ANALYSIS OF SKIN PREPARATIONS

by

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ABSTRACT

Skin-lightening products are widely used in South Africa by the Indian and African communities. The main purpose of these products is to lighten the skin as well as to even out skin tone or treat pigmentation disorders such as freckles, melasma, pregnancy marks and age spots. These products are widely available as over-the-counter cosmetics and only a few are available by prescription only. The active ingredients in these products act on the tyrosinase enzyme to prevent melanin formation. Different skin-lightening agents (SLA) have different effects on the skin; some are harmful to the skin whereas some can be used without detrimental effects.

In some countries, those SLA which are harmful to the skin have either been banned or had limits imposed on the amounts which can be used. These include compounds containing mercury, glucocorticoids and hydroquinone. An alarming number of women present themselves at dermatology clinics with permanently damaged skin as a result of the use of over-the-counter preparations and products sold by street vendors. In this study a number of preparations bought in the market in Durban were investigated.

Reversed-phase high performance liquid chromatography (HPLC) with both ultraviolet and fluorescence detection was used. Phenolic and steroid standards were used to qualitatively identify the active ingredients. A method was developed for the extraction of SLA in the creams and also for their quantification by means of HPLC. A total number of 35 skin-lightening preparations were qualitatively analysed, and it was found that 11 samples had skin-lightening agents based on the standards that were used.

From the samples analysed it was found that one sample contained phenol, five samples contained arbutin, and three samples contained benzoquinone and hydroquinone, whereas two samples were found to contain niacinamide. The samples containing hydroquinone were found to also contain benzoquinone because the hydroquinone hydrolysed into benzoquinone. The benzoquinone concentrations could not be quantified as benzoquinone was not fully resolved at a detection wavelength of 240 nm.

The concentration of phenol found in sample 1 was 0.655 ± 0.026% (m/m). The amounts of arbutin found in the samples (5, 6, 8, 9, and 16) were found to be between 0.291-2.498% (m/m). The amount of niacinamide was found to be 0.751 ± 0.025 and 1.049 ± 0.225% (m/m) for sample 27 and 26 respectively. The concentration of hydroquinone was found to be, 1.255 ± 0.070, 2.224 ± 0.019 and 1.853 ± 0.029% (m/m) for sample 22, 23 and 24 respectively. The skin-lightening agents, phenol and hydroquinone, are banned in South Africa therefore those samples containing this SLAs are illegal. Limits for niacinamide have not been determined and arbutin was within the limits. The %RSD of the samples was between 0.009-8.900% which is acceptable. This shows that the method can be reproducible for the different samples with a good precision.

A method for gas chromatography coupled with a mass detector (GC-MS) was developed. The method was used to analyse phenolic standards which were first derivatised by using N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA). The method was able to separate a mixture containing resorcinol, hydroquinone, kojic acid and arbutin. The method failed to detect the SLAs in the skin-lightening products. GC-MS failed to confirm the presence of phenolic SLAs detected with HPLC.

The second part of the research involved an investigation of the clays which are used by Xhosa and Zulu women as sunscreens and also for skin-lightening purposes. Two types of clays were analysed: a red and a white coloured clay. The functions of clays are determined by their different physical properties. The compositions of the clays were determined by means of powder x-ray diffraction (XRD) and x-ray florescence (XRF). XRD gave two different diffactograms. XRF gave the major oxides in the clays. The clays had the same oxides but in different amounts. The red clay contained more iron(III) oxide, while the white clay contained much less but had a higher amount of quartz. The results from XRD were in agreement with those from XRF. It was found that the clays were not only made of clay minerals, but also contained impurities. They contained a significant amount of titanium dioxide which is used in sunscreens. The clays were identified as kaolinite (white clay) and iron stained kaolinite (red clay). Further confirmations of the composition of the clays were obtained by using thermogravimatric analysis (TGA), infrared spectroscopy (IR) and

transmission electron microscopy (TEM). The sun protection factor of these clays was low (approximately 4) but they offer a cheap alternative to the more expensive products of sun protection.

PREFACE

The experimental work described in this dissertation was carried out in the School of Chemistry and Physics, University of KwaZulu-Natal, Durban 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, Funanani Thelma Nevondo, 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

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5. This thesis 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.

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DECLARATION 2 - PUBLICATIONS

DETAILS OF CONTRIBUTION TO PUBLICATIONS that form part and/or include research presented in this thesis (include publications in preparation, submitted, *in press* and published and give details of the contributions of each author to the experimental work and writing of each publication)

Publication 1

Title: Chemical analysis and *in vitro* UV-protection characteristics of clays traditionally used for sun protection in South Africa

Authors: Ncoza C. Dlova, Funanani T. Nevondo, Elizabeth M. Mwangi, Beverley Summers, Joyce Tsoka-Gwegweni, Bice S. Martincigh and Dulcie A. Mulholland

Journal: Photodermatology, Photoimmunology & Photomedicine 2013, 29: 164-169

Contribution: I performed the analyses and interpreted the results.

Publication 2

A manuscript on the analysis of skin-lightening agents in skin-lightening creams available in Durban is being prepared for publication.

Poster presentation

Authors: Funanani.T Nevondo, Bice S. Martincigh and Ncoza C. Dlova

Title: An investigation of skin-lightening preparations

Conference: 4th SEANAC Conference, Maputo, Mozambique, 8-11 July 2012

A poster was presented at the College of Agriculture, Engineering, and Science Postgraduate Research Day, 29 October 2012 at UKZN PMB campus.

Signed:…….

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To my family who continually support me when I want to achieve my dreams, I say thank you. It is great to know you allow me to reach as many stars as I want. Edzani: thank you for all your worrying and support. It's been good having you close during this time. To my friends who never ceased to pray for me and never failed to encourage thank you. Buyisile, Mothusi and Nteseng your willingness to always avail yourself to helping me was truly love in action. My family in His People Church Durban, you have grown me into a leader and a strong woman.

"Mme anga Vho- Edzisani Nevondo, zwothe zwine vha nnyitela ndi a zwi vhona. Ndi a livhuwa vho ntutuwedza uri ndi fhedze musi ndo latela thavhula. Lufuno lwavho ndi luhulu. Ahuna munwe mme mubebi ane anga vhone."

And to my Lord Jesus Christ, it is through your grace I am here and only through you this was possible.

"[9] But he said to me, "My grace is sufficient for you, for my power is made perfect in weakness." Therefore I will boast all the more gladly of my weaknesses, so that the power of Christ may rest upon me. [10] For the sake of Christ, then, I am content with weaknesses, insults, hardships, persecutions, and calamities. For when I am weak, then I am strong." 2nd Corinthians 12: 9- 10 (ESV)

Thank you to UKZN for the fee remission and NRF for sponsoring me.

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Chapter 1

Introduction

In life people want to look their best. In particular, women want to appear beautiful and this has led to the growth of the cosmetic industry and it continues to grow each year. The beauty industry is composed mostly of cosmetics and skin care products.

Westerners consider a tanned skin beautiful [1] whereas for African women beauty is associated with light skin. When a women is dark skinned, society has made them believe that they are ugly and need to be light to be beautiful. This has led to an obsession with beauty, more especially with women all over the world and has made the market for skin-lightening creams big and profitable. The production and marketing of skin-lightening products (SLPs) has become a multi-billion-dollar global industry [2]. Women in Asia are deeply influenced by the concept that a lighter complexion has the power to hide a number of faults [3]. In South Africa the concept of "Yellow bones", which refers to very light skinned ladies, has been making the rounds as men now prefer those ladies compared to the dark skinned ones. A women's worth is judged based on the way she looks, and skin colour is used to judge the attractiveness of women [2]. Khessal is the process of voluntary depigmentation which is widespread in sub-Saharan Africa [4].

Companies spend a lot of money in research and development so as to find ways to lighten the skin that are not harmful in the long run. A lot of money is also used in advertising and marketing of skin-lightening creams (SLCs) [2]. This industry has also given way for some smugglers, transitional migrants and petty traders to take the opportunity to illegally produce, transport and sell unregulated lightening products [2].

1.1 Skin-lightening creams and their uses

Skin-lightening creams are creams made with the purpose of lightening the skin. They usually contain an active ingredient that does this. These are called skin-

lightening agents (SLAs). Skin-lightening agents have different mechanisms of action to lighten skin depending upon what class of compound they are.

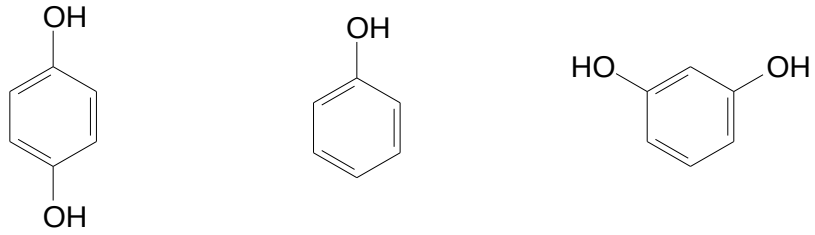
People use these creams because they have been led to believe that light skin is more beautiful than dark skin, so obsession with beauty is their main motivation. Other reasons for using skin-lightening agents include to even out skin tone and to treat pigmentation disorders such as freckles, melasma, pregnancy marks and age spots [5]. Such pigmentary disorders have an impact on people's quality of life [6]. Some skin-lightening creams claim to have oil catchers and as a result some women use them to prevent their face from being shiny throughout the whole day. Skin-lightening creams are commonly used by the sub-Saharan African [7] and Asian populations. People continue applying the creams so as to ensure their skin does not go back to the previous state [8], and therefore there is a practice of long-term use.

1.2 Skin lightening agents

Skin-lightening agents (SLAs) are compounds which are used to treat skin pigmentation disorders like hyperpigmentation and freckles. Brown spots and skin blemishes occur as a negative result of ultraviolet (UV) radiation and therefore make the production of skin-lightening agents important to help reduce such skin problems [5]. SLAs are classified into different classes based on the type of compounds they are made of.

1.2.1 Phenolics

Phenolics are compounds which have a phenol group in them (Figure 0.1) and are used as skin-lightening agents. Compounds like phenol, hydroquinone and resorcinol are a few examples that have been used in skin-lightening creams. Hydroquinone, for example, has been proven to be successful as a skin-lightening agent in the treatment of post-inflammatory hyperpigmentation, melasma and other disorders of hyperpigmentation.



Hydroquinone Phenol Resorcinol

Figure 0.1 Examples of some phenolics used as SLAs: hydroquinone, phenol and resorcinol.

Hydroquinone is the gold standard for skin-lightening and it is the most frequently prescribed in the world [9]. It has been widely used for the treatment of melasma, inflammatory hyperpigmentation and other hyperpigmentation disorders [10]. Depigmentation of the skin is usually observed within 4 to 6 weeks of using products containing hydroquinone as the only SLA in the product [10]. It can be found in products in combination with tretinoin, glycolic acid, vitamin C, retinol and fluorinated steroids [10].

1.2.2 Steroids

Steroids are useful in treating some of skin disorders, such as eczema and psoriasis. They are found to be effective but their unmonitored use may lead to many problems. They are usually used in high dosages to treat such skin disorders and it is essential that doctors' orders are followed in terms of their use. The strongest topical steroids (Figure 0.2) used include betamethasone dipropionate, clobetasol propionate and fluocinonide [8]. Betamethasone, dexamethasone, hydrocortisone, fluocinolone, triamcinolone and other corticosteroids are used in combination with other skin-lightening agents to decrease their side-effects or enhance their activity [11].

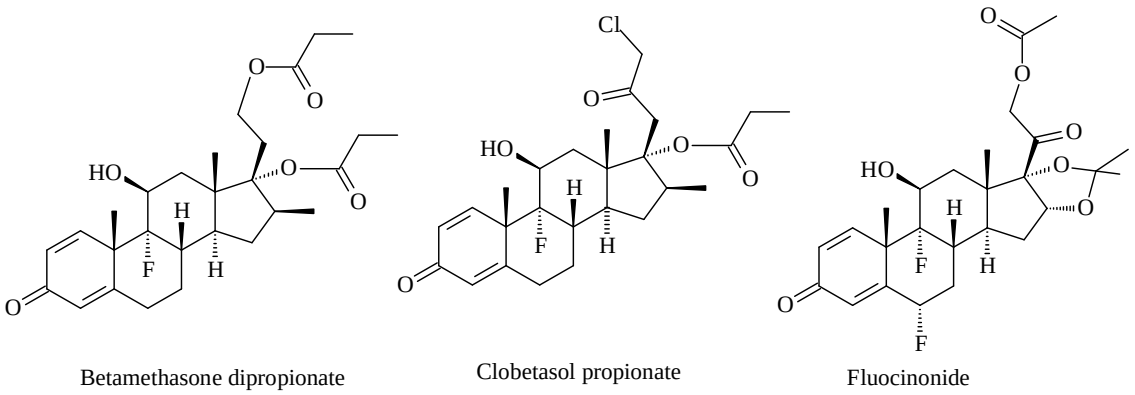


Figure 0.2 Structures of some of the strongest topical steroids used in the SLPs market: betamethasone, clobetasol propionate and fluocinonide.

1.2.3 Plant extracts

Plant extracts are used in skin-lightening creams as the industry attempts to move towards a more natural solution. There are contrary effects associated with the use of artificial chemicals and this has made the move towards the development of natural ingredients in skin care products [12]. These compounds are usually found in nature. Kojic acid and arbutin (Figure 0.3) are some of the compounds that have been found in plant extracts and have the ability to lighten skin. Kojic acid is derived from fungi species whereas arbutin can be derived from the bearberry plant, certain herbs and pear trees [10]. Melanin formation inhibitors, which are more effective than hydroquinone, have been found to be these plant extracts which do not exhibit cytotoxicity or mutagenicity of melanocytes [13]. These compounds and their derivatives are currently used in commercial products.

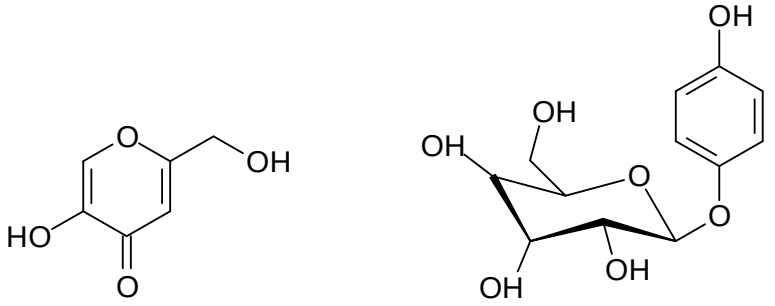


Figure 0.3 Some of the SLAs found in plant extracts used in SLP: kojic acid and arbutin.

Some of the natural SLAs include aloesin, azelaic acid, glycolic acid (from sugar cane), licorice extract and soy. Aloesin is a natural derivative of aloe vera [14] which is still an experimental product and is not available clinically [10]. Aloesin has a unique ability as it acts as a competitative inhibitor of Dopa oxidation and as a noncompetitor to tyrosine hydroxylase activity [10, 14].

Azealic acid is a naturally occurring dicarboxylic acid derived from *pityrosporum ovale* [10]. Azealic acid has a great effect on heavily pigmented melanocytes but has a minimal effect on normal pigments [10]. It can be used for extended periods as it is well tolerated by the skin [10, 11]. It is available by prescription in the strength of 15 to 20 % in the United States [10].

Glabridin is an active compound in licorice extract and it is used in concentrations of 10 - 40 % in SLCs [10]. It has fast action as depigmentation results can be seen within a week of use [10].

Natural soyabeans contain Bowman-Birk inhibitor and soy-bean trypsin inhibitor, two serine protease inhibitors that interfere with the protease-activated receptor 2 path way [10]. It is safe and effective [10]. Unpasteurized soy milk needs to be used for it to be effective [10]. It is available in the market in products that improve mottled hyperpigmentation and solar lentigines [10].

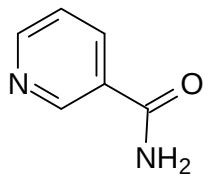
There are botanical extracts which contain a combination of two or more classes of compounds that achieve skin-lightening by working synergistically [15]. Botanicals are from nature, and this makes then more acceptable to people [15]. Botanical extracts could even be from indigenous knowledge which is passed down generations. This has led to products being labelled exotic because they have certain botanical extracts [15]. It should be noted, however, that not all natural extracts are stable and can be compatible in SLP formulations [15].

1.2.4 Vitamins

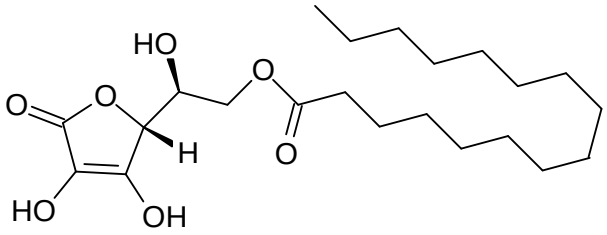
The research into skin care products that are more effective has resulted in products that will not only improve the appearance of the skin, but the health of the skin as well [16]. This has resulted in research into the incorporation of vitamins and

antioxidants in cosmetics. There are some vitamins which are used as SLAs in cosmetics creams. Niacinamide, a form of vitamin B3 (Figure 0.4), is among one of the most used SLAs in creams. It has been shown to improve acne due to its anti-inflammatory properties[16]. It has also been suggested that it can possibly also have some photoprotective effects due to its antioxidant activity [16].

Vitamin C, which is very important due to its ability to quench uv-induced free radicals directly, is also considered as an antiaging ingredient [16]. In cosmetics it is found in three forms. It can be found as ascorbyl palmitate (Figure 0.4) which is considered a lipophilic whitening agent [1, 16, 17]. It is also found as magnesium ascorbyl phosphate and L-ascorbic acid [16, 17] which are hydrophilic whitening agents [1]. These compounds are used in the chemical peeling process, which entails their application as a therapeutic agent for damaged facial skin arising from acne, melasma, and common warts and so on [1]. This results in the skin becoming lighter and therefore the chemo-therapeutic agent is also called a whitening agent [1].



Niacinamide (Vitamin B_3)



Ascorbyl palmitate

Figure 0.4 Structures of niacinamide and ascorbyl palmitate some of the vitamins used as SLAs.

1.2.5 Combination of SLA therapy

Skin-lightening agents need to be effective and also have little to no side-effects [18]. The major problem encountered with the use of one SLA is that they fail to meet the requirements of being a good SLA. This has led to the development of combinations of SLAs which produce a synergistic hypopigmenting effect [18]. The combinations of SLAs have been determined by clinical trials. When the use of hydroquinone is alternated with any plant extract, combined with steroids or in sequential applications, the side effects caused by hydroquinone are minimised [18].

Hydroquinone 5%, tretinoin 0.1% and dexamethasone 0.1% (Kligman's formula) is the most commonly used combination in treatments [18]. Dexamethasone decreases the side-effects of hydroquinone [18]. Tretinoin reduces the side-effects caused by using corticosteroids and improves the penetration of SLAs into the stratum corneum [18]. There are many more combinations that are have been developed via clinical trials [18].

1.2.6 Mercury

Mercury has been used in SLPs. Mercury exists in the inorganic, elemental and organic form. Mercury is poisonous and can be absorbed as vapour or through the skin and gastrointestinal tract as finely dispersed granules which can be excreted through the kidneys and colon [8]. During the first decade of the 20th century, mercurious chloride, oxide and ammoniated mercury were introduced as active ingredients in toiletries and cosmetics [8]. Eventually, they became popular skin-lighteners [8].

1.3 Mechanism of skin lighteners

Pigmentation of the skin is caused by the amount, quality and distribution of melanin, which is the naturally occurring pigment in the skin [4, 19]. Melanin is synthesised by large dendric cells known as melanocytes which are located at the epidermal-dermal junction [20]. Melanin production is the major source of skin colour and plays an important role in protecting human skin from the harmful effects of UV radiation from the sun [3]. Solar UV radiation, toxic drugs, hormones and chemicals are some of the environmental factors than can increase melanin production [20]. There are also genetic factors that affect its production. The major source for skin pigmentation is UV radiation.

Melanin in the skin is catalysed by solar UV radiation. It causes the cytokine growth factors and other inflammatory factors to be released by fibroblasts to stimulate melanin production [3]. The melanocytes increase the production of intracellular nitric oxide (NO), which triggers signal transduction cascades to initiate melanogenesis through the tyrosinase enzyme, a copper-containing oxidase [3]. Tyrosinase in melanocytes is a key enzyme in the synthesis of melanin pigments (Figure 0.5) [5, 19, 20]. Melanogenesis is the whole process which leads to the formation of dark macromolecular pigments (Figure 0.5), i.e. melanin [21].

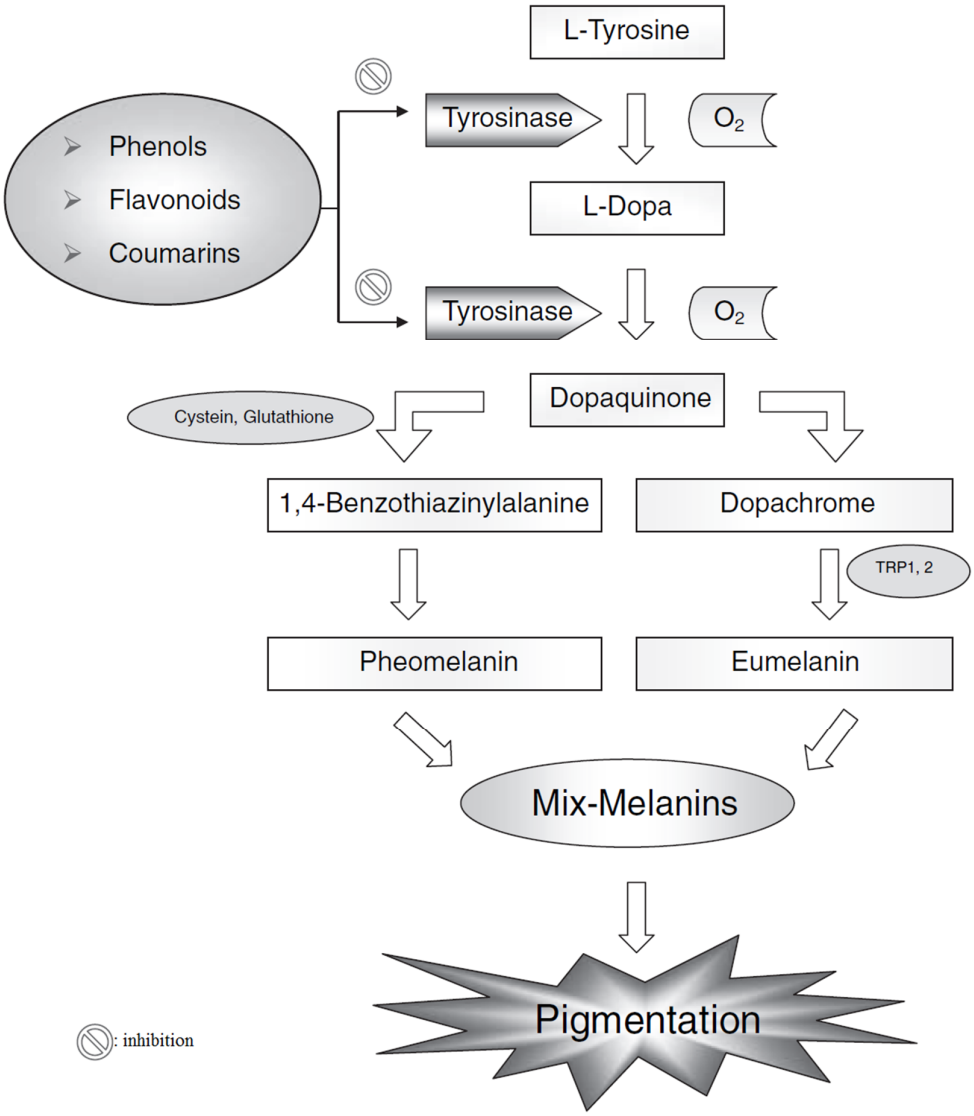


Figure 0.5 Scheme of melanogenesis [3].

Although melanin mainly has a photoprotective function in human skin, the accumulation of an abnormal amount of it in different specific parts of the skin resulting in more pigmented patches might become an aesthetic problem and cause a dermatological issue [3, 19]. Examples of conditions caused by over production of melanin include, but are not limited to, tanning, melasma, chloasma, freckles, age spots or uneven skin tone [4, 20]. It is due to this factor that skin-lightening agents where developed and are now used throughout the world. The function of the SLAs is to prevent and block the accumulation of melanin [19].

Although there are a lot of mechanistic points that can be targeted, tyrosinase inhibition is the most common approach to achieve skin whitening. The development of successful whitening skin care products depends on the use of effective whitening or depigmenting ingredients that inhibit melanin formation in melanocytes [20] but do not stop the production of melanin indefinitely [19].

Hydroquinone inhibits melanin production by acting as a substrate for tyrosinase and inhibiting sulfhydryl groups [8, 19, 22]. The mode of action of mercury includes the inactivation of the sulfhydryl enzymes called mercaptans [8]. Mercaptans are able to capture mercurial ions which then compete and replace copper in the tyrosinase molecule thereby deactivating the molecule [8]. This process interrupts the production of melanin [8].

Corticosteroids inhibit endogenous steroid production and thus decrease the propiocortin hormone thereby lightening the skin [8].

Arbutin works as a skin-lightening agent by inhibiting the conversion of tyrosine to melanin [23]. It hinders the oxidation of L-dopa to L-dopaquinone by competing with L-dopa for the receptor site on tyrosinase [24].

Kojic acid's activity is attributed to the ability to bind to copper and thereby reducing tyrosinase activity [22].

1.4 Regulations governing the use of skin-lightening creams

There are regulations which have been set in terms of what skin-lightening agents are allowed and in what quantity they are allowed. The National Food and Drug Agency of Nigeria has banned all bleaching agents in cosmetics and toiletries due to the long-term effects they have on the skin and also the noncompliance in terms of labelling requirements [8].

1.4.1 Hydroquinone

Hydroquinone has set limitations all over the world. A set limit of 2% for over-the-counter cosmetics has been set in the Republic of South Africa [25] and the United States of America, whereas in the European Union it has been banned from all over-the-

counter products and can only be found in prescription products [4]. It is stated that hydroquinone products have been banned in Nigeria and South Africa [2].

1.4.2 Phenol

Phenol is used as a skin disinfect in a solution of 3-5% [4]. Phenol is banned in South Africa.

1.4.3 Arbutin

Arbutin can be classified as a plant extract as it can be isolated from the bearberry plant [13, 23]. It is a glycoside derivate of hydroquinone [5, 13] and therefore has a chemical structure that is similar to that of hydroquinone. The allowable amount of arbutin is 7% [23].

1.5 Side-effects of prolonged usage of skin-lightening creams

Hydroquinone is the most commonly used skin-lightening agent which is effective [4], however, it is very unstable as it is oxidised rapidly [18]. Prolonged usage of hydroquinone may cause exogenous ochronosis which is in fact further pigmentation of the skin [2, 4, 8, 10, 13, 18]. People that use the hydroquinone products and live in sunny climates are more at risk of getting exogenous ochronosis [10]. Other reported dermatological complications include dermatitis, cataracts, pigmented colloid milia, sclera disorders, nail pigmentation and patch depigmentation [4, 8, 13, 18]. It is due to these side-effects that there are regulations set. When such complications arise, people tend to use the SLAs more in the hope that the complications will clear out [8]. The most common side-effect of using hydroquinone are skin irritations and contact dermatitis, which can be treated with topical steroids [10]. It is for this reason that some products containing hydroquinone as an active ingredient also have topical steroids so as to minimise any side-effects from using the product.

Phenol has a minor degree of whitening effect [4]. Prolonged exposure greatly irritates and harms the skin [4].

Arbutin in acidic conditions hydrolyses to D-glucose and hydroquinone and therefore presents the same health effects of those of hydroquinone which are not desired [24].

Topical steroids when misused as skin-lightening agents have secondary effects which include skin thinning and also increased infection rates [26]. This is due to the fact that steroids only relieve inflammation on skin but do not kill bacteria; in fact the breeding of bacteria is actually promoted [26]. Skin thinning results in the swelling of fine blood vessels (telangiectasia), temporary loss of pigment in the areas of treated skin, perioral dermatitis (rash around the mouth), permanent stretch marks (striae) and enlarged blood vessels (telangiectasia) [26, 27]. The side-effects of steroids are dependent on the nature of the skin problem, the site treated, the strength of the steroid and the length of application [26, 27]. The application of potent to moderately potent glucocorticosteroid preparations to the neck, facial skin and scrotum or vulva for a period of one month can result in steroid addiction syndrome [8]. Severe burning occurs when steroids are no longer applied to such skin tissues which can only be stopped by further applications of steroids [8]. This is because those skin tissues are addicted to the steroids [8].

Mercury is highly toxic. The use of mercury as a skin-lightening agent can lead to its accumulation in the body and this can cause neurological damage and kidney disease [2].

1.6 Methods of analysis of skin-lightening agents

Skin-lightening agents such as mercury and hydroquinone have been analysed for many years since their side-effects where first recorded. An increase in new skin-lightening creams and agents has led to a demand of constantly new procedures to quantify such compounds. Methods which have been used over the years for analysis of skin-lightening agents include, gas chromatography (GC), spectrophotometry, thin layer chromatography (TLC), capillary electrophoresis (CE), capillary electrochromatography (CEC), micellar electrokinetic chromatography (MEKC), electrochemistry, differential pulse voltammetry (DPV) and high performance liquid chromatography (HPLC) [28-

30]. Mass spectrometry is also used for the analysis of skin-lightening agents coupled with either HPLC or GC [29].

Chisvert *et al.* proposed a method to determine the quantity of kojic acid, azelaic acid, arbutin, resorcinol and hydroquinone in skin-whitening agents in cosmetics using GCMS [31]. The target analytes were derivatised due to their low volatility. It was reported that this was the first official analytical method in literature except for those proposed by the EU commission and the United States Pharmacopeia [31].

Gaudiano *et al.* have reported on an HPLC method for the determination of six different glucocorticoids simultaneously and the method was used to identify counterfeit products from Europe [28]. HPLC is one of the most used instrumentation methods in the analysis of SLAs. The method is important as it allows for the simultaneous analysis of six glucocorticoids.

A flow-injection method has been reported for the analysis of arbutin in SLPs and also extracts [5]. The method has been reported as being fast, accurate, sensitive and simple [5].

1.7 Extraction of skin-lightening agents

Before SLAs can be identified and quantified they need to be quantitatively extracted from the product by a suitable method. Vortex mixing of the samples in the mobile phase has been a common way to extract the SLAs in the creams [5, 12, 32]. Depending on the SLA of interest, samples would undergo filtering or centrifugation [32]. Vortex mixing has been coupled with ultrasound and heating in a water bath in order to assist with the extraction [28, 33]. Depending on the SLAs of interest, the sample will undergo some or all of the steps once or several times.

Samples are also homogenised in specific solvents and then ultra-sonication is used to assist in the extraction of the SLAs in the samples [17, 23]. Heating a dissolved sample in a water bath and then followed by centrifugation has also been used to extract SLAs [30]. All samples prepared for HPLC are filtered through a 0.45 µm syringe filter before injection into the HPLC.

1.8 HPLC methods of analysis for skin-lightening agents

The instrumental method chosen to separate, identify and quantify the SLAs in the selected products in this work was reversed phase HPLC. A number of methods have been previously reported.

Gaudiano *et al*. reported on an HPLC method where six glucocorticoids (methyl-prednisolone acetate, fluocinonide, dexamethasone, clobetasol propionate, betamethasone dipropionate and fluocinolone acetonide) were analysed [28]. The samples were extracted by using methanol as a solvent. Chromatographic separation was obtained by isocratic elution with a Symmetry C_{18} column (Waters Corporation, MA, USA) with a mobile phase of water/acetonitrile (50:50, v/v). The detection wavelength was 240 nm. The method was reported to be robust, linear over a wide concentration range and sufficiently accurate and precise [28]. The method can be used to simultaneously analyse for the presence of the six glucocorticoids in commercial products.

Huang *et al.* developed a method for the simultaneous determination of five whitening ingredients (magnesium ascorbyl phosphate, ascorbyl glucoside, kojic acid, arbutin and hydroquinone decomposed from arbutin in cosmetics) [23]. Samples were extracted with 0.05 M KH_2PO_4 buffer solution (pH 2.5) and analyzed with a Cosmosil 5 C_{18}-AR-II column [23]. A mixture of 0.05 M KH_2PO_4 buffer solution (pH 2.5) and methanol (99:1, v/v) was used as mobile phase [23]. The UV detector was set at 280 nm [23]. Recoveries of these five ingredients spiked into a sample ranged from 93.5% to 103.3% [23]. The relative standard deviations of average recoveries were less than 1.3% [23]. The limits of quantitation in cosmetics were 80.0, 20.0, 3.0, 15.0 and 10.0 µg/mL for magnesium ascorbylphosphate, ascorbyl glucoside, kojic acid, arbutin and hydroquinone, respectively [23]. The purpose of this study was to develop a rapid and simple quantitative method for the simultaneous determination of above five ingredients in cosmetic samples using HPLC with a photodiode array detector (PDA) [23].

Sotofattori *et al*. reported on an HPLC method for the simultaneous determination of magnesium ascorbyl phosphate, imidazolidinylurea, a mixture of methyl-, ethyl-, propyl-, butyl- parabens dissolved in phenoxyethanol, and ascorbyl palmitate, by using

a cyano-propyl column and a methanol gradient at 220 and 240 nm [33]. Linear regression analysis of the data demonstrated the efficiency of the method in terms of precision and accuracy [33]. They claimed that this was the first time in literature where the simultaneous HPLC determination of such ingredients is reported [33].

Balaguer *et al.* developed a method they called environmentally friendly for the determination of ascorbic acid (vitamin C) and its derivatives (ascorbyl phosphate (as magnesium or sodium salts) and ascorbyl palmitate, and ascorbyl glucoside) in skin-whitening cosmetics [17]. A C_{18} stationary phase and a mobile phase gradient of ethanol: 50 mM phosphate buffer at different pHs (containing 0.1 M NaCl) were used [17]. Detection was carried out with a UV/visible spectrometry detector set at different wavelengths [17]. The method was able to separate all analytes at good resolution [17].

Lin *et al.* used microdialysis sampling combined with HPLC for the determination of hydroquinone [34]. HPLC was conducted with a Hypersil Fluophase PFP column, the mobile phase was a solution of methanol (40%, v/v) in phosphate buffer (pH 5.50, 0.020 M) [34]. The proposed method has easy sample pretreatment, rapid isolation and it is said to have a lower organic solvent consumption as compared to other methods for analysis of hydroquinone in cosmetic samples [34].

García *et al.* reported on an HPLC and an ultraviolet derivative spectrophotometric (UVDS) method for the quantitative determination of hydroquinone in gels and creams containing this compound as a unique active ingredient [35]. The HPLC was carried out by using a mobile phase constituted of methanol and water (20:80, v/v), and detection was made at 289.0 nm [35]. A Lichrospher® 100 RP-C_{18} column (125 mm × 4 mm i.d., 5 µm particle size) (Merck®) was used [35]. This method was developed for samples which contain hydroquinone as the only active SLA since kojic acid and arbutin can cause interference in both methods [35].

Majmudar *et al.* developed a method for the determination of kojic acid in SLPs by using HPLC [20]. The chromatographic conditions consisted of a C_8, ODS-5, 150 × 4.5 mm column and a mobile phase composed of water:acetonitrile (20:80 v/v). The wavelength of detection was 254 nm [20]. This method was developed to evaluate the toxicity of SLAs and SLPs before they can be tested on humans [20].

Gagliardi *et al*. developed a method where a tandem thin-layer HPLC was used for the identification and determination of corticosteroids in cosmetic products [36]. The analytical column used was a 250 mm × 4.6 mm i.d stainless steel column packed with 5 µm endcapped Purospher RP-C_{18} (Merck, Darmstadt, Germany) [36]. Three eluting conditions were adopted: (A) isocratic elution with the mobile phase acetonitrile-water, in the ratio 60:40 (v/v); (B) gradient elution from acetonitrile –water in the ratio 25:75 (v/v) to 90% acetonitrile in 30 min, the final composition was maintained for 10 min before re-equilibrating the column; and (C) isocratic elution with acetonitrile [36]. The detection wavelength used was 239 nm [36]. There were 51 corticosteroids which were analysed [36]. The method has been applied to the analysis of 80 cosmetic products marketed within the European Union [36].

Reepmeyer *et al.* reported a method for the determination of clobetasol propionate. Chromatographic separation was obtained by using reversed-phase and normal-phase chromatography [37]. The analytical column used for reversed-phase chromatography was a Nova-Pak C_{18} column (150 × 3.9 mm, 4 µm particle size, Waters Associates) [37]. The mobile phase was acetonitrile-water (1:1) [37]. The analytical column used for normal-phase chromatography was a Chromegasphere SI 60 column (250×4.6 mm, 5 µm, E.S. Industries) [37]. The mobile phase was isopropanol-heptane (1:4) [37]. The signal was scanned from 200 to 350 nm in order to record the UV spectra and the chromatogram were monitored at 240 nm [37].

Lin *et al.* combined HPLC with online microdialysis sampling for the simultaneous determination of ascorbyl glucoside, kojic acid and niacinamide in skin-whitening cosmetics [38]. Separation was achieved on a 5 µm, 250 mm × 4.6 mm i.d. Hypersil Fluophase PFP column (Cheshire, England) and the analytes were detected through UV absorption at 254 nm [38]. The HPLC eluent was 40% (v/v) methanol in 0.020 M phosphate buffer [38]. The method was used to analyse six commercial cosmetic products. On-line microdialysis sampling was used because the authors claim the method gives a more simple sample pretreatment and a lower degree of sample contamination [38]. The method is also less time consuming [38].

1.9 Aim and objectives of this study

A large number of women have been visiting dermatologists with skin problems. A number of products containing skin-lightening agents have been banned by some African countries such as Kenya, Gambia and Burkina Faso [28]. Despite these bans, products containing hydroquinone, glucocorticoids and mercury soaps are still advertised and sold [28].

Regulations have been established for cosmetics, product evaluation, chemical analyses and safety testing to protect consumers [12]. Regulations require that the product ingredients be listed on the product label with the exception of fragrances and flavours [12, 33]. Some of the cosmetic products in the South African market list the ingredients but most of them do not state the quantity of the SLAs in the creams. The consistency of the contents of the products with those of the ingredients on the label is a concern as some of the creams give rise to adverse effects which are not consistent with the product label [12].

In this project, a method was developed for the analysis of skin-lightening agents in skin-lightening creams by using HPLC. This was achieved with the following specific objectives.

1.9.1 Specific objectives

- Various standards of SLAs were obtained.
- The standards were analysed by UV and fluorescence spectrophotometry so as to determine their UV absorbance wavelength and their excitation and emission wavelengths for florescence.
- Method development was performed by using the results obtained by UV and fluorescence spectrophotometry to detect the analytes of interest in HPLC.
- Once a suitable method was identified, the SLP samples were analysed to see if there was separation of the different components.
- Optimisation of the extraction time and solvent was evaluated.
- Qualitative analysis of the samples was done by HPLC and the various available standards.

- Calibration plots were obtained for the standards that were qualitatively found to be present in the samples.
- The samples were analysed in triplicate and the data was analysed to obtain the concentrations of the SLAs in the SLPs.
- GC was also attempted in order to verify the results obtained by HPLC.
- Phenolic compounds were derivatised with BSTFA to make the standards volatile.
- Qualitative analysis of the samples was done by using GC-MS.

The HPLC method was used to qualitatively identify the skin-lightening agents in the samples and thereafter quantify them provided their standards were available. The method was used to analyse 35 different skin-lightening creams purchased at the market in Durban, KwaZulu-Natal.

Public education has become important as cosmetics have a number of different chemicals in them which may impact negatively on the skin or be banned or restricted. This work is necessary so that women can know which skin-lightening creams are good for their skins and which ones possess harmful compounds that can be detrimental for their skin.

1.10 Dissertation overview

The following dissertation is composed of 2 sections. Section A covers the analysis of SLA in SLP, and this is from Chapter 1 to Chapter 4. Section B concerns the analysis of clays used by South African women on their faces as a sunscreen. This can be obtained from Chapter 5 to Chapter 8.

Section A

Chapter 1 provides an overview of what skin lightening agents are, why people use them and how they function on the skin. It includes regulations and side-effects of some commonly used SLAs in the market. Various methods which have been used in the analysis of SLAs are reported. Different HPLC methods reported by different authors are reviewed for the analysis of SLAs. The specific objectives and aims of the study are discussed.

