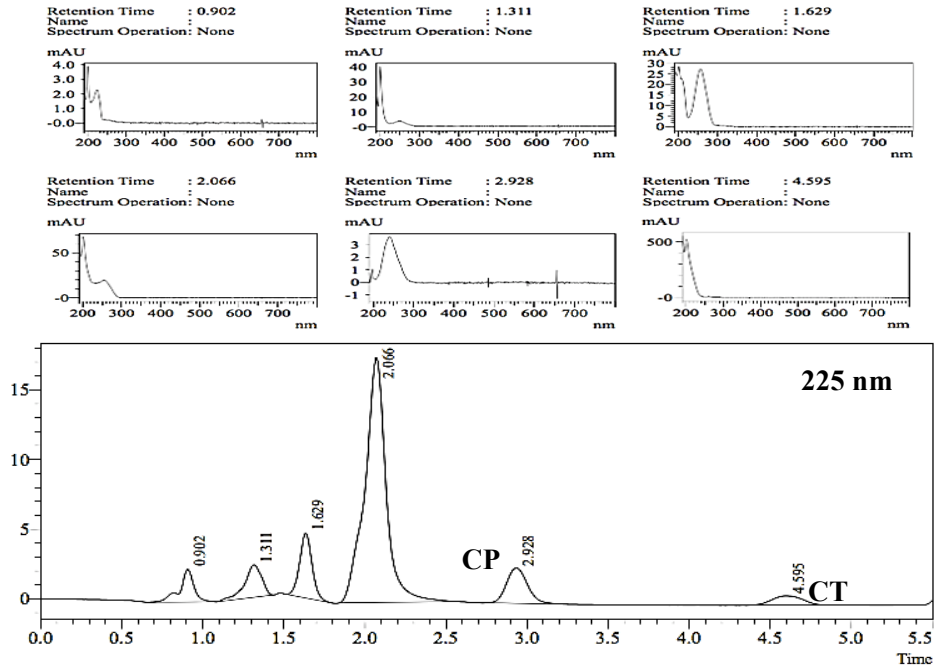


Figure E26: Chromatogram of SLP 20. The active ingredients detected are CP and CT. The retention times are 2.928 and 4.595 minutes respectively.

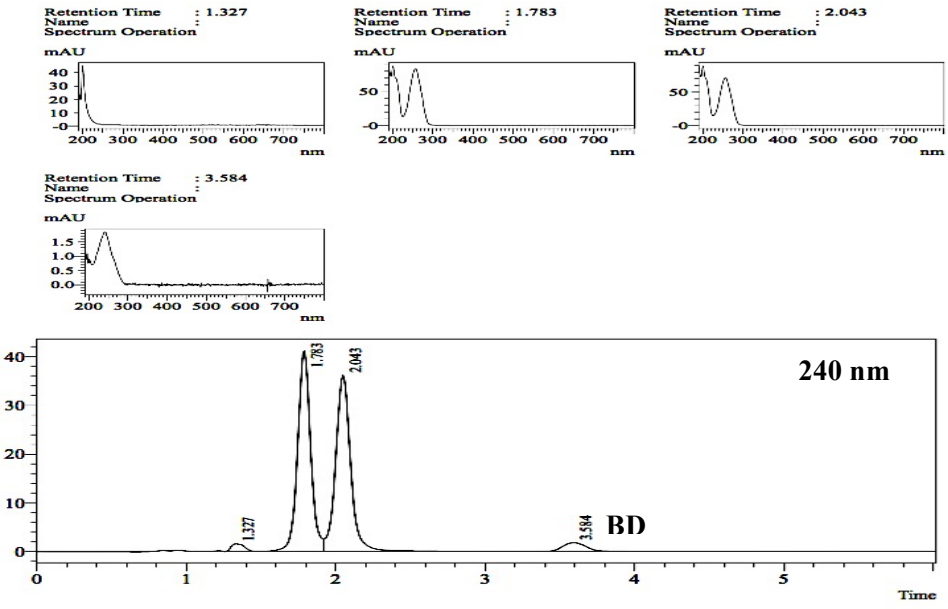


Figure E27: Chromatogram of SLP 25. The active ingredient detected is BD. The retention time is 3.584 minutes.

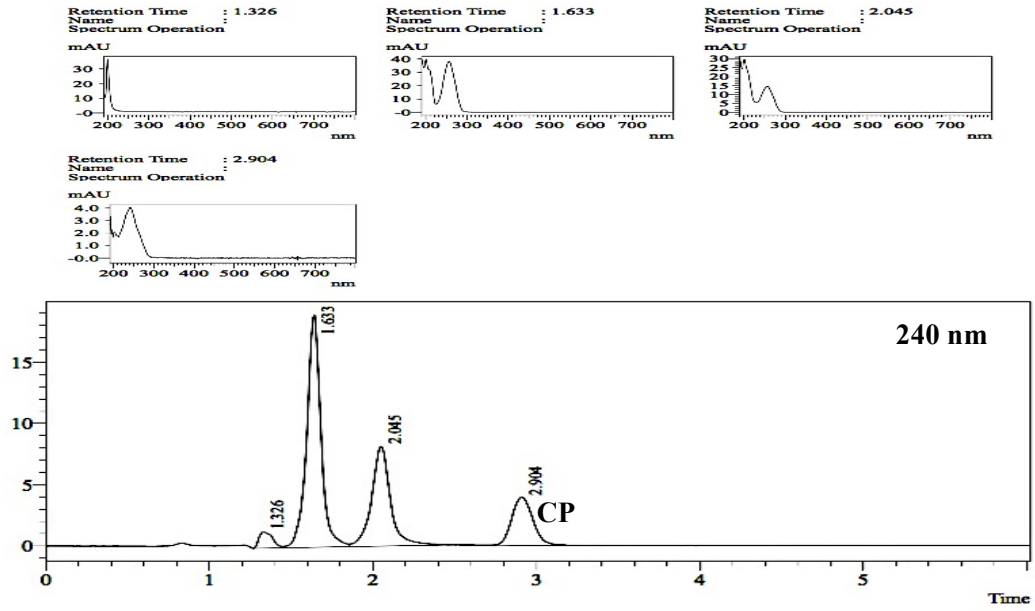


Figure E28: Chromatogram of SLP 26. The active ingredient detected is CP. The retention time is 2.904 minutes.

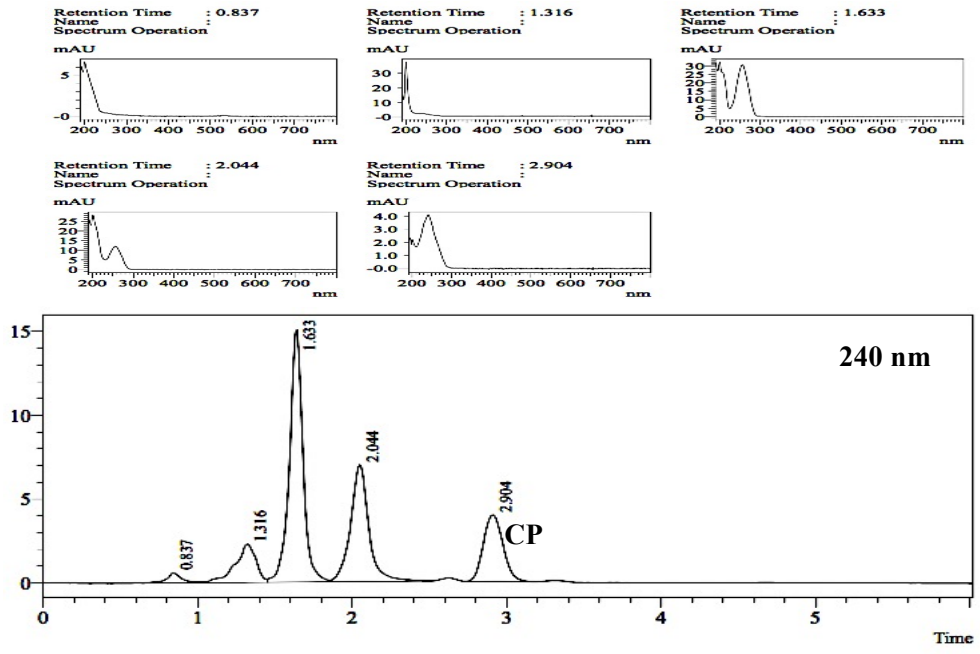


Figure E29: Chromatogram of SLP 27. The active ingredient detected is CP. The retention time is 2.904 minutes.

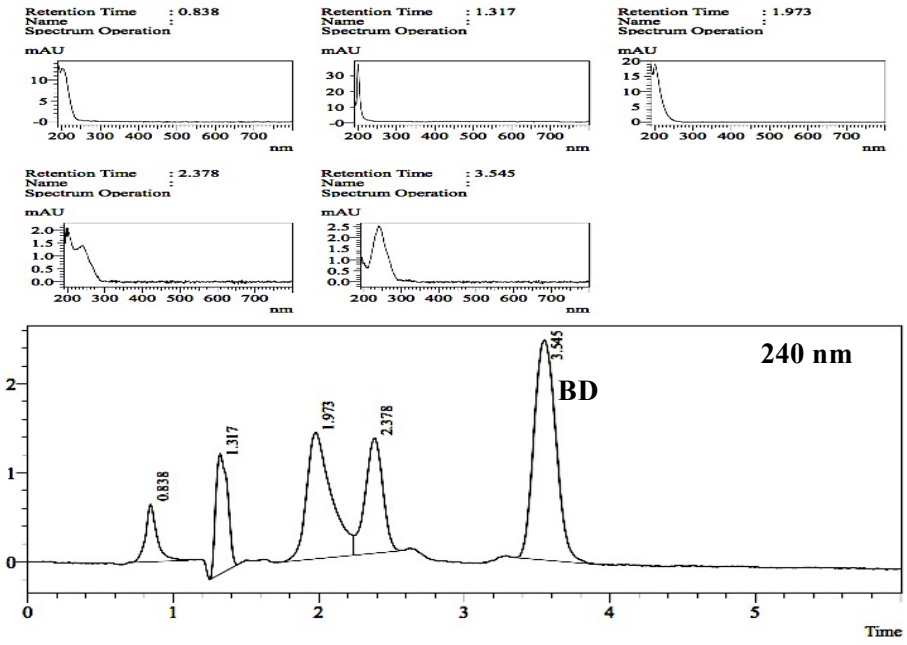


Figure E30: Chromatogram of SLP 28. The active ingredient detected is BD. The retention time is 3.543 minutes.