Chapter 2 covers the experimental part of the HPLC and GC-MS work carried out. The theory of the instrumentation is covered as well as the experimental procedures carried out. This chapter also includes precautions to be taken when using HPLC, and derivatisation of the standards and samples in order to be able to analyse them by using GC-MS.

Chapter 3 presents the results and discussion of the analysis. It includes difficulties experienced during the research. It discusses the process of the method development. It was found that some of the SLPs contained SLAs which are banned in over-the-counter products and some had amounts that exceed the limits allowed in South Africa. The results obtained by GC-MS were not conclusive.

Chapter 4 is where the conclusion of the first section on the SLAs is drawn.

Section B

Chapter 5 gives a detailed overview about clays and their uses. It defines clays and what is required for a material to be called clay. It also covers the white and red clays used by South African women to protect their skin.

Chapter 6 outlines the experimental method and different principles of the instrumentation used.

Chapter 7 covers the results from the experimental and the discussion. The characteristics of the clays based on the obtained results are discussed.

Chapter 8 contains the conclusion for section B. However, it was noted that further studies should be done to assess the toxicity of the clays as this is not currently known.

The references are listed after Chapter 8 and Appendices A to E provide details of the chemicals used, the UV and fluorescence spectra of the standards as well as the HPLC and GC-MS chromatograms. The calibration graphs are also shown there.

Chapter 2

Experimental

This chapter describes the materials, equipment and methods used to identify skin-lightening agents in commercially available skin lightening creams and also their quantitative analysis.

2.1 Materials

The chemicals used are listed in Appendix A including their chemical grades and manufacturers information. All the reagents used were of analytical or HPLC reagent grade. Methanol, 2-propanol and THF were all HPLC grade solvents. Pyridine was analytical grade. Water was purified by a Millipore Milli-Q system.

The skin-lightening products were provided by Dr N Dlova of the University of KwaZulu-Natal Medical School. They were stored at room temperature in a dark cupboard before extraction. The extracted samples were stored in the refrigerator prior to the analysis. A list of all samples analysed can be found in Table 2.1 with product information and names of the manufactures.

2.2 Ultraviolet and visible (UV/vis) spectrophotometry

A number of skin-lightening agents have been analysed by means of HPLC, with a UV detector. This requires knowledge of the wavelength of maximum absorption for each of the possible analytes. By using these different wavelengths that the compounds absorb, one can differentiate them based on their UV spectrum and retention times with HPLC. The HPLC used in this project had a photodiode array (PDA) detector which made it possible to obtain the UV spectrum of each individual peak in the chromatogram. This made UV and HPLC to be compatible and of importance for this project.

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

Product Label	**Brand name**	**Country of manufacture**	**Company**	**Active ingredients declared on product label**	**Side effects or warnings given on packaging label**
1	BCO original skin repair cream	South Africa	Champion Cosmetics	-	-
2	Gentle magic lotion	-	-	-	-
3	Unknown	-	-	-	-
4	Beta cream	South Africa	Mthatha plaza & Triangle Pharmacies	-	Avoid sunlight exposure

5	Pure perfect parfait (6)	South Africa	Pure perfect cc	Kojic acid dipalmitate Arbutin	Apply broad spectrum sunscreen of at least SPF 15 while using product and a week after discontinued use.
6	Pure perfect parfait (7)	South Africa	Pure perfect	Kojic acid dipalmitate Arbutin	Apply broad spectrum sunscreen of at least SPF 15 while using product and a week after discontinued use.
7	Pure perfect parfait (8)	South Africa	Pure perfect	Kojic acid	-
8	Pure perfect parfait (24)	South Africa	Pure perfect	Kojic acid dipalmitate Arbutin	Apply broad spectrum sunscreen of at least SPF 15 while using product and a week after discontinued use.

9	Pure perfect parfait (25)	South Africa	Pure perfect	Kojic acid dipalmitate Arbutin	Apply broad spectrum sunscreen of at least SPF 15 while using product and a week after discontinued use.
10	Quick and clear blemish cream	South Africa	-	-	Avoid excessive exposure to sunlight while using the product.
11	Bioclear vanishing cream	South Africa	-	-	If no adverse effects occur continue to apply as directed.
12	Bioclear nourishing cream	South Africa	-	-	If no adverse effects occur continue to apply as directed.
13	Hypercreme plus Beauty toning cream	Switzerland	Hypercreme	-	Avoid contact with eyes. If irritation occurs, discontinue use.

14	Skin spor G.S	South Africa	Mthatha Pharmacy	-	-
15	Fair and white whitening cream	France	Labo Derma	-	-
16	Dabur Uveda complete fairness cream	India	Dabur India Ltd	-	-
17	Fem bleach cream	India	Fem Care Pharma Ltd	Hydrogen peroxide	-
18	Fem post bleach	India	Fem Care Pharma Ltd	-	-
19	Fem pre-bleach	India	Fem Care Pharma Ltd	Hydrogen peroxide	-
20	Magic amabala lotion	-	-	-	-
21	Hypoderm day cream	South Africa	Cosmetic Warehouse, Epping	Vitamin C derivate	-

22	Hypoderm night cream	South Africa	Cosmetic Warehouse, Epping	-	-
23	Hypoderm cream with moisturiser	South Africa	Cosmetic warehouse, Epping	-	-
24	Caro light	Democratic Republic of the Congo	Angel Cosmetics	2% Hydroquinone	-
25	Eraser antimarks cream	India	IPSA labs (p) Ltd.	-	Discontinue use if irritation occurs.
26	Ponds perfect colour complex beauty cream normal to oily skin	India	Ponds Institute	Niacinamide	Apply a broad spectrum sunscreen of at least SPF 15 while using and after a week of discontinued use of the product.
27	Fair and radiant	India	Fem Care Pharma Ltd	-	Apply the cream half an hour before going into the sun so the cream is well

					absorbed.
28	Paw paw fortuneapple whitening anti-spot facial cleanser	People's Republic of China	Fortuneapple	Arbutin	-
29	Paw paw fortuneapple freckle remover night cream	People's Republic of China	Fortuneapple	Arbutin	-
30	Paw paw fortuneapple freckle remover day cream	People's Republic of China	Fortuneapple	Arbutin	-
31	Gold aloe whiten moisturizing-cream	People's republic of China	Beckon (manufactured by Shantou herbal-hall cosmetics Co., Ltd	-	Do not apply to healthy skin. Special skin produces swollen suppuration.
32	La Belle beauty masks (mask cloth)	China	La Belle	Anti-melanin element extract	Do not use if allergic to any of the ingredients. Avoid contact with eyes. Do not apply to broken or inflamed skin.

33	La Belle beauty masks (gel in mask)	China	La Belle	Anti-melanin element extract	Do not use if allergic to any of the ingredients. Avoid contact with eyes. Do not apply to broken or inflamed skin.
34	Diproson	India	Shalina	Betamethasone dipropionate	Systemic absorption of diproson has produced reversible hypothalamic-pituitary-adrenal (HPA) axis suppression. If it is noted withdraw from the cream, or reduce frequency of application.
35	Cuticura vanishing cream	South Africa	Novartis Consumer Health SA	-	-

2.2.1 Theory and instrumentation

Ultraviolet and visible (UV/vis) spectroscopy provides information about compounds based on their ability to absorb radiation in the UV/vis region. Ultraviolet and visible light have enough energy to promote an electron from one molecular orbital to another of a higher energy, this is known as an electronic transition [39]. The energy needed for the electronic transition will determine whether the compound absorbs either ultraviolet or visible light. A UV spectrum is obtained if a compound absorbs UV light and a visible spectrum is obtained if a compound absorbs visible light. Compounds that absorb visible light are coloured, whereas those that absorb UV light do not need to be coloured. Ultraviolet light has wavelengths ranging from 180 to 400 nm whereas visible light has wavelengths ranging from 400 – 780 nm.

The wavelength at which an organic compound absorbs depends on how tightly its atoms in a molecule are held, but the wavelength is inversely proportional to the energy of radiation as seen in the equation below.

$$E = \frac{hc}{\lambda}$$

where E is the energy, h is plank's constant, c is the speed of light and λ is the wavelength of absorption.

Carbon-carbon bonds or carbon-hydrogen bonds have shared electrons in single bonds that are tightly held such that their excitation requires energies corresponding to wavelengths below 180 nm, which is known as the vacuum UV region [40].

The compounds of interest were organic compounds and were not coloured. This made the compounds to be inactive in the visible region of the spectrum. Electrons in double and triple bonds are not so tightly held compared to single bonds, and therefore can be more easily excited by radiation. Ultraviolet light has only enough energy to cause electronic transitions from a nonbonding (lone pair) electron (*n)* into an π^* antibonding molecular orbital, and also the electronic transition from a π bonding molecular orbital into a π^* anti-bonding molecular orbital [39]. Thus, only organic

compounds with π bonding electrons can produce UV spectra because both transitions promote electrons into a π^* anti bonding molecular orbital.

The UV spectra were measured by means of a double-beam Perkin Elmer Lambda 35 UV/vis spectrophotometer. The light from the source which is usually a deuterium lamp for UV and a tungsten lamp for the visible region passes through a monochromator, which filters the light to a narrow wavelength. The filtered radiation then splits into two light beams, where one passes through the blank (solvent) and the other passes through the sample. The output from the detector is the absorbance of the sample having accounted for that of the solvent from the reference cell.

According to Beer's law, absorbance (A) is directly proportional to the concentration (c) of the absorbing sample and the optical pathlength (b) through which the sample absorbs the light. The proportional constant is referred to as the absorptivity (ε) in cm^{-1} $mol^{-1} \times dm^3$.

$$A = \varepsilon bc$$

The concentration of the samples must be low in order for Beer's law to be obeyed [40]. The absorbance of the standard mixtures was kept below a value of 1.5.

2.2.2 Preparation of standards and analysis

Standard solutions were prepared by weighing pure samples of known skin-lightening agents and dissolving in methanol to make solutions of ~1×10^{-3} mol L^{-1} in 50.00 mL volumetric flasks. The standard solutions were then diluted qualitatively to give UV absorption of not more than 1.5. A scan of the standards between the wavelengths of 200 - 500 nm was obtained. Quartz cuvettes with a pathlength of 1 cm were used for the analysis. The wavelength of maximum absorbance for all standards were noted and used in HPLC analysis.

2.3 Fluorescence spectroscopy

This is a photoluminescence process in which molecules are excited by absorption of electromagnetic radiation [40]. The excited species relax to the ground state, and

give up their excess energy as a photon; this takes a few nanoseconds. This technique has a higher sensitivity compared with absorbance spectroscopy.

Fluorescence is just one of the pathways a molecule can take to return to the ground state. All compounds have the potential to fluoresce, but some structures provide radiationless pathways for relaxation which occur at a faster rate than fluorescence, hence they do not fluoresce [40]. Aromatic rings in compounds give the most intense molecular fluorescence emission [40]. Certain highly conjugated double bonded structures, and aliphatic and alicyclic carbonyls, have the ability to fluoresce [40]. Fluorescence is measured by first exciting the sample at the absorption wavelength, and then measuring the emission which usually happens at longer wavelengths than absorption. In order to prevent self-absorption and the inner filter effect the concentration of the solutions used to measure fluorescence spectra should have concentrations such that their absorbance is less than 0.05 at the excitation wavelength.

Standard solutions were prepared as described in Section 2.2.2. To obtain the fluorescence spectra, very dilute samples had to be used since fluorescence has higher sensitivity. The standards were qualitatively diluted to give the best spectra.

The cuvettes used were quartz and the instrument was a Perkin Elmer LS50B luminescence spectrometer. The standards were excited at the wavelengths according to Table 2.2 and their spectra obtained.

Table 2.2 Excitation wavelengths used for the determination of fluorescence spectra of standards in methanol.

Standard	**Excitation wavelength/nm**
hydroquinone	278
resorcinol	268
p-benzoquinone	230
N-acetyl-L-cysteine	210
p-arbutin	278
kojic acid	256

phenol	260
4-hydroxyanisole	295
dexamethasone	230
4-propoxyphenol	280
hydrocortisone	230
hydrochinon-dimethylether	275
6α-methylprednisolone	230
fluocinolone acetonide	225
4-phenoxyphenol	218
clobetasol propionate	220

The values obtained where used in the fluorescence detector of the HPLC in order to determine the presence of SLAs in SLPs.

2.4 High performance liquid chromatography

HPLC was used for the analysis of the samples. This method is one of the most widely used for the analysis of skin-lightening agents.

2.4.1 Theory and instrumentation

HPLC is an elution chromatography that separates compounds based on their polarity. It requires a mobile phase, stationary phase, pump and detector. The stationary phase is in a column with a small diameter and the particle sizes are very small. There are different kinds of stationary phases which allow for different kinds of chromatography based on the separation mechanism.

The mobile phase is also an important component of HPLC. The mobile phase must be compatible with the stationary phase since it must not damage or dissolve it. It must also be able to dissolve the analyte of choice, since precipitation of the analyte in the column can lead to damage.

For the mobile phase to be able to pass through the small column, pressure needs to be applied. This allows for fast separation compared to normal liquid

chromatography. There are two types of elutions: isocratic and gradient elution. Isocratic elution is when the composition of the mobile phase stays constant with time, whereas for gradient elution the mobile phase changes with time.

A detector is needed for the analysis of the compounds in the analyte. To minimize extra column band-broadening the detector must have a low dead volume. UV/Vis detectors are the widely used in HPLC systems, although these are not the only detectors that can be coupled with HPLC. Other detectors that can be coupled with HPLC include the PDA, mass spectrometers, fluorescence, etc.

In this project a column with a reversed phase packing was used. This is called a C_{18} column, because its stationary phase is composed of silica bonded with octyldecyl groups. By introducing the long carbon chain, the polarity of the silicon (normal phase) is changed and we obtain a nonpolar stationary phase. The mobile phase used is normally relatively polar such as water, methanol and acetonitrile. These are the most used mobile phases in reversed phase chromatography. The most polar compounds are eluted first and increasing the polarity of the mobile phase decreases the elution time [40].

Two instruments were used for the analysis of SLAs. The first HPLC instrument used for this research was a Shimadzu which consisted of: Prominence auto-sampler (SIL-20A), Prominence degasser (DGU-$20A_5$), Prominence liquid chromatograph (LC-20AT), Prominence diode array detector (SPD-M20A) and a fluorescence detector (RF-10AXL). The instrument had to be changed due to the instrument becoming contaminated and several clean-up methods could not sufficiently clean the instrument. This instrument was used for the method developments described in Section 2.4.5.

The second HPLC instrument was a Shimadzu-UFLCXR which consisted of a Prominence auto sampler (SIL-20AXR), Prominence degasser (DGU-$20A_3$), Prominence liquid chromatograph (LC-20ADXRX2), Prominence diode array detector (SPD-M20A), Prominence column oven (CTO-20A) and a Prominence communications bus module (CBM). This instrument was used for the qualitative analysis of standards,

recovery analysis, intra-day and inter-day analysis, and for the quantification of the SLAs in the creams.

2.4.2 Precautions while using HPLC

The use of HPLC requires the use of HPLC grade solvents. These solvents contain HPLC-grade on their labels. The water used must be Millipore water with a resistance below 18 Ω. The solvents always have to be filtered through a 0.45 µm filtering system to insure there are no contaminants. The HPLC used in this project contained a degasser. The mobile phase must be purged with helium gas in cases were the instrument does not have a built-in degasser. Air bubbles interfere with the analysis and can increase the pressure of the pumps. One must ensure that the pressure of the pumps does not exceed the maximum pressure allowed for the instrument. Most instruments these days are equipped with a built-in warning which switches off the pump if the pressure limit is exceeded.

When using acidic or basic mobile phases one must make sure the column used is compatible with such pHs, for example, at very low pHs the C_{18} column loses its stationary phase. It is essential that no carryovers from the previous runs are obtained. This can be checked for by running blanks in between each run. In instruments with automatic rinses, one must make sure the rinsing solvent bottle always has sufficient solvent and that the instrument is rinsing the injector.

2.4.3 Column performance test

A column performance test was done to check if the instrument was functioning well and also the state of the column. The column used was brand new, which was a Perkin Elmer Brownlee Pecosphere C_{18} (150 ×4.6 mm I.D., 5 µm) column. The column did not come with a certificate of analysis chromatogram. A test mixture was obtained from the manufacturer which was made up of uracil, naphthalene, acetophenone, toluene and benzene. The mixture was an already made mix. It was made in 75% methanol and 25% acetonitrile. The concentration of the mixture was stated as 1 mg L^{-1}. The column performance test was done by using 60% methanol with 40% water as the mobile phase. The flow rate was set at 1 mL min^{-1}, the injection volume was 5 µL, the detection wavelength was 210 nm and the run time was set at 30 minutes.

The chromatogram obtained was compared with the chromatogram which came with the test mix. The results of the column performance test are discussed in Section 3.2.

2.4.4 Standards and sample preparation

Standard solutions were prepared by dissolving a specific mass of pure skin lightening agents in methanol to give a concentration of ~1×10^{-3} mol L^{-1}. A total of 35 standards were prepared and analysed.

Samples were prepared by weighing ~0.05 g of skin-lightening cream into a beaker, adding 4 ml of methanol and sonication for 15 minutes in an ultrasonic bath. The samples were quantitatively transferred into a 10.00 mL volumetric flask and made to the mark by using methanol. All samples were filtered through a 0.45 µm disc syringe filter directly into HPLC 1.5 mL vials before analysis.

2.4.5 Method development

Sixteen standard solutions prepared according to the method in Section 2.4.4 where used for method development. The standards used were hydroquinone, resorcinol, *p*-benzoquinone, N-acetyl-L-cysteine, *p*-arbutin, kojic acid, phenol, 4-hydroxyanisole, dexamethasone, 4-propoxyphenol, hydrocortisone, hydrochinon-dimethylether, 6α-methylprednisolone, fluocinolone acetonide, 4-phenoxyphenol and clobetasol propionate.

The standards were analysed individually and as a mixture, by using different solvents systems. Isocratic and gradient elutions were investigated. Although not all standards had base line resolution, the mobile phase composition was narrowed down to 60% methanol in water (v/v) and 50 % methanol in water (v/v), isocratic elution. The mobile phase was prepared manually and the filtered through a 0.45 µm filtering system. The water used was Millipore water from a Millipore Milli-Q water purification system with conductivity of less than 18.2 Ω cm^{-1} at 25 ºC while the methanol was HPLC-grade.

An analytical column Brownlee Pecosphere C_{18} (150 × 4.6 mm I.D., 5 µm) column was used for the method development. The flow rate of all runs was maintained

at 1 mL min^{-1} and the injection volume used was 10 µL. The results were collected at three wavelengths 240, 270 and 289 nm, which were determined to be appropriate for all standards in Section 2.2.2.

Prepared samples were then analysed by using the two best mobile phase compositions. Based on the separation of the components in the SLCs samples, it was found that the 50% methanol in water mobile phase gave the better separation compared with 60% methanol in water.

2.4.6 Qualitative analysis of samples

A total of 35 samples (Table 2.1) were analysed by using the parameters given in Section 2.4.5. The results obtained were compared with the chromatograms of the individual standards, by comparing the retention times and UV spectra from the PDA. Spiking of some samples was done to positively identify the peaks.

2.4.7 Determination of optimum extraction method for samples

The extraction of the skin-lightening agents (SLA) in the creams is also an important factor in their analysis. The extraction method must be able to extract the maximum amount of the SLA in the creams.

Two different parameters were investigated: the extraction solvent and the extraction time. Three different solvents were used, namely, methanol, 2-propanol and 50% methanol in water (v/v). Two samples were analysed to test the parameters.

The samples were prepared according to the method described in Section 2.4.4, replacing methanol with the appropriate extraction solvent. All samples were extracted for 15 and 30 minutes to investigate the best extraction time.

The column used for this analysis was a Brownlee C_{18} (100 × 4.6 mm I.D., 3 µm particle size) analytical column. The column was changed from the Brownlee Pecosphere C_{18} (150 × 4.6 mm I.D., 5 µm) due to peak-tailing experienced. The Brownlee C_{18} (100 × 4.6 mm I.D., 3 µm particle size) analytical column was used for the quantification of the skin lightening agents.

The best extraction solvent was found to be 2-propanol for an extraction time of 15 minutes.

2.4.8 Quantitative analysis of samples

An external calibration curve method was used to quantify the SLA identified from the qualitative analysis of the SLCs. It was found that phenol, hydroquinone, *p*-arbutin and benzoquinone could be identified based on their retention times and UV spectra.

Multi-standard stock solutions were accurately prepared by weighing approximately, phenol (0.0030 g), arbutin (0.0120 g) and dexamethasone (0.0040) into a 10.00 mL volumetric flask to give a concentration of approximately 300, 1200 and 1400 mg L^{-1} respectively. The standards were grouped such that there is no co-elution in HPLC. Phenol, arbutin and dexamethasone were grouped together whereas benzoquinone, niacinamide and hydroquinone were prepared separately. A mass of niacinamide (0.0080 g) was accurately weighed into a 10.00 mL volumetric flask to give a concentration of 800 mg L^{-1}. A mass of benzoquinone (0.0125 g) was weighed in a 25.00 mL volumetric flask to give an approximate concentration of 500 mg L^{-1}. A mass of hydroquinone (0.0090 g) was weighed in a 10.00 mL volumetric flask to make a solution of approximately 900 mg L^{-1}. The stock solutions and methanol were filtered through a 0.45 µm disc filter.

Working from the stock solutions, volumes of 4 µL, 15 µL, 25 µL, 45 µL, 70 µL, 85 µL and 100 µL were measured into individual HPLC vials. To each vial methanol was added to make the final volume of the standards 1000 µL by taking 996 µL, 985 µL, 975 µL, 955 µL, 930 µL, 915 µL and 900 µL respectively into the HPLC vial. In some cases instead of using 4 µL of the standard solutions, 10 µL was used and then 990 µL of methanol was added to that HPLC vial. Dilution of the standards was done with a Perkin Elmer series 200 auto-sampler.

A mass (0.05 g) of each of the samples was weighed into a beaker in which a volume (4 mL) of methanol was added. The mixture was then sonicated in an ultra-sonic bath for 15 minutes. The supernatant was transferred into a 10.00 mL volumetric flask being careful not to take the solid which did not dissolve in the solvent. The

samples were made to the mark with methanol. A small aliquot was filtered through a 0.45 µm syringe filtering disc into a HPLC vial and then analysed.

2.4.9 Recovery

Recoveries were performed to determine the accuracy of the developed method. This was done by spiking samples with the SLA standards. The samples spiked were those which were determined not to have any peaks close to those of the standard to be added. Recovery was done for hydroquinone (0.0015, 0.0045 and 0.0080 g in a 100 mL volumetric flask), niacinamide (0.0006, 0.0016 and 0.0035 g in a 50 mL volumetric flask), arbutin (0.0006, 0.0030 and 0.0050 g in a 50 mL volumetric flask), phenol (0.0006, 0.0015 and 0.0025 g in a 100 mL volumetric flask) and dexamethasone (0.0006, 0.0017 and 0.0024 g in a 100 mL volumetric flask)

High, medium and low masses of the standards were weighed directly into a pre-weighed sample of the cosmetic cream. The samples were then treated as described in Section 2.4.8. All samples were prepared in triplicate. The mass of SLA weighed into the sample was different for each SLA.

2.4.10 Inter-day and Intra-day analysis

Inter-day (over a period of 3 days) and intra-day (same day) analysis was done to determine the accuracy and precision of the instrument. The reproducibility of the results was done by using standards. The standards were prepared as described in Section 2.4.8. The HPLC method described in Section 2.4.8 was used to analyse the standards.

Intra-day analysis was done by running the standards 3 times over a period of 1 day. Each standard was injected 3 times. Inter-day analysis was done by analysing the standard every day for a period of 3 days. This was done to determine the accuracy of the HPLC method developed. The results are reported and discussed in Section 3.6

2.5 Gas chromatography-mass spectroscopy

Gas chromatography coupled to a mass spectrometer (GC-MS) is a good method to identify compounds which are unknown. Consequently, GC-MS was attempted in this work in order to identify unknown compounds in the SLP samples.

2.5.1 Theory and instrumentation

Gas chromatography (GC) separates compounds based on their boiling points. The sample is vaporised and then partitioned between a gas and a stationary phase. The mobile phase in this case is a gas, which does not interact with the analyte, but just transports it through the column and since the carrier gas does not interact with the analyte, it must be chemically inert. Some of the gases used in GC are hydrogen, helium, argon and nitrogen. Just like with HPLC, GC also has different columns which determine the kind of separation obtained.

The sample is injected into an injector port which is set at a certain temperature, usually higher than the boiling point of the analyte of interest. The injection of the sample is important since reproducibility is of interest. The sample injected must not be too large as this could result in overloading of the column. Injection must be fast; slow injections cause band-broadening which is undesirable.

Temperature programming is important as it affects the separation of the compounds. For samples with a broad boiling range, temperature programmes can be used. This involves increasing the column temperature in steps or continuously during elution [40].

Mass spectrometry is used as a detector in GC. The combination of GC and MS is called GC-MS. The mass spectrometer measures the mass to charge ratio of the analyte that has been fragmented. Different compounds have different fragmentation patterns, which allows for the compounds to be identified based on the pattern. This has allowed for libraries with GC-MS data of different compounds to be developed. In a GC-MS system, the analyte which has been separated by the GC enter the mass detector. In the mass spectrometer, the molecules enter the ionisation source, which ionises the sample. This source is powerful enough to break the chemical bonds of the molecules, but they do not decompose it. This is how fragments of the molecules are obtained. The fragments then enter an analyser where a current is employed, such that the uncharged molecules will be discarded and only the charged fragments will pass through to the ion detector.

The GC-MS allows for the mass spectrum of each peak on the chromatogram from the GC to be obtained and identified. The instrumentation used was a Agilent 6890 (Palo Alto, CA, USA) gas chromatograph (GC), coupled to a 5973N series mass spectrometer (MS) operated in electron impact (EI) ionization mode. The column was a DB5 which was 30 meters in length.

2.5.2 Preparation of standards and samples

The standards used for qualitative analysis were composed of mostly phenolic compounds, which are not sufficiently volatile for GC-MS analysis. Standards and samples had to be derivatised before they could be analysed. The derivatisation agent to be used was N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA).

The temperature programme was set as, 50 °C held for 2 minutes, then an increase of 20 °C min^{-1} to 295 °C then held there for 10 minutes. The split ratio was set at 1:50. The injection volume used was 1 µL. Standards were prepared directly into a GC vial to make a multi-standard, hydroquinone (0.0022 g), resorcinol (0.0047 g), arbutin (0.0017 g) and kojic acid (0.0010 g). Pyridine (100 µL) was added followed by BSTFA (100 µL). The sample vial was closed and heated in an oil bath at a temperature of about 65-70 °C for 30 minutes. The standard was allowed to cool before analysis. The same standards were analysed separately to identify the individual components in the chromatogram of the mixture and to obtain the individual mass spectra.

Qualitative analysis of all standards that had an –OH group which could be derivatised where analysed. The following standards where analysed: hydroquinone (18 µg mL^{-1}), kojic acid (13 µg mL^{-1}), resorcinol (24 µg mL^{-1}), phenol (194 µg mL^{-1}), 4-propoxyphenol (25 µg mL^{-1}), 4-phenoxyphenol (18 µg mL^{-1}) and 4-hydroxyanisole (13 µg mL^{-1}). Some of the steroids were also prepared, but the column oven could not be heated high enough to enable their analysis.

Samples were prepared by weighing 0.5 g into a beaker; 5 mL of pyridine was added. The mixture was ultrasonicated for 30 minutes, transferred quantitatively to a 10.00 mL volumetric flask and then made up to the mark with pyridine. A small volume was filtered through a 0.45 µm syringe filtering disc. A volume of 200 µL of

the filtered sample was then added into a sample vial, and 250 µL of BSTFA was added. The samples were derivatised by using the same procedure as described above. Liquid samples which could not be weighed were prepared by adding 100 µL of the sample into the vial in which 100 µL pyridine was added to dilute the samples. The volume of the derivatisation agent added was the same as for solid samples, i.e. 250 µL. The samples where then derivatised and analysed by using GC-MS.

The results of the experimental described here are presented and discussed in Chapter 3.

Chapter 3

Results and discussion

Skin-lightening creams have been in the market for many years and there continues to be research into finding new SLAs that do not harm the skin. Restrictions have been imposed on the use of hydroquinone since occurrences of side-effects associated with its use were reported. South Africa was the first country to limit the use of hydroquinone in over-the-counter products to 2% (m/m), and later other countries followed suit [9]. Other SLAs have been developed as researchers try to find a suitable agent that can replace hydroquinone. Today there are many SLAs and some of these are detrimental to the skin. There have been reports of SLPs having side-effects that correspond to the use of steroids. Some products falsely list their ingredients, while some vendors sell products containing banned SLAs. It is therefore important that the SLAs in SLPs be known. There need to be strong laws that prohibit the sale of illegal SLAs. It is also important that the public knows what they are putting on their skin and the consequences of their actions in terms of side-effects.

This research develops two methods to qualitatively and quantitatively determine SLAs in SLPs. These experiments are detailed in Chapter 2. Levels of hydroquinone, phenol, niacinamide and arbutin were qualitatively and quantitatively identified in a number of skin-lightening products which are readily accessible without the use of a prescription. The investigation was done with the use of HPLC equipped with a PDA and a fluorescence detector. GC-MS was also used to qualitatively identify the SLAs in the creams.

3.1 Optimum detection wavelengths

The UV-visible spectra of different SLA standards were measured by dissolving the standards in methanol. The wavelength of maximum absorption of distinguishable peaks were observed and recorded (see Table 3.1). Most compounds have high absorptions at low wavelengths, making them indistinguishable. By using the

wavelength of the peak with the second highest absorbance allowed for the compounds to have different wavelengths for analysis, even though they were not the maximum wavelengths. This was important since selectivity was needed and the compounds were sensitive at the other wavelengths as well. Therefore, selectivity and sensitivity was achieved this way.

Table 3.1 UV/vis absorbance and fluorescence emission wavelengths of SLA standards dissolved in methanol obtained by using a UV/vis spectrophotometer and a luminescence spectrophotometer respectively

Standards	**Absorption wavelength/nm**	**Fluorescence emission wavelength/ nm**
hydroquinone	293	329.18
resorcinol	276	308.44
p-benzoquinone	243	329.52
N-acetyl-L-cysteine	200	-
p-arbutin	286	321.06
kojic acid	269	-
phenol	272	299.96
4-hydroxyanisole	291	322.87
dexamethasone	239	458.08
4-propoxyphenol	292	324.85
hydrocortisone	241	458.57
hydrochinon-dimethylether	289	318.52
6α-methylprednisolone	243	458.57
fluocinolone acetonide	237	-
4-phenoxyphenol	227	323.61
clobetasol propionate	238	-

Thus, for HPLC the PDA detector was set to acquire chromatograms at three wavelengths to cover the wavelengths in Table 3.1, namely, at 240, 270 and 289 nm. The fluorescence detector was set at an excitation wavelength of 280 nm and an

emission wavelength of 320 nm. These wavelengths were chosen such that they could cover all the compounds that fluoresce based on the results in Table 3.1. The wavelengths used for each analyte for the PDA detector are listed in Table 3.2. Although some of the SLAs had maximum wavelengths of 200 nm, this value could not be used as many other analytes as well as solvents absorb at this wavelength. The wavelengths chosen allowed for sensitivity at a specific wavelength.

Table 3.2 The UV absorbance wavelength used for the detection of the SLAs in HPLC

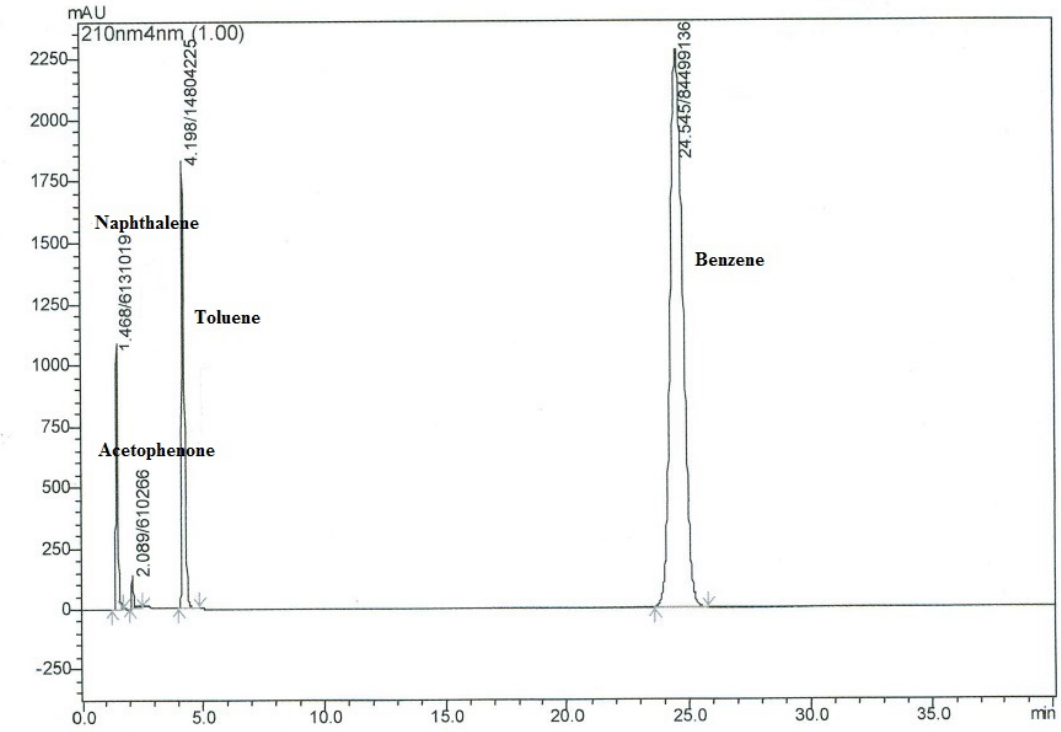
Standard	**Detection wavelength/nm**
hydroquinone	289
resorcinol	270
p-benzoquinone	240
N-acetyl-L-cysteine	240
p-arbutin	289
kojic acid	270
phenol	270
4-hydroxyanisole	290
dexamethasone	240
4-propoxyphenol	289
hydrocortisone	240
hydrochinon-dimethylether	289
6α-methylprednisolone	240
fluocinolone acetonide	240
4-phenoxyphenol	240
clobetasol propionate	240
fluticasone propionate	240
mometasone furoate	240
triamcinolone acetonide	240
beclomethasone dipropionate	240

nicotinamide (niacinamide)	240
betamethasone 17-valerate	240
betamethasone 17,21-dipropionate	240

3.2 Column performance test

A column performance test was done according to the method described in Section 2.4.3. The results will be discussed here. The column performance mixture was run and the chromatogram was obtained. The chromatogram obtained was compared with that of the chromatogram that came with the mixture (Figure 3.1).

The mixture was labelled as having 5 components (1 mg L^{-1} of uracil, naphthalene, acetophenone, toluene and benzene in 75% methanol in 25% acetonitrile), but the chromatogram displayed 4 peaks. This was also what was observed when the mixture was analysed (Figure 3.2). The results from the column displayed a very small peak which was not on the test mix chromatogram. This was acceptable since the mixture was supposed to have 5 components. It was assumed based on the results that the column and the instrument were working well since the separation of the peaks was obtained. The column was then used for the method development of the SLAs.



HPLC Efficiency check solution

1 mg/L in 75% Methanol in 25% acetonitrile prepared: 01/09/2009

Components

1. Uracil
2. Naphthalene
3. Acetophenone
4. Toluene
5. Benzene

Mobile phase: 60% methanol in water
Injection volume: 5 µL
Flow rate: 1 ml/min
Detection wavelength: 210 nm

Figure 3.1 Reference chromatogram for the mixture used to test column performance of a Perkin Elmer Brownlee analytical column, Pecosphere C_{18} (150 × 4.6 mm, ID) with a 5 µm particle size, injection volume 5 µL, mobile phase methanol:water (60:40, v/v), flow rate of 1.0 mL min^{-1} and the detection wavelength of 210 nm

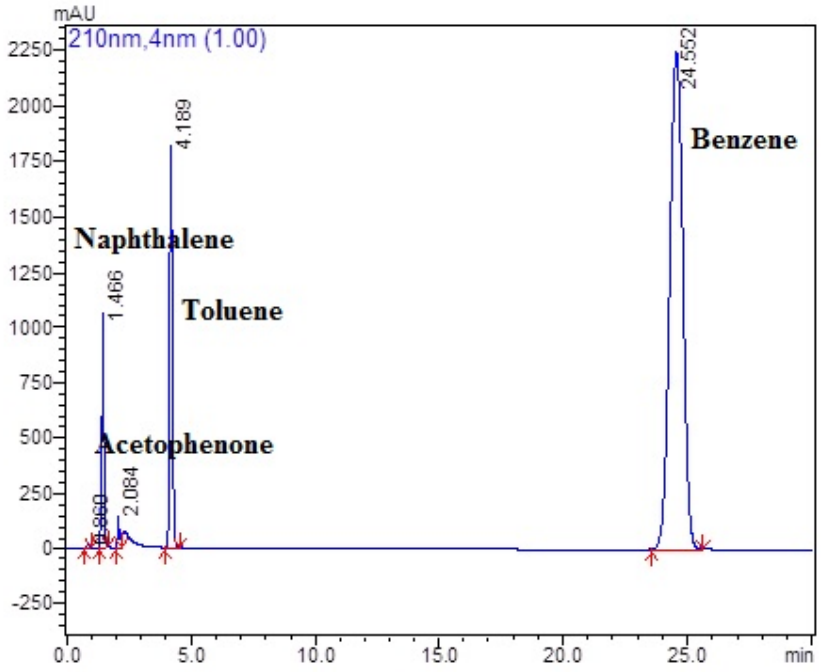


Figure 3.2 Chromatogram of column performance of the Perkin Elmer Brownlee analytical column, Pecosphere C_{18} (150 × 4.6 mm, ID.) with a 5 µm particle size, injection volume 5 µL, mobile phase methanol:water (60:40, v/v), flow rate of 1.0 mL min^{-1} and the detection wavelength of 210 nm.

3.3 Optimum HPLC mobile phase

Once the detector wavelengths had been determined, the mobile phase now needed to be optimised. The column used for the optimisation of the method was a new Perkin Elmer Brownlee analytical column, Pecosphere C_{18} (150 × 4.6 mm, ID.) with a 5 µm particle size.

The SLAs in Table 2.2 were used for the optimisation. The standards were analysed individually to obtain the retention times, and then mixed to prepare a multicomponent solution that was used to test adequate separation of the compounds. Gagliardi *et al.* reported a method where isocratic separation of corticosteroids was obtained by using acetonitrile-water 60:40 (v/v) as a mobile phase [36]. This was chosen as a starting point, but methanol was used instead of acetonitrile because it is cheaper. The multicomponent standard was composed of 13 standards chosen based on the retention times. The standards mixed were hydroquinone, resorcinol, benzoquinone, kojic acid, phenol, 4-hydroxyanisole, dexamethasone, 4-propoxyphenol,

hydrocortisone, 6α-methylprednisolone, fluocinolone acetonide, 4-phenoxyphenol and clobetasol propionate. The results were obtained by using the PDA and the fluorescence detector.

It was observed that there was no baseline resolution between some of the compounds with the aforementioned mobile phase (Figure 3.3) thus different mobile phases were investigated (Figure 3.4). Both isocratic and gradient elution were investigated. It was found that from all the mobile phase combinations attempted, 50% methanol in water and 60% methanol in water gave reasonable separation of the SLA. Since not all of the SLAs in Table 3.2 are simultaneously obtained in SLCs, samples had to be analysed to determine which mobile phase gave the best separation of the SLCs.

By using SLC samples, it was observed that 50% methanol in water (v/v) gave the best results, and this mobile phase was chosen as the mobile phase for the analysis of SLAs in SLCs. This mobile phase was then used to obtain the retention times of different steroid standards.

Once the optimum mobile phase was selected, samples were analysed to qualitatively identify which SLAs were present in the creams. This was done by comparing the retention times and UV spectra from the PDA, and confirmation which was obtained by spiking the sample. A chromatogram of a sample containing arbutin and spiked can be observed in Figure 3.5. It was found that phenol, arbutin, niacinamide, hydroquinone and benzoquinone were the only SLA that could be identified. None of the steroids standards tested were identified in the samples.

3.4 Linearity and limit of detection

An external standard calibration method was used to obtain calibration curves for the SLAs identified in the creams. It was assumed that when the same analyte concentration is present in the sample then the same response would be obtained with the standards. The standards where prepared in different sets. One multi-standard was prepared with a composition of arbutin, phenol and dexamethasone. The standards that were grouped together had different retention times and different detection wavelengths as seen in Table 3.3.

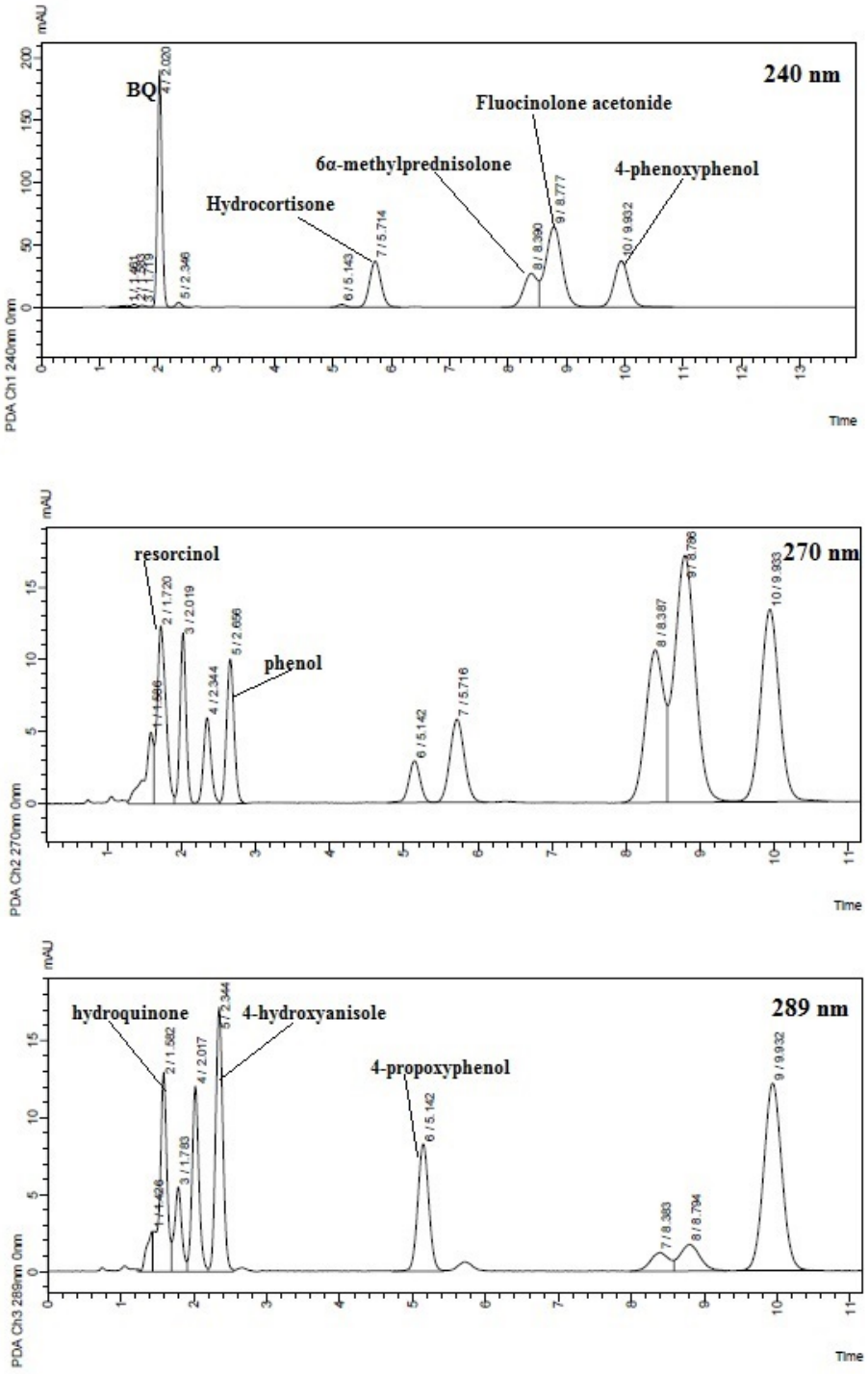


Figure 3.3 Separation of 13 SLAs mixture with detection at 240, 270 and 289 nm. The chromatographic conditions used were: Brownlee C_{18} (150 × 4.6 mm I.D., 5 µm particle size) column, injection volume 10 µL, mobile phase methanol:water (60:40, v/v), flow rate of 1.0 mL min^{-1}

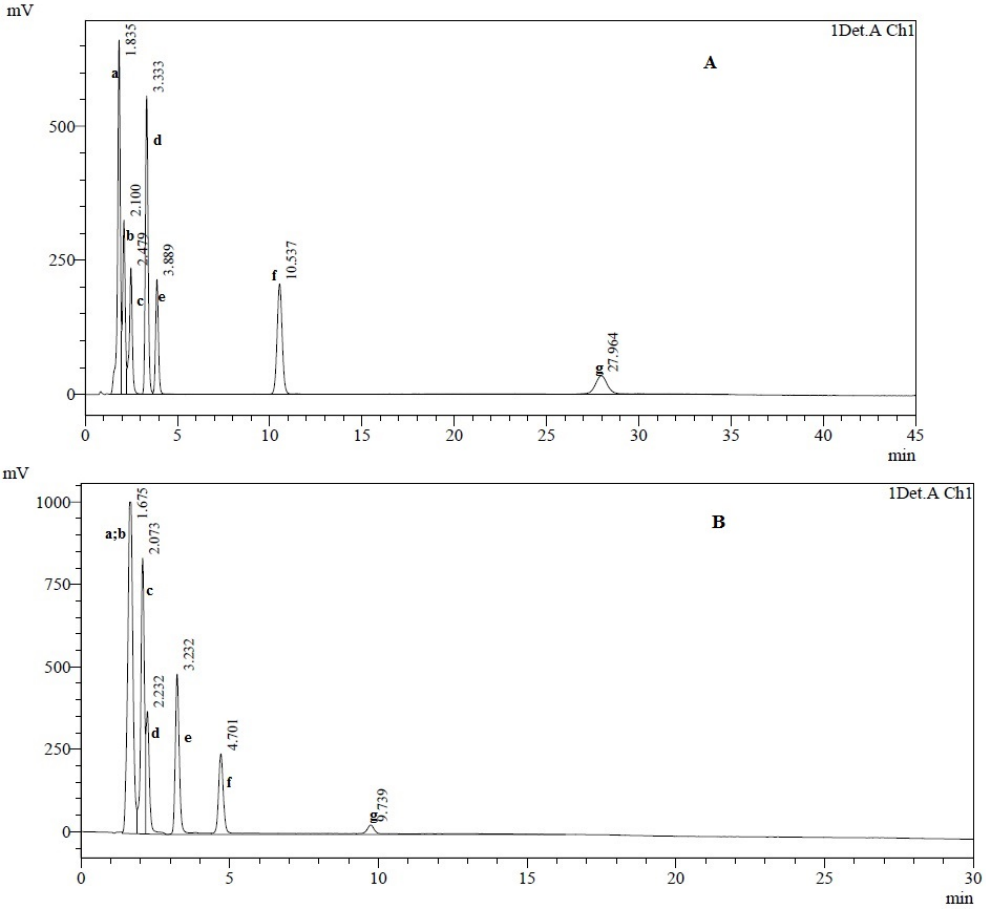


Figure 3.4 Chromatogram of HPLC-fluorescence with a mobile phase of A) 50% methanol in water and B) 70% methanol in water giving peaks of a) hydroquinone, b) resorcinol c) benzoquinone d) 4-hydroxyanisole, e) phenol, f) 4-propoxyphenol g) 4- phenoxyphenol. The chromatographic conditions used were: Brownlee C_{18} (150 × 4.6mm I.D., 5 µm particle size) column, injection volume 10 µL, flow rate of 1.0 mL min^{-1} and excitation wavelength used for detection was 320 nm.

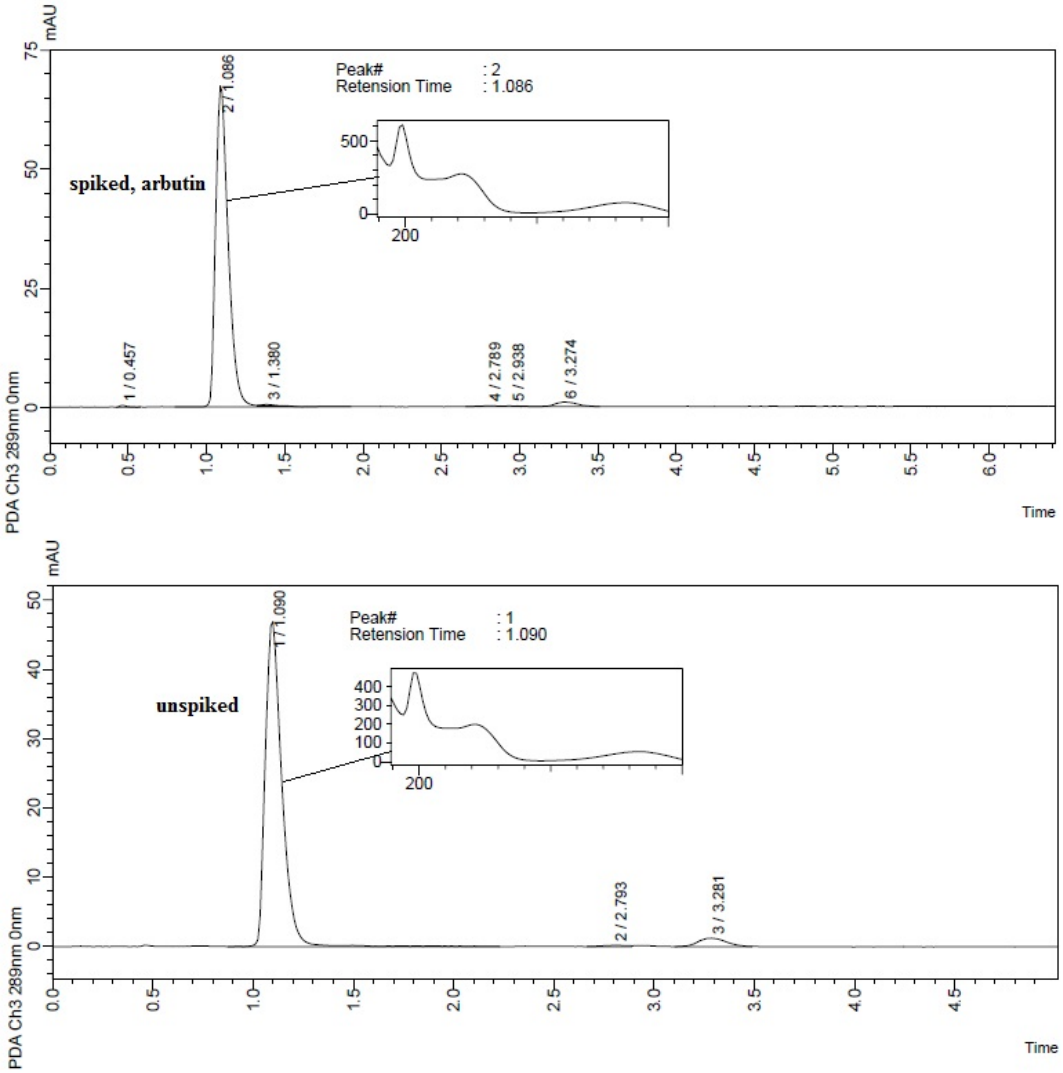


Figure 3.5 HPLC chromatogram of sample 6 confirming the presence of arbutin by spiking. The chromatographic conditions used were: Brownlee C_{18} (100 × 4.6 mm I.D., 3 µm particle size) column, injection volume 10 µL, mobile phase methanol:water (50:50, v/v), flow rate of 1.0 mL min^{-1} and the detection wavelength of 289 nm.

Table 3.3 Retention times for standards used to obtain calibration data with HPLC and their detection wavelengths.

	Retention time/minutes	**Detection wavelength/nm**
Arbutin	1.021- 1.049	289
Niacinamide	1.119- 1.139	240
Hydroquinone	1.158- 1.171	289
Benzoquinone	1.384- 1.392	240
Phenol	1.998- 2.190	270
Dexamethasone	6.690- 9.884	240

Figure 3.6 displays dexamethasone at its highest peak intensity when detected at 240 nm. This is the wavelength that was used for dexamethasone analysis. Although other peaks could be seen at this detection wavelength, they were not quantified at this wavelength.

Phenol has a higher absorbance at 270 nm (Figure 3.7), as can be seen by the increase of the peak in comparison to that in Figure 3.6. Consequently, phenol was quantified at this wavelength.

At a detection wavelength of 289 nm phenol and dexamethasone hardly absorb (Figure 3.8) and therefore their peaks are barely visible but arbutin is at its maximum absorbance at this wavelength and was quantified at 289 nm.

Hydroquinone, benzoquinone and niacinamide were prepared separately on their own because their retention times were too close to each other and there was no clear resolution of the peaks (Table 3.3).

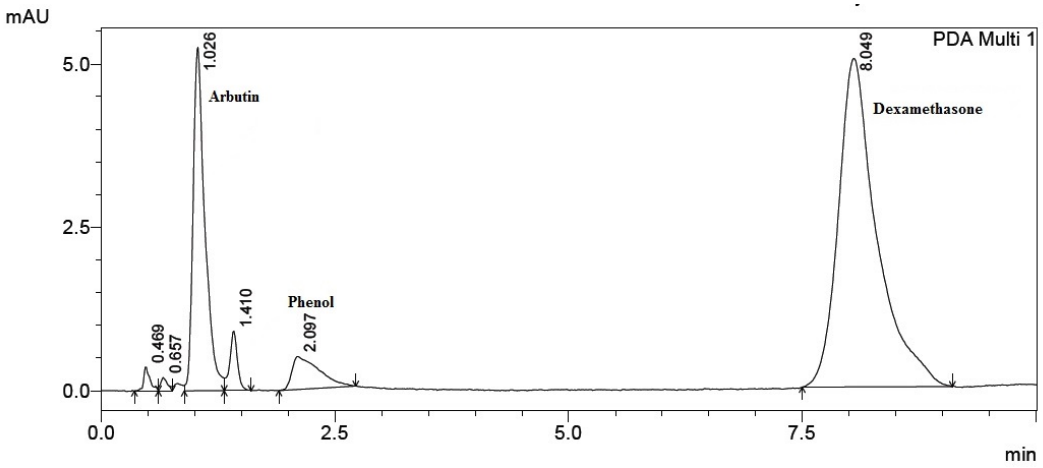


Figure 3.6 Chromatogram of arbutin, phenol and dexamethasone obtained at a detection wavelength of 240 nm. The chromatographic conditions used were: Brownlee C_{18} (100 × 4.6 mm I.D., 3 µm particle size) column, injection volume 10 µL, mobile phase methanol:water (50:50, v/v), flow rate of 1.0 mL min^{-1}.

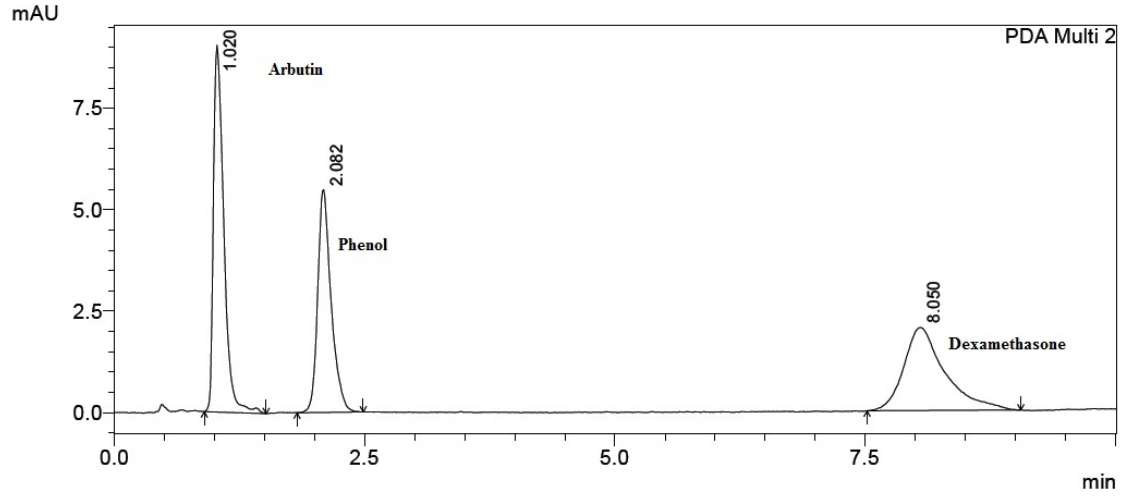


Figure 3.7 Chromatogram of arbutin, phenol and dexamethasone obtained at a detection wavelength of 270 nm showing the increase of the phenol peak and the decrease of the dexamethasone peak. The chromatographic conditions used were: Brownlee C_{18} (100 × 4.6 mm I.D., 3 µm particle size) column, injection volume 10 µL, mobile phase methanol:water (50:50, v/v), flow rate of 1.0 mL min^{-1}.

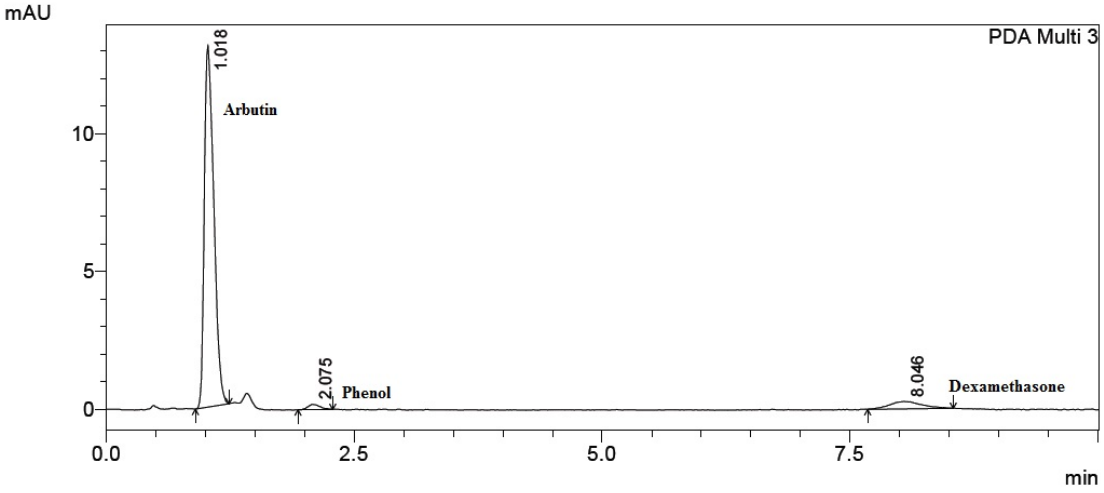


Figure 3.8 Chromatogram of arbutin, phenol and dexamethasone at 289 nm showing that arbutin is the dominant peak at this wavelength. The chromatographic conditions used were: Brownlee C_{18} (100 × 4.6 mm I.D., 3 µm particle size) column, injection volume 10 µL, mobile phase methanol:water (50:50, v/v), and flow rate of 1.0 mL min^{-1}.

Three replicates of each of the seven standard solutions were prepared and each concentration was injected three times. The calibration curves were constructed by plotting the peak area against the concentration. Linear regression of the calibration data for each standard was performed by using Microsoft Excel 2010. The linear regression graph of phenol is given in Figure 3.9. All calibration plots with their residual plots can be obtained in Appendix C.

Good linearity was observed for all the standards over the concentration range tested. The results of the linear regression analysis and the limits of detection and quantification are given in Table 3.4.

The correlation coefficients of the different standards were all above 0.95. The hydroquinone standard had the lowest correlation coefficient at 0.965 whereas dexamethasone had the highest at a value of 0.999. The normal probability plots, line fit plots and residuals were constructed at the 95% confidence level.

The residuals give the difference between the predicted value and the experimentally determined value. The closer the values are to zero, the more close the linear regression is to the predicted values. Regression also gives an idea of whether

assumptions that were made while making the calibration plot are valid. A good regression is that which has residual values close to zero and with a random distribution.

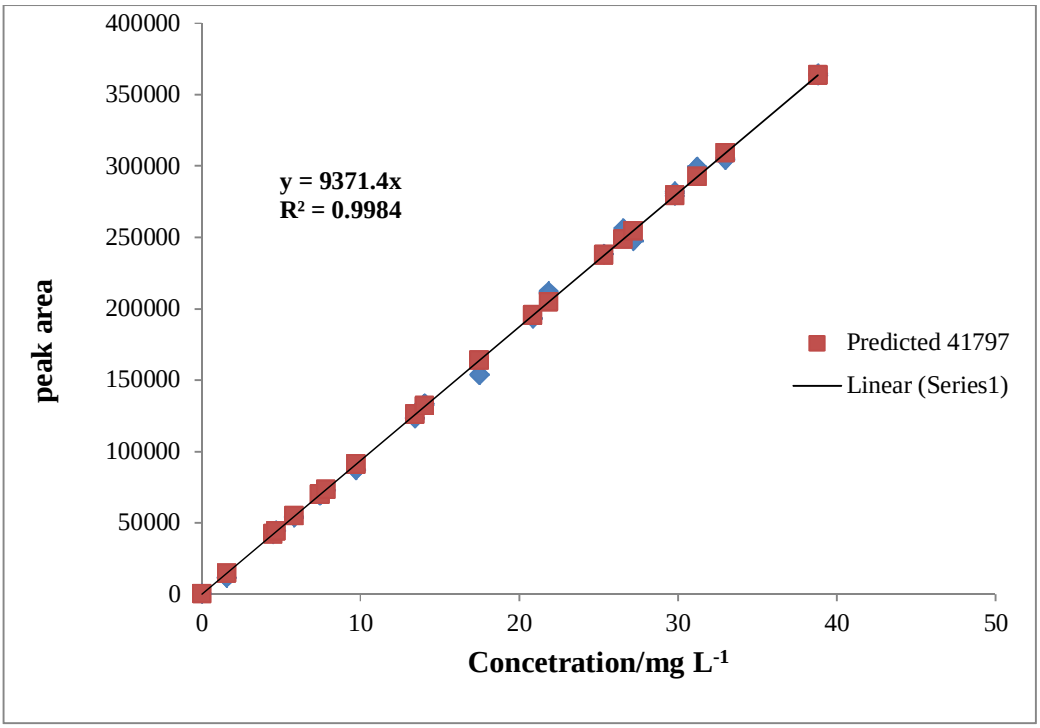


Figure 3.9 Calibration curve for phenol. The blue diamond points depict the experimental data and the solid line shows the linear regression model fitted.

Residuals for the calibration curves of phenol, niacinamide, benzoquinone and dexamethasone were found to be random. The residuals of the calibration curve for niacinamide are plotted in Figure 3.10. It can be seen that the smaller concentration show a better fit for the linear regression as they are closer to zero the larger concentrations. The residual plots for phenol, benzoquinone and dexamethasone can be obtained in Appendix C.

The residual plot for arbutin was random at high concentrations, however at concentrations below 100 mg L^{-1} all the residuals were below zero (Figure 3.11). This could indicate the presence of systematic errors in the smaller concentrations.

The residuals for hydroquinone gave a U-shape (Figure 3.12). This indicates that the linear regression method is not valid for hydroquinone. Therefore, the assumption made that the peak area of the standard will increase linearly with

concentration did not fit the model as well as for the other standards and this was also reflected in the lower value of the correlation coefficient of 0.965 (Table 3.4).

The limit of detection (LOD) is found by using the error of the slope of the analyte calibration curve at a specific confidence level [41]. It was determined by using the following equation:

$$\text{LOD} = kS_a/\text{b}$$

where S_a is the error of the slope, b is the slope of the calibration graph. The value of k = 3 was used which is associated with the confidence level of 99.86% [42].

The value obtained gives the lowest concentration of an analyte that can be reliably detected by an analytical process [42]. The highest limit of detection was that of hydroquinone at 0.071 mg L^{-1}, with the lowest that of dexamethasone at 0.0066 mg L^{-1}. The other LODs where found to be in-between these values and can be found in Table 3.4.

The concentration amount below which an analyte cannot be quantified is known as the limit of quantification (LOQ). It is associated with the limit of detection and the noise of the instrument. Below the LOQ, it can be safely assumed that the analyte peak is due to noise. This value is calculated by using the following equation [43] at a noise to signal ratio of 10.

$$LOQ = 3.33 \times LOD$$

The LOQs of the standards are reported in Table 3.4, which shows that dexamethasone has the lowest LOQ at 0.022 mg L^{-1}. Therefore, the lowest concentration of dexamethasone that can be quantified by using the HPLC method reported here is 0.022 mg L^{-1}.

Jeon *et al.* reported the LODs for niacinamide and arbutin as 0.12 and 0.75 mg L^{-1} respectively [44]. The LOQ was found to be 0.41 and 2.48 mg L^{-1} for niacinamide and arbutin respectively at 260 nm detection wavelength [44]. However, Lin *et al.* developed a method for quantifying niacinamide with a LOD of 0.07 mg L^{-1} [38]. Desmedt *et al.*, had a method that could quantify a number of SLAs simultaneously.

The LOD of niacinamide, arbutin, hydroquinone and dexamethasone were determined to be 0.03503, 0.02121, 0.29935 and 0.06555 mg mL^{-1} respectively [45]. The LOQ values were 0.05560, 0.05656, 0.89827 and 0.15732 mg L^{-1} for niacinamide, arbutin, hydroquinone and dexamethasone respectively [45].

The reported LODs for niacinamide are between 0.12 [44] and 0.03503 [45] mg L^{-1}. The value obtained for the method reported here is 0.029 mg L^{-1}, making it better than that of any reported value thus far. The LOQ of other methods were 0.41 and 0.0556 mg L^{-1}. Niacinamide had a LOQ of 0.095 mg L^{-1}. This value is between the values reported in literature.

Arbutin had a LOD and LOQ of 0.010 and 0.033 mg L^{-1} respectively. Both the LOD and LOQ for arbutin are lower than reported values. This method is able to detect lower concentrations of arbutin compared with other methods.

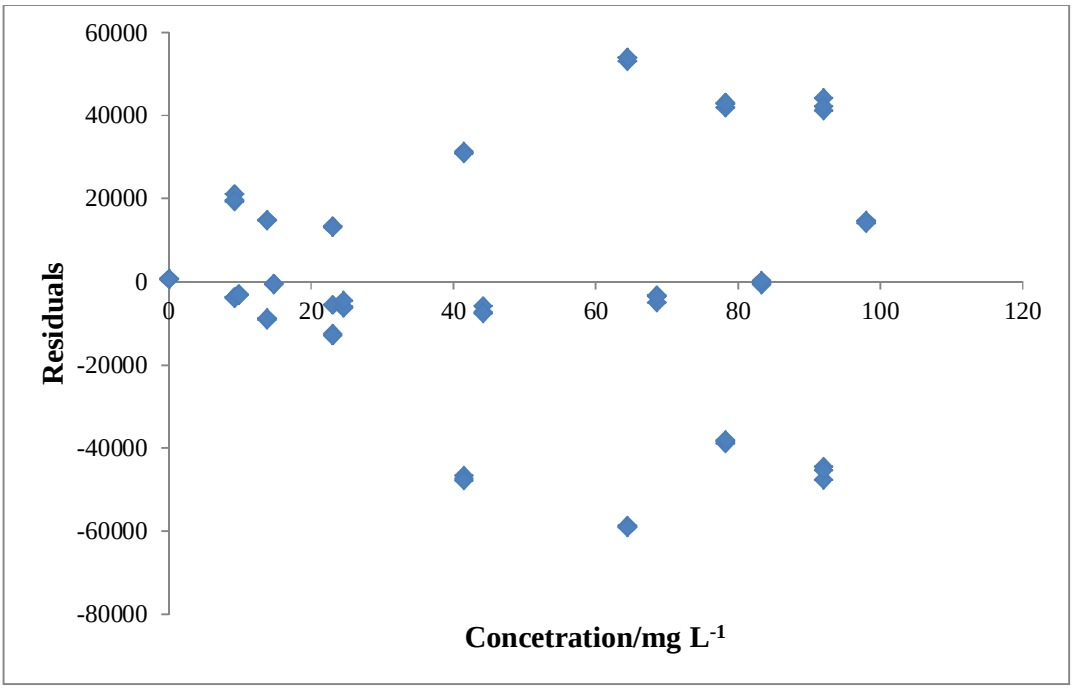


Figure 3.10 Residual plot for niacinamide displaying a random pattern.

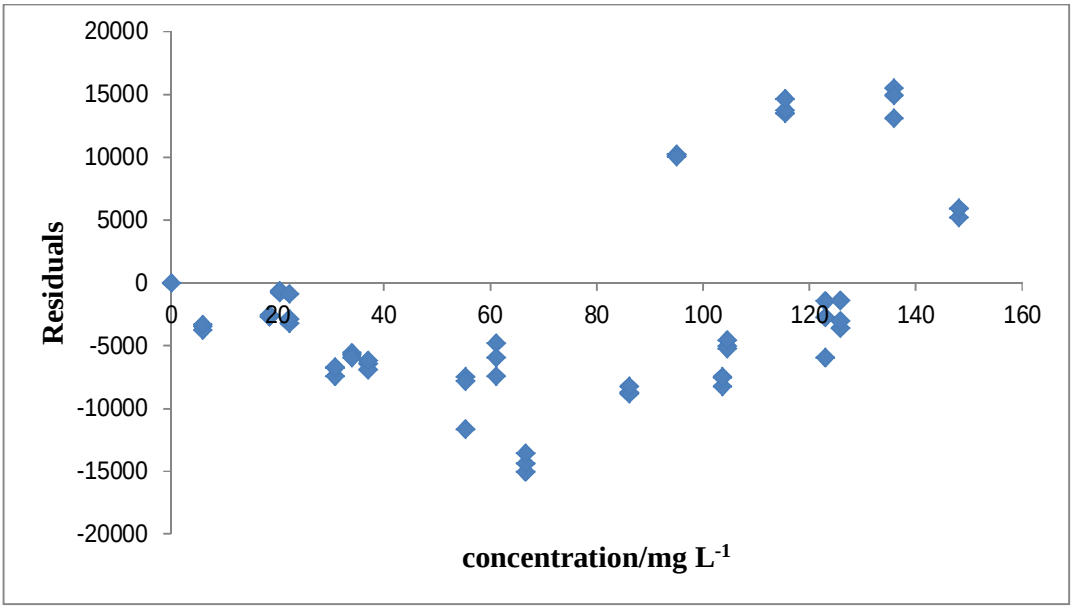


Figure 3.11 Residual plot for arbutin showing two different patterns.

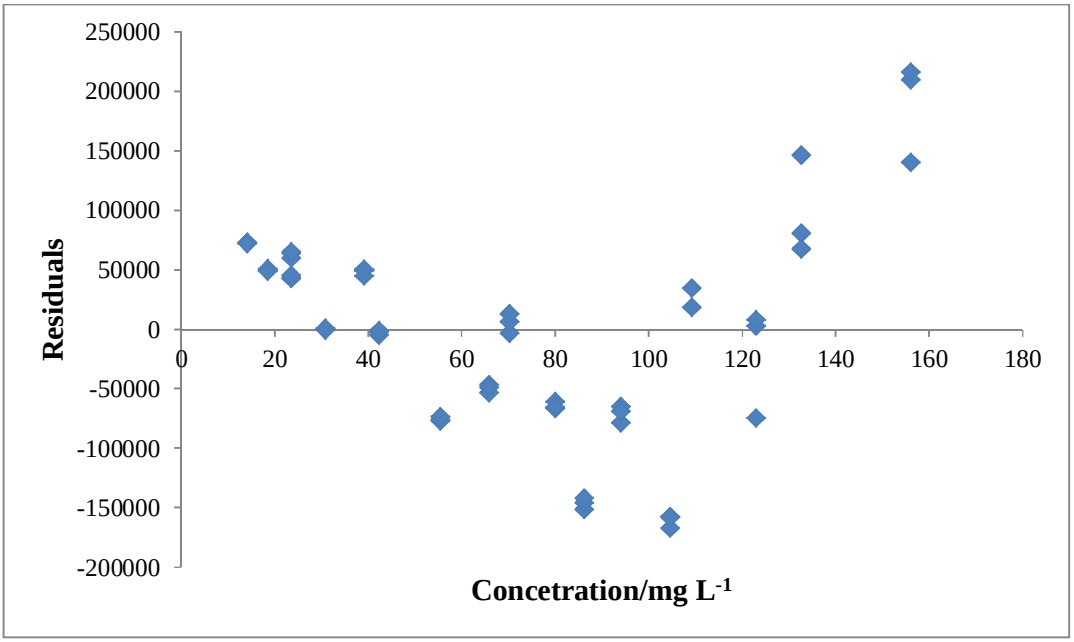


Figure 3.12 Residual plot for hydroquinone showing a U-shape.

Table 3.4 Results of regression analysis of the calibration data for hydroquinone, benzoquinone, arbutin, phenol, niacinamide and dexamethasone using HPLC. The chromatographic conditions used were: Brownlee C_{18} (100 × 4.6 mm I.D., 3 µm particle size) column, injection volume 10 µL, mobile phase methanol:water (50:50, v/v), flow rate of 1.0 mL min^{-1} and detection wavelengths of 240, 270 and 289 nm.

	Hydroquinone	**Benzoquinone**	**Arbutin**	**Phenol**	**Niacinamide**	**Dexamethasone**
Concentration range/mg L^{-1}	14.1 - 132.6	7.44 - 60.2	5.92 - 148	1.552 - 38.8	9.2 - 98	1.18 - 30. 40
Regression equation	y = 12248x	y = 53075x	y = 3693x	y = 9371.4x	y = 11819x	y = 23348x
correlation coefficient (R^2)	0.965	0.986	0.998	0.998	0.994	0.999
Standard error of slope (S_a)	287.94	468.14	12.328	27.533	112.50	51.391
Limit of Detection (LOD)/mg L^{-1}	0.071	0.026	0.010	0.0088	0.029	0.0066
Limit of quantification (LOQ)/mg L^{-1}	0.23	0.088	0.033	0.029	0.095	0.022

The LOD of hydroquinone was 0.071 mg L^{-1}. Gao *et al*. reported the LOD of hydroquinone to be 0.15 mg L^{-1} [46] and 0.2 mg L^{-1} [4], by using different sampling methods . Garcia *et al*. had the LOD at 0.08 mg L^{-1}. All the values stated here are higher than that which was obtained by the HPLC method reported here. The LOQ was also found to be 0.235 mg L^{-1}, which is in-between the values reported in literature.

For dexamethasone the LOD and LOQ were 0.0066 and 0.022 mg L^{-1} respectively. These values are much lower than those reported in literature [45].

Gao *et al*. had reported different LOD for phenol based on the method of sample treatment. The LOD was found to be 0.5 [4] and 0.2 mg L^{-1} [46]. The LOD determined here is 0.0088 mg L^{-1} which is lower than those reported in literature.

The results reported in Table 3.4 indicate that the analytes of interest are sensitive to the HPLC method reported here, and are even better than some of those reported in literature. The chromatographic conditions used for the HPLC method were: Brownlee C_{18} (100 × 4.6 mm I.D., 3 µm particle size) column, injection volume 10 µL, mobile phase methanol:water (50:50, v/v), flow rate of 1.0 mL min^{-1} and the detection wavelengths on the PDA detector were 240, 270 and 289 nm.

3.5 Determination of extraction time and solvent for samples

The mobile phase and extraction time was determined in order to get the best extraction of the SLAs in the creams. This was done by using the experimental method described in Section 2.4.5. The results obtained were plotted into a graph (Figure 3.13) in order to easily interpret them. The best extraction solvent was found to be 2-propanol, followed by methanol, and 50% methanol in 50% water was found to be the least effective solvent for the extraction.

The extraction method of analysis involves sonication of the sample for a particular period of time. By using 30 minutes and 15 minutes the extraction time was also optimised to give the best SLA extraction. It was found that the best extraction time was 15 minutes for 2-propanol and 30 minutes for both methanol and 50% methanol in water (v/v). However, the methanol extraction gave results that were similar.

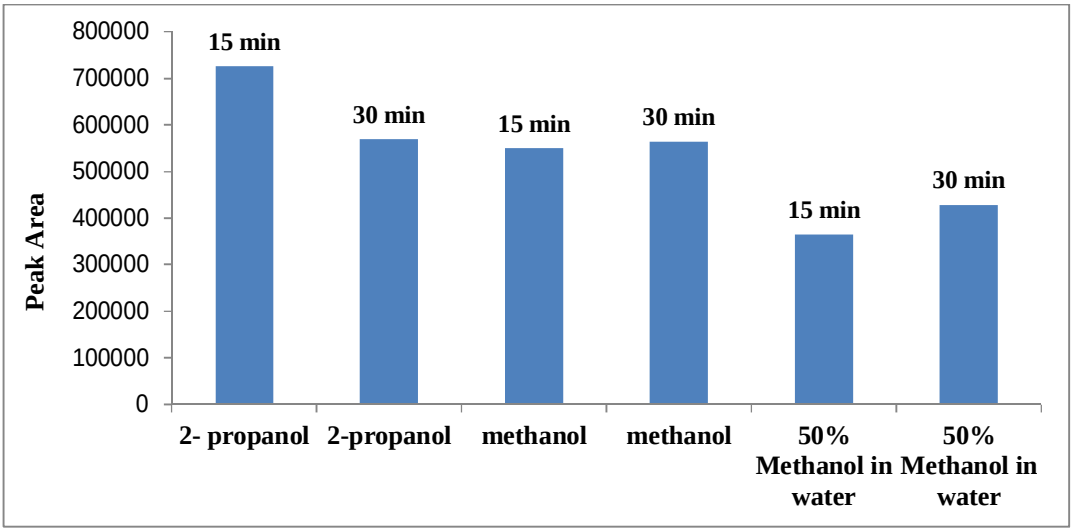


Figure 3.13 Optimisation of the HPLC extraction solvent and time using a sample containing the SLA hydroquinone. The HPLC column used was a Brownlee C_{18} (100 × 4.6 mm) column, the mobile phase 50% methanol in water (v/v) at a detection wavelength of 289 nm.

The best extraction solvent was determined to be 2-propanol with an extraction time of 15 minutes. There were problems encountered with the use of 2-propanol as an extraction solvent (discussed in Section 3.8.2), and it was therefore replaced with methanol. The extraction time was still kept at 15 minutes.

3.6 Recovery and precision

The accuracy of the method was determined by performing a recovery test. Recovery was done for different amounts of samples to eliminate any bias in terms of the sample size. A small, medium and large amount of sample was used in samples that did not contain the analyte of interest.

The recoveries of the standards were different for each standard and also for each amount. The recovery ranged from 70.1 to 227.1% with the lowest and highest recoveries coming from hydroquinone and arbutin respectively (Table 3.5). The recovery obtained should always be lower than 100% as this is the maximum amount of the sample added. Niacinamide, dexamethasone, arbutin and phenol had values which were higher than 100% recovery. A trend was observed for dexamethasone, arbutin and niacinamide which indicated that as the amount of standard is increased, the recovery becomes closer to 100%. This could be interpreted as that for best results in

dexamethasone, arbutin and niacinamide determination, higher amounts of samples must be used so as to obtain the best SLA extraction for the samples.

Phenol had the highest recovery at the lowest amount added, but this recovery was 134.3 ± 39.6% which is above the amount added. The lowest recovery for phenol was that of the medium amount of standard added. This was the case for benzoquinone as well. Hydroquinone had a low recovery of 70.1 ± 4.5% at the lowest amount of standard added (1.8 ± 0.2 mg L^{-1}) and the highest recovery at the medium amount of standard added (see Table 3.5). The method was found to be reasonably accurate and could therefore be used in the analysis of the real samples.

Table 3.5 Recovery of standards added to SLCs and analysed by the proposed HPLC method (n = 3).