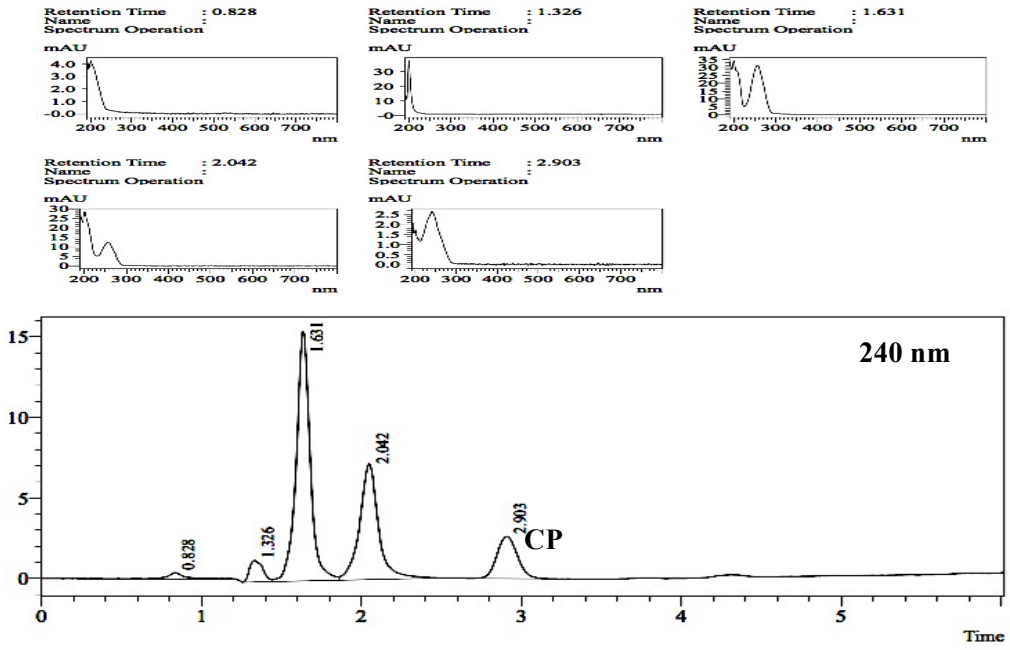


Figure E31: Chromatogram of SLP 29. The active ingredient detected is CP. The retention time is 2.903 minutes.

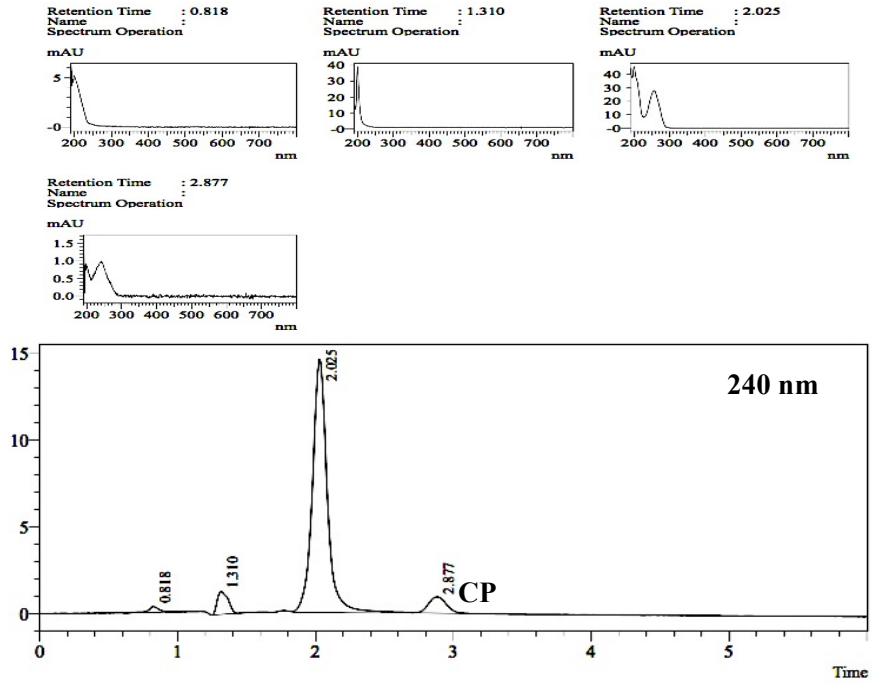


Figure E32: Chromatogram of SLP 30. The active ingredient detected is CP. The retention time is 2.877 minutes.

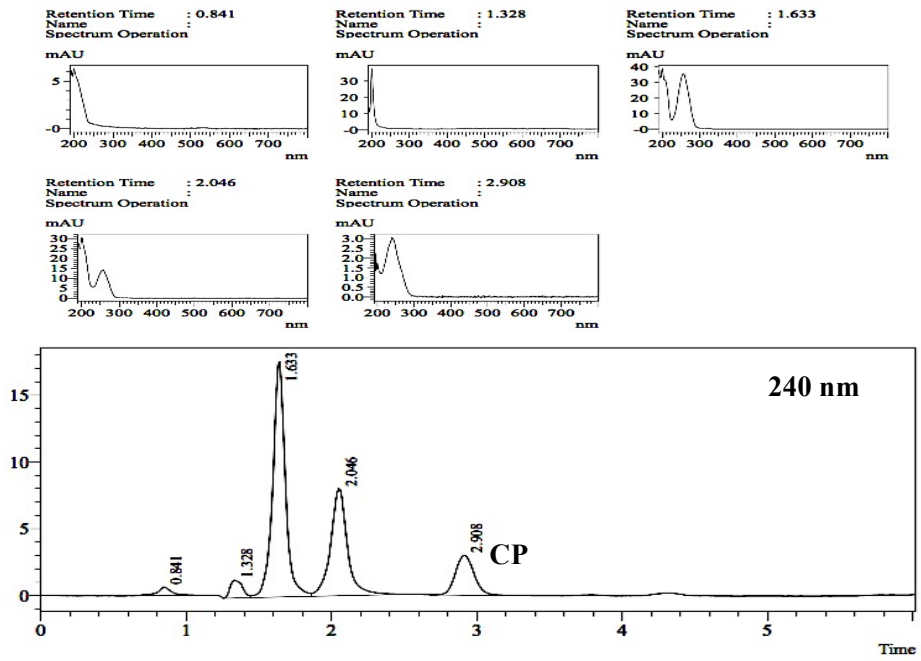


Figure E33: Chromatogram of SLP 31. The active ingredient detected is CP. The retention time is 2.908 minutes.

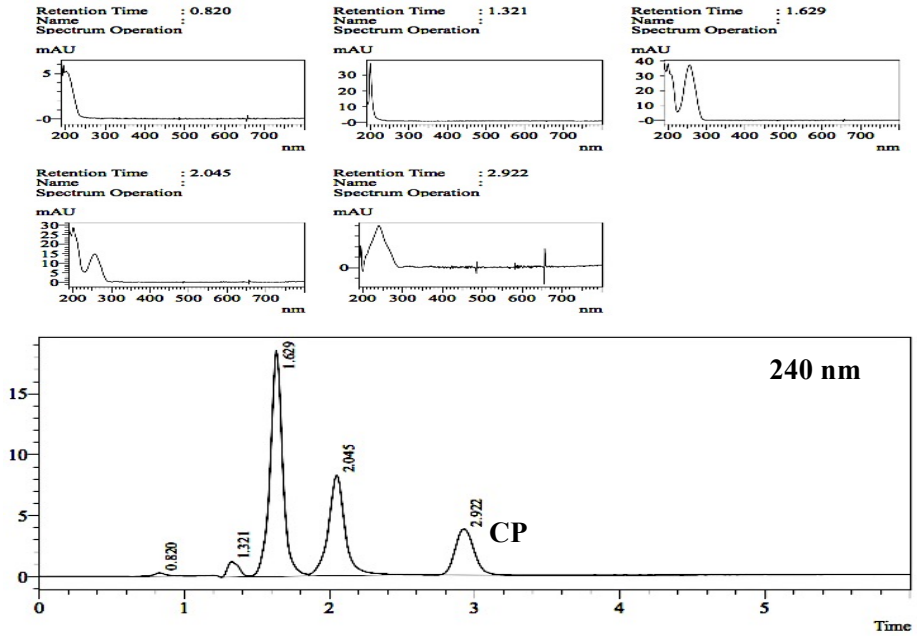


Figure E34: Chromatogram of SLP 32. The active ingredient detected is CP. The retention time is 2.922 minutes.