Sample	Amount of standard added/mg	Amount of standard found/mg	% Recovery
Phenol	0.5 ± 0.1	0.6 ± 0.2	134.3 ± 39.6
	1.7 ± 0.1	1.6 ± 0.1	95.3 ± 5.7
	2.6 ± 0.2	2.5 ± 0.1	96.1 ± 7.0
Dexamethasone	0.6 ± 0.1	0.7 ± 0.1	110.0 ± 19.2
	1.7 ± 0.1	1.9 ± 0.1	108.8 ± 6.3
	2.6 ± 0.1	2.6 ± 0.1	100.0 ± 7.3
Arbutin	0.8 ± 0.1	1.7 ± 0.2	227.1 ± 16.0
	3.0 ± 0.1	4.1 ± 0.2	136.3 ± 2.1
	6.1 ± 0.3	6.5 ± 0.3	108.1 ± 7.3
Hydroquinone	1.8 ± 0.2	1.3 ± 0.1	70.1 ± 4.5
	4.6 ± 0.3	3.6 ± 0.2	76.9 ± 1.1
	8.3 ± 0.2	6.1 ± 0.3	74.9 ± 1.8

Benzoquinone	1.0 ± 0.2	0.9 ± 0.3	88.4 ± 31.2
	2.4 ± 0.1	1.9 ± 0.1	79.8 ± 5.1
	5.1 ± 0.6	4.2 ± 0.7	82.1 ± 3.8
Niacinamide	0.8 ± 0.1	1.0 ± 0.2	137.8 ± 33.6
	1.5 ± 0.1	1.7 ± 0.2	108.9 ± 11.9
	3.9 ± 0.1	3.6 ± 0.1	90.9 ± 1.6

Precision of the method was determined from the intra-day (same day) and inter-day (over a period of three days) analysis. Precision analysis expresses the closeness of the agreement between the average values obtained from repeated measurements. It measures the relative errors of the instrument. Precision is expressed as relative standard deviation.

The intraday precision analysis is reported in Table 3.6. The %RSD for hydroquinone, arbutin, phenol, niacinamide and dexamethasone were 0.71-1.93, 0.31-12.98, 0.1-0.61, 0.07-1.08 and 0.18-1.20% respectively. The inter-day precision was found to be 2.23-37.70, 1.60-4.20, 0.54-2.65, 0.14-1.18 and 0.43-2.58 %RSD for hydroquinone, arbutin, phenol, niacinamide and dexamethasone respectively (see Table 3.7).

The inter-day analysis for hydroquinone was high. It was noticed that in the hydroquinone standard there was a presence of benzoquinone (Figure 3.14). This could mean that the hydroquinone standard does not stay the same over time. The longer it stays, the more unstable it is and the more benzoquinone is observed which then interferes with the hydroquinone analysis. The intra-day analysis of hydroquinone was good and acceptable.

The inter-day and intra-day values were different for each SLA. The best precision was observed for phenol in the intraday analysis. The best inter-day precision was that of niacinamide. The values for intra- and inter-day were acceptable for all

standards except for the intraday of hydroquinone. To ensure precision all standards and samples containing hydroquinone were prepared and analysed at the same time so as to minimise the decomposition of the hydroquinone. The HPLC method developed gives precise results.

Table 3.6 Determination of precision by using the intra-day analysis of the standards (n = 9).

Compound	**Concentration/mg L^{-1}**	**Intra-day/mg L^{-1} ± SD**	**% RSD**
Hydroquinone[a]	2	1.87 ± 0.03	1.34
	6	5.83 ± 0.11	1.93
	24	22.52 ± 0.16	0.71
Arbutin	12.92	12.97 ± 0.13	12.98
	32.30	30.17 ± 0.43	1.44
	63.18	60.77 ± 0.97	1.6
	109.82	111.74 ± 0.35	0.31
Phenol	4.10	4.65 ± 0.03	0.61
	10.25	10.58 ± 0.03	0.26
	18.45	19.50 ± 0.02	0.10
	34.85	38.13 ± 0.14	0.35
Niacinamide	9.80	9.48 ± 0.01	0.07
	34.65	36.79 ± 0.24	0.66
	68.60	68.35 ± 0.11	0.16
	98.00	100.51 ± 1.08	1.08
Dexamethasone	3.90	3.41 ± 0.04	1.20
	9.75	8.68 ± 0.04	0.50
	27.30	25.51 ± 0.06	0.23
	39.00	38.06 ± 0.07	0.18

[a] n = 5

Table 3.7 Precision analysis of proposed HPLC method by using the inter-day analysis (n = 9).

Compound	Concentration/mg L^{-1}	Inter-day/mg L^{-1} ± SD	%RSD
Hydroquinone	18.45	14.98 ± 0.51	3.41
	42.3	37.49 ± 0.84	2.23
	94	99.18 ± 11.78	11.87
	156	182.88 ± 4.44	2.43
Arbutin	20.37	20.13 ± 0.43	2.15
	61.11	59.42 ± 1.48	2.49
	95.06	94.80 ± 2.09	2.21
	135.80	135.29 ± 2.88	2.13
Phenol	4.47	4.51 ± 0.03	0.77
	13.41	13.39 ± 0.23	1.7
	27.16	27.46 ± 0.24	0.86
	38.80	39.27 ± 0.46	1.16
Niacinamide	22.00	25.02 ± 0.12	0.46
	40.05	47.86 ± 0.29	0.61
	74.80	90.77 ± 0.25	0.27
	89.00	109.07 ± 0.49	0.45
Dexamethasone	4.44	4.49 ± 0.12	2.58
	11.70	11.70 ± 0.10	0.89
	21.28	21.04 ± 0.07	0.34
	30.40	30.73 ± 0.29	0.95

3.7 Determination of real samples

Skin-lightening creams obtained from the local shops where analysed. Qualitative analysis was done on a wide number of samples. These samples were prepared and analysed by the proposed HPLC method reported here. A total of 35 samples where qualitatively analysed with the proposed HPLC method. The chromatograms for these samples can be seen in Appendix D.

The samples which were found to contain SLAs and had good resolution of the analyte peaks were quantified. It was found that 11 samples out of the original 35 could be quantified. The results of the quantified samples can be obtained in Table 3.8.

From the samples analysed it was found that one sample contained phenol, five samples contained arbutin, and three samples contained benzoquinone and hydroquinone, whereas two samples were found to contain niacinamide. The samples containing hydroquinone were found to also contain benzoquinone because the hydroquinone hydrolysed into benzoquinone. The benzoquinone concentrations could not be quantified as they were not fully resolved at their detection wavelength of 240 nm as seen in the chromatogram in Figure 3.15. It was found that at this wavelength the peak for hydroquinone could also be observed and co-eluted with that of benzoquinone. Hydroquinone was quantified at a detection wavelength of 289 nm where benzoquinone absorption is low.

Hydroquinone has been banned in South Africa [2] and therefore the three samples containing hydroquinone are illegal in this country, but as seen from the results these products are still in circulation and sold without any medical prescription.

Phenol has been reported to have a minor degree of whitening effect and it is used as a 3-5% solution to disinfect the skin [4] and it has been banned in South Africa. Sample 1 was found to have 0.655 ± 0.026% (m/m) of phenol. This means this sample by law should not be sold in South Africa as it has been banned.

The amounts of arbutin found in the samples were found to be between 0.291-2.498% (m/m). This range is below the allowed amount of arbutin which is said not to exceed (7% (m/m)) [3].

The allowed amount of niacinamide in skin-lightening products has not yet been established. The amount of niacinamide in the samples was found to be 0.751 ± 0.025 and 1.049 ± 0.225% (m/m) for sample 27 and 26 respectively.

There were samples which were labelled to contain specific SLAs, but when those samples were analysed the specific agents stated on the label were not detected. Sample 24 was labelled to contain 2% (m/m) hydroquinone. No hydroquinone was

detected upon analysis of this sample. Although there are many other hydroquinone derivatives which are used in creams, because of the lack of standards they could not be identified. Sample 24 could maybe have one of the derivatives of hydroquinone although the label said hydroquinone.

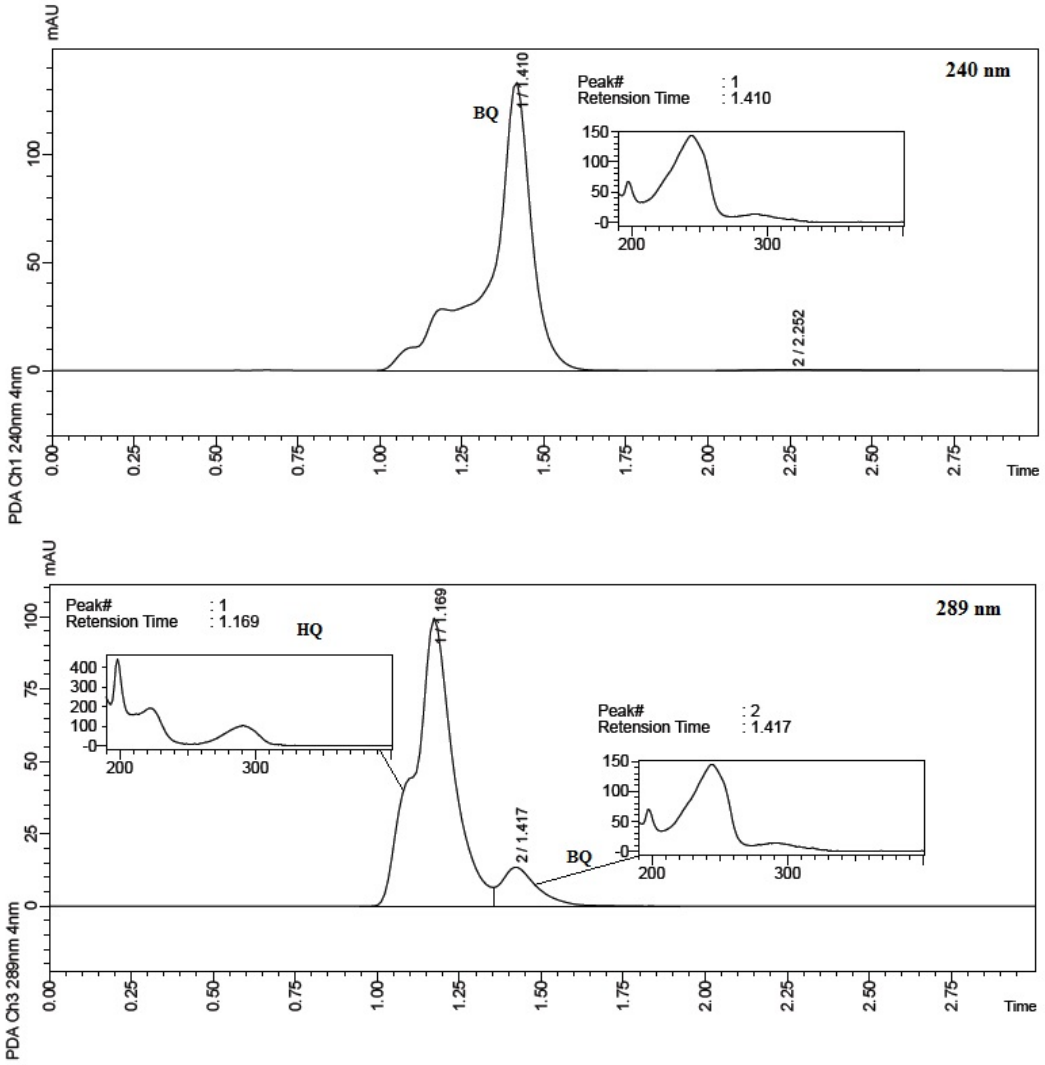


Figure 3.14 HPLC chromatogram with PDA results of a hydroquinone (HQ) standard displaying the presence of benzoquinone (BQ) at 240 nm and at 289 nm where it interferes with the hydroquinone analysis. The chromatographic conditions used were: Brownlee C_{18} (100 × 4.6 mm I.D., 3 µm particle size) column, injection volume 10 µL, mobile phase methanol:water (50:50, v/v), flow rate of 1.0 mL min^{-1}.

Table 3.8 Concentrations of SLA determined in commercial skin-lightening products available in the Durban market.

Samples	SLA detected	concentration of analyte/%m/m	%RSD
1	Phenol	0.655 ± 0.026	0.039
5	Arbutin	2.118 ± 0.039	1.820
6	Arbutin	2.498 ± 0.073	2.912
8	Arbutin	2.318 ± 0.035	1.491
9	Arbutin	2.220 ± 0.114	5.130
16	Arbutin	0.291 ± 0.026	8.900
21	Benzoquinone	NR*	
	Hydroquinone	1.255 ± 0.070	0.056
22	Benzoquinone	NR*	
	Hydroquinone	2.224 ± 0.019	0.009
23	Benzoquinone	NR*	
	Hydroquinone	1.853 ± 0.029	0.016
27	Niacinamide	0.751 ± 0.025	0.166
26	Niacinamide	1.049 ± 0.225	0.214

* NR no full resolution of the peak

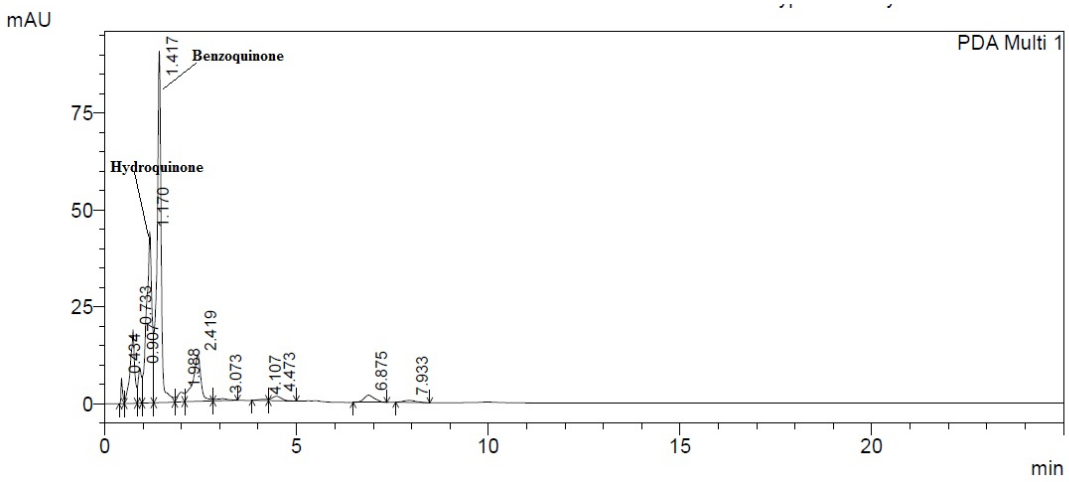


Figure 3.15 Chromatogram of sample 22 showing the co-elution of benzoquinone and hydroquinone at the wavelength of 240 nm. The chromatographic conditions used were: Brownlee C_{18} (100 × 4.6 mm I.D., 3 µm particle size) column, injection volume 10 µL, mobile phase methanol:water (50:50, v/v), and flow rate of 1.0 mL min^{-1}.

Samples 28, 29 and 30 were labelled to contain arbutin as a SLA. The analysis of the creams produced no peak for arbutin. The UV spectra of the peaks obtained in the creams did not match that of arbutin. The sample was labelled to also contain paw-paw extract.

From all the samples analysed no sample was determined to contain steroids. Although samples 5, 6, 7, 8 and 9 were analysed because they were giving patients side-effects associated with steroid use. Sample 35 was bought because it was labelled that it contained the steroid betamethasone dipropionate. Extraction and analysis of this sample with the method developed did not show the presence of this steroid. The method developed was determined to be active even for steroids by the good recovery (see Table 3.5 for recovery data) obtained for the steroid dexamethasone.

Samples 5, 6, 8 and 9 were labelled to contain a derivative of kojic acid, kojic dipalmitate. The derivative was not analysed for because of the lack of a standard. Sample 7 was labelled to contain kojic acid. Kojic acid could not be analysed because it was not detected when a standard was analysed.

The %RSD of the samples was between 0.009-8.900%. This shows that the method can be reproducible for the different samples with a good precision.

3.8 Problems encountered with HPLC

HPLC analysis produces peaks that are symmetrical. The peaks should not have shoulders if there is no co-elution with any other compound in the sample. The column performance test which is discussed in Section 3.2 was done to see the performance of the instrument as well as of the column. This test was done in between runs as time progressed during analysis.

3.8.1 Contamination of HPLC tubing

A column performance test mixture was injected and the separation of the standards was observed. However, the peaks contained peak shoulders and this problem needed to be addressed (Figure 3.16). The universal mixture contained (in their elution order): sodium chloride, benzene, toluene, ethylbenzene, isopropylbenzene, t-butylbenzene and antracine. The column was cleaned by using 100% methanol to remove anything that could have been stuck to the stationary phase.

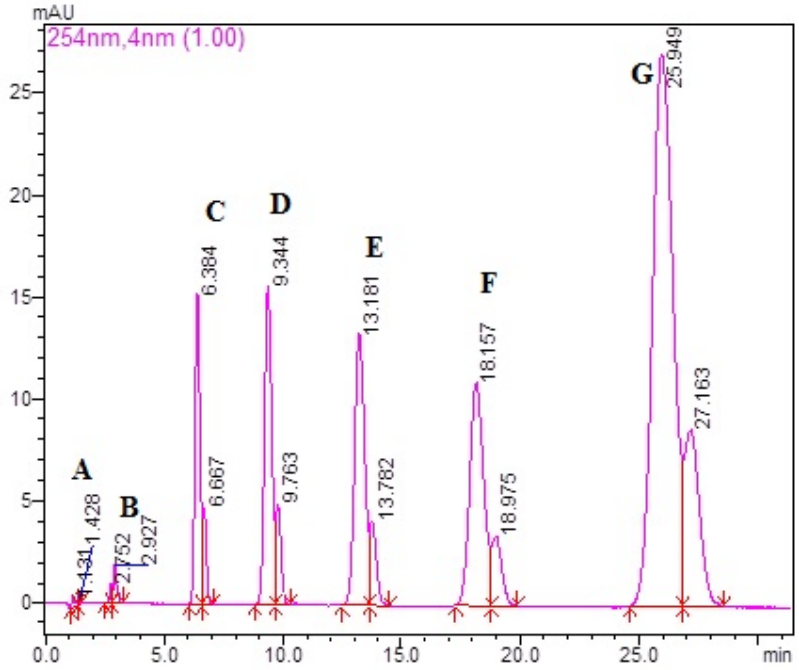


Figure 3.16 Chromatogram of universal mixture showing peak splitting with the Perkin Elmer Brownlee analytical column, Pecosphere C_{18} (150 × 4.6 mm, ID.) with a 5 µm particle size, injection volume 6 µL, mobile phase methanol:water (70:30, v/v), flow rate of 2.0 mL min^{-1} and the detection wavelength of 254 nm. The peaks are A) sodium chloride, B) benzene, C) toluene, D) ethylbenzene, E) isopropylbenzene, F) t-butylbenzene and G) antracine

A no injection blank was also run as this gives an idea of where the impurity in the peak could be coming from. The column was removed from the Shimadzu instrument and put into a Perkin Elmer series 200 instrument. The column performance test mixture was run again and the peaks were observed to be symmetrical. It was then determined that the Shimadzu instrument with the fluorescence detector was the problem. It was suspected that the tubing of the instrument was contaiminated. The tubing was cleaned by running several solvents through it. The solvents used were water containing nitric acid, methanol, 2-propanol and tetrahydrofuran (THF). The clean-up was done without installing the column. The column was put back and the peaks symmetry was resolved. The test mixture no longer had some of the components as they are volatile and evaporated, but from the observed peaks, peak symmetry was resolved.

3.8.2 Problems encountered with the injector

To make sure there was no sample carry overs, blanks were run before each sample run. The blanks used were dependant on the solvent used for the sample preparation. During the analysis of samples prepared by extraction with the solvent 2-propanol, there were peaks observed in the blank after analysis. The peak in the solvent grew bigger in intensity as the injection volume of the sample was increased. A no injection blank was done and the column was determined to be clean. The increase of the peak intensity with the injection volume increase made it safe to assume that the injector must be contaminated.

The clean-up method involved the use of different solvents to purge the injector. High injection volumes of 2-propanol were also used to clean the injector. The injector loop was removed from the instrument and cleaned by putting it in different solvents and sonication for an hour per solvent. The solvents used were methanol, acetonitrile, 2-propanol and hexane. A range of solvents were used on order to cover a range of polarities.

Sonication of the injector loop decreased the peak of 2-propanol. The peak was noticed to be low in methanol and even lower in 50:50 methanol:water, v/v. Purging the injector also decreased the peak intensity, but this did not always work. It was

noticed during the purge that the solvent was not moving from the solvent reservoir to the instrument. The purge only worked sometimes.

After several blank runs of 2-propanol which did not get the instrument clean, the instrument was changed. This meant that the fluorescence detector could no longer be used as the UFLC Shimadzu is only equipped with a PDA detector. The peaks observed in the blanks were not UV-active and did not match any of the SLAs. Although this was the case, the extraction solvent was changed to methanol as a precaution that the UFLC Shimadzu also did become contaminated.

The UFLC instrument was purged after each batch of runs to ensure no contamination. It was ensured that rinsing liquid was always available and methanol was used.

3.9 GC-MS analysis of standards

Gas chromatography coupled with mass spectrometry was used to develop a method to separate SLA in SLPs. The SLAs are mostly composed of phenolic compounds and steroids. The GC-MS used for the analysis could only detect masses up to 550 m/z. Phenolic compounds are not very volatile, therefore, to be analysed by GC-MS they had to be derivatised.

GC-MS was used for the qualitative analysis of SLAs in SLPs. A method using BSTFA as a derivatising agent was used. Pyridine was used as a solvent. A mixture containing four standards was run. The mixture contained resorcinol, hydroquinone, kojic acid, and arbutin (Figure 3.17). The total ion chromatogram obtained had many peaks. The peaks were then identified by using their mass spectra (Figure 3.18). Resorcinol, hydroquinone, kojic acid and arbutin peaks were identified.

To further confirm the GC retention times of the observed peaks, each standard was run individually. The retention time for resorcinol, hydroquinone, kojic acid and arbutin were 9.03, 9.17, 10.94 and 14.77 minutes respectively. The GC chromatogram of arbutin contained a lot of peaks which could not be explained. The individual chromatograms with their mass spectra can be obtained in Appendix E.

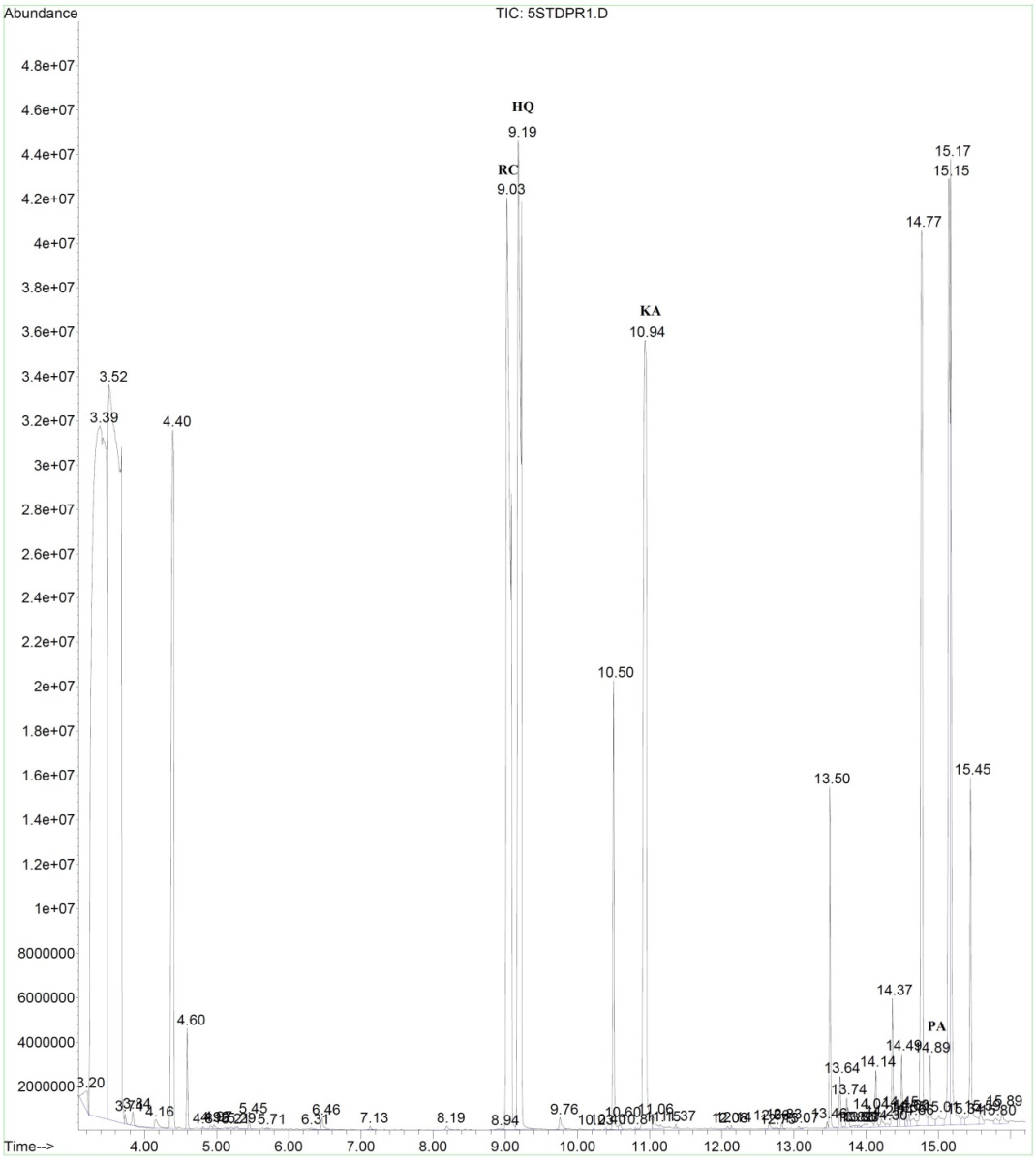


Figure 3.17 Total ion Chromatogram of a mixture containing resorcinol (RC), hydroquinone (HQ), kojic acid (KA), and arbutin (PA). The temperature programme was set as 50 °C held for 2 minutes, then an increase of 20 °C min^{-1} to 295 °C then held there for 10 minutes. The split ratio was set at 1:50. The injection volume used was 1 µL. The column used was DB5 30 metres

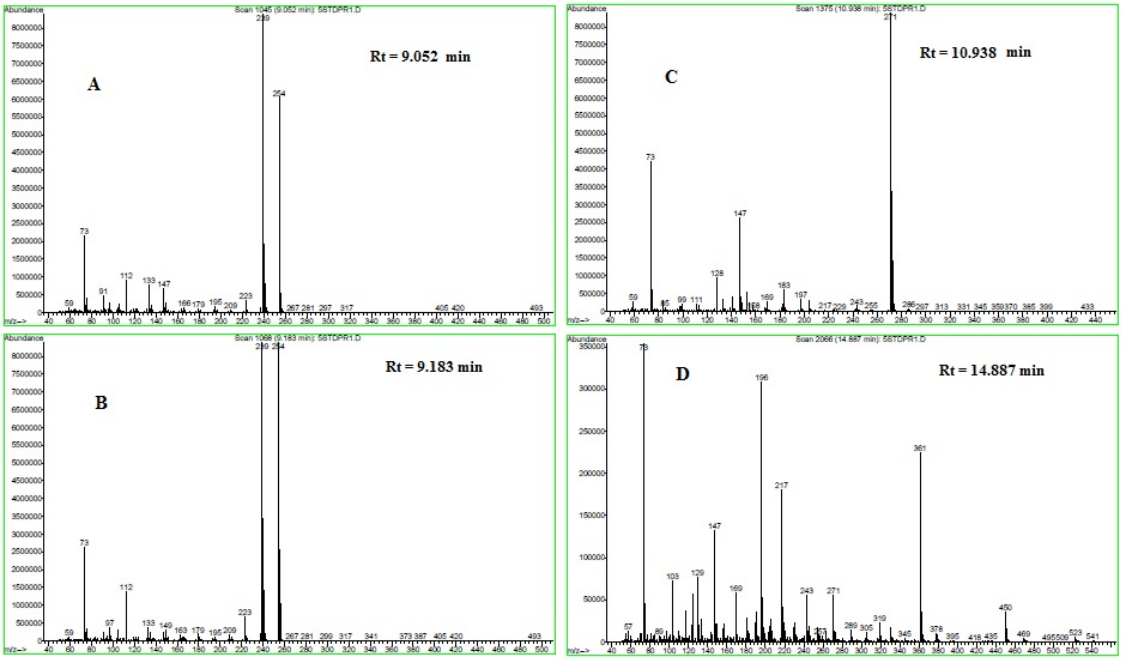


Figure 3.18 Mass spectra of peaks separated by GC-MS A) resorcinol, B) hydroquinone, C) kojic acid and D) arbutin.

Having been successful in obtaining a GC-MS chromatogram, further analysis of standards which contained an –OH group in their structures was done. The –OH group is important for the derivatisation using BSTFA.

Phenol, 4-hydroxyanisol, 4-propoxyphenol and 4-phenoxyphenol were found to have retention times of 5.391, 7.315, 8.401, and 10.402 minutes respectively. N-acetyl-L-cysteine was found to have two peaks to which the mass spectra did not coincide with its molecular mass.

There were steroids which were run but no GC-MS chromatograms were obtained. This means the steroids could not be derivatised. The steroids analysed were hydrocortisone, fluticasone propionate, clobetasol butyrate, betamethasone 17, 21-dipropionate, triamicinolone acetonide, 6α-methylprednisolone acetonide, fluocinolone acetonide, dexamethasone, beclomethasone dipropionate, and momentasone fuorate.

Different samples were prepared and analysed by the method described in Section 2.5.2. It was found that none of the samples contained the SLAs of interest. The GC chromatograms of the samples can be obtained in Appendix E.

Chapter 4

Conclusions

In this study a number of skin-lightening preparations bought in the market in Durban were investigated. Reversed-phase high performance liquid chromatography (HPLC) with both ultraviolet and fluorescence detection was used. Phenolic and steroid standards were used to qualitatively identify the active ingredients. A method was developed for the extraction of SLAs in the creams and also for their quantification by means of HPLC.

A total number of 35 skin-lightening preparations were qualitatively analysed, and it was found that 11 samples had skin-lightening agents based on the standards that were used. Arbutin, phenol, benzoquinone, hydroquinone and niacinamide were the active ingredients found in some of the skin-lightening preparations. An external method of calibration was used to prepare the calibration plots for hydroquinone, arbutin, phenol, dexamethasone and benzoquinone. All standards had a linear calibration curve over the concentration ranges tested. The correlation coefficients for the different calibration curves were all above 0.95. The hydroquinone standard had the lowest correlation coefficient at 0.965 whereas dexamethasone had the highest at a value of 0.999. The residual plots showed random distribution for dexamethasone, benzoquinone, phenol and niacinamide. Arbutin exhibited a systematic error at low concentrations and random errors at high concentrations. Hydroquinone had a U-shape pattern. This meant that for hydroquinone linear regression was a poorer choice. This is consistent with the low correlation coefficient obtained and the fact that it hydrolyses to benzoquinone.

The limits of detection and quantification were calculated and hydroquinone, benzoquinone, arbutin, phenol, niacinamide and dexamethasone had limits of detection of 0.071, 0.026, 0.010, 0009, 0.029 and 0.007 mg L^{-1} respectively. The limit of quantification for hydroquinone, benzoquinone, arbutin, phenol, niacinamide, and dexamethasone were 0.235, 0.088, 0.033, 0.029, 0.095 and 0.022 mg L^{-1} respectively.

This values were found to be in line with literature with some even better than those reported in literature. Recovery tests were used to test the accuracy of the method.

The recoveries ranged from 70.1 to 227.1% with the lowest and highest recoveries coming from hydroquinone and arbutin respectively. The recovery obtained should be close to 100% as this is the maximum amount of the sample added. Niacinamide, dexamethasone, arbutin and phenol had values which were higher than 100% recovery. Intra-day and inter-day analysis was done to determine the precision of the method. The method was found to be precise.

The samples which were found to contain skin-lightening agents and had good resolution of the analyte peaks were quantified. From the samples analysed it was found that one sample contained phenol, five samples contained arbutin, and three samples contained benzoquinone and hydroquinone, whereas two samples were found to contain niacinamide. The samples containing hydroquinone were found to also contain benzoquinone because the hydroquinone hydrolysed into benzoquinone. The benzoquinone concentrations could not be quantified as they were not fully resolved at their detection wavelength of 240 nm. It was found that at this wavelength peaks of hydroquinone could also be observed and co-eluted with the benzoquinone. The %RSD of the samples was between 0.009-8.900% which is acceptable. This shows that the method can be reproducible for the different samples with a good precision.

GC-MS was able to separate some phenolic standards, but analysis of the samples did not detect any SLAs in the samples.

There is a need for strict regulations with the sale of SLPs and samples containing phenol and hydroquinone, as found here are not allowed in South Africa and their sale should be stopped.

4.1 Future plans

The analysis of the samples should be done with liquid chromatography - mass spectrometry so that all peaks can be identified based on the mass spectra. There is a need to improve the GC-MS method and the sample preparation so that SLAs can be extracted and analysed. There are always new SLPs in the market, so further work needs to be done to cover more of the SLAs found over the counter.

Section 2

Analysis of clays used by Xhosa and Zulu women on their faces

Chapter 5

Introduction

The skin is the largest organ that humans have, and it is important that it is protected from the harsh climate that we have in South Africa. In particular in Durban, South Africa, the solar irradiance is high whether it is during winter or summer [47]. With the beaches in Durban people tend to spend a lot of time outdoors enjoying the hot weather. The skin needs to be protected from solar ultraviolet radiation because it causes a number of detrimental effects. Regardless of the skin colour of a person, the skin needs to be protected.

5.1 Solar UV radiation

Sunlight is composed of ultraviolet (UV), visible and infrared electromagnetic radiation [48]. UV solar radiation is in a range between 290 to 400 nm and it is just shorter than visible light (400-700 nm) [48]. UV is divided into UVA (320-400 nm), UVB (280-320 nm) and UVC (200-280 nm). UVC does not reach the earth as it is blocked by the stratospheric ozone. The ozone absorbs most of the radiation with wavelengths below 310 nm [49]. UVA and some of the UVB however do reach the surface of the earth. The amount of UVA and UVB that reaches the earth is enough to cause biological consequences to the skin and the eyes [48].

UV solar radiation is predominantly composed of UVA. UVA penetrates the deepest into the skin. It generates radicals, such as reactive oxygen species, which can damage membranes, DNA and other cellular constituents [49]. UVA is weakly carcinogenic and causes aging and wrinkling of the skin [48]. UVA is an important toxic factor from UV solar radiation [49].

UVB solar radiation is absorbed into the skin. It is absorbed by DNA and proteins and it is enough to damage and kill unprotected cells [49]. UVB is more phototoxic compared to UVA [50]. It has been proven to be most effective in inducing skin cancer in animals [50]. In addition to this, UVB solar radiation also produces burns and erythema [48].

The main cause of cancer has been the continuing exposures to sunlight [50, 51]. It is therefore important that we are protected from the solar UV radiation. Some of the ways to protect the skin from solar radiation include the use of a broad-spectrum sunscreens, wearing protective clothing and avoiding exposure to the sun. The daily use of sunscreens to avoid acute skin damage is important as repeated damage to the skin may lead to photoaging and skin cancer [50, 51].

There are many sunscreens which have been developed throughout the years. The research into safe and effective sunscreens is growing. There is a need to find sunscreen agents that will protect the skin from both UVA and UVB. Sunscreens that seem to be most effective and safe on the skin are very expensive, therefore, there is a need to develop cheap and effective sunscreen products.

Women in South Africa spend a lot of time outside in the sun. This is because of households chores which could include fetching water from the river and collecting firewood. In the cities women also spend a lot of time outdoors working in the garden, selling as street vendors, and also working for the municipality whereby they sweep the city streets and collect garbage in the sun. These women are mostly affected by the sun as they work in the sun the whole day. The women have been using red and white clays on their faces to protect the skin from the harsh solar UV radiation. The red and white clays are very cheap and can be bought from street vendors at about R10 per packet for a quantity of about 1 kg. These clays can last them for long periods of time. The clays are not only used as sunscreens. In some cultures in South Africa clays are used during the initiation ceremony when boys are said to go into manhood. The boys in the Xhosa tradition are covered in white clay all over their bodies during the ceremony. Clays are also used for decorative purposes. This can be seen in Xhosa women who paint dots on their faces with these clays.

5.2 What are clays?

Clay is a deposit which is distributed extensively over the surface of the earth [52]. Clays of different purity can be found everywhere. They are rarely found in their pure composition, rather they can be found in large portions of impure clay [52]. The

definition was first formalised in 1546 by Agricola and has been revised many times since then [53]. Clays are produced by the decomposition of various rocks [52].

The term clay has been said to refer to naturally occurring material which is primarily composed of fine-grained minerals [54, 55]. This definition defines clays as naturally occurring and therefore would exclude all synthesised materials [53]. They are primarily inorganic materials excluding peat, muck, and some soils that contain large quantities of organic materials [53, 55]. Its characteristics are that it is generally plastic at appropriate water contents and hardens when dried or fired [54].

Clay has also been defined purely on the basis of particle/crystallite size, without compositional association [56, 57]. According to Hurst and Pickering, all definitions of clay have the common denominator of clay consisting of fine material which have particles finer than 74 μm [56].

The fine graininess of clay cannot be quantified because a specific particle size is not a property that is universally accepted by all disciplines [53]. Most geologists and soil scientists use particle sizes less than 2 μm whereas sedimentologists use 4 μm and chemists use 1 μm for clay particle size [53, 56, 57]. Engineers use 74 μm as the maximum particle size [56]. Clay is a term used by sedimentologists to denote a specific grain size only [53, 57].

From the chemical and mineralogical point of view clay can be defined as a finely divided rock that is composed essentially of clay minerals, usually together with smaller amounts of other crystalline and amorphous minerals besides organic matter [54, 56, 57]. Clay minerals are composed of crystalline hydrated aluminosilicates and normally contain considerable amounts of iron, alkaline metals and alkaline earth metals [56, 57].

5.3 Characteristics of clays

For a compound to be considered as clay the Association Internationale Pour L`étude des Argiles (AIPEA) have put up the following criteria:

1) Natural, 2) fine-grained, 3) plasticity at appropriate water content, 4) hardening on drying and firing, and 5) containing phyllosilicates as principal constituents [54].

Pure clay is composed of silicates of aluminium and when found in large quantities it is called kaolin [52]. Kaolin typically has the formula of $2SiO_2$, Al_2O_3, $2H_2O$. The pure composition of kaolin would be SiO_2, 41.7%; Al_2O_3, 39.2%; H_2O, 13.7% [52, 58]. Impure clays contain feldspar such as orthoclase K_2O, Al_2O_3, $6SiO_2$ or balbite Na_2O, Al_2O_3, $6SiO_2$ and there will be also present mica having the formula K_2O, $3Al_2O_3$, $4SiO_2$ and quartz SiO_2 [52].

5.4 Examples of clays and their functions

Kaolinite, smectite, palygorskite and sepiolite are the world's most important and useful industrial clay minerals [59]. Their applications in industry are different. Their structure and composition give them different physical and chemical properties [59]. Although kaolins, smectites and palygorskite-sepiolite have octahedral and tetrahedral sheets as their basic building blocks; their structures and composition are different [59]. The difference in the clay properties of the clay minerals is based on the composition and arrangement of the octahedral and tetrahedral sheets [59]. This in turn affects their difference in applications.

Some of the properties of the clay minerals that affect their applications are the particle size, shape and distribution [59]. Surface chemistry, surface charge and surface area are important properties of the clay minerals [59]. The application of interest will determine the many more properties of the clay minerals to be considered. Clays and clay minerals in their applications are not just inert components but they are functional in the system [59].

5.4.1 Kaolins

Kaolin is usually white in colour. It has low base exchange capacity and minimal layer charge because it has very little substitution in the structural lattice [59]. The kaolin group of clay minerals is mostly composed of the mineral kaolinite (Figure 5.1) [56, 59]. The kaolinite crystals are pseudo-hexagonal along the plate, some larger books and vermicular stacks [59]. The geological conditions under which the kaolin is formed, the total mineralogical composition of the kaolin deposit, and the physical and chemical properties, are some of the important factors which influence their uses in

industry [59]. Kaolin deposits can be residual, sedimentary or hydrothermal and they all have different properties [59].

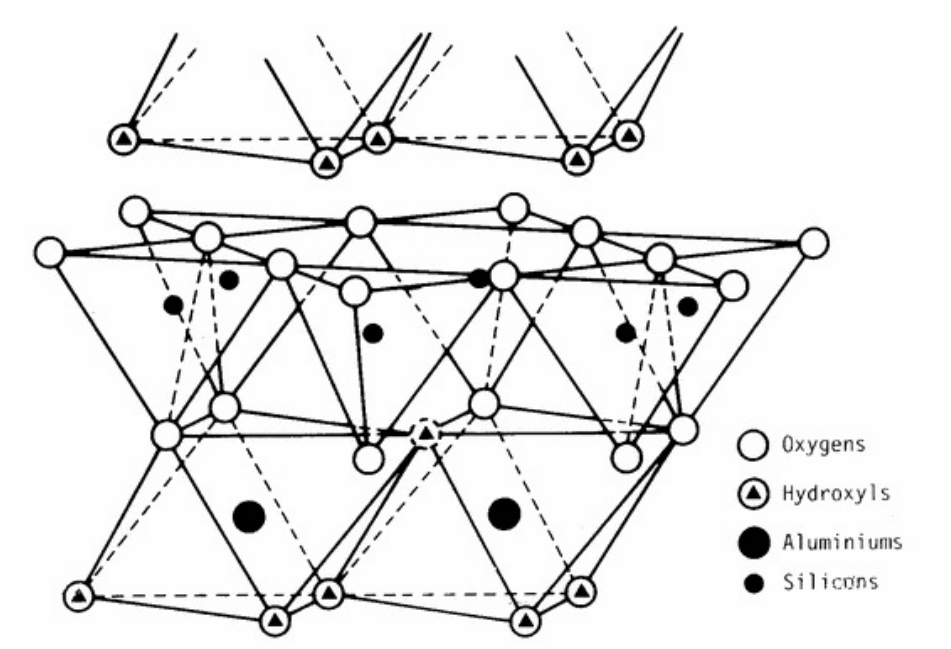


Figure 5.1 Diagram of kaolinite [60].

The largest use of kaolin is in the coating of paper [59]. Some of the other traditional uses include but are not limited to: cracking catalysis, pharmaceuticals, adhesives, crayons, ceramic ingredient and fibre glass [59].

5.4.2 Smectites

The smectite group of clay minerals has several clay minerals, with sodium montmorrillonite and calcium montmorrillonite being the most important in industry [59]. They can have substitutions in both the octahedral and tetrahedral sheets therefore creating a charge imbalance in the 2:1 layer (see Figure 5.2) [59]. In the octahedral sheet, magnesium and iron substitute for aluminium [59]. In the tetrahedral sheets, alumina substitutes for silica [59].

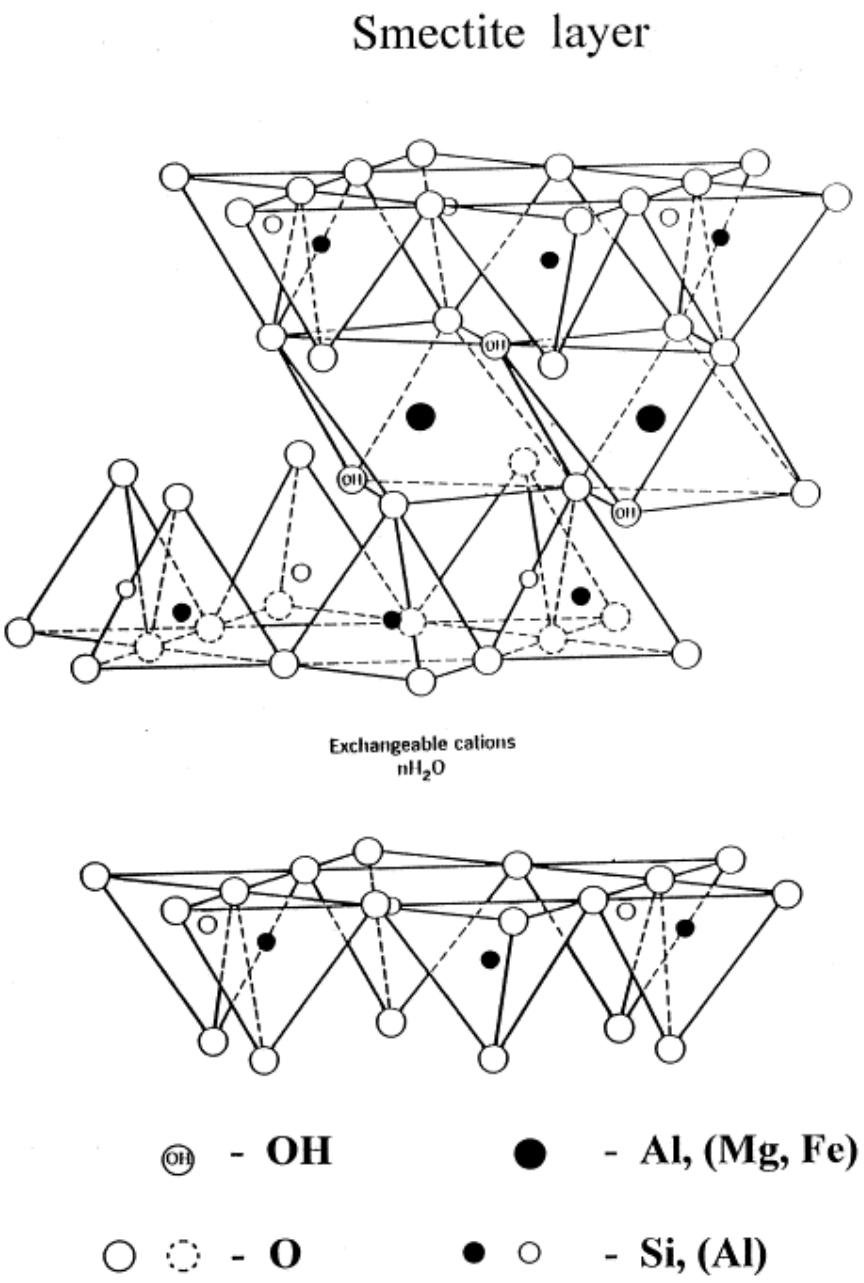


Figure 5.2 Diagram of smectite layers [61].

Traditional applications of smectites include but are not limited to the following: pelletizing iron ore, bleaching clay, cosmetics, pharmaceuticals and animal feed bonds [59]. The applications are affected by the high layer charge, the thin flakes, high cation exchange capacity, very fine particle size and the high surface area, which determine the chemical and physical properties of the smectite group of clay minerals [59].

5.4.3 Palygorskite-sepiolite

Palygorskite (Figure 5.3A) is a hydrated magnesium aluminium silicate mineral [59]. It is almost chemically and structurally identical to sepiolite (Figure 5.3B) except that sepiolite has a slightly larger unit cell [59]. Their applications include: suspension fertilizers, paper, paint, drilling fluids and as anticracking agents [59]. The major controlling physical property is its elongate shape [59].

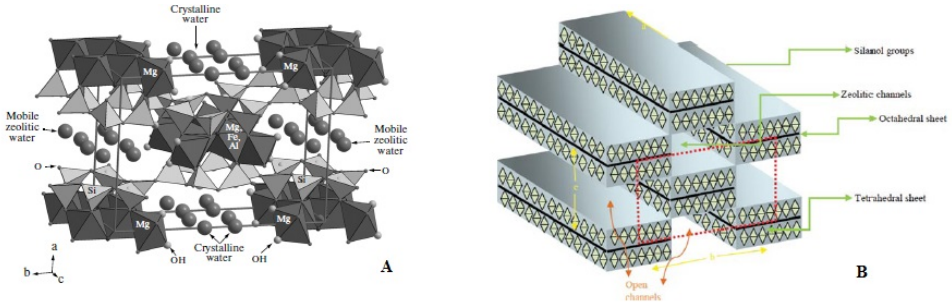


Figure 5.3 A) Structural scheme of palygorskite [62] and B) structure of sepiolite [63].

5.5 Use of clays in cosmetics or for skin treatment

The process of using clays for medicinal purposes has been around for many years. It has been reported that there are indications that ochres mixed with water and different types of mud have been used to soothe irritations, cure wounds, as a method of cleansing the skin, etc., by *Homo Erectus and Homo Neanderthalensis* [64]. This process of using clay minerals has been proven in ancient Egypt and Mesopotamia [64]. Clay minerals are used for therapeutic purposes [64]. They are used in aesthetic medicine, pharmaceutical formulations and in spas [64].

Talc, palygorskite, smectites and kaolin are clays minerals that are used in pharmaceutical formulations. These clays have a high specific area and sorptive capacity, chemical inertness, rheological properties and low or no toxicity for the patients which make them useful in pharmaceutical formulations [64].

Clay minerals are used as what are called dermatological protectors. These are ointments, creams and powders used to protect the skin. Kaolinite, smectites and talc are capable of adhering to the skin and form a film which protects the skin against

external chemical and physical agents. This is due to their absorbent power. They also absorb the skin's secretions which results in having a refreshing action, creating a large surface for their evaporation which in turn promotes a gentle antiseptic action as it creates a water-poor medium, which is unfavourable for the development of bacteria [64]. This action is reinforced by the mineral's capacity to absorb dissolved and suspended substances, such as greases, toxins and even bacteria and viruses [64].

Clays have a high adsorbency level of toxins, grease, etc,. and as a result are used as active ingredients in cosmetics and face masks [64]. They are also used as antiperspirants and to give the skin opacity, remove shine and cover blemishes [64]. Clay minerals are widely used in spas. It is necessary to study the presence of toxic elements in the clay-water mix used in spas such as Hg, Pb, As, Sb, Cu, Zn, Cd, Se, etc. [64]. It is very important that their bioavailability is also studied to avoid possible intoxications [64].

The UV protection of clays has been investigated by Hoang-Minh *et al*. [65]. They investigated different clays in comparison to a sunscreen of SPF 20 [65]. It was shown that clays could absorb UV radiation without a need for additives [65]. The clay creams studied showed higher UV-transmission values than those of a commercial sunscreen of SPF 20 [65]. The clays that displayed high UV-protection potential were red in colour and were not well suited for sunscreen formulations [65]. Combination of the clays with a material that has favourable sunscreen properties was also investigated [66]. Clays combined with *Ganoderma pfeifferi* extract were examined based on their proctection ability against UV solar radiation [66]. The results showed that the mixtures had high UV-protection potential when compared with creams which did not contain the plant extract [66].

5.6 Aim and objectives of the project

This project focused on the use of clays by women in South Africa on their faces. Beauty is one of the most important things to every woman; everyone wants to look beautiful and presentable. To a woman her skin, especially her face determines how people think about her when it comes to beauty, it is for this purpose that cosmetics are important. South African women have been wearing red and white clays on their faces as a form of cleansing product and also as a sunscreen. In this project the composition

of those clays was determined with respect to the minerals present in the clays and their influence on skin. The particle sizes of these clays were also determined to see if this has an effect on their light reflecting properties. In addition, the SPF of these clays was determined.

5.6.1 Objectives

- The composition of the clays was determined by means of X-ray fluorescence.
- The composition of the clays was further identified by powder X-ray diffraction.
- Confirmation of the phases and presence of the minerals in clays was done by means of infrared spectroscopy and thermogravimetric analysis.
- Transmission electron microscopy was used to see the shape of the minerals in the clays.
- The particle sizes of the clays were determined to see how suitable the material is for sun protection.
- The sun protection factor of the clays was determined to see how effective these clays are as sunscreens.

Chapter 6 will describe the experiments performed to characterise the red and white clay. The results of these experiments will be presented and discussed in Chapter 7.

Chapter 6

Experimental

Characterisation of these commonly used clays is important in order to determine their composition and thereby understand the way they function as sunscreens. The composition and quantity of the elements in the samples was determined by using X-ray fluorescence (XRF). The samples were further analysed by means of X-ray diffraction (XRD) to determine the minerals present and their phases. Infrared (IR) spectroscopy was also used to further confirm the identity of the clay minerals present in the samples. Further confirmation of the clay minerals in the samples was obtained by thermogravimetric analysis (TGA). The morphology of the samples was determined by transmission electron microscopy (TEM). The particle size distribution of the samples was obtained by laser light scattering as the particle size affects the effectiveness of the clays in their use as sunscreens. Additionally, the SPF values of the clays were determined at the Medunsa Photobiology laboratory.

6.1 X-ray fluorescence

X-ray fluorescence (XRF) is a technique used for the elemental analysis of a material. Different materials can be analysed in the detection range of parts per million to 100% [67]. The materials can be in liquid, powder, solid, filtered or other form [67].

In XRF analysis materials are exposed to short wavelength X-rays or gamma rays [67]. Ionization occurs when an atom is exposed to radiation with energy greater than its ionization potential. Ionization is the process whereby a material is exposed to radiation and it undergoes ejection of one or more electrons from its atoms [67]. When tightly held electrons are expelled, the electron structure of the atom becomes unstable [67]. There is a hole left behind, which is filled by the electrons in the higher orbital which fall into the lower orbital [67]. Energy in the form of a photon is released and this energy is equal to the energy characteristics of the atoms present [67].

When absorption of higher radiation energy results in the re-emission of lower radiation energy the occurrence is called fluorescence [67]. Measuring the energies of the radiation emitted by the sample makes it possible for the qualitative determination of the elements present in the sample [67]. Quantitative analysis of the elements present in the sample is done by measuring the intensity of the energies emitted by the samples [67].

6.1.1 Sample analysis

Two clay samples (red and white) were submitted to the School of Agriculture, Earth and Environmental Sciences Geological Department for analysis.

6.2 X-ray diffraction

Materials such as clays are made of atoms in particular arrangements. The knowledge of how the atoms are arranged into crystal structures and microstructures is the foundation of the understanding of the structure and properties of materials [68].

Powder X-ray diffraction (XRD) was done to determine the chemical composition of the clays. This technique is powerful in phase identification and chemical analysis of the samples [69]. The technique involves the use of diffraction of X-rays. To date, diffraction experiments have been used to gain knowledge on the spatial arrangements of atoms in materials [68].

One of the most important uses of powder XRD is for identifying unknown crystals in a sample of material. This is because every crystal will always produce a unique pattern and in a mixture of substances the obtained pattern will be the combination of individual crystals in the substance. This is done by matching the positions and intensities of the peaks in the sample diffraction pattern to a known pattern of peaks from a standard sample or from calculations [69]. The sample diffactogram can be matched by finger-printing. The International Centre for Diffraction Data (ICDD, formerly the Joint Committee of Powder Diffraction Standards, JCPDS) holds a database of diffraction patterns from more than one hundred thousand inorganic and organic materials [69].

Software packages are available to identify peaks in the experimental diffraction pattern and then search the ICDD database to find candidate materials. This is good

when a sample contains a mixture of phases and their chemical combinations are uncertain. This has made XRD an important tool for identifying, quantifying and characterising minerals in composite mineral accumulations [70].

6.2.1 Instrumentation

The instrument used was a XRD Bruker D8 Advance, equipped with a Cu Kα (λ= 1.540 Å) source. Diffractograms were collected over the 2θ range of 15- 90º.

6.3 Infrared analysis

The bond length between two atoms reported is the average bond length [39]. This is because bonds in a molecule behave like vibrating springs, vibrating with both bending and stretching motions [39]. The bending vibration does not occur along the line of the bond and it changes the bond angles [39]. The stretching vibration changes the bong length and occurs along the line of the bond [39]. The bending and stretching vibrations occur with a characteristic frequency of a given bond [39].

Infrared radiation has frequencies which correspond to the frequencies of the bending and stretching vibrations [39]. When a molecule is exposed to radiation of a frequency that matches the frequency of one of its vibrations, it absorbs the energy [39]. The bonds of the molecule will stretch and bend more due to the energy absorbed [39]. The wavenumbers of the energy absorbed are determined to give the kinds of bonds present in the compound [39].

The spectrum obtained is a plot of the percent transmission of radiation versus the wavenumber of the radiation transmitted [39]. The instrument used to obtain the spectrum is called an infrared (IR) spectrometer.

A Fourier transform IR (FTIR) spectrometer was used to analyse the clays. This type of instrument has higher sensitivity and speed compared with the conventional IR spectrometer [39]. It simultaneously scans through all frequencies instead of sequentially scanning them; therefore this makes it more sensitive [39]. IR is often used in conjunction with other techniques to identify a compound. IR is specific to the functional groups and bonds on a molecule. There are libraries of compounds which can be used to identify the material of interest.

6.3.1 Instrumentation

The FTIR spectra of the clays were obtained with a Perkin Elmer Precisely FTIR spectrometer fitted with a Universal attenuated total reflectance (ATR) sampling accessory.

6.4 Thermogravimetric analysis

Thermogravimetric analysis (TGA) is a technique used to measure the mass of a sample as a function of temperature [71]. It is used to determine the fraction of volatile components in a sample and also determine the thermal stability of the sample by monitoring the weight change as the sample is heated. The sample is in a controlled atmosphere such as air or in an inert atmosphere such as helium or argon [72].

The mass change of the material associated with the degradation and transition process can be quantified by using TGA [72]. TGA data provides characteristic curves for a given material because each material has its own unique pattern of reaction at specific temperatures [72].

6.4.1 Instrumentation and conditions

The instrument used was the Universal TA Instruments Q600 thermal analyser.

The samples contained in alumina pans were run under air at the flow rate of 50 mL min^{-1}. They were heated from room temperature to 1000 ºC at a heating rate of 10 ºC min^{-1}.

6.5 Transmission electron microscopy

Transmission electron microscopy (TEM) is a tool that has been used widely to characterise different materials. It is used to give information on the particle size, size distribution and the morphology of materials. Some of the materials that have been analysed by means of TEM are metals, ceramics, polymers, alloys, glasses, semiconductors, wood, textiles and concrete [73].

TEM uses high energy electrons in a focused beam. The beam of electrons passes through a material, therefore scattering electrons. Electromagnetic lenses are used to

focus the scattered electrons into an image. Therefore this makes TEM have higher resolution compared with light-based imaging techniques.

6.5.1 Instrumentation

The instrument used for TEM analysis was a high-resolution transmission electron microscopy (HR-TEM, JOEL JEM-1010, Tokyo, Japan).

6.6 Particle size distribution

Clays are made of particles and the size of the particles is important. The particle size distribution is important because it determines the possible applications and effectiveness of the material.

6.6.1 Instrumentation

The instrument used was a Malvern Instruments, Mastersizer 2000. The instrument uses diffraction of laser light to measure the particle sizes. A small sample of the clays was directly placed into the water of the instrument. The clay particles are then dispersed in the water. As the laser light passes through the dispersed particulate sample, light is scattered [74]. Measuring the intensity of the scattered light allows for the size of the particles to be calculated based on their scattering pattern [74] and the particle size distribution of the sample is obtained.

6.7 Sun protection factor

The sun protection factor (SPF) is used as an indicator of the efficiency of a sunscreen product to protect the skin against UVB radiation [75]. The food and drug administration (FDA) of the USA defines SPF as the UV energy required to produce a minimal erythema dose on protected skin divided by the UV energy required to produce minimal a erythema dose on unprotected skin [76]. Protected skin is that which there has been application of a sunscreen at 2 mg cm^{-2}, and unprotected skin is skin to which no sunscreen product has been applied [76].

The SPF of the clays (red and white) was determined by the University of Limpopo Medunsa Campus, Department of Pharmacy and Photobiology Laboratory. An in vitro SPF testing procedure developed by Diffey and Robson was used to analyse

the clay. The Optometrics SPF 290 analyser instrument was used. The wavelength range of analysis was 290 to 400 nm.

The results of the experiments described in this chapter are presented and discussed in Chapter 7.

Chapter 7

Results and discussion

The clay samples obtained from the local river bank next to Inanda district in Durban were analysed by using different techniques. It is of great importance to know the composition of the clays as these products are continually used by many South African women as a beauty care product and as a sunscreen. Once the composition of the clays is known, their efficacy and any toxicity can be assessed. The experiments conducted for the analysis of the clays are described in Chapter 6.

7.1 Use of the clays

Two clays are typically used, a clay which is white in colour and one which is red in colour. The white clay is locally known as *umcaku* (IsiZulu/isiXhosa) and the red clay is called *ibomvu* (isiZulu/isiXhosa) [77]. The clays are used individually and the colour of the clays used by the women is a personal preference. The clays for application are prepared by mixing the clay (100 g) with water (125 mL) and glycerine (20 mL) [77]. This produces a paste which is the applied to the face. The appearance of women wearing the clays is shown in Figure 7.1.

Figure 7.1 South African women working in construction with applied white and red clays on their faces.

7.2 XRF analysis

The major elements in the clays were determined by means of XRF. The results are presented in Table 7.1 as weight percentages of the oxides. The major oxides present were found to be those of silicon and aluminium. The red clay was found to have a substantial amount of iron oxide compared with the white clay. This was expected as the iron is responsible for the red colour of the clay. The clays were found to have some titanium oxide which is used in sunscreens as a physical blocker (that is, to scatter and reflect incident radiation). The clay samples also contained some other oxides in smaller amounts: these are MnO, Na_2O, MgO, K_2O, P_2O_5 and Cr_2O_3. The loss on ignition was 10.58 and 24.46 wt% for the white and red clay respectively. Loss on ignition (L.O.I) gives the loss of moisture and organics in the sample upon ignition. The results show that the red clay contained more organics compared with the white clay.

Table 7.1 Major oxides present expressed as mass percentages of the white and red clays as determined by XRF.

	SiO_2	Al_2O_3	Fe_2O_3	MnO	MgO	CaO	Na_2O	K_2O	TiO_2	P_2O_5	Cr_2O_3	NiO	Total	L.O.I
White clay	63.4	32.85	0.49	0.000	0.200	0	0.02	1.13	1.525	0.02	0.086	0	99.7	10.6
Red clay	46.7	33.50	17.81	0.025	0.045	0	0.15	0.21	1.25	0.05	0.053	0	100	24.5

7.3 Powder XRD analysis

Powder XRD was performed to determine the mineral phases present in the clays. The mineralogy of the white clay interpreted from the XRD patterns shows that it is composed of kaolinite [$Al_2(Si_2O_5)(OH)_4$] as the dominant mineral phase and also some quartz (SiO_2) (see Figure 7.2). This was done by comparing the XRD pattern obtained with those previously reported [78-80]. The unique crystal structure of each solid phase of a compound allows for XRD to be used to identify them. Each phase has a unique XRD pattern, which has resulted in libraries of spectra for ease of identification.

The red clay was found to contain kaolinite [$Al_2(Si2O5)(OH)_4$], quartz (SiO_2), hematite (α-Fe_2O_3) and maghemite (γ-Fe_2O_3) (see Figure 7.3). Hematite and maghemite are iron oxides which are responsible for the red colour of the clay. The XRD results

are consistent with what was observed in the XRF. The red clay can therefore be identified as iron-stained kaolinite.

The diffactogram of the white clay was more clear and defined compared with that of the red clay. This could be due to the crystallinity of the material. The more crystalline the material, the sharper are the peaks in the diffactogram.

7.4 Infrared spectroscopic analysis

Fourier transform infrared spectroscopy (FTIR) was used to further identify the composition of the clays. It was found that the red clay and white clay showed similar functional groups with just a few shifts in adsorption frequencies (Figure 7.4). Peaks due to the Al_2O_3 of the kaolin were observed between 3950-3620 cm^{-1} [79, 81]. The white clay had two very weak peaks in the region between 2000-1600 cm^{-1} which were attributed to the presence of quartz [81]. The red clay had one peak in this region which was weak.

Quartz and kaolin have doublets at 780-800 cm^{-1} which overlap each other [82]. These doublets where observed in both clays. The doublet at 900-1000 cm^{-1} in both clays is due to kaolin [82]. Thus, FTIR confirmed the presence of the phases identified by powder XRD.

7.5 Thermogravimetric analysis

Thermogravimetric analysis (TGA) was performed to further identify the clays. It was observed that the clays exhibited a loss in mass in the TGA curve just after 100 °C which is due to the loss of moisture in the clays (Figure 7.5). Dehydroxylation of the kaolinite ($Al_2Si_2O_5(OH)_4$) to meta-kaolinite ($Al_2Si_2O_7$) in the temperature range 400-500 °C gives a weight loss in the TG curves and a rise to endothermic peaks in the DTA curve (Figure 7.5) [79, 83], these isotherms were observed at 507 ºC in the case of the white clay and 481 ºC for the red clay. The red clay had a bigger loss in mass in the range 100-350 °C. This may be attributed to the higher content of absorbed water in the red clay as compared to the white clay [83] and to the change of phases of the iron oxides in the red clay. Evaluation of the crystallinity of the samples can also be done by using the curves. It can be deduced from the TGA graph that the white clay (Figure 7.5A) is more crystalline than the red clay (Figure 7.5B) by the weight lost at the temperature of

dehydroxylation (400-500 ºC) [79]. The DTA shows a sharper slope for the dehydroxylation of the white clay compared with that of the red clay which indicates that the white clay is more crystalline than the red clay [79]. This further confirms what was observed with the powder-XRD.

7.6 Transmission electron microscopy

The microstructures of the two clays were observed by means of transmission electron microscopy (Figure 7.6). Kaolin has been shown to display pseudo-hexagonal structures [84, 85] which can be seen in the micrographs of the red and white clays shown in Figure 7.6. The red clay has a less regular shape (see Figure 7.6B). This corresponds with what was observed with powder-XRD and TGA showing that it is not pure kaolinite and that the material is less crystalline.

The kaolinite clays as identified above have been used in South Africa for a number of years. Ekkose *et al.* has reported on kaolin deposits in Inanda (where the clays were obtained) and the Ndwedwe region which are close to each other [86]. The clays are mixed with water to make a paste which is then applied to the face as a mask. The use of geological minerals or clays for the therapy of skin is known as geotherapy. It has been known to treat dermatological diseases such as blackheads, spots, seborrhoea and many more [64]. Kaolinite is a good mineral to use as it is a good carrier and releaser of important minerals in the body. This is due to its large surface area and adsorption capacity [87]. The effects of the clays can be observed by physically looking at the skin texture therefore making the skin more beautiful. Silicon is found in kaolinite which is responsible for skin softness [88].

7.7 Particle size distribution

The particle size distribution of the red and white clays was determined with a Malvern Mastersizer 2000 instrument that employs laser light scattering. An average of three results was taken to produce the graphs shown in Figure 7.7 and Figure 7.8 for the white and red clay respectively.

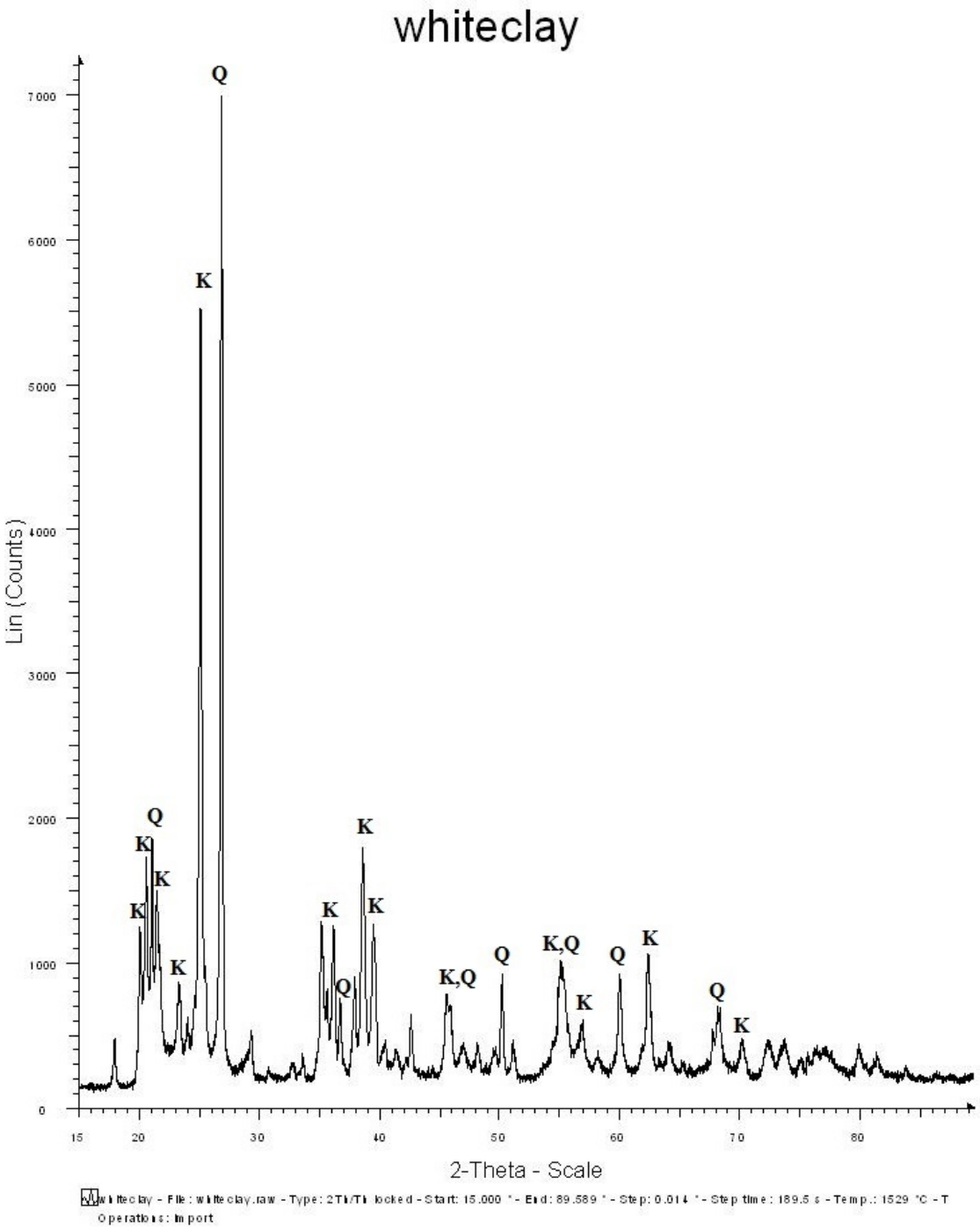


Figure 7.2 Powder X-ray diffractogram of white clay displaying kaolinite (K) and quartz (Q) phases.

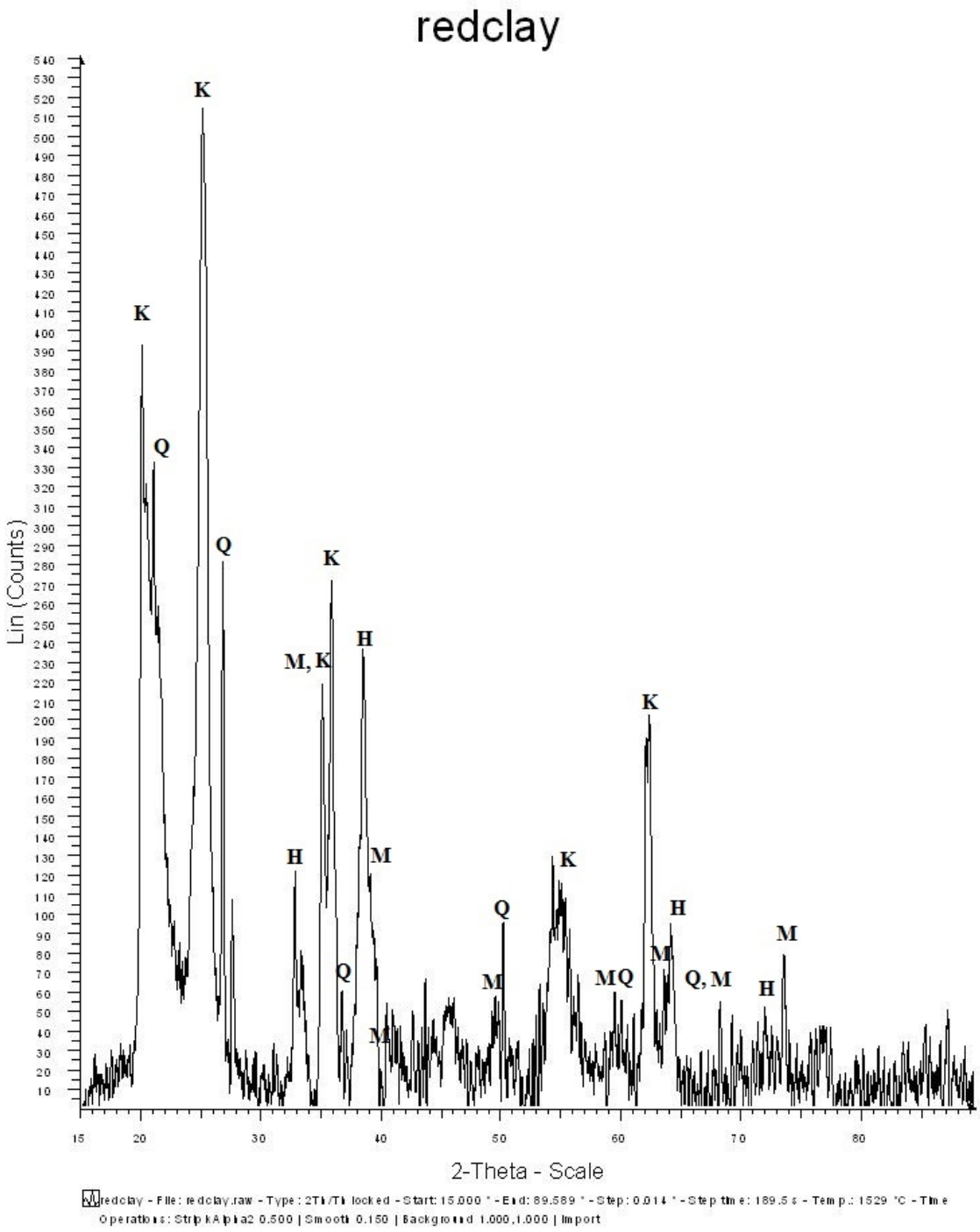


Figure 7.3 Powder X-ray diffractogram of red clay showing kaolinite (K), quartz (Q), hematite (H) and maghemite (M) as the dominant phases in the clay.

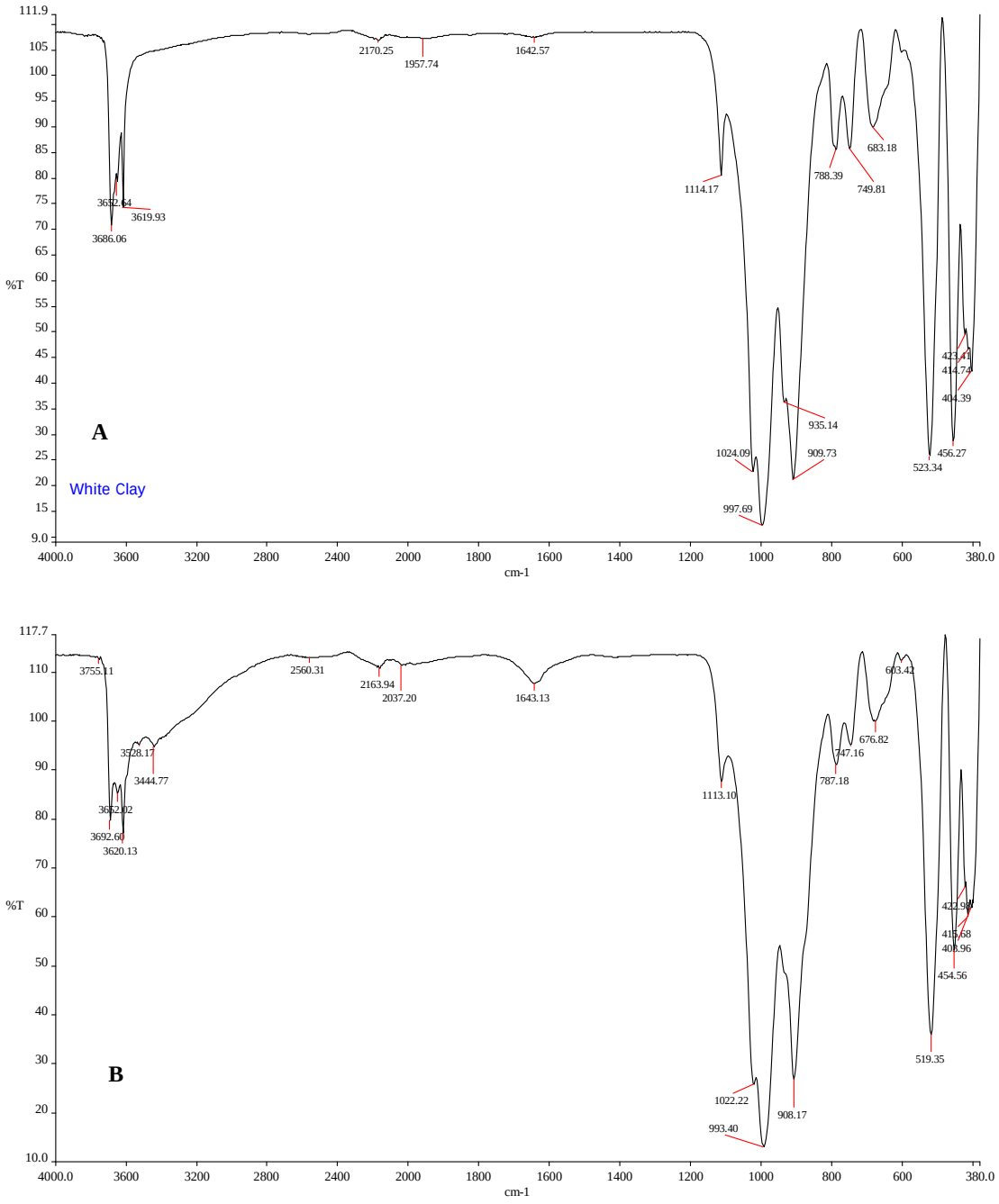


Figure 7.4 Infrared spectra of A) white clay and B) red clay

Geologists and soil scientists use a particle size of less than 2 µm to define clays [53]. Clays are therefore expected to be uniform in terms of their particle size distribution. This is because they are mostly composed of particles of the same size. Consequently, it is expected that the particle size distribution graph will show the particles to be almost all the same size and the curve not to be broad.

The results obtained show that the particles in our clay samples are not uniform. The red clay (Figure 7.8) has particle sizes ranging from 0.317 - ~2000 µm; with most of the clay particles ranging from 1.626 - 70.963 µm. That is a very wide range for particles which are supposed to be of the same size. The white clay (Figure 7.7) had particles sizes ranging from 0.448 - ~2000 µm; the range for most of the particles was between 1.416 - 1261.915 µm.

The samples were analysed as purchased by the customer and where not fractioned into components of more uniform sizes. The particle size analysis results indicate that these samples should be more appropriately termed “river silt” (2-63 µm). The presence of the peak at larger particle sizes is indicative of the sand (quartz) present in these samples.

The white clay appears to be coarser than the red clay. The particle size distribution for the white clay is very large and this can also affect how light is reflected by the particles.

The particle size of the clays is important when used as a sunscreen. Their effectiveness as a sunscreen is due to the ability to reflect and scatter ultraviolet (UV) radiation from the skin surface. The mechanism by which the sunscreen agents will protect the skin is different depending on the particles size. Particles which are nano-size absorb UV radiation while those that are pigmentary size (µm range) reflect and scatter the radiation [89]. The micron range of the particles in the clays makes them visible when applied to the skin. Sunscreens which contain metal oxides do so in the micronized form with the particles in the nanometre range. This makes the metal oxides to appear almost invisible when applied but they have a good UV blocking ability.