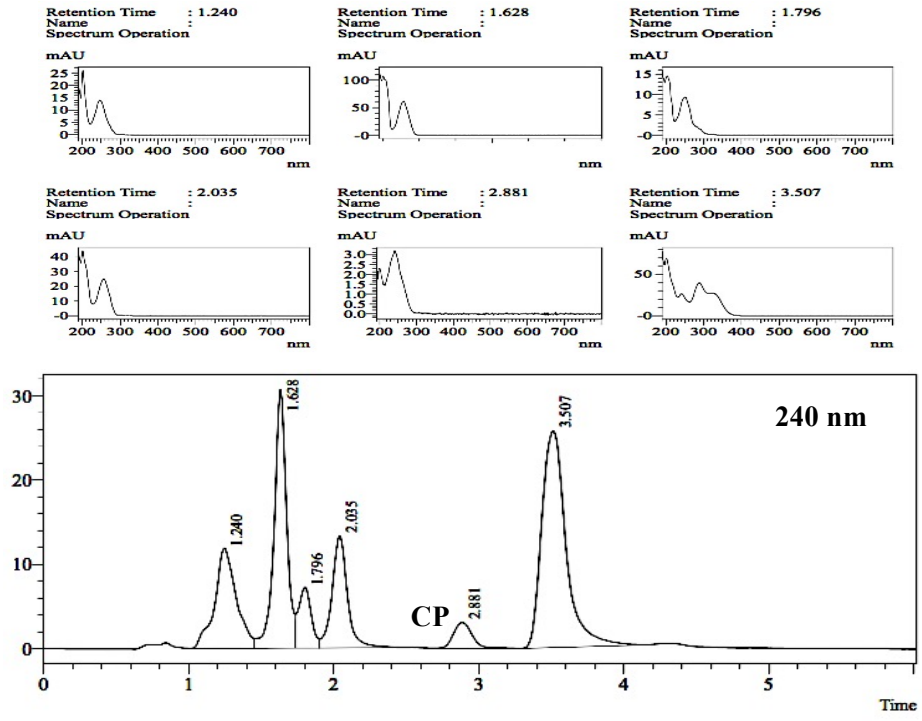


Figure E35: Chromatogram of SLP 33. The active ingredient detected is CP. The retention time is 2.881 minutes.

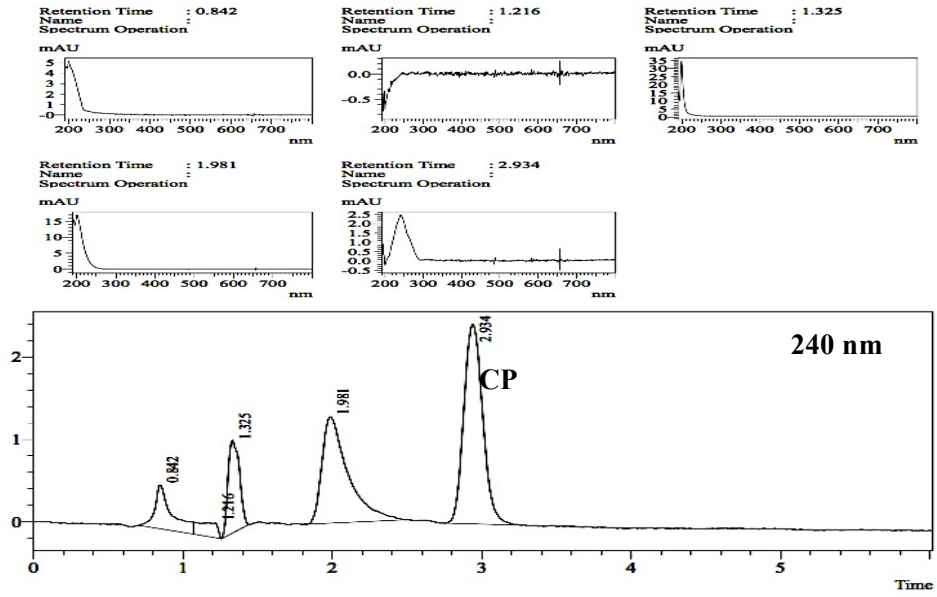


Figure E36: Chromatogram of SLP 34. The active ingredient detected is CP. The retention time is 2.934 minutes.

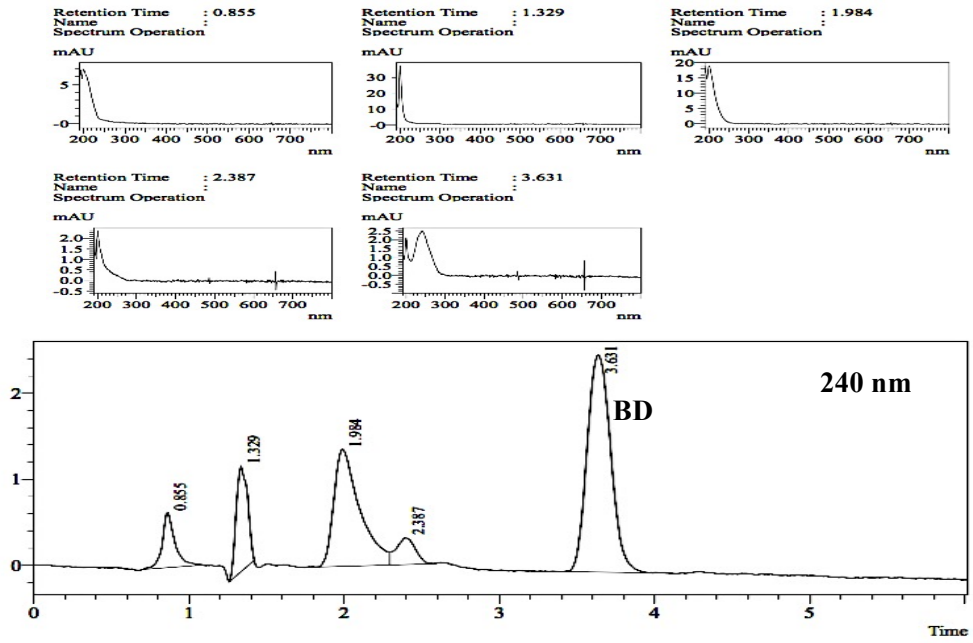


Figure E37: Chromatogram of SLP 35. The active ingredient detected is BD. The retention time is 3.631 minutes.

Appendix F

RAW DATA FOR SKIN-LIGHTENING PRODUCTS

This following tables gives the HPLC peak areas of the detected active ingredients in skin-lightening products. The mass of each product analysed was 0.05 g.

SLP1 HQ	SLP1 KA	SLP1 NC	SLP2 HQ	SLP2 KA	SLP3 NC	SLP3 HQ
721838	618803	15241	555600	344515	36548	1020158
706539	610842	15629	555472	340478	34687	1026687
709943	608294	14872	555678	338366	34787	1026687
702431	605418	17892	542029	335507	37310	1017666
713853	602316	18573	548604	334959	35523	1016093
699164	606199	17629	547966	334798	35472	1021539
698544	598238	16880	522027	330715	35111	1019342
697907	601286	16950	523133	330094	35449	1012430
696650	601840	16994	516341	327272	35493	1013760

SLP3 KA	SLP3 NC	SLP4 HQ	SLP4 KA	SLP5 HQ	SLP5 KA	SLP8 HQ
736145	26444	617787	660678	1105280	209905	10768
736900	27881	613291	659710	1107130	212475	9938
730189	26872	616319	658861	1108778	213340	10170
722006	28092	599520	650878	1084052	215091	9937
723306	27838	596606	649159	1078371	213889	9829
732888	27951	596427	650997	1070224	213127	9916
718429	28831	584134	648930	1071570	211729	10209
718187	29163	587775	648159	1069604	209156	9892
717849	27294	585271	645492	1072695	209087	9997

SLP8 KA	SLP9 CP	SLP9 CT	SLP10 HQ	SLP11 HQ	SLP12 HQ	SLP12 BQ
1060520	39317	17263	974992	944473	657681	30859
1059959	39106	17050	970614	945941	654640	29504
1054661	40268	17369	974102	945771	656533	30357
1116260	38617	18166	958888	929479	647841	27378
1110279	40453	16687	954675	925394	644763	27700
1115774	40110	17823	957208	928039	649535	27370
1060127	39622	16381	940544	923914	648497	26507

1054867	39640	16444	943519	921504	644461	27583
1061047	38978	16808	941446	922835	647537	28076

SLP13 HQ	SLP13 BQ	SLP14 HQ	SLP14 BQ	SLP15 KA	SLP17 KA	SLP18 HQ
1031006	15628	1453277	20573	1946580	24489	4301542
1051079	14977	1443216	19989	1966659	23830	4291433
1039586	15314	1444007	19090	1948886	23262	4298915
1008018	13700	1430744	18198	1927769	23534	4084166
1005811	13891	1429711	18649	1926448	23454	4089610
1009152	13409	1430644	18046	1923683	23425	4062487
987991	13448	1389701	14523	1921777	24029	4120771
996372	13449	1388655	14313	1922439	23480	4069215
982152	13745	1386605	14649	1919654	23212	4067305

SLP18 BQ	SLP19 KA	SLP20 CP	SLP20 CT	SLP21 HQ	SLP22 HQ	SLP22 BQ
9452	930544	33674	9114	1164657	2413728	10154
9400	925890	33014	9019	1140037	2416790	9625
9708	926029	33071	10956	1146627	2417796	9690
9006	904426	32990	8876	1090789	2409746	9392
8287	887731	33122	8791	1066674	2407573	9523
9692	889094	33220	9067	1058817	2397989	9075
13010	913321	33110	8755	1100825	2436873	10380
13059	897890	33098	8959	1090397	2424235	10385
14098	899171	33128	8361	1095979	2427706	10418

SLP23 HQ	SLP23 BQ	SLP24 NC	SLP25 BD	SLP26 CP	SLP27 CP	SLP28 BD
2237655	18534	1039284	19147	34827	34080	26930
2199600	18948	1033020	18939	35970	35323	25904
2191544	19555	1047378	18805	35820	37797	25718
2148236	18399	1044395	19203	34955	34879	25530
2143456	15047	1052379	19183	35137	38948	27038
2133775	15291	972911	19283	35949	36985	25854
2150146	16614	1043345	19478	35129	36474	25998
2147944	15122	1040507	19833	35490	38028	25142
2143602	15865	1039984	19664	35803	36461	26257

SLP29 CP	SLP30 CP	SLP31 CP	SLP32 CP	SLP33 CP	SLP34 CP	SLP35 BD
23339	8662	26586	34980	27813	21332	26691
23532	8484	26628	36555	27274	21221	27946
23274	8382	26310	35096	32086	21110	27829
23441	8441	26354	36587	33814	21573	26699
23328	8244	26553	34009	29998	21372	27798
23319	8425	26512	34138	37967	21376	28134
23639	8164	26211	35226	28232	20971	26886
23531	8159	26405	34010	26805	21591	27580
23693	8202	26762	35788	28565	21360	27473

Appendix G

CALIBRATION DATA FOR HEAVY METALS

The calibration data for all the heavy metals analysed is given in this section.