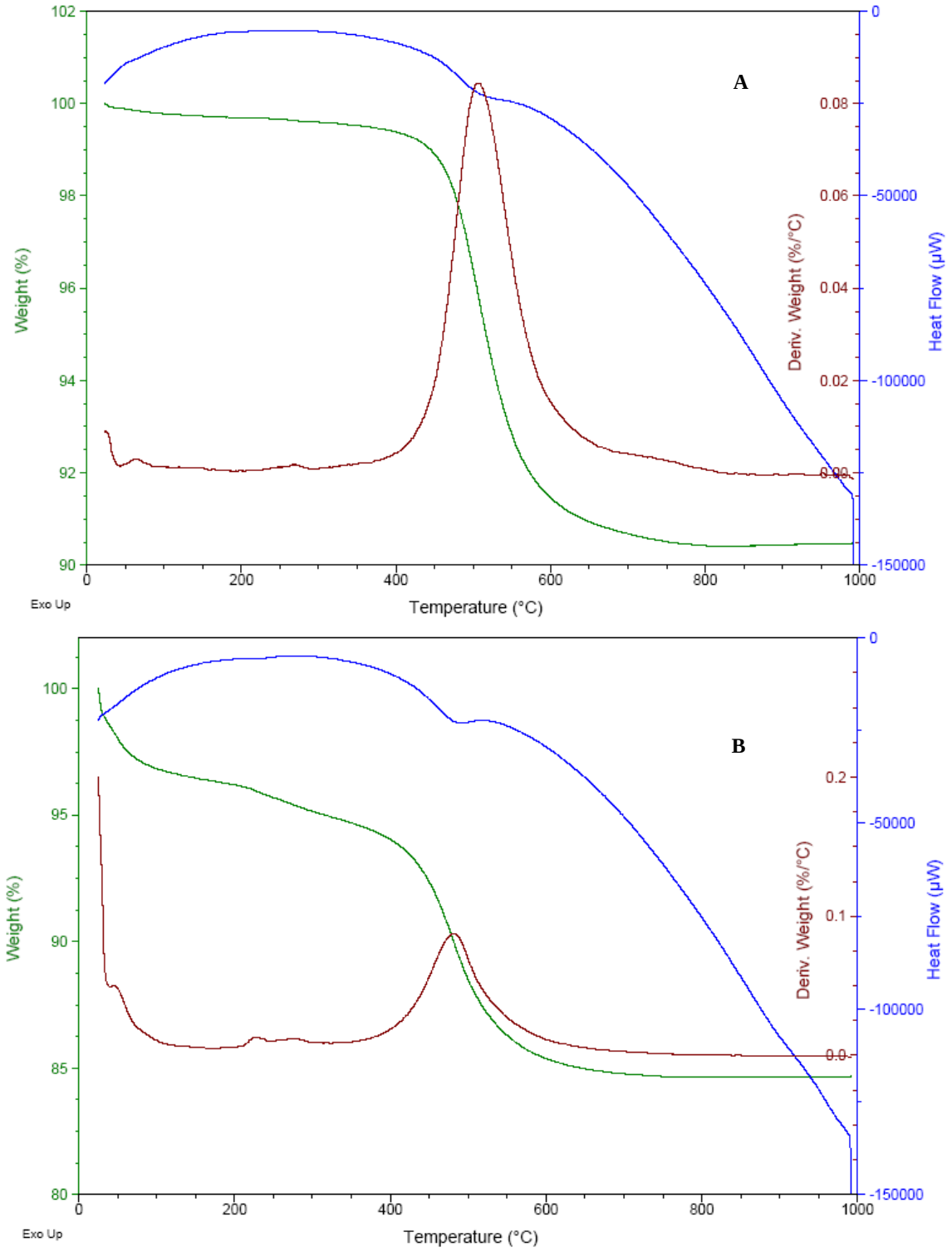


Figure 7.5 Thermogravimetric analysis curves of A) white and B) red clay.

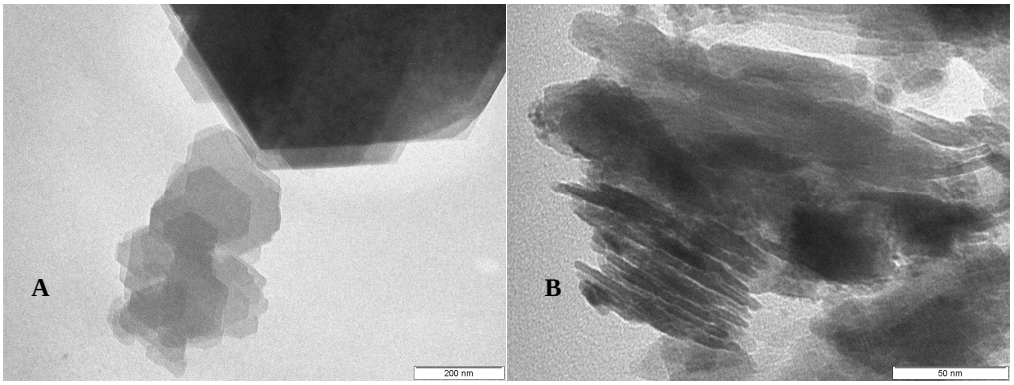
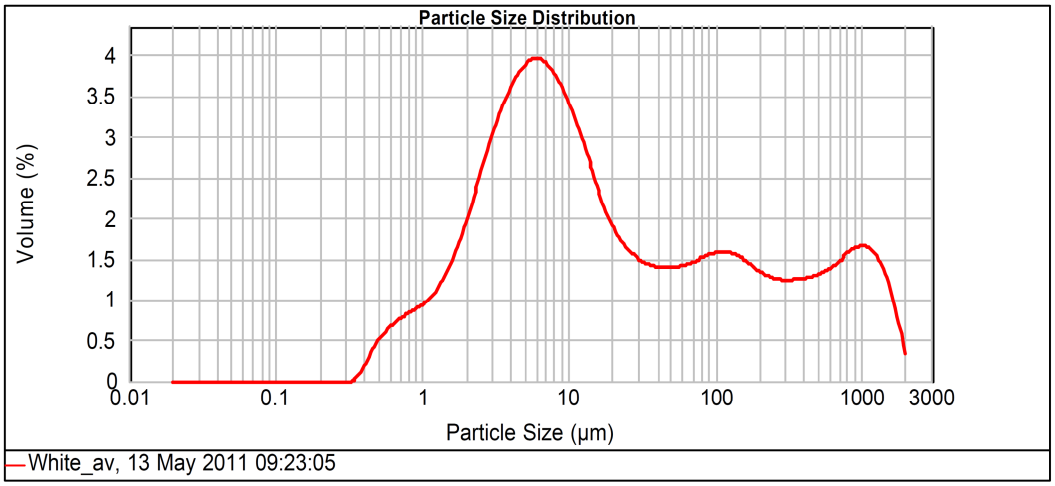
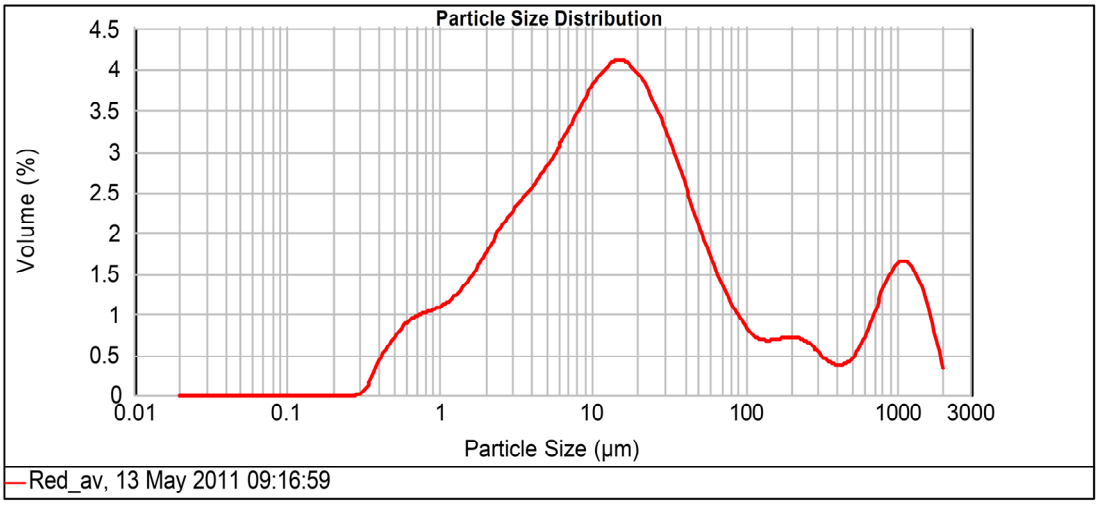


Figure 7.6 TEM micrographs of A) white clay and B) red clay.



Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %	Size (µm)	Volume In %
0.020	0.00	0.142	0.00	1.002	0.72	7.096	2.89	50.238	1.05	355.656	0.94
0.022	0.00	0.159	0.00	1.125	0.79	7.962	2.79	56.368	1.07	399.052	0.95
0.025	0.00	0.178	0.00	1.262	0.88	8.934	2.65	63.246	1.09	447.744	0.97
0.028	0.00	0.200	0.00	1.416	1.01	10.024	2.48	70.963	1.12	502.377	1.00
0.032	0.00	0.224	0.00	1.589	1.17	11.247	2.29	79.621	1.15	563.677	1.04
0.036	0.00	0.252	0.00	1.783	1.36	12.619	2.09	89.337	1.17	632.456	1.09
0.040	0.00	0.283	0.00	2.000	1.57	14.159	1.88	100.237	1.19	709.627	1.14
0.045	0.00	0.317	0.00	2.244	1.80	15.887	1.70	112.468	1.19	796.214	1.20
0.050	0.00	0.356	0.08	2.518	2.02	17.825	1.53	126.191	1.17	893.367	1.24
0.056	0.00	0.399	0.17	2.825	2.24	20.000	1.39	141.589	1.14	1002.374	1.25
0.063	0.00	0.448	0.33	3.170	2.44	22.440	1.27	158.866	1.09	1124.683	1.20
0.071	0.00	0.502	0.41	3.557	2.63	25.179	1.19	178.250	1.04	1261.915	1.10
0.080	0.00	0.564	0.50	3.991	2.77	28.251	1.13	200.000	1.00	1415.892	0.93
0.089	0.00	0.632	0.56	4.477	2.88	31.698	1.09	224.404	0.96	1588.656	0.66
0.100	0.00	0.710	0.61	5.024	2.95	35.566	1.07	251.785	0.94	1782.502	0.39
0.112	0.00	0.796	0.64	5.637	2.98	39.905	1.05	282.508	0.93	2000.000	
0.126	0.00	0.893	0.68	6.325	2.95	44.774	1.05	316.979	0.93		
0.142	0.00	1.002		7.096		50.238		355.656			

Figure 7.7 Average particle size distribution of white clay.



Size (µm)	Volume In %
0.020	0.00
0.022	0.00
0.025	0.00
0.028	0.00
0.032	0.00
0.036	0.00
0.040	0.00
0.045	0.00
0.050	0.00
0.056	0.00
0.063	0.00
0.071	0.00
0.080	0.00
0.089	0.00
0.100	0.00
0.112	0.00
0.126	0.00
0.142	0.00

Size (µm)	Volume In %
0.142	0.00
0.159	0.00
0.178	0.00
0.200	0.00
0.224	0.00
0.252	0.00
0.283	0.01
0.317	0.08
0.356	0.22
0.399	0.37
0.448	0.48
0.502	0.58
0.564	0.65
0.632	0.71
0.710	0.75
0.796	0.78
0.893	0.80
1.002	0.83

Size (µm)	Volume In %
1.002	0.83
1.125	0.87
1.262	0.94
1.416	1.02
1.589	1.13
1.783	1.25
2.000	1.36
2.244	1.48
2.518	1.58
2.825	1.68
3.170	1.78
3.557	1.87
3.991	1.96
4.477	2.06
5.024	2.16
5.637	2.28
6.325	2.41
7.096	2.54

Size (µm)	Volume In %
7.096	2.54
7.962	2.68
8.934	2.80
10.024	2.92
11.247	3.01
12.619	3.07
14.159	3.10
15.887	3.08
17.825	3.03
20.000	2.94
22.440	2.81
25.179	2.66
28.251	2.48
31.698	2.29
35.566	2.09
39.905	1.88
44.774	1.68
50.238	1.48

Size (µm)	Volume In %
50.238	1.48
56.368	1.29
63.246	1.11
70.963	0.94
79.621	0.80
89.337	0.68
100.237	0.59
112.468	0.54
126.191	0.51
141.589	0.51
158.866	0.53
178.250	0.54
200.000	0.54
224.404	0.52
251.785	0.48
282.508	0.42
316.979	0.35
355.656	0.30

Size (µm)	Volume In %
355.656	0.30
399.052	0.28
447.744	0.31
502.377	0.38
563.677	0.51
632.456	0.68
709.627	0.87
796.214	1.04
893.367	1.17
1002.374	1.24
1124.683	1.22
1261.915	1.10
1415.892	0.92
1588.656	0.63
1782.502	0.37
2000.000	

Figure 7.8 Average particle size distribution of red clay.

7.8 Sun protection factor

The sun protection factor of the clays was determined according to the South African Bureau of Standards (SABS) sunscreen 1557 method by Dr Beverly Summers at the School of Pharmacy, University of Limpopo, Medunsa Campus. The white clay displayed an SPF of 3.6, a UVA/UVB ratio of 0.9 and a critical wavelength of 387.6 nm. The red clay had an SPF of 4, a UVA/UVB ratio of 1 and a critical wavelength of 388.9 nm. The critical wavelengths of the clays were greater than 370 nm, which makes the clays to give broad-spectrum protection. The red clay had smaller particle sizes which improves its light scattering and light absorbing ability and hence the marginally larger SPF value and critical wavelength. Regardless of the low SPF values of the clays, they provide a degree of UVA protection. UVA constitutes the greater amount of incident solar UV radiation and has been implicated in skin cancer. To achieve good

sun protection, sunscreen formulators specifically pay attention to the particle dispersion of the material [77]. The home preparation of mixing the clays with water or glycerine makes particle aggregation a limiting factor in improving the SPF of the formulations.

Application density is an important factor in sunscreen usage. When testing commercial sunscreens, an application density of 2 mg cm^{-2} is used, but it is well known that typically usage patterns are less than half this amount. With the use of the clays, it is unlikely to be the case because of the 100 g of clay mixed; about 5 g is used to cover the face, which is greater than the amount advocated for sunscreens.

Chapter 8

Conclusions

The clays used in South Africa provide a cheap way in which skin can be taken care of. The clays are dug out of river beds for free and are sold at low prices without any further processing. The clays have been identified as kaolinite and iron-stained kaolinite for white and red clay respectively. Their SPF is low, but they do provide some UV protection. The use of clays in cosmetics to enhance the sun protecting factor of the creams is continually being researched [65]. Clays are a natural alternative to the substances that are currently used in the market. In addition, they do not suffer from the lack of photostability exhibited by some of the organic absorbers found in sunscreen products. The safety and toxicity of these clays has not been determined, but those who use them have not reported any side-effects apart from the red colour that is left on the skin after the use of the red clay. There is still, however, need for the safety and toxicity of the clays to be determined.

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Appendices

Appendix A

Materials

Reagents used in Chapter 2 are listed here.

Table A 1 Reagents used in the qualitative and quantitative analysis of SLAs with HPLC and GC.

Chemical	**Grade**	**Manufacturer**
Hydroquinone	≥99 % HPLC	Fluka Analytical
Resorcin (1,3 dihydroxybenzol)		Riedel-de Haën
p-benzoquinone	≥99.5 % (HPLC)	Fluka Analytical
N-acetyl-L-cysteine	99 % TLC, sigma grade	Sigma
p-arbutin	≥96.0 % (HPLC)	Fluka Biochemika
Kojic acid		Sigma
Phenol	99.5 % (GC)	Riedel-de Haën
4-hydroxyanisole (4-methoxyphenol)	99 % (GC)	Sigma Aldrich
Dexamethasone		
4-propoxyphenol	96%	Aldrich
Hydrocortisone		
Hydrochinondimethylether (1,4-dimethoxybenzene)	99% (GC)	Aldrich
6α-methylprednisolone	≥98%	Sigma Life Science
Fluocinolone acetonide	Minimum 98%	Fluka Analytical
4-phenoxyphenol	99%	Aldrich
Clobetasol propionate	Minimum 98%	Fluka Analytical
Fluticasone propionate	≥98% (HPLC)	Sigma Life Science
Mometasone furoate	≥98% (HPLC)	Sigma Life Science
Betamethasone 17,21-dipropionate		Sigma Life Science

Triamcinolone acetonide	Analytical standard	Fluka Analytical
Beclomethasone dipropionate	Analytical standard, for drug analysis	Fluka Analytical
Nicotinamide (niacinamide)		Supelco Analytical
Betamethasone 17-valerate	Analytical standard area 99.6% (HPLC)	Fluka Analytical
Dexamethasone	≥98% (HPLC)	Sigma Life Sciences
Hydrocortisone Vetranal	Area 98.7% (HPLC)	Fluka Analytical
Methanol	Chromasolv for HPLC, ≥99.9%	Sigma Aldrich
2-propanol	99.5%, HPLC grade	Aldrich
pyridine	Analytical grade	
BSTFA	Derivatization Grade	Supelco

Appendix B

UV and fluorescence spectra of SLA standards

The UV and fluorescence spectra of SLA standards determined. The UV spectra were obtained by using a UV/vis spectrophotometer, and the fluorescence spectra by using a luminescence instrument. All the standards were dissolved in methanol and methanol was used as a blank.

B.1 UV spectra of SLAs standards

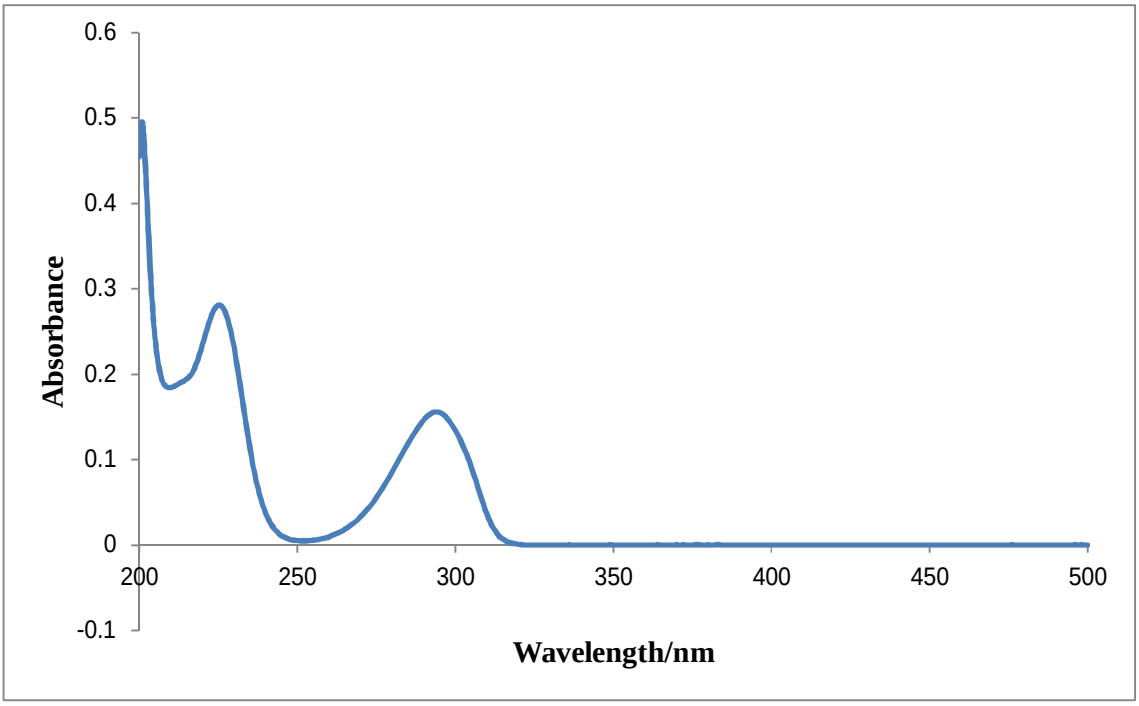

Figure B 1 UV spectrum of hydroquinone.

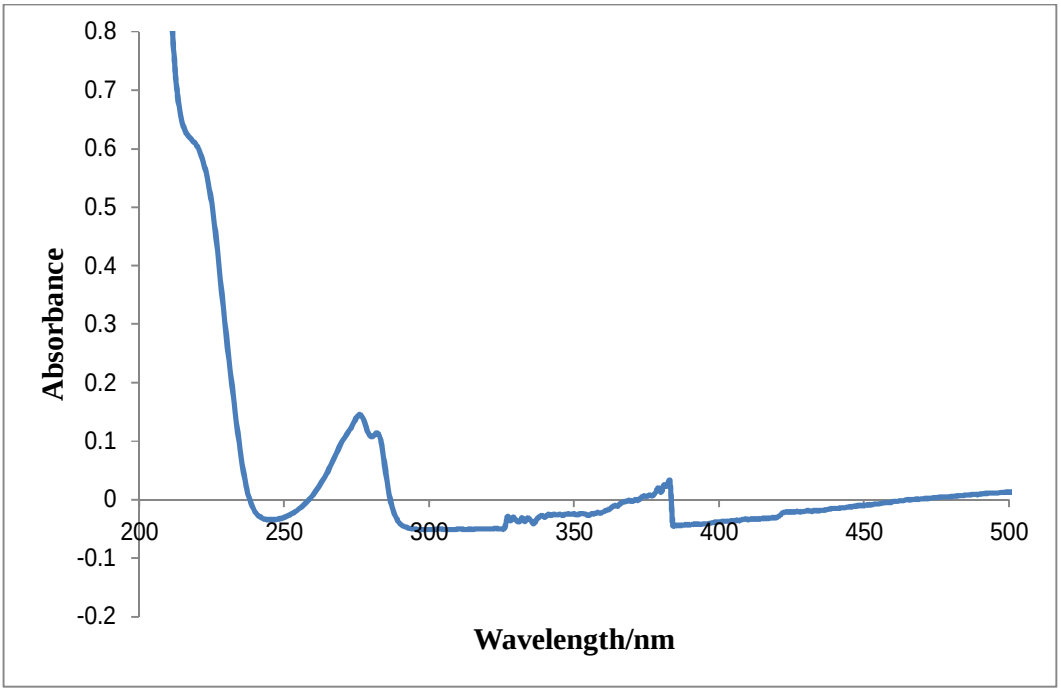

Figure B 2 UV spectrum of resorcinol.

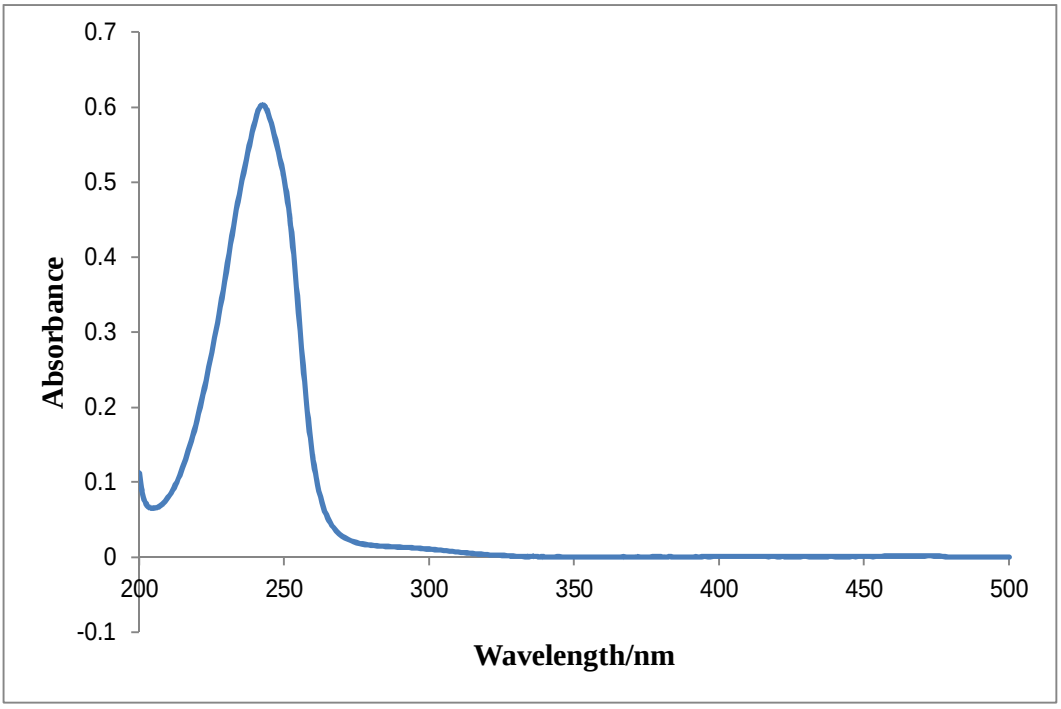

Figure B 3 UV spectrum of benzoquinone in methanol.

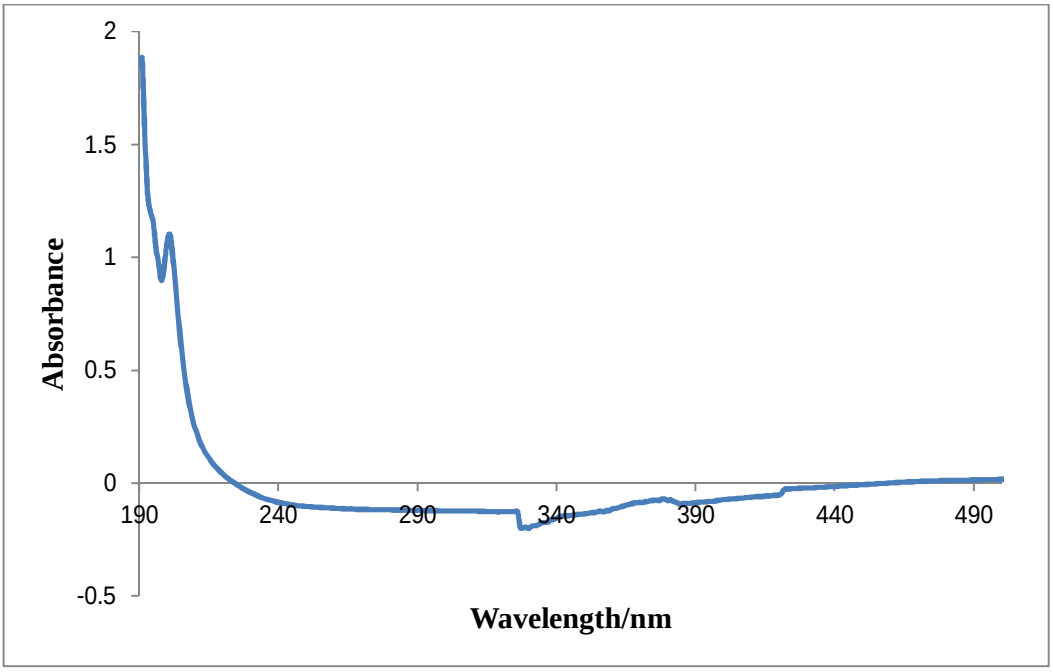

Figure B 4 UV spectrum of N-acetyl-L-cysteine.

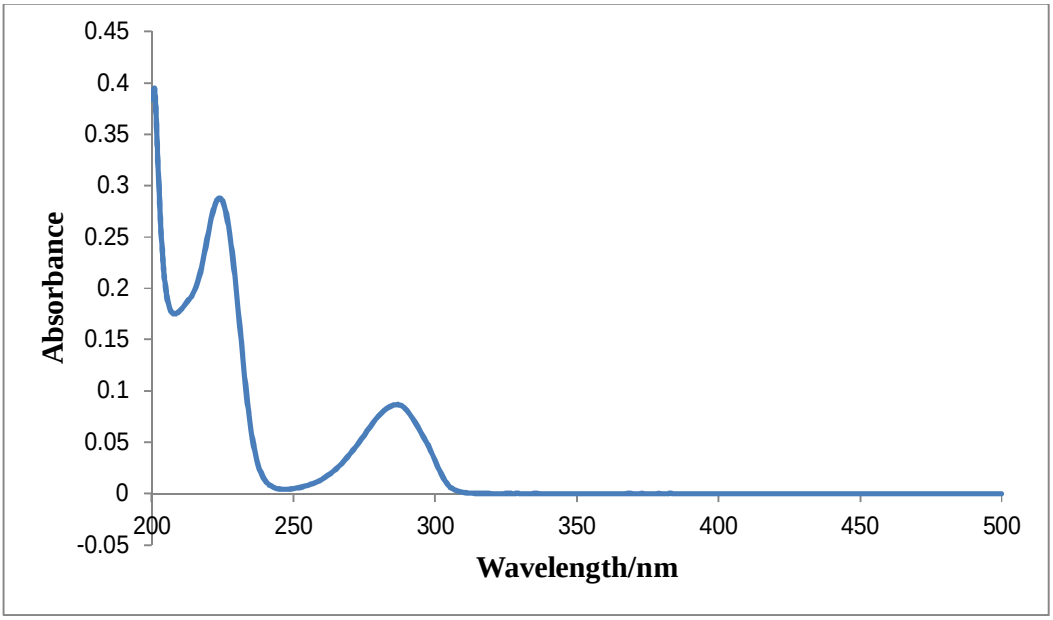

Figure B 5 UV spectrum of arbutin.

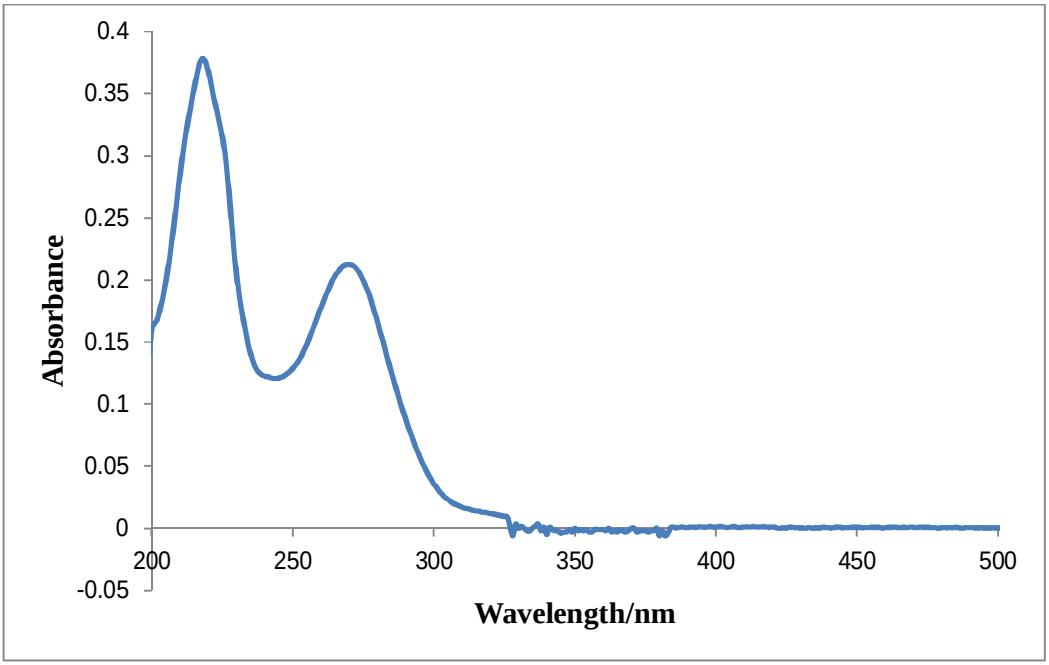

Figure B 6 UV spectrum of kojic acid.

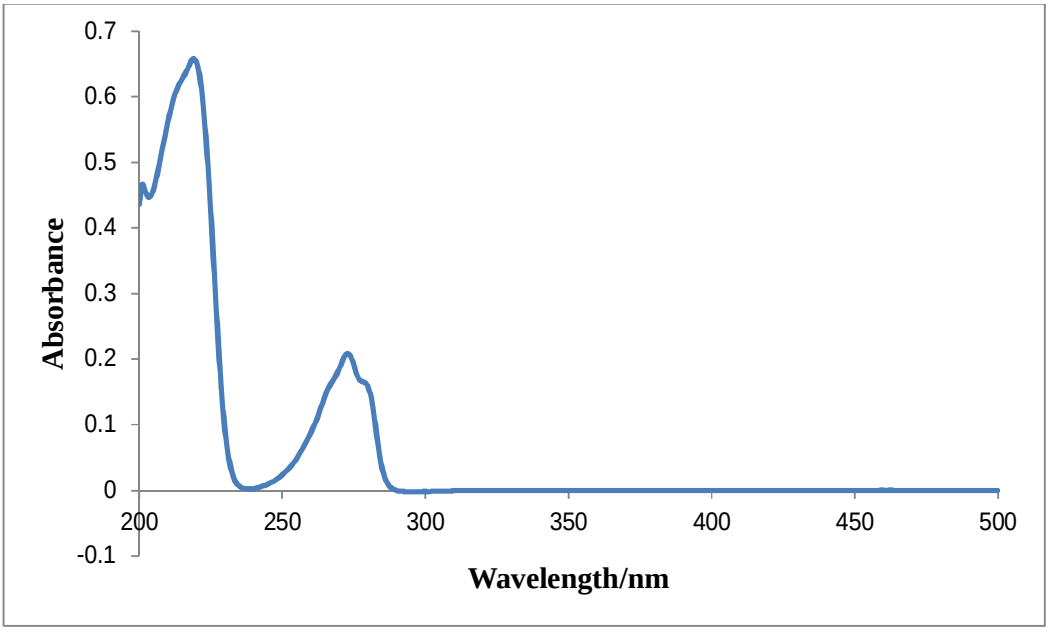

Figure B 7 UV spectrum of phenol.

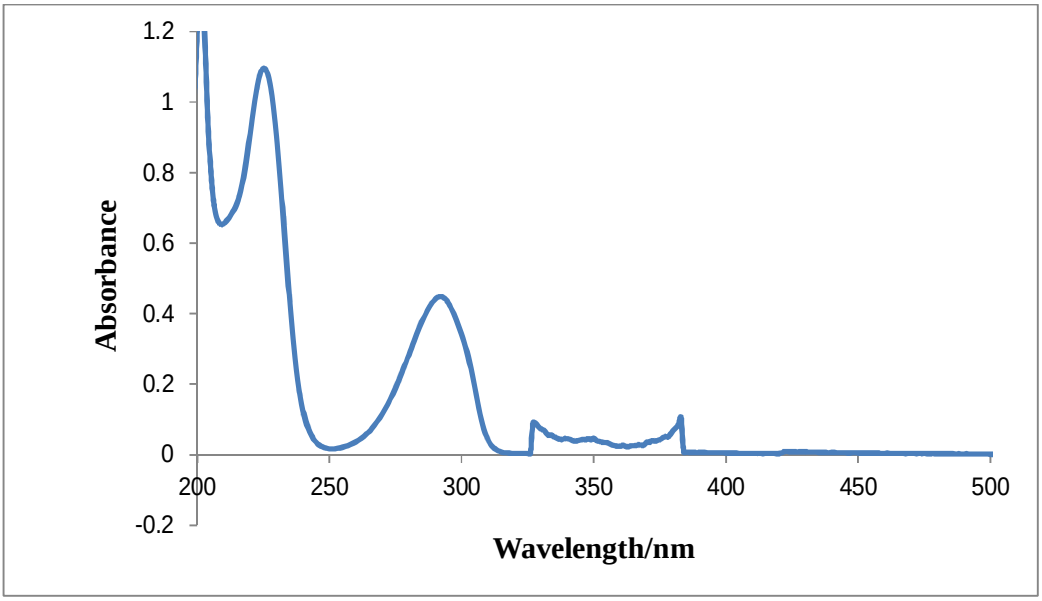

Figure B 8 UV spectrum of 4-hydroxyanisole.

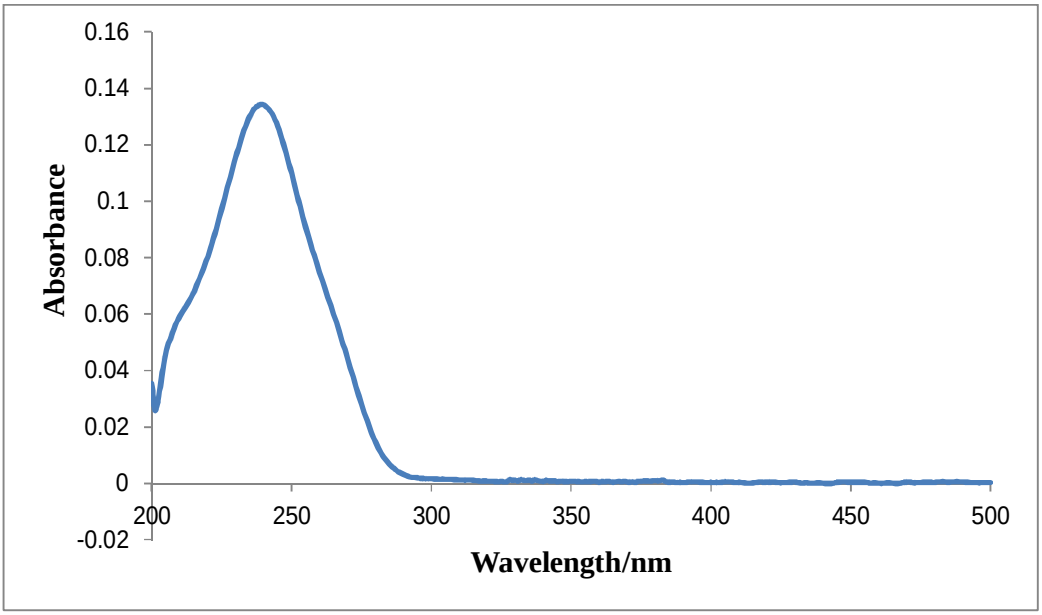

Figure B 9 UV spectrum of dexamethasone.

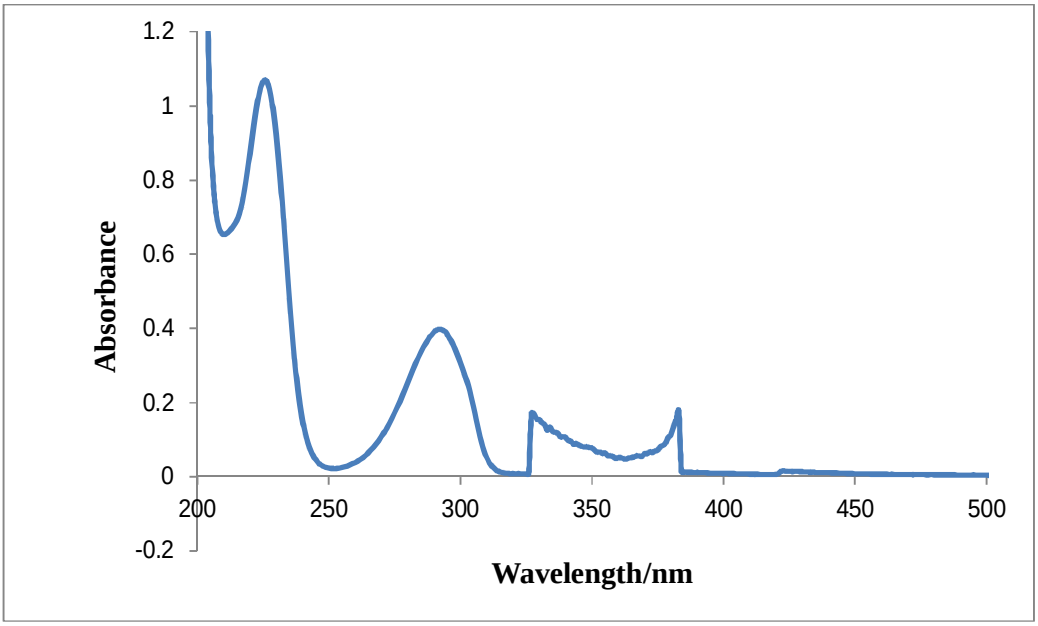

Figure B 10 UV spectrum of 4-propoxyphenol.

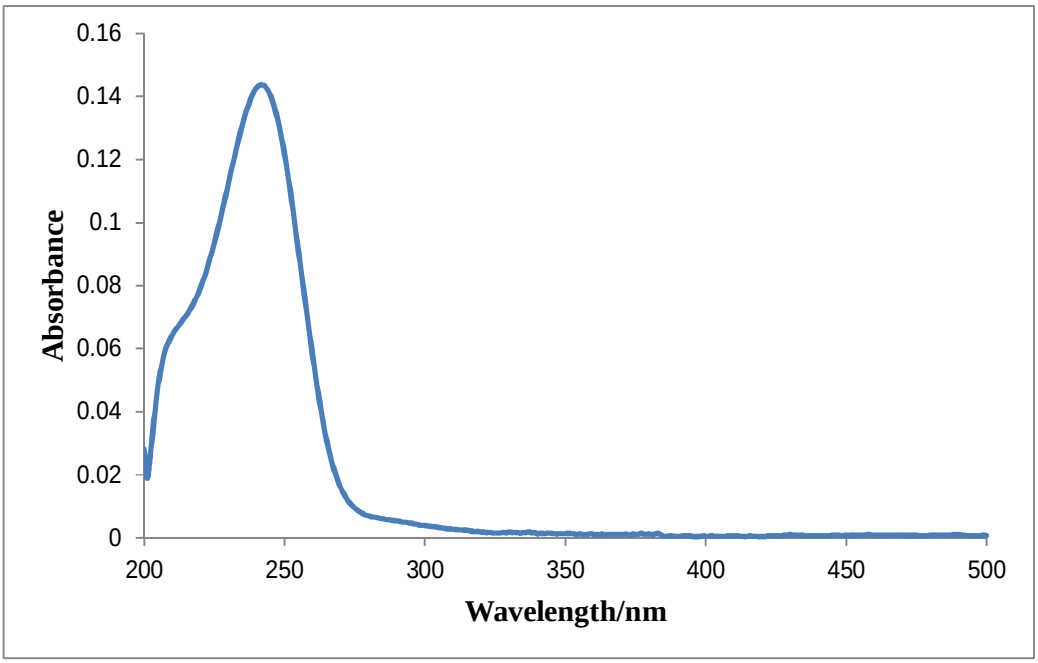

Figure B 11 UV spectrum of hydrocortisone.