Table G1: Calibration data for the determination of aluminum (Al).

Concentration/mg L^{-1}	0.01	0.04	0.07	0.10	0.40	0.70	1.00	1.30	1.60
1	409.8	1749.0	3723.0	6009.0	24626.4	41544.6	57591.6	79374.5	96096.7
2	348.4	1701.5	3843.7	6050.9	24631.7	41208.0	57944.6	76937.5	97026.6
3	396.4	1813.2	3829.9	5984.2	24765.2	40354.6	59113.2	77358.8	96642.0
Mean	384.9	1754.6	3798.9	6014.7	24674.4	41035.7	58216.6	77890.3	96588.4
Std Dev	32.3	56.1	66.1	33.7	78.7	613.4	796.4	1302.5	467.3
RSD%	8.4	3.2	1.7	0.6	0.3	1.5	1.4	1.7	0.5

Table G2: Calibration data for the determination of chromium (Cr).

Concentration/mg L^{-1}	0.01	0.04	0.07	0.10	0.40	0.70	1.00	1.30	1.60
1	276.5	1320.6	2552.4	4017.8	15507.6	27471.2	39027.7	51219.1	62213.5
2	299.8	1336.9	2575.1	3986.9	15382.0	27498.9	39120.7	51203.1	61427.1
3	286.8	1333.5	2630.6	3984.1	15227.0	27479.7	39314.2	50971.1	61067.0
Mean	287.7	1330.3	2586.0	3996.3	15372.2	27483.3	39154.2	51131.1	61569.2
Std Dev	11.7	8.6	40.2	18.7	140.6	14.2	146.2	138.8	586.3
RSD%	4.1	0.6	1.6	0.5	0.9	0.1	0.4	0.3	0.9

Table G3: Calibration data for the determination of copper (Cu).

Concentration/mg L^{-1}	0.01	0.04	0.07	0.10	0.40	0.70	1.00	1.30	1.60
1	2687.3	11743	18471	27934	91752	155008.0	212363.7	295999.2	347650.8
2	2594.5	12011	18260	27724	91057	154177.9	212447.9	292484.5	345815.2
3	3031.0	12622	17974	28047	92346	151557.3	214919.0	291580.5	344257.3
Mean	2770.9	12125	18235	27901	91718	153581.1	213243.5	293354.7	345907.8
Std Dev	229.9	450.5	249.4	163.9	645.2	1801.1	1451.6	2334.4	1698.6
RSD%	8.3	3.7	1.4	0.6	0.7	1.2	0.7	0.8	0.5

Table G4: Calibration data for the determination of iron (Fe).

Concentration/mg L^{-1}	0.01	0.04	0.07	0.10	0.40	0.70	1.00	1.30	1.60
1	80.0	786.0	2016.7	3542.5	20055.5	40330.1	58242.3	79905.7	98769.7
2	88.0	816.3	2055.2	3537.5	20082.1	39957.8	58070.6	79695.5	97555.3
3	82.6	815.0	2119.0	3557.6	20059.7	39656.6	58401.8	79210.3	97118.4
Mean	83.5	805.8	2063.6	3545.9	20065.8	39981.5	58238.2	79603.8	97814.5
Std Dev	4.1	17.1	51.7	10.5	14.3	337.4	165.6	356.6	855.6
RSD%	4.9	2.1	2.5	0.3	0.1	0.8	0.3	0.4	0.9

Table G5: Calibration data for the determination of manganese (Mn).

Concentration/mg L^{-1}	0.01	0.04	0.07	0.10	0.40	0.70	1.00	1.30	1.60
1	1676	10790	18065	25764	113357	200232	280982	383087	454975
2	1602	10952	18038	25865	112903	198842	281022	382282	450026
3	1738	10949	18084	25873	114252	196395	282266	380837	446693
Mean	1672	10897	18062.3	25834	113504	198489	281423	382068	450564
Std Dev	68.1	92.7	23.1	60.8	686.4	1942.6	730.0	1140.1	4167.2
RSD%	4.1	0.9	0.1	0.2	0.6	0.9	0.3	0.3	0.9

Table G6: Calibration data for the determination of nickel (Ni).

Concentration/mg L^{-1}	0.01	0.04	0.07	0.10	0.40	0.70	1.00	1.30	1.60
1	129.0	721.4	1240.8	1939.5	7534.5	13370.2	19241.9	25910.6	31754.3
2	116.2	726.5	1243.3	1925.5	7511.9	13363.5	19348.3	25881.8	31789.3
3	121.8	753.7	1265.7	1992.5	7429.1	13365.9	19538.3	25918.1	31597.1
Mean	122.3	733.9	1249.9	1952.5	7491.8	13366.5	19376.2	25903.5	31713.6
Std Dev	6.4	17.5	13.7	35.3	55.5	3.4	150.2	19.2	102.4
RSD%	5.2	2.4	1.1	1.8	0.7	0.03	0.8	0.1	0.3

Table G7: Calibration data for the determination of lead (Pb).

Concentration/mg L^{-1}	0.01	0.04	0.07	0.10	0.40	0.70	1.00	1.30	1.60
1	25.1	32.4	74.4	114.8	498.2	815.5	1127.3	1538.8	1849.2
2	25.1	42.5	78.1	112.3	489.9	818.4	1155.3	1539.2	1869.6
3	25.1	36.4	53.1	130.6	489.9	809.3	1137.2	1511.2	1869.4
Mean	25.1	37.1	68.5	119.2	492.7	814.4	1139.9	1529.7	1862.7
Std Dev	4.4×10^{-15}	5.1	13.5	9.9	4.8	4.6	14.2	16.1	11.7
RSD%	1.7×10^{-14}	13.7	19.7	8.3	0.9	0.6	1.2	1.0	0.6

Table G8: Calibration data for the determination of zinc (Zn).

Concentration/mg L^{-1}	0.01	0.04	0.07	0.10	0.40	0.70	1.00	1.30	1.60
1	391.4	1567.5	2343.9	3720.4	14375.3	25345.0	35827.4	48458.0	58951.0
2	377.1	1546.4	2342.3	3712.6	14353.2	25329.8	36679.6	48516.5	58413.3
3	371.0	1530.9	2327.2	3779.5	14390.5	25434.8	36045.0	48388.6	58379.5
Mean	379.8	1548.3	2337.8	3737.5	14373	25369.9	36184	48454.4	58581.3
Std Dev	10.5	18.4	9.2	36.6	18.8	56.7	442.8	64	320.6
RSD%	2.8	1.2	0.4	0.9	0.1	0.2	1.2	0.1	0.5

Table G9: Calibration data for the determination of mercury (Hg).

Mass /ng	100	200	300	400	500	600	700	800
1	0.078	0.145	0.235	0.307	0.384	0.448	0.508	0.567
2	0.080	0.144	0.237	0.309	0.38	0.447	0.505	0.565
3	0.081	0.149	0.232	0.313	0.379	0.442	0.501	0.569
Mean	0.079	0.146	0.235	0.309	0.381	0.446	0.505	0.567
Std Dev	0.0015	0.0026	0.0025	0.0031	0.0026	0.0032	0.0035	0.0020
RSD%	1.9	1.8	1.1	0.9	0.7	0.7	0.7	0.4

Appendix H

CALIBRATION CURVES AND RESIDUAL PLOTS FOR HEAVY METALS

The calibration curves and residual plots for all the heavy metals analysed by ICP-OES and CV-AAS are provided in this Appendix.

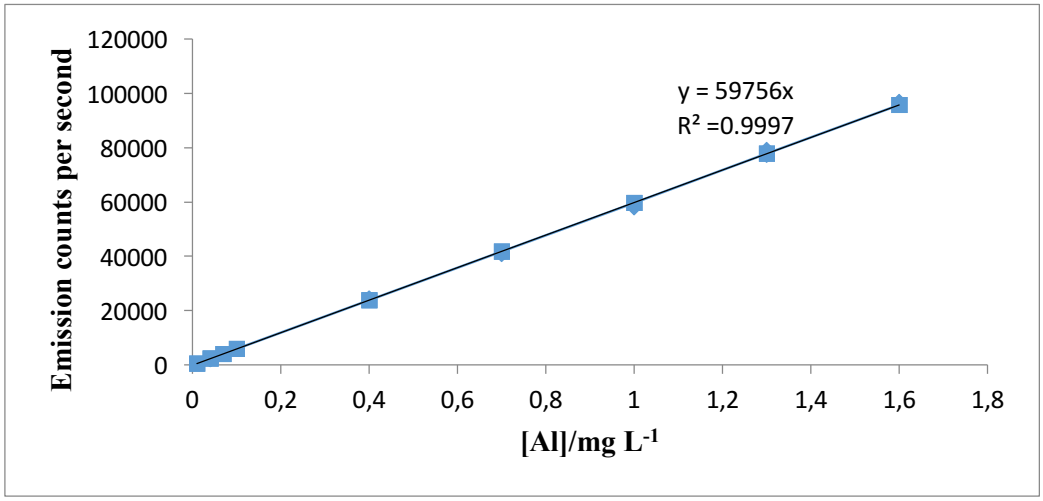


Figure H1: Calibration graph for the determination of aluminium at 396.153 nm.

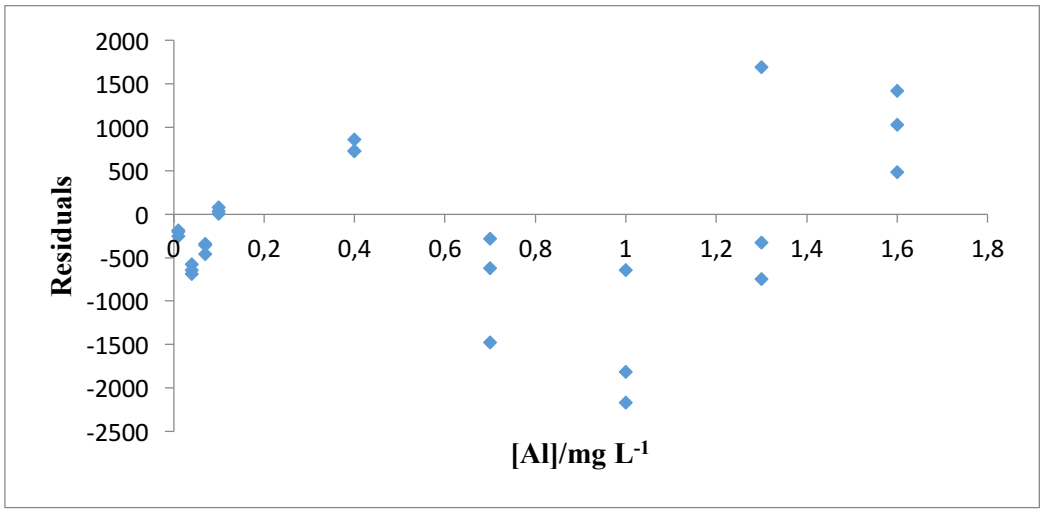


Figure H2: Residual plot for the calibration graph of aluminium.

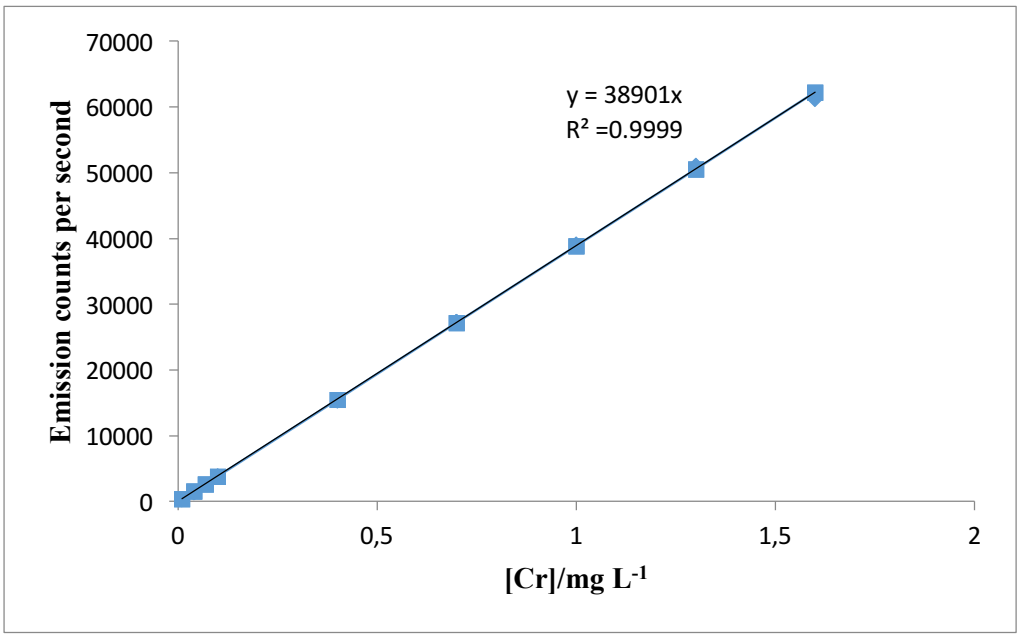


Figure H3: Calibration graph for the determination of chromium at 267.716 nm.

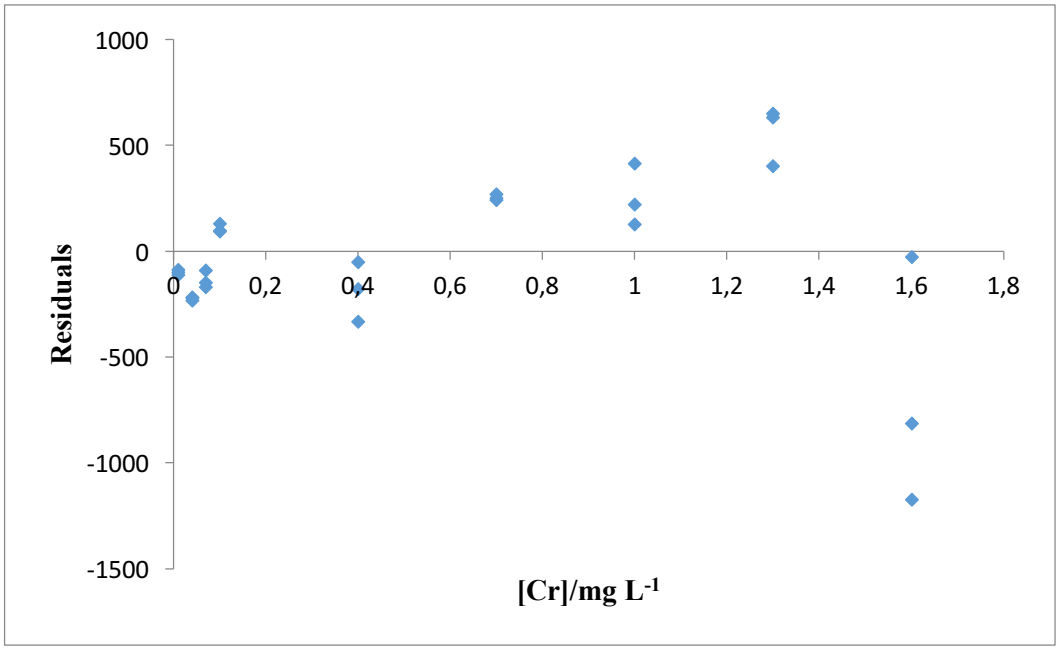


Figure H4: Residual plot for the calibration graph chromium.

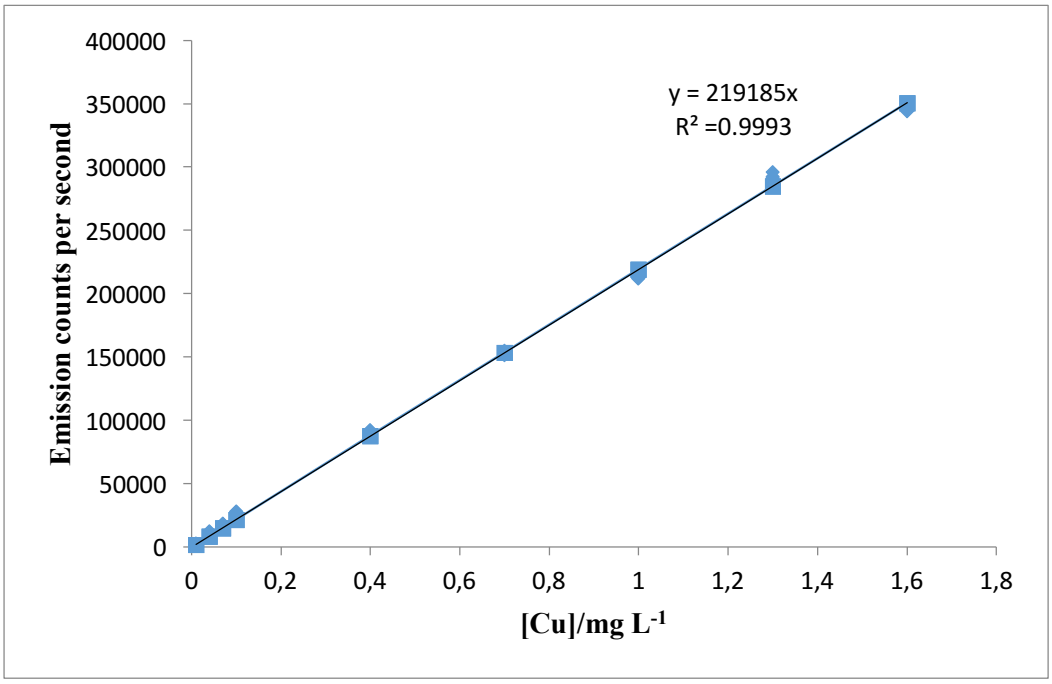


Figure H5: Calibration graph for the determination of copper at 324.752 nm.