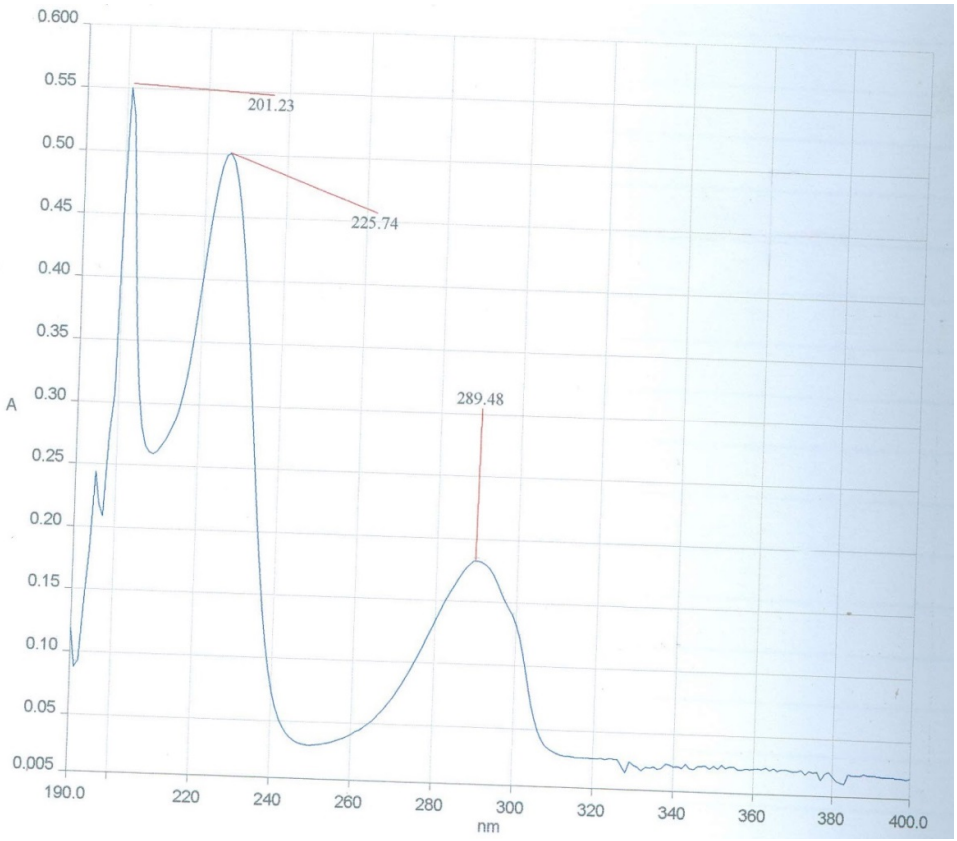

Figure B 12 UV spectrum of hydrochinon-dimethylether.

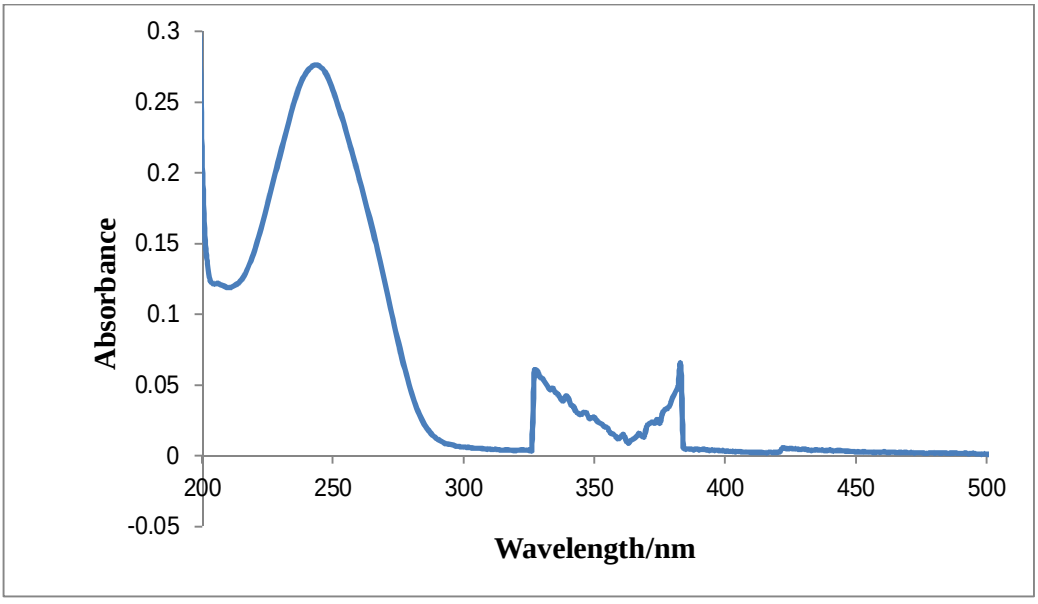

Figure B 13 UV spectrum of 6α-methylprednisolone.

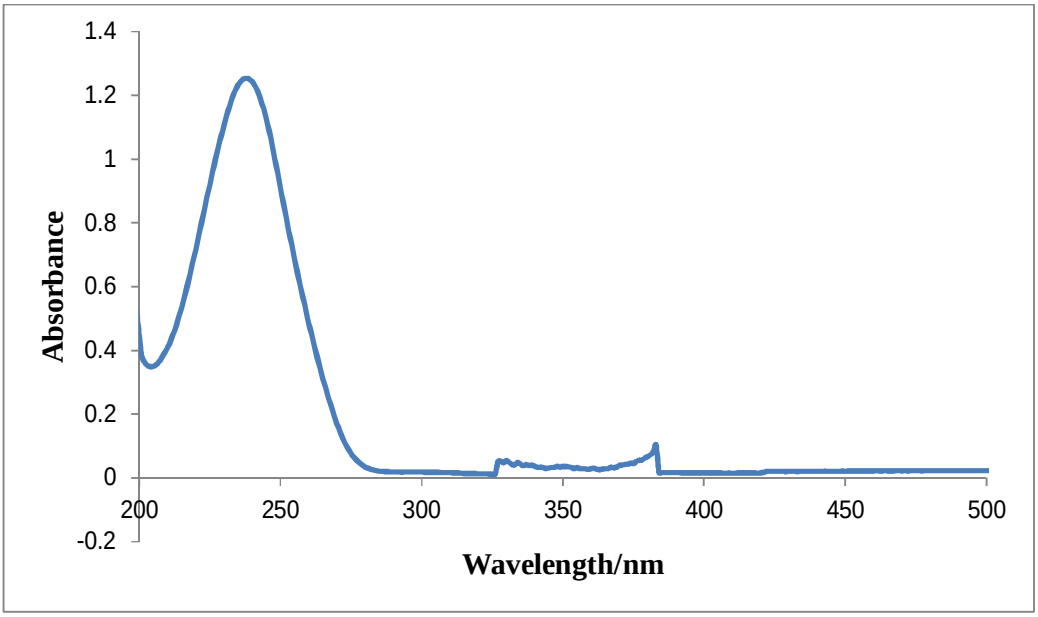

Figure B 14 UV spectrum of fluocinolone acetonide.

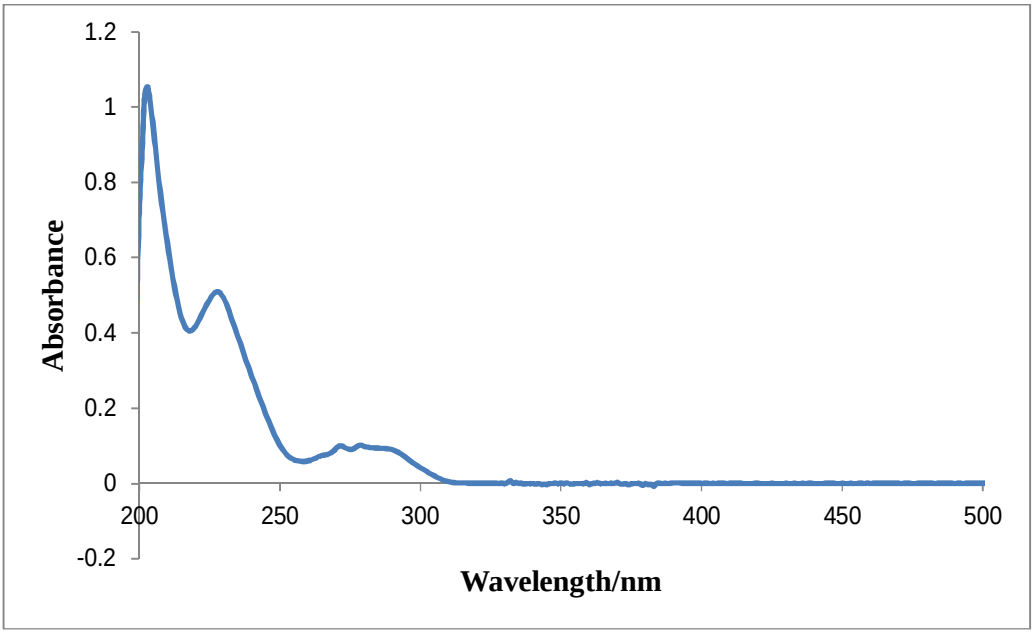

Figure B 15 UV spectrum of 4-phenoxyphenol.

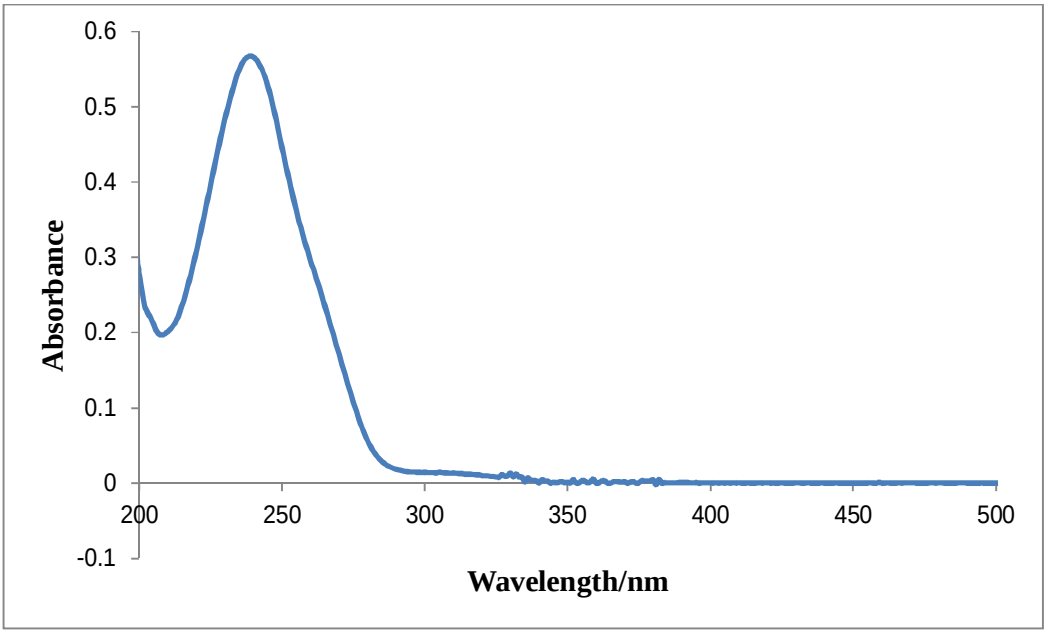

Figure B 16 UV spectrum of clobetasol propionate.

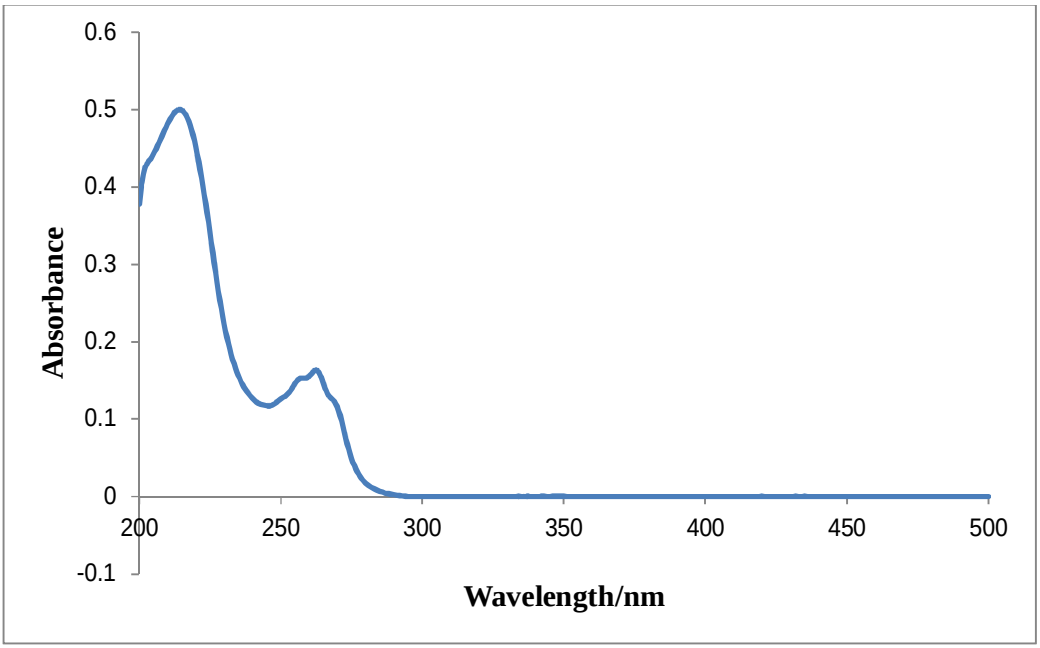

Figure B 17 UV spectrum of niacinamide.

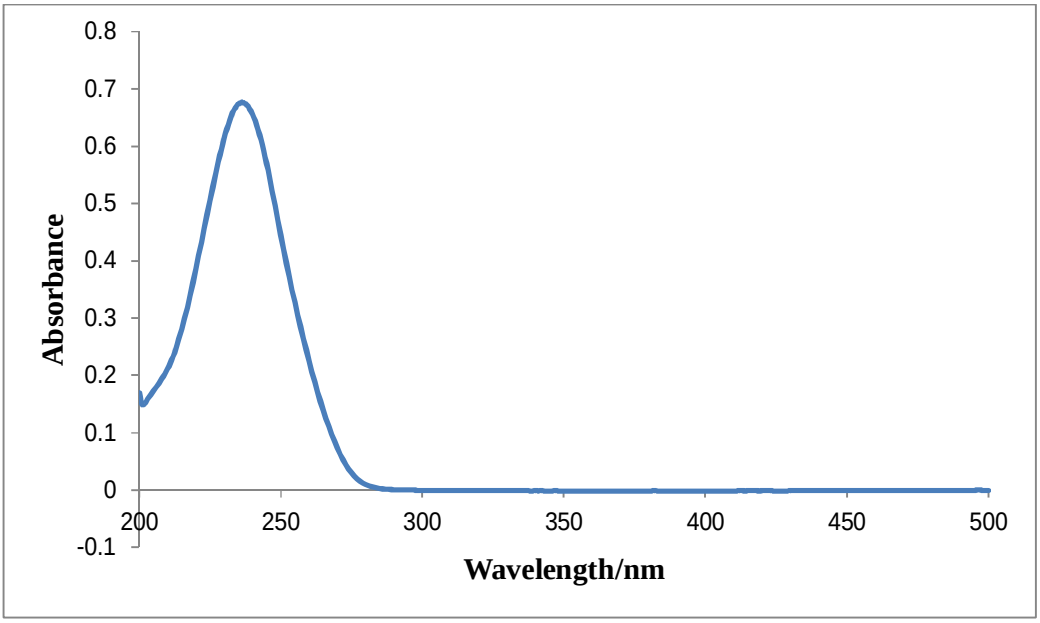

Figure B 18 UV spectrum of fluticasone propionate.

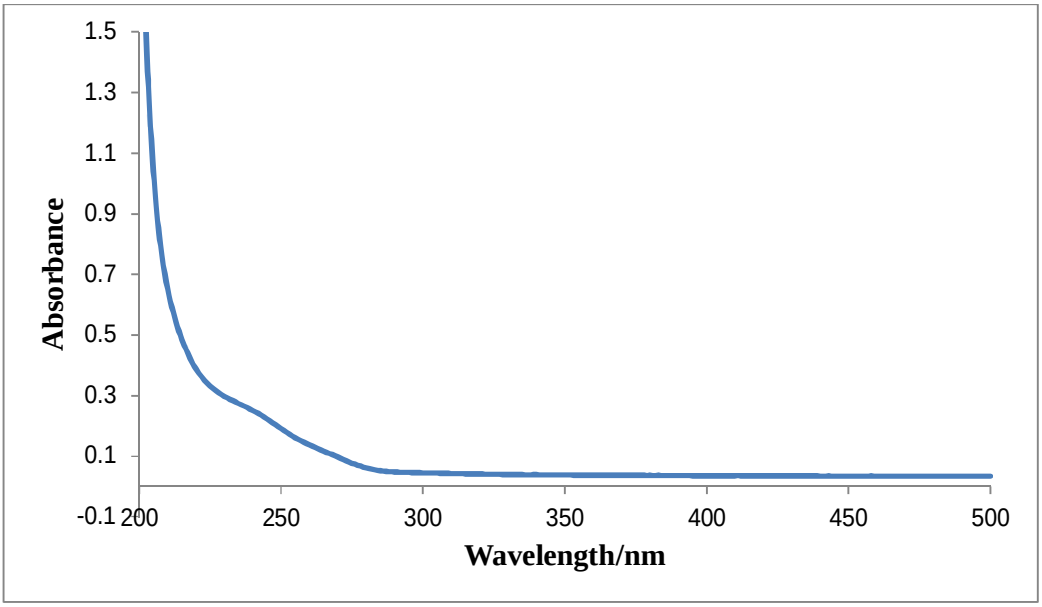

Figure B 19 UV spectrum of triamcinolone acetonide.

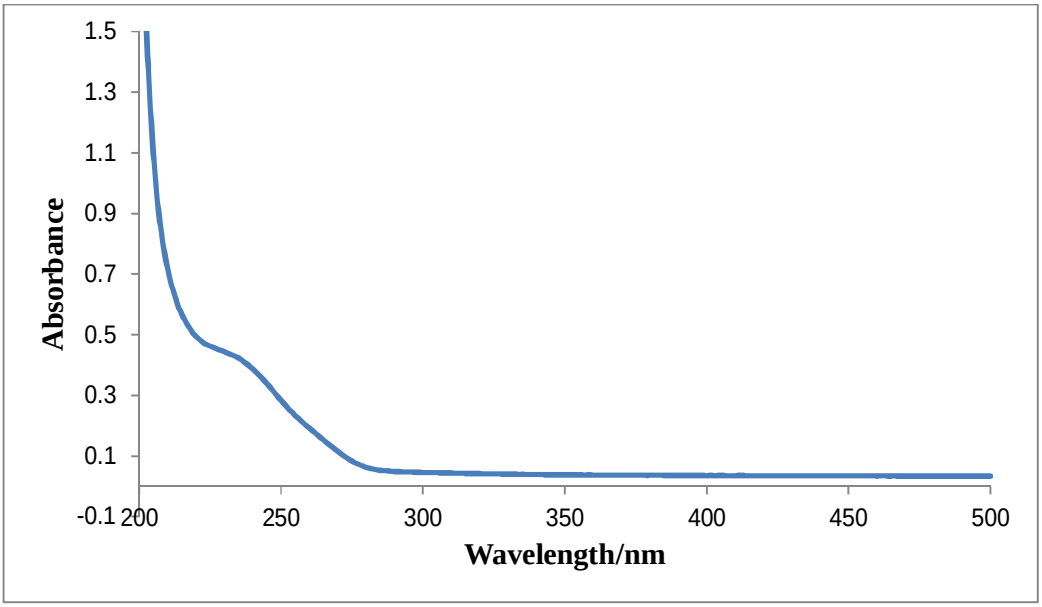

Figure B 20 UV spectrum of clobetasone butyrate.

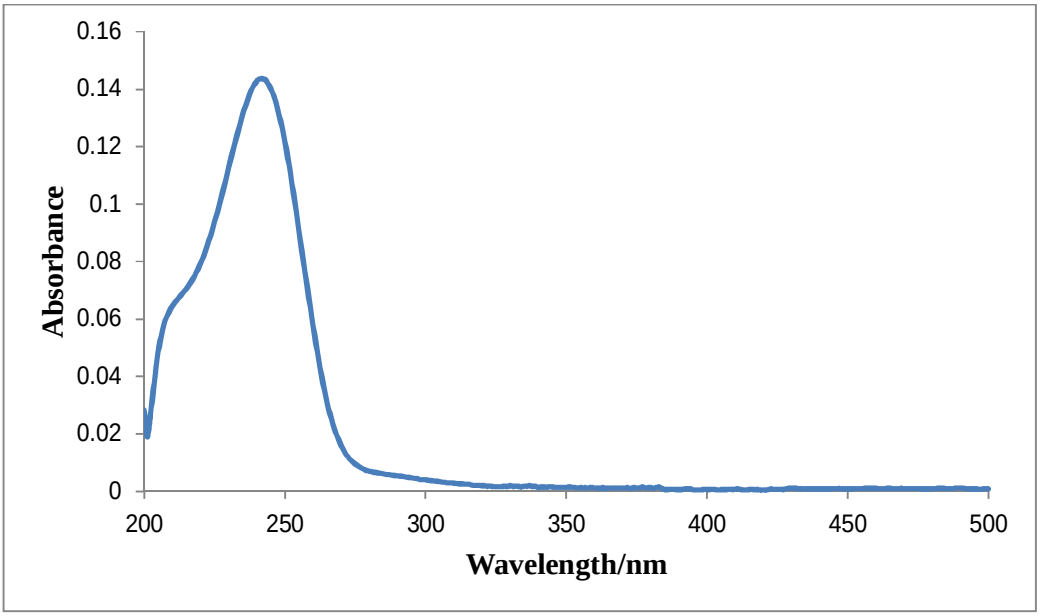

Figure B 21 UV spectrum of hydrocortisone.

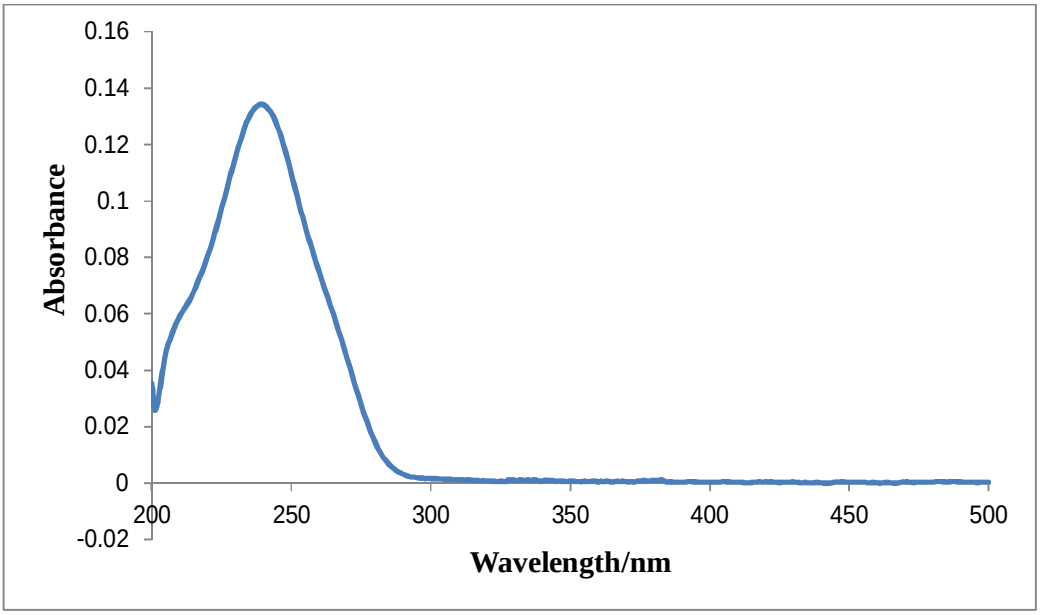

Figure B 22 UV spectrum of dexamethasone.

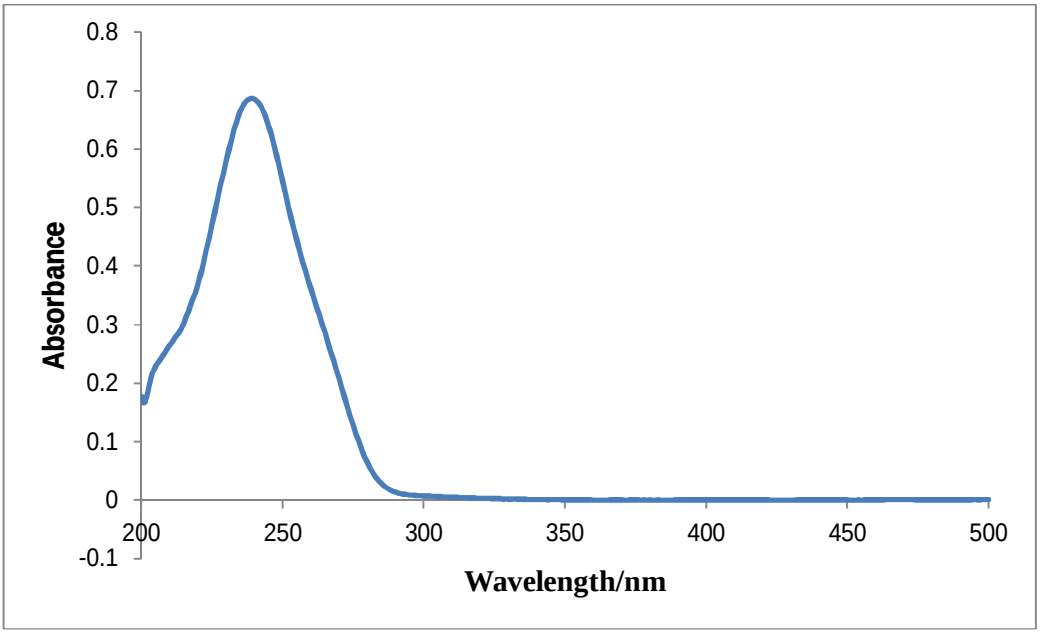

Figure B 23 UV spectrum of betamethasone 17, 21- dipropionate.

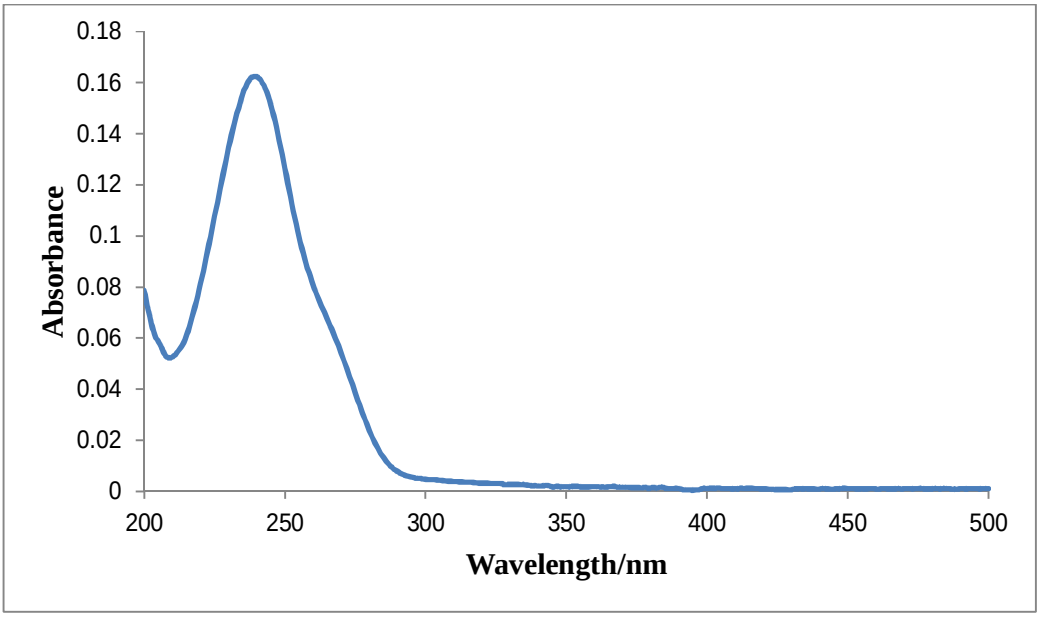

Figure B 24 UV spectrum of beclomethasone dipropionate.

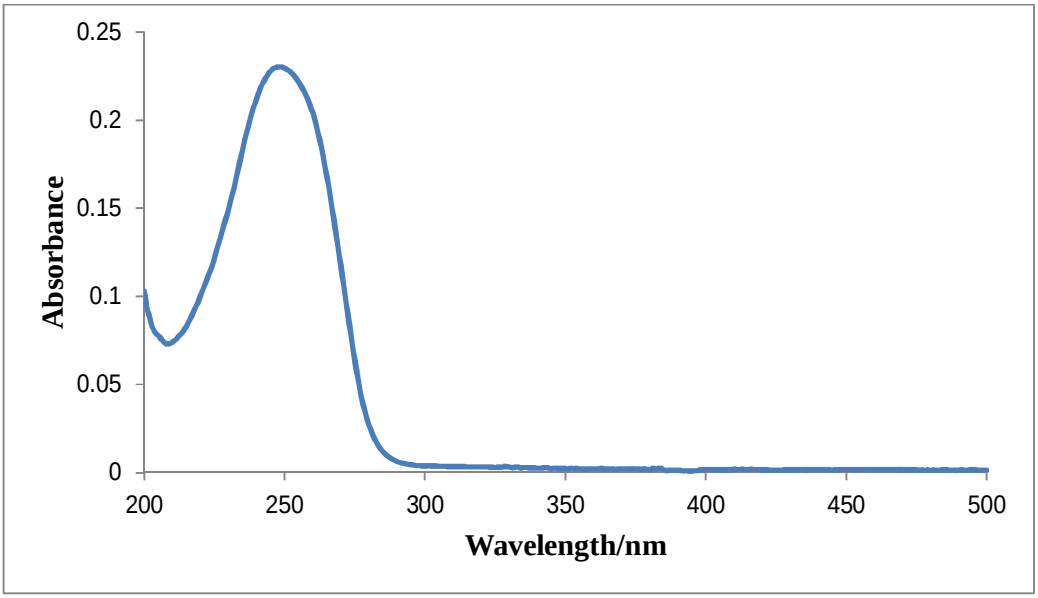

Figure B 25 UV spectrum of mometasone furoate.

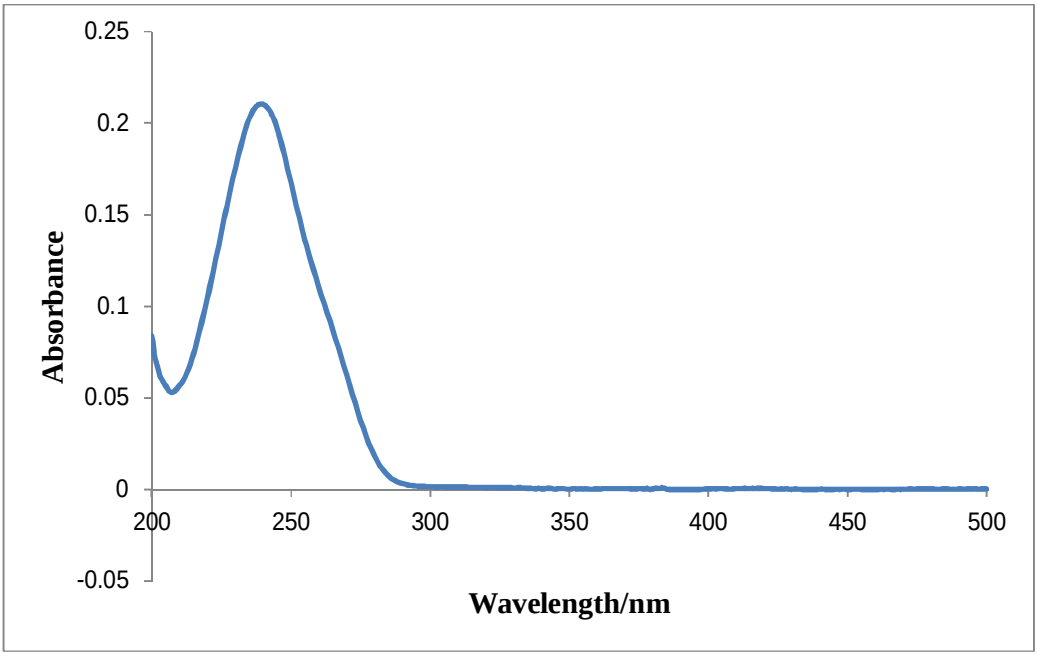

Figure B 26 UV spectrum of betamethasone 17- valerate.

B.2 Fluorescence spectra of SLA standards

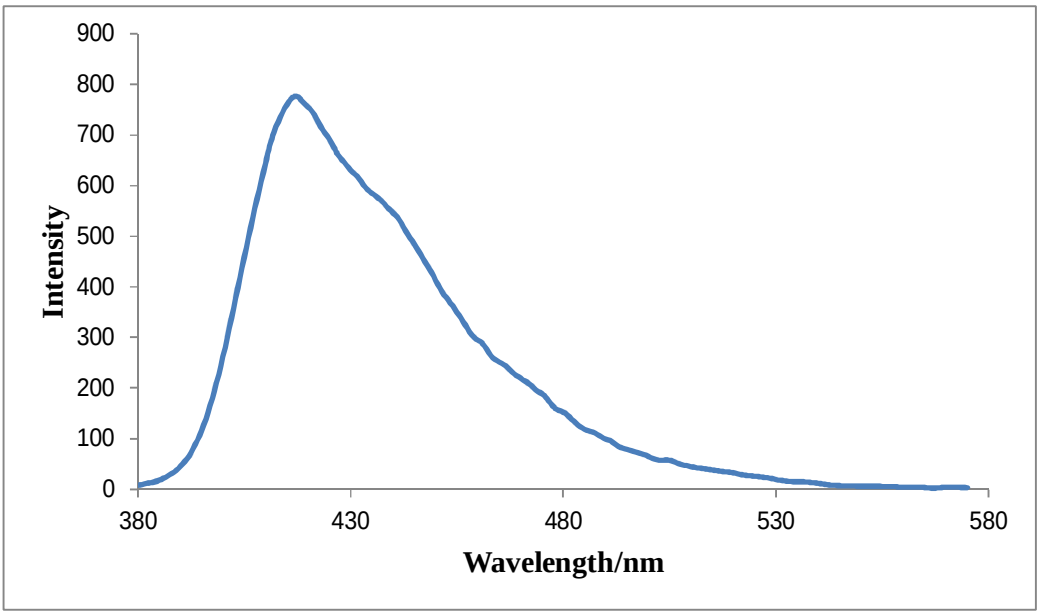

Figure B 27 Fluorescence spectrum of hydroquinone.

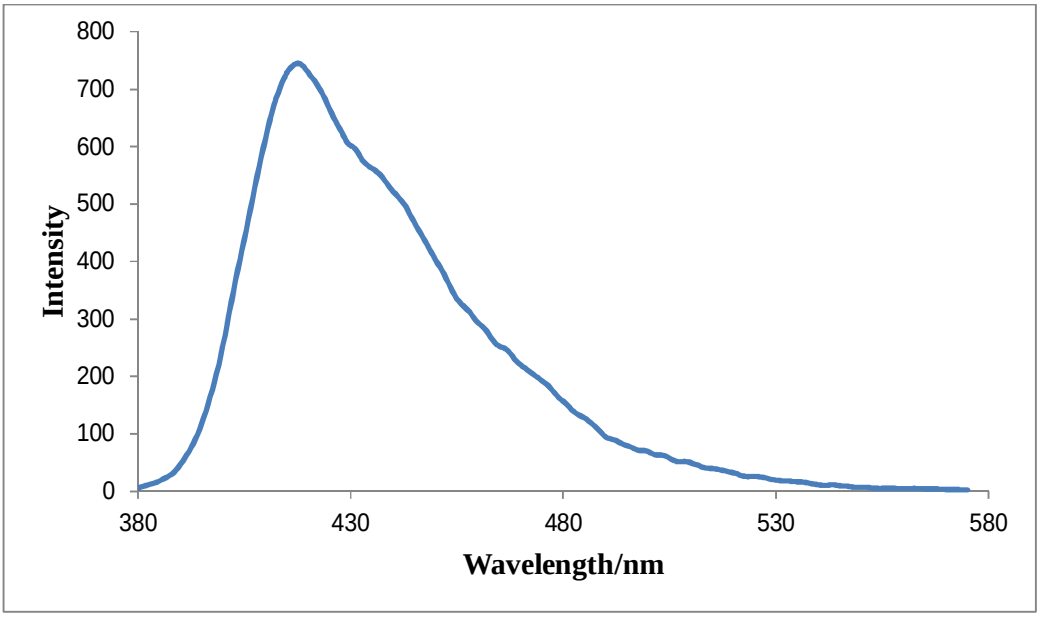

Figure B 28 Fluorescence spectrum of resorcinol.

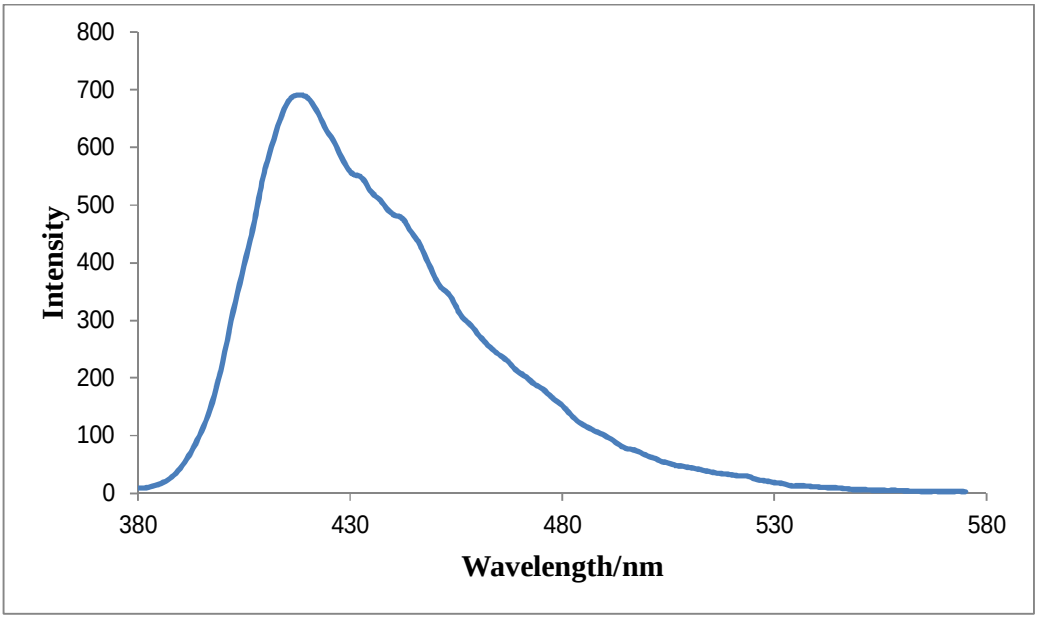

Figure B 29 Fluorescence spectrum of benzoquinone.