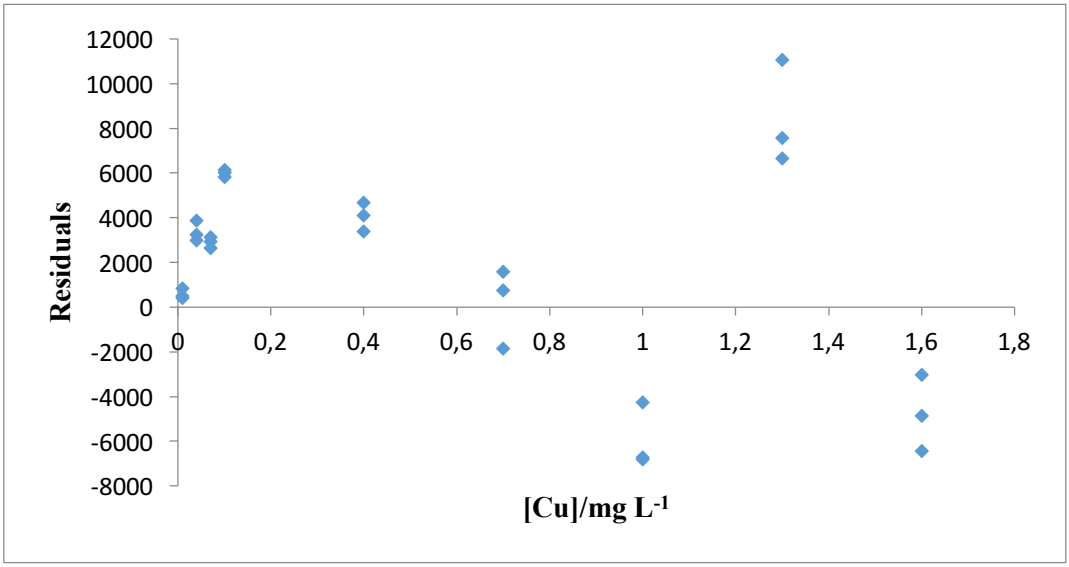


Figure H6: Residual plot for the calibration graph of copper.

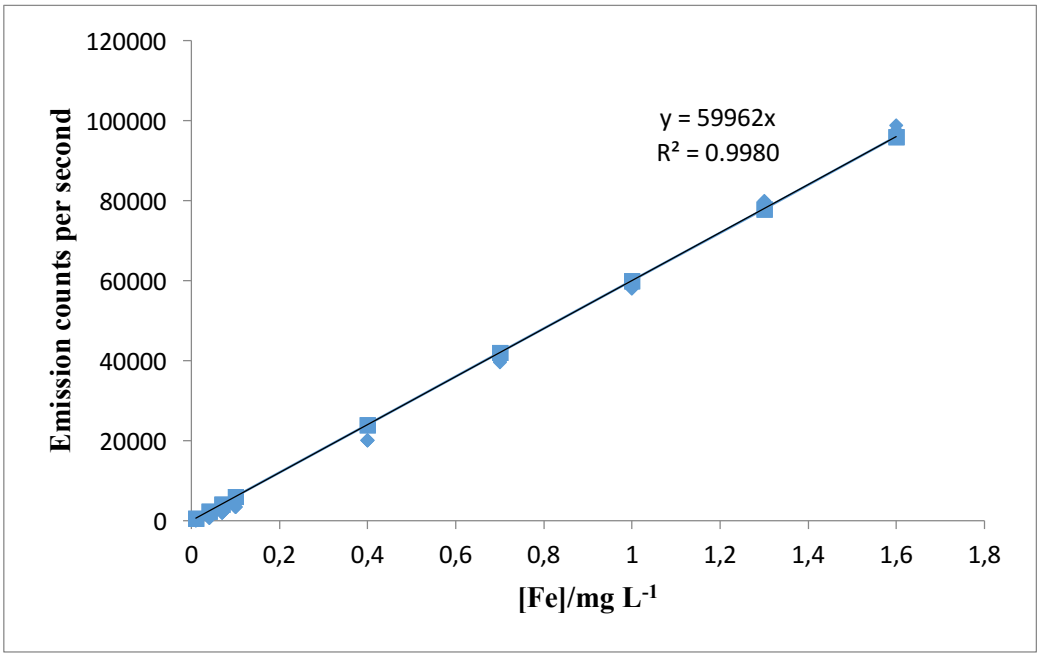


Figure H7: Calibration graph for the determination of iron at 259.939 nm.

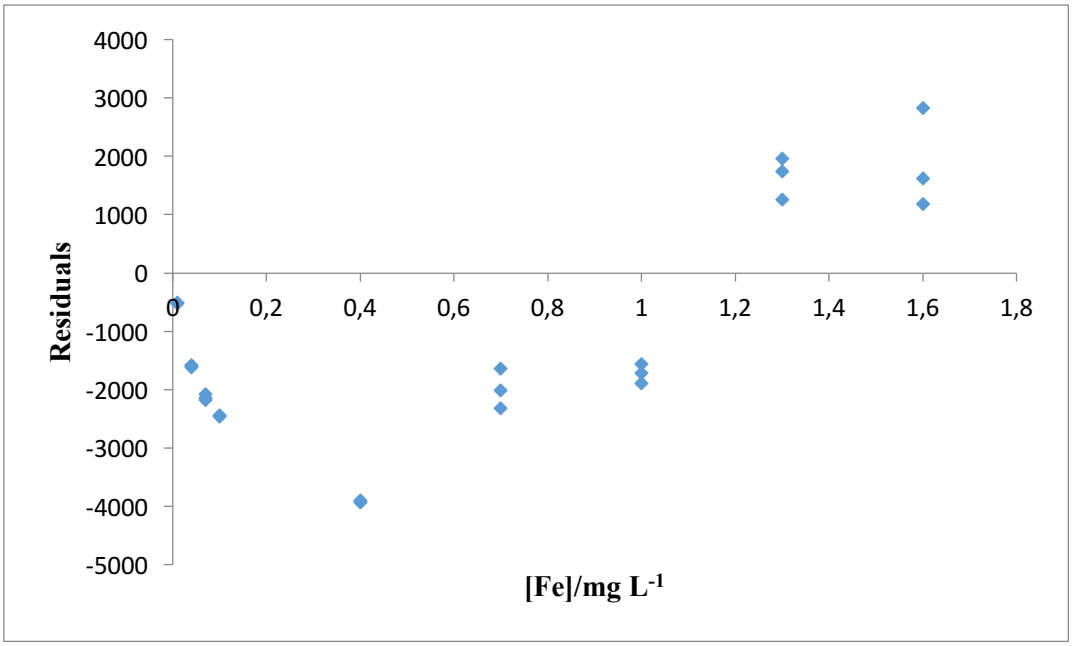


Figure H8: Residual plot for the calibration graph of iron.

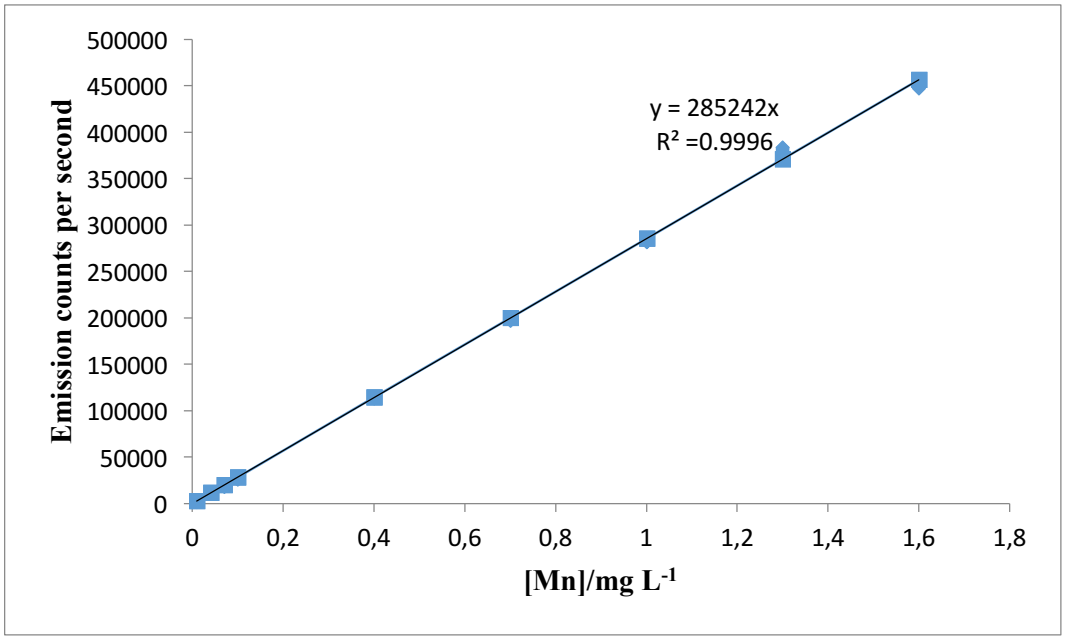


Figure H9: Calibration graph for the determination of manganese at 257.610 nm.

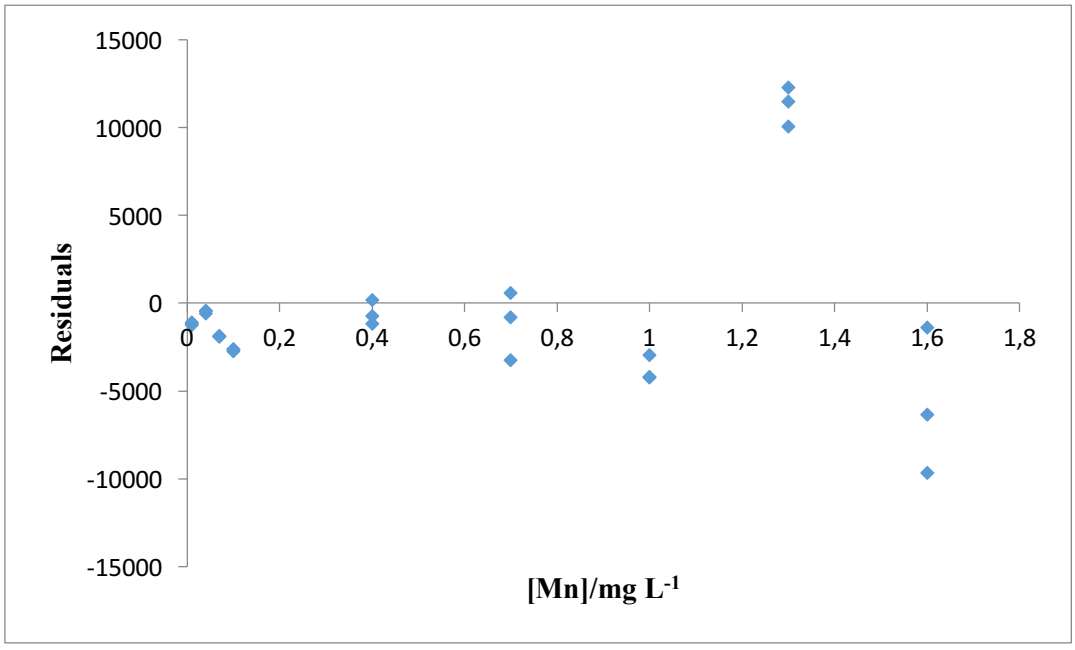


Figure H10: Residual plot for the calibration graph of manganese.

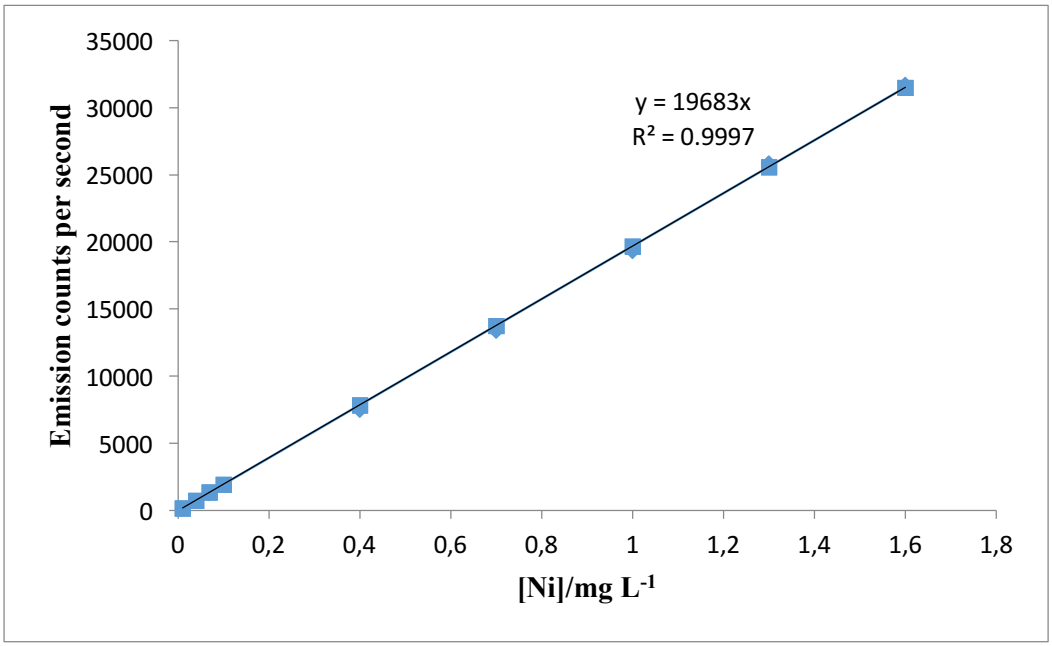


Figure H11: Calibration graph for the determination of nickel at 231.604 nm.

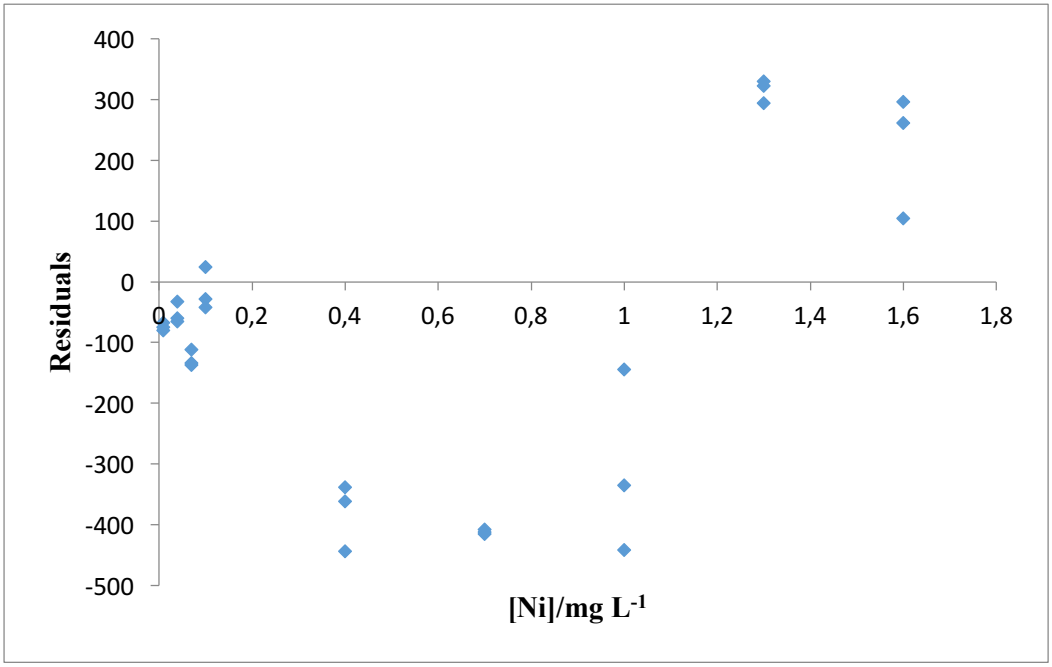


Figure H12: Residual plot for the calibration graph of nickel.

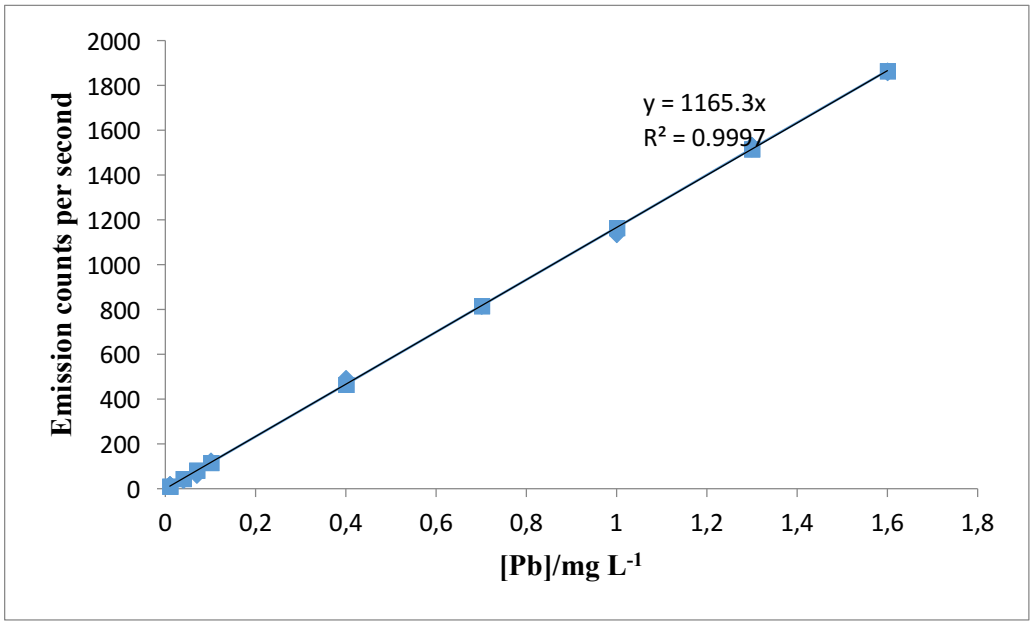


Figure H13: Calibration graph for the determination of lead at 217.000 nm.

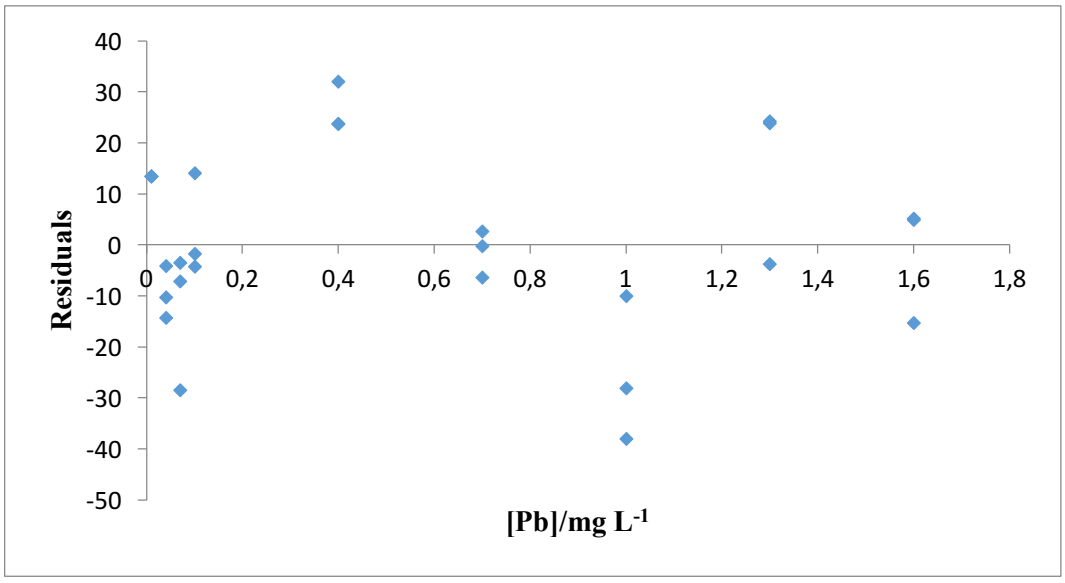


Figure H14: Residual plot for the calibration graph of lead.

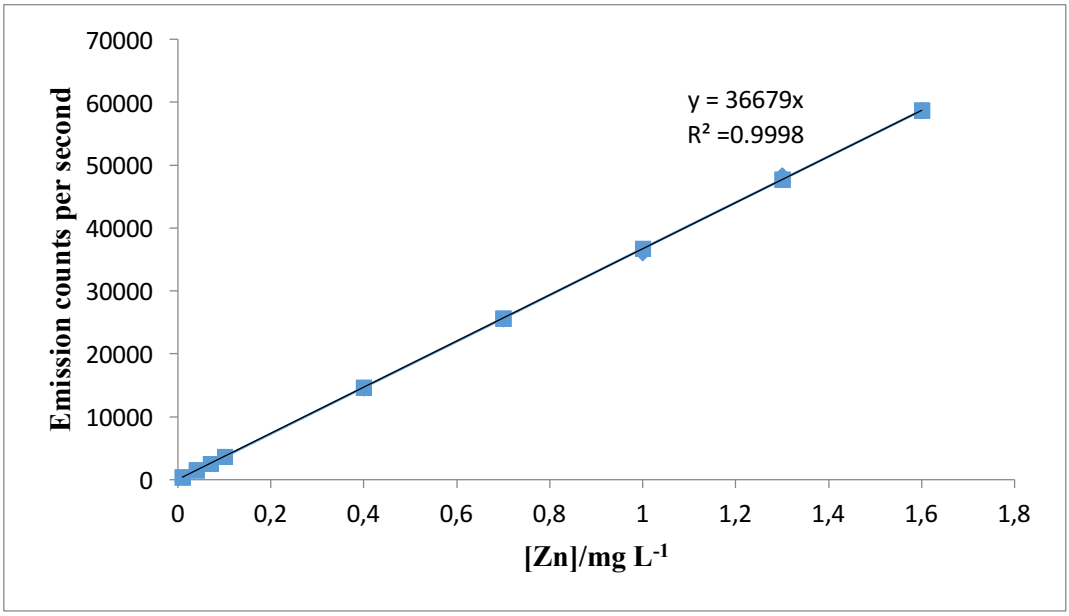


Figure H15: Calibration graph for the determination of zinc at 213.857 nm.

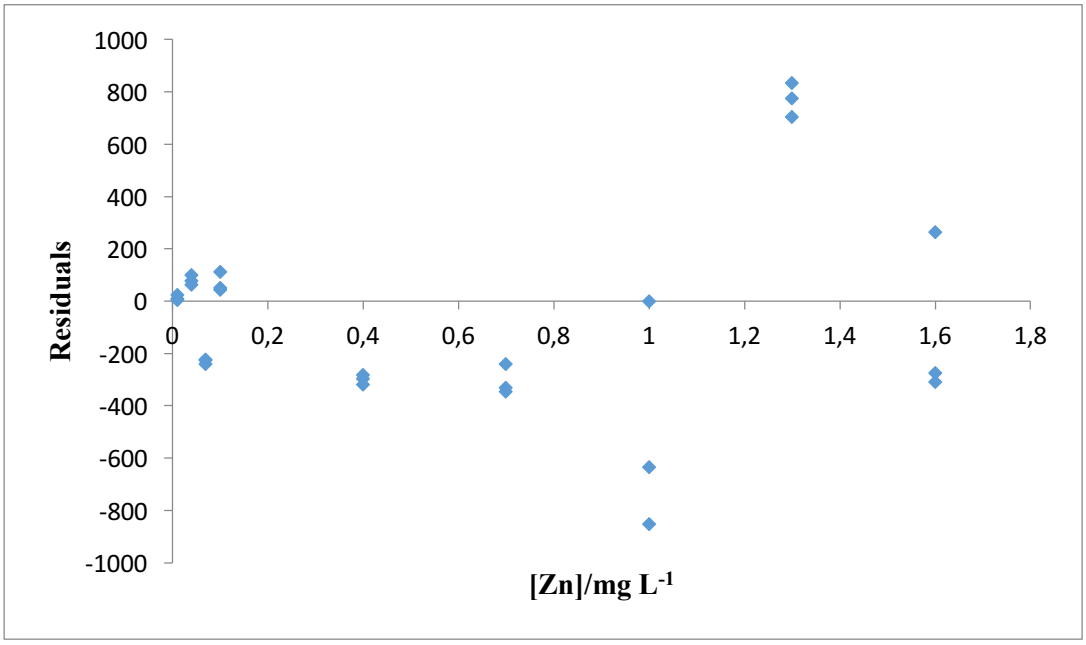


Figure H16: Residual plot for the calibration graph of zinc.

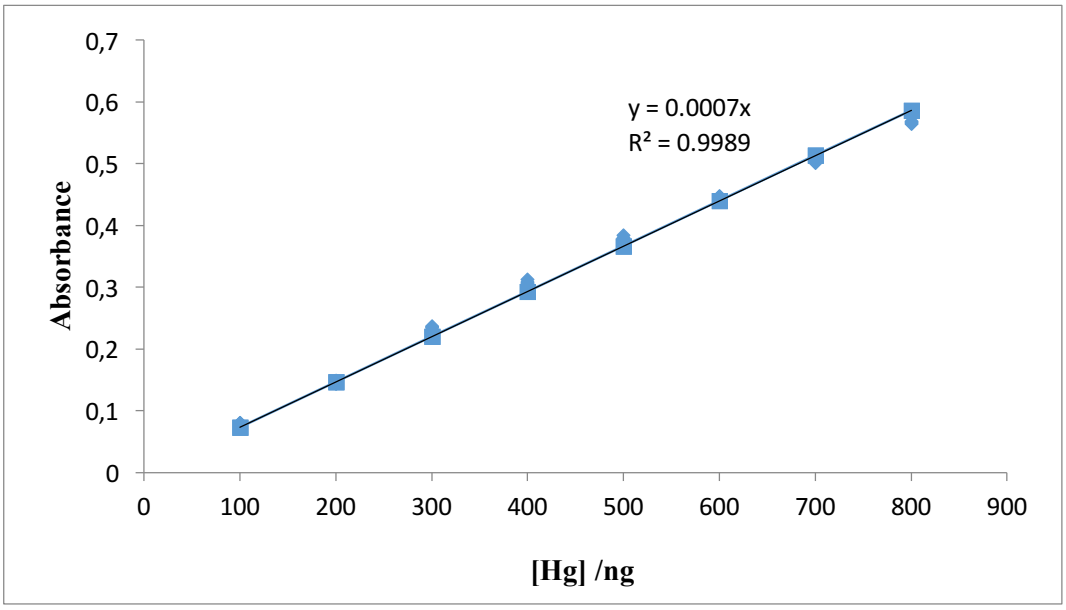


Figure H17: Calibration graph for the determination of mercury at 253.7 nm.

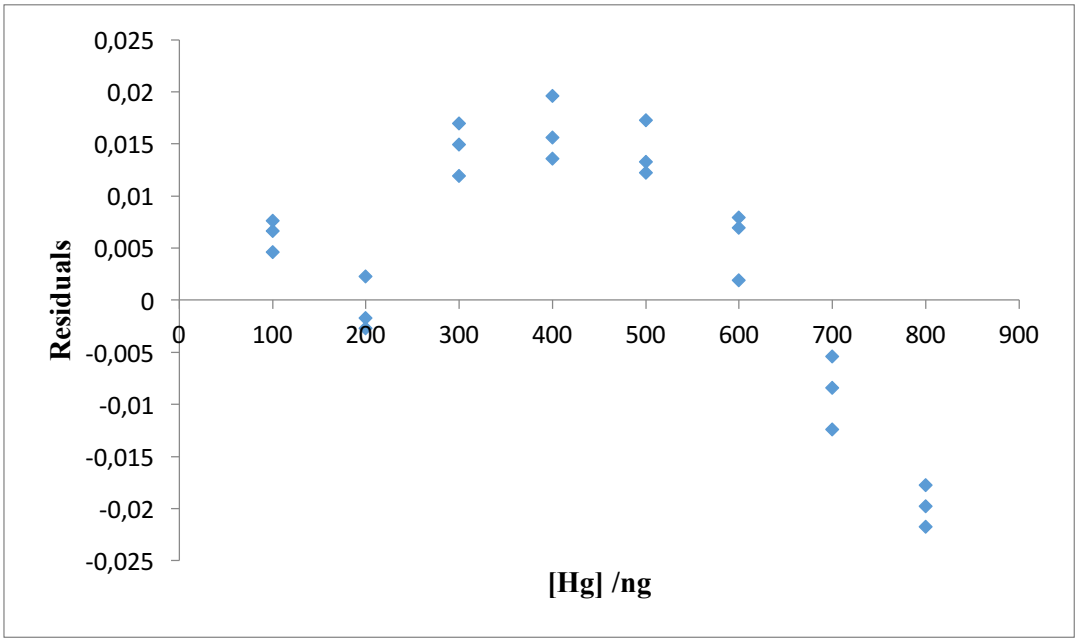


Figure H18: Residual plot for the calibration graph of mercury.