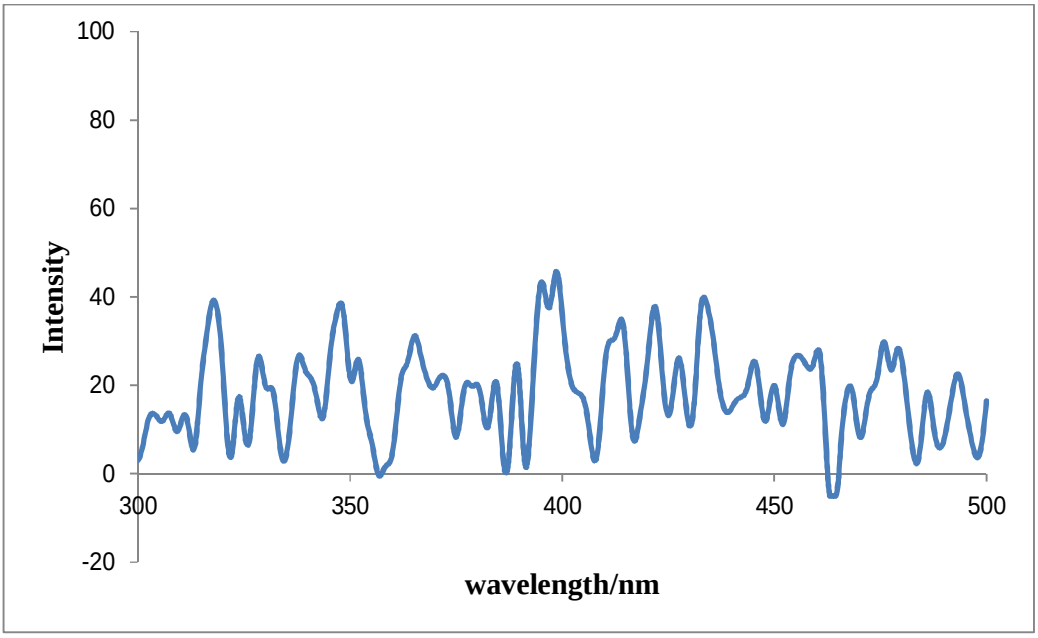

Figure B 30 Fluorescence spectrum of N-acetyl-L-cysteine.

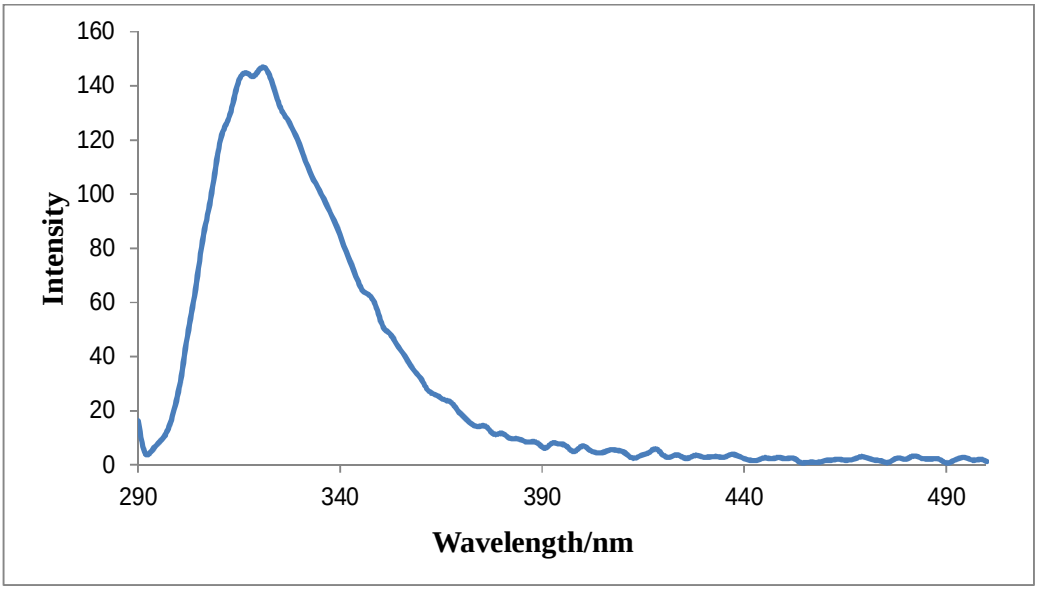

Figure B 31 Fluorescence spectrum of arbutin.

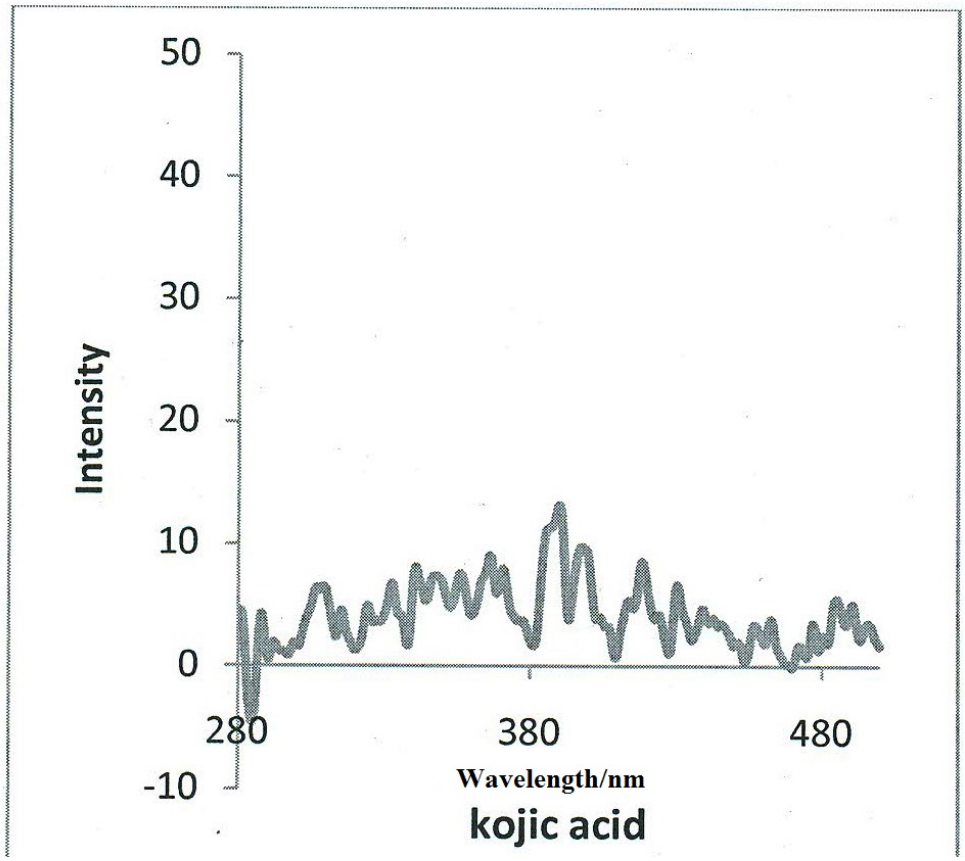

Figure B 32 Fluorescence spectrum of kojic acid.

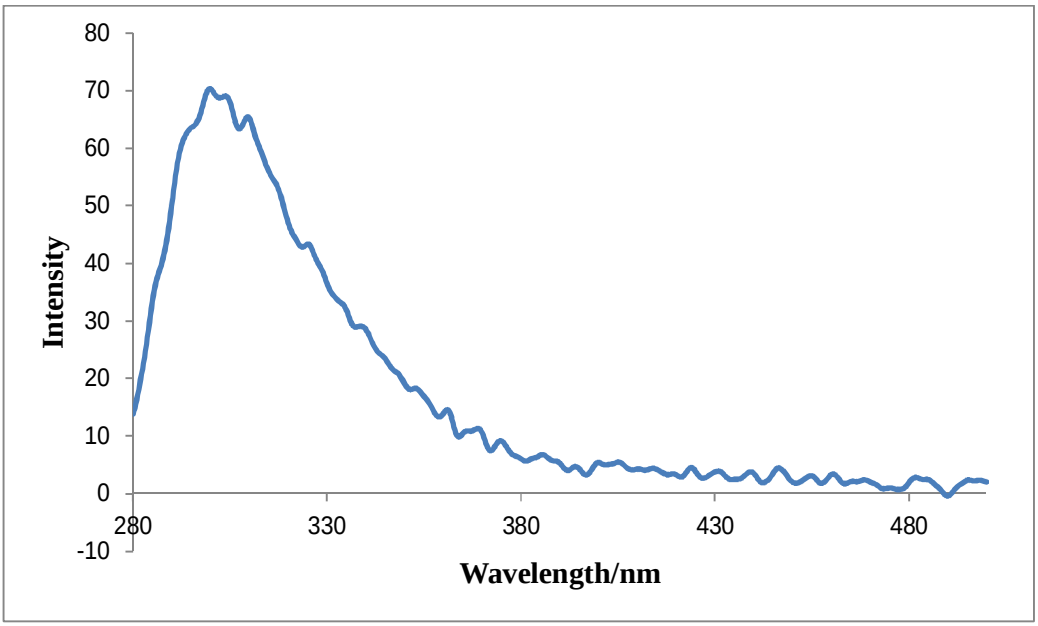

Figure B 33 Fluorescence spectrum of phenol.

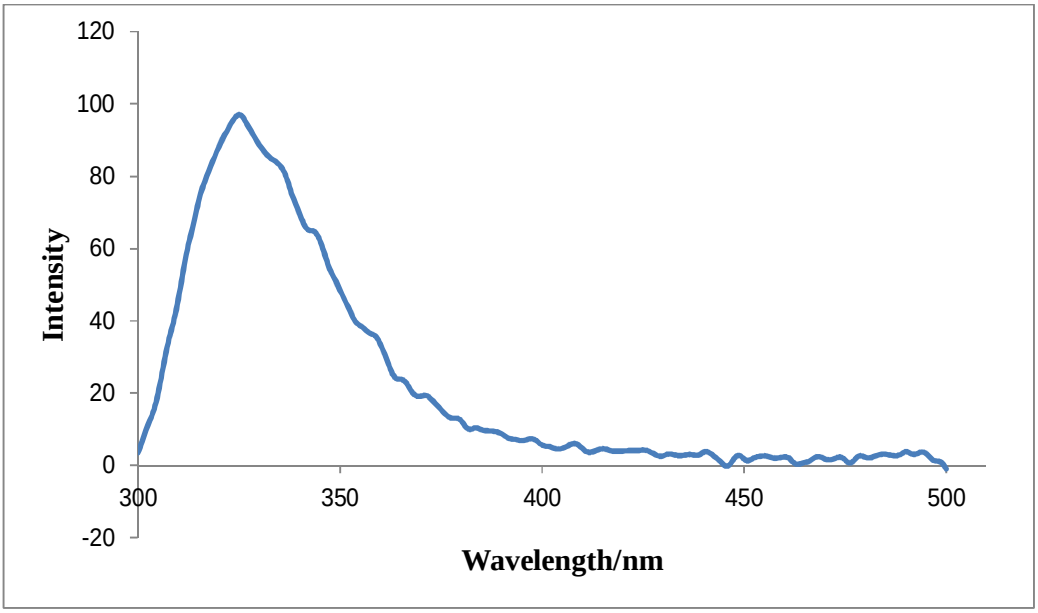

Figure B 34 Fluorescence spectrum of 4-propoxyphenol.

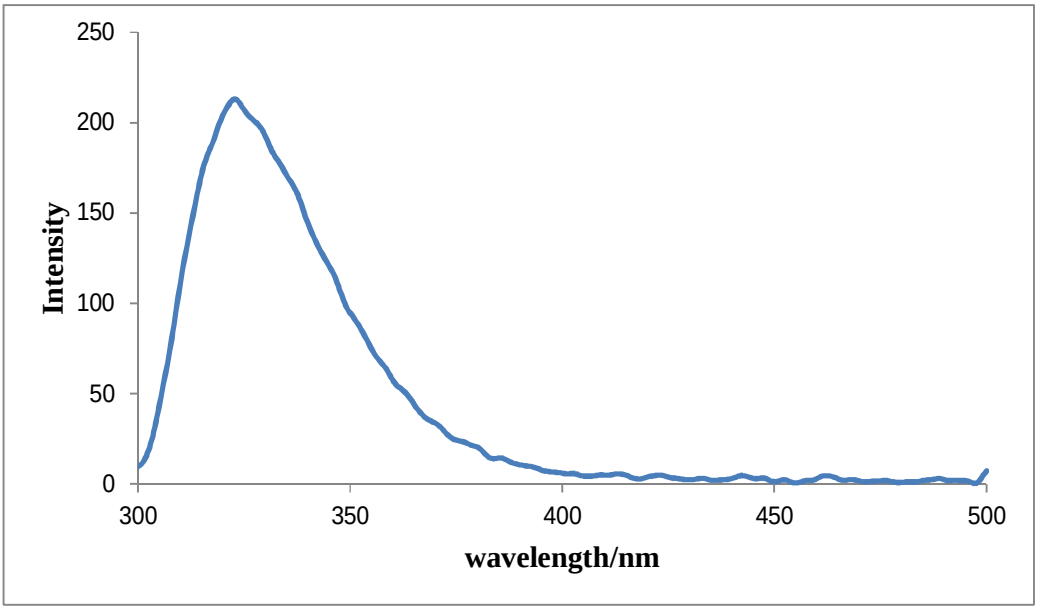

Figure B 35 Fluorescence spectrum of 4-hydroxyanisole.

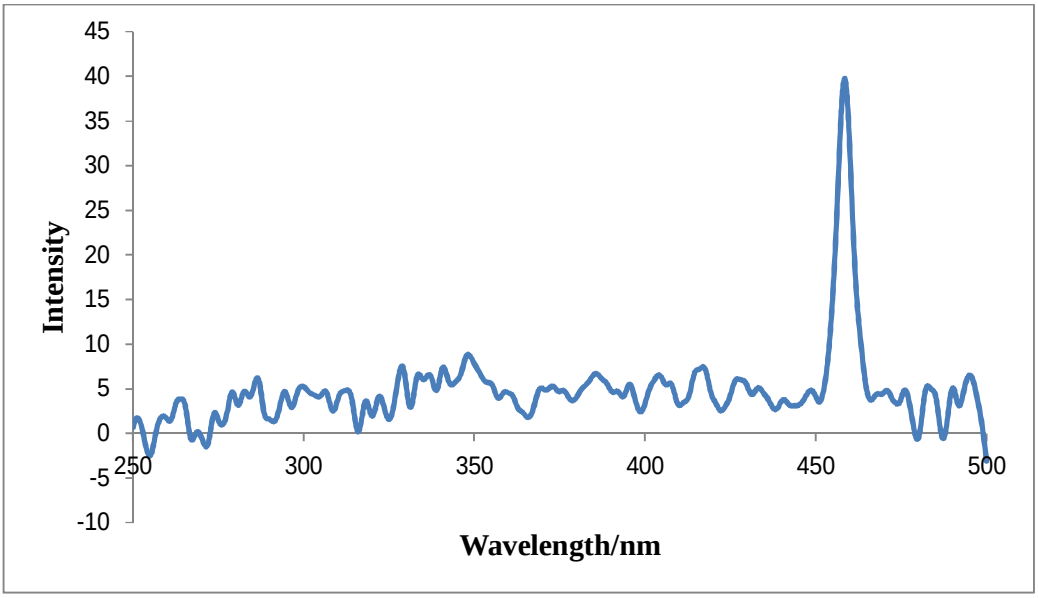

Figure B 36 Fluorescence spectrum of dexamethasone.

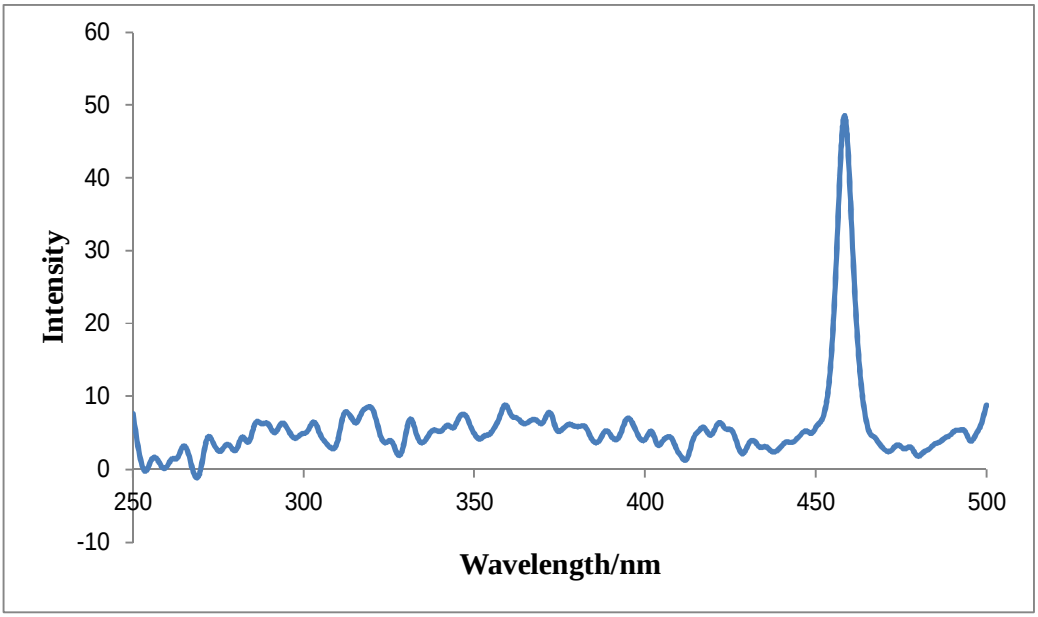

Figure B 37 Fluorescence spectrum of hydrocortisone.

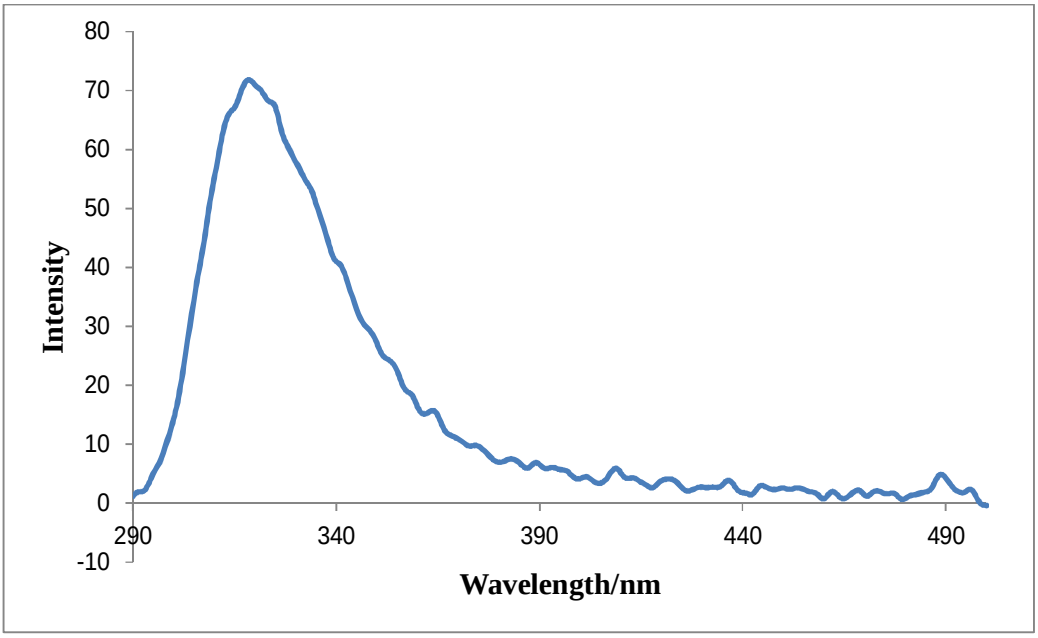

Figure B 38 Fluorescence spectrum of hydrochinon-dimethylether.

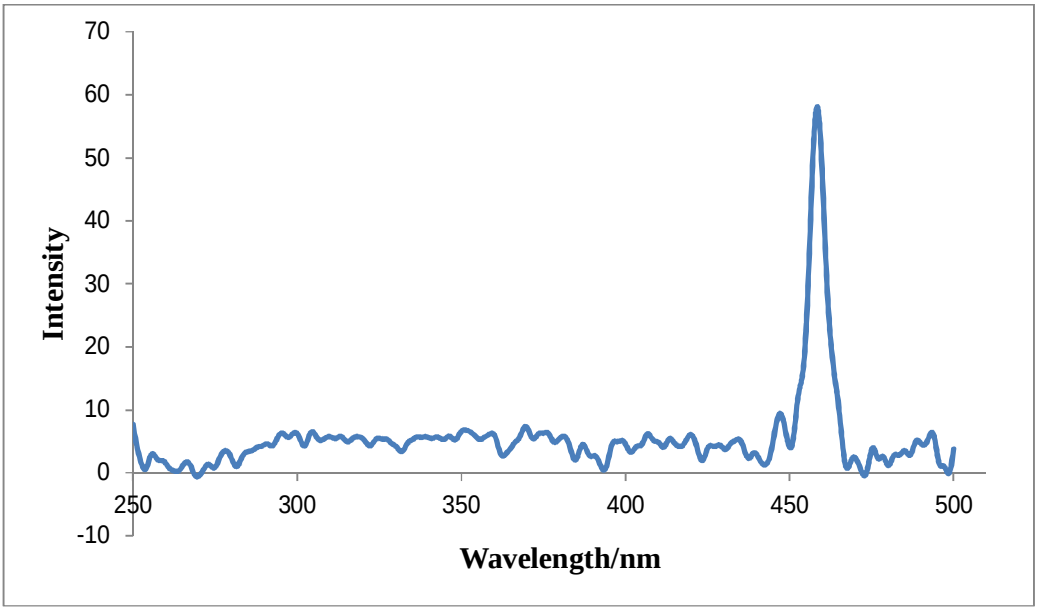

Figure B 39 Fluorescence spectrum of 6α-methylprednisolone.

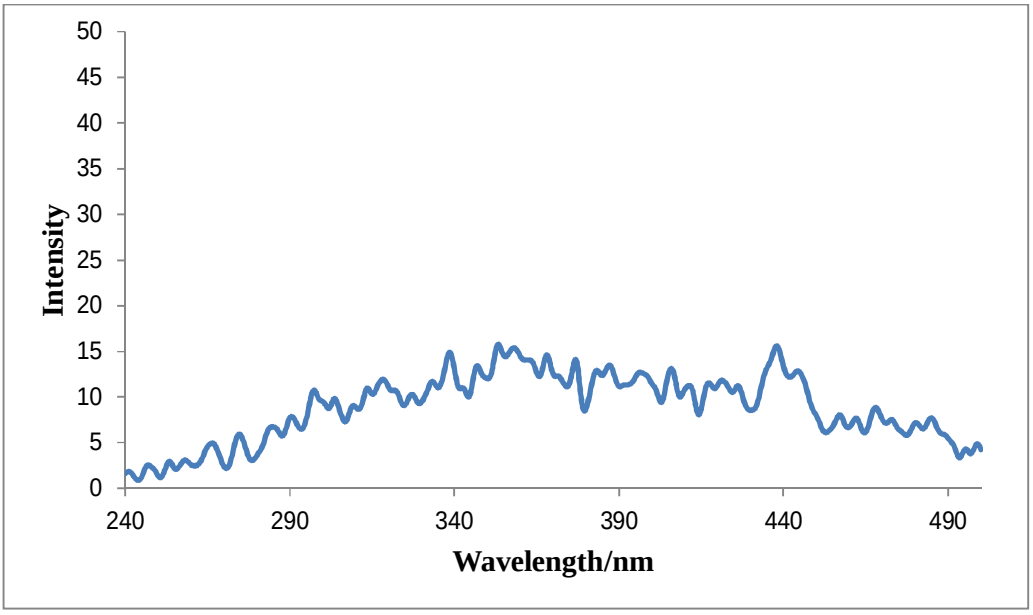

Figure B 40 Fluorescence spectrum of fluocinolone acetonide.

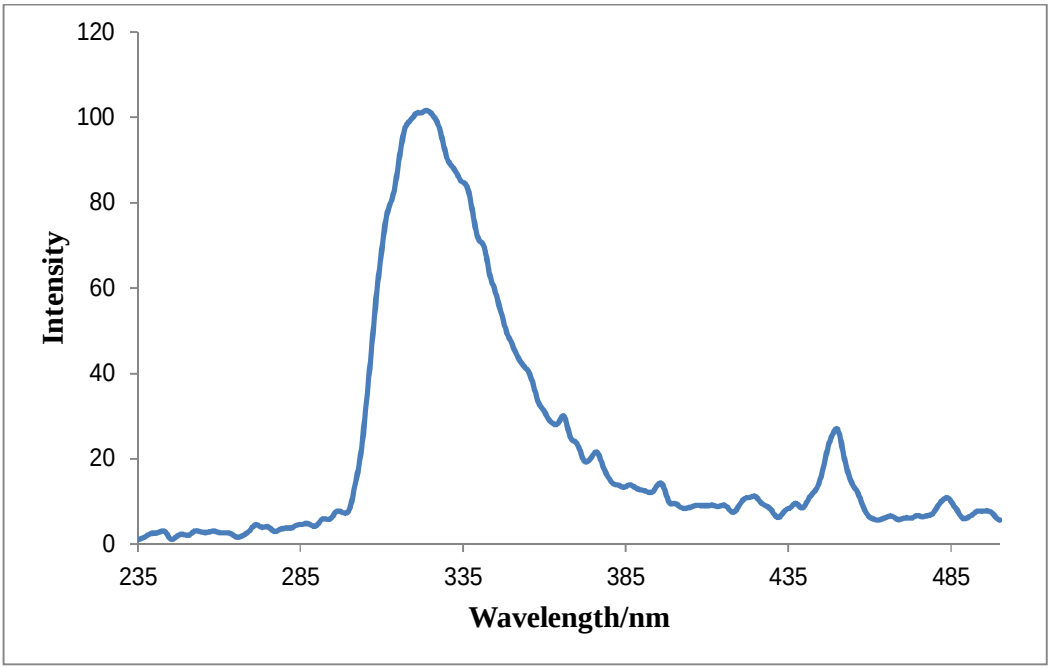

Figure B 41 Fluorescence spectrum of 4-phenoxyphenol.

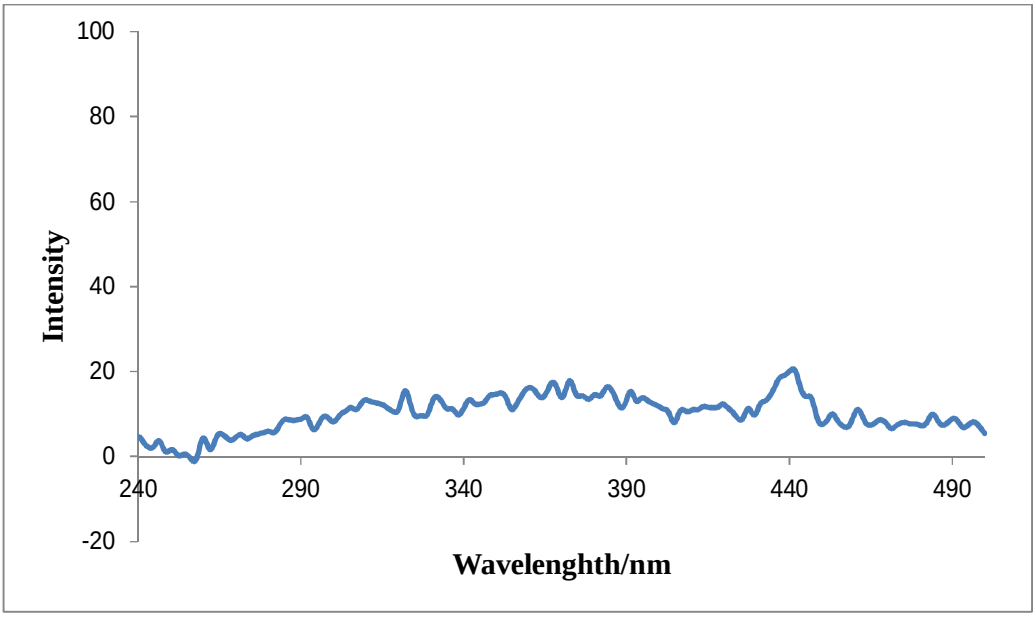

Figure B 42 Fluorescence spectrum of clobetasol propionate.

Appendix C

Linear regression and residual plots

The calibration graphs of the standards analysed are presented here together with the residual plots.

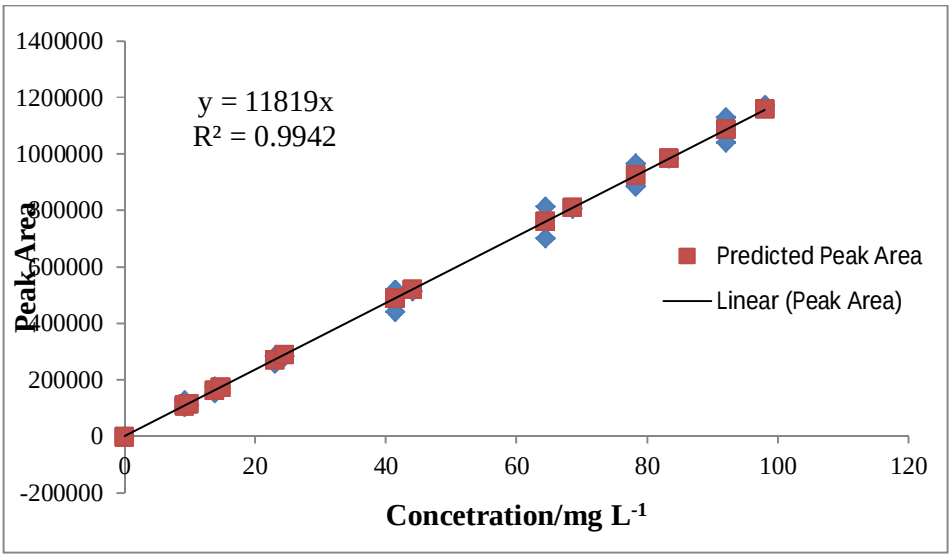

Figure C 1 Calibration curve for niacinamide. The diamond points depict the experimental data and the solid line shows the linear regression model fitted.

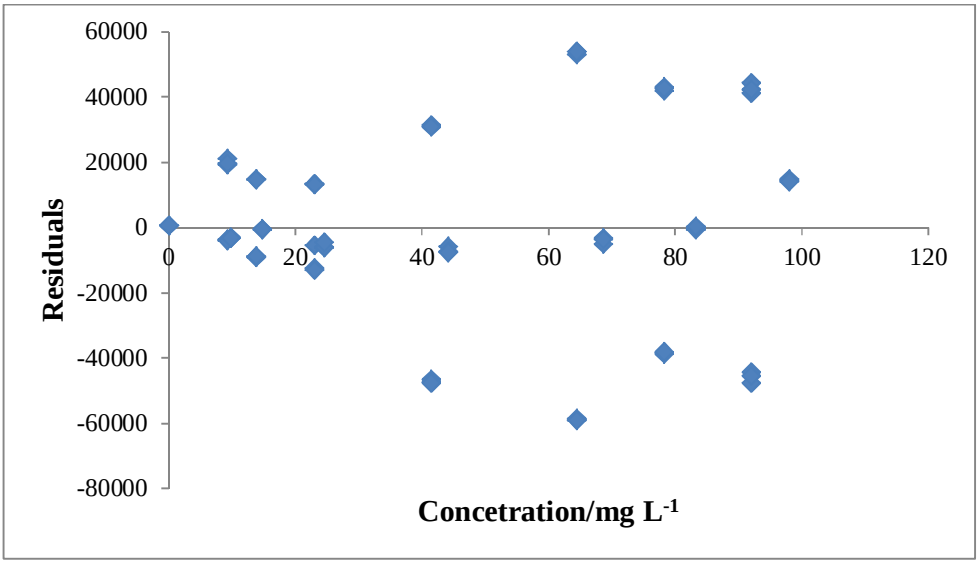

Figure C 2 Residual plot for niacinamide.

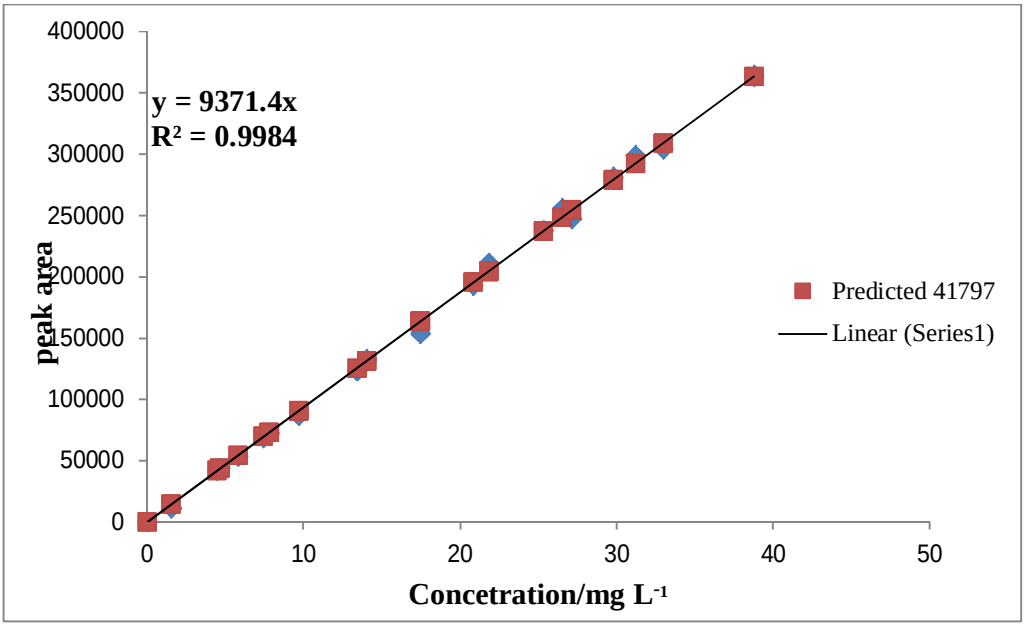

Figure C 3 Calibration curve for phenol. The diamond points depict the experimental data and the solid line shows the linear regression model fitted.

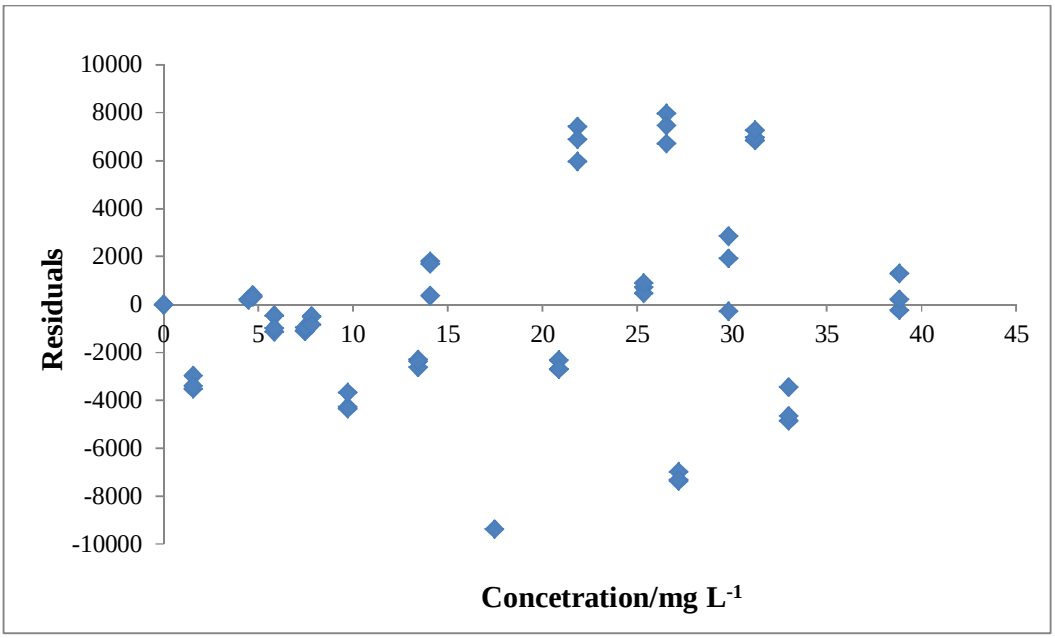

Figure C 4 Residual plot of phenol.

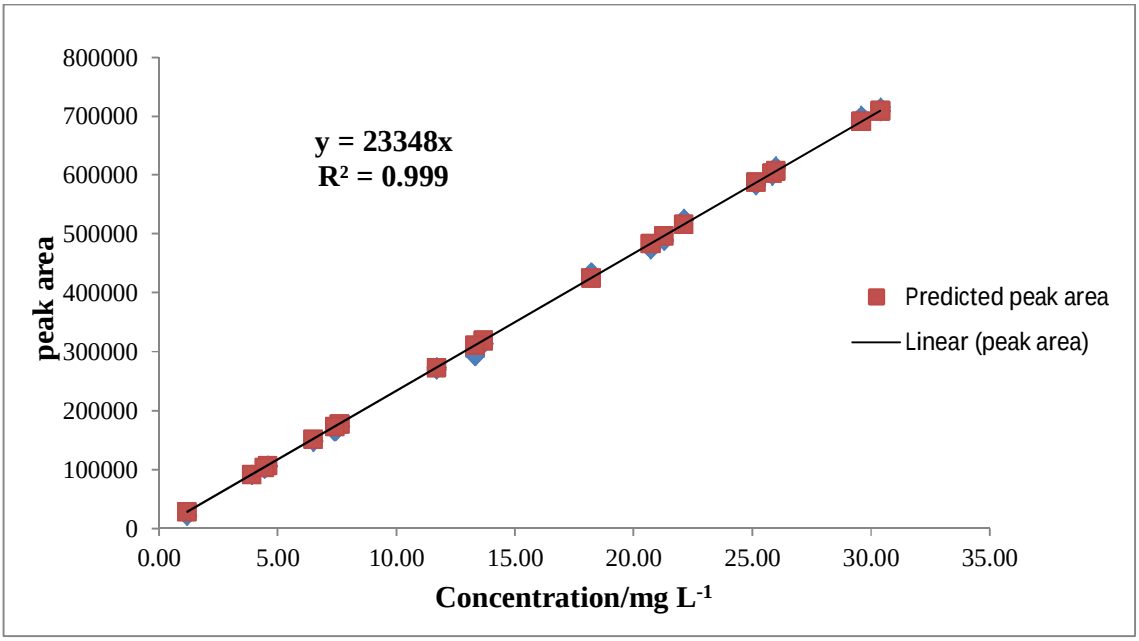

Figure C 5 Calibration curve for dexamethasone. The diamond points depict the experimental data and the solid line shows the linear regression model fitted.

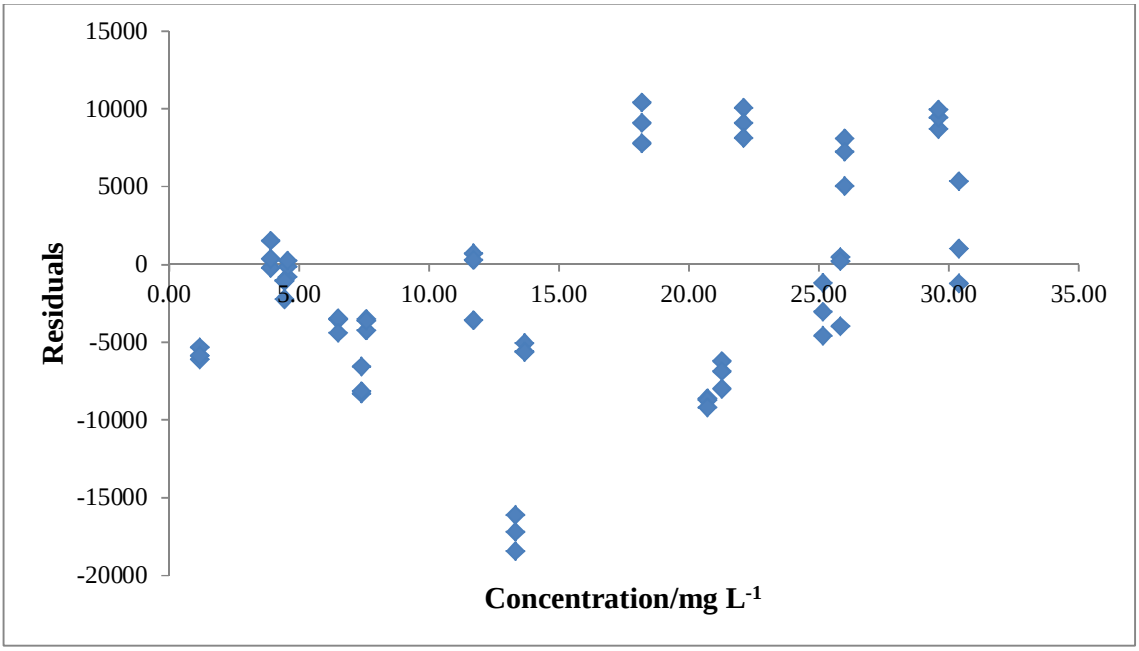

Figure C 6 Residual plot of dexamethasone.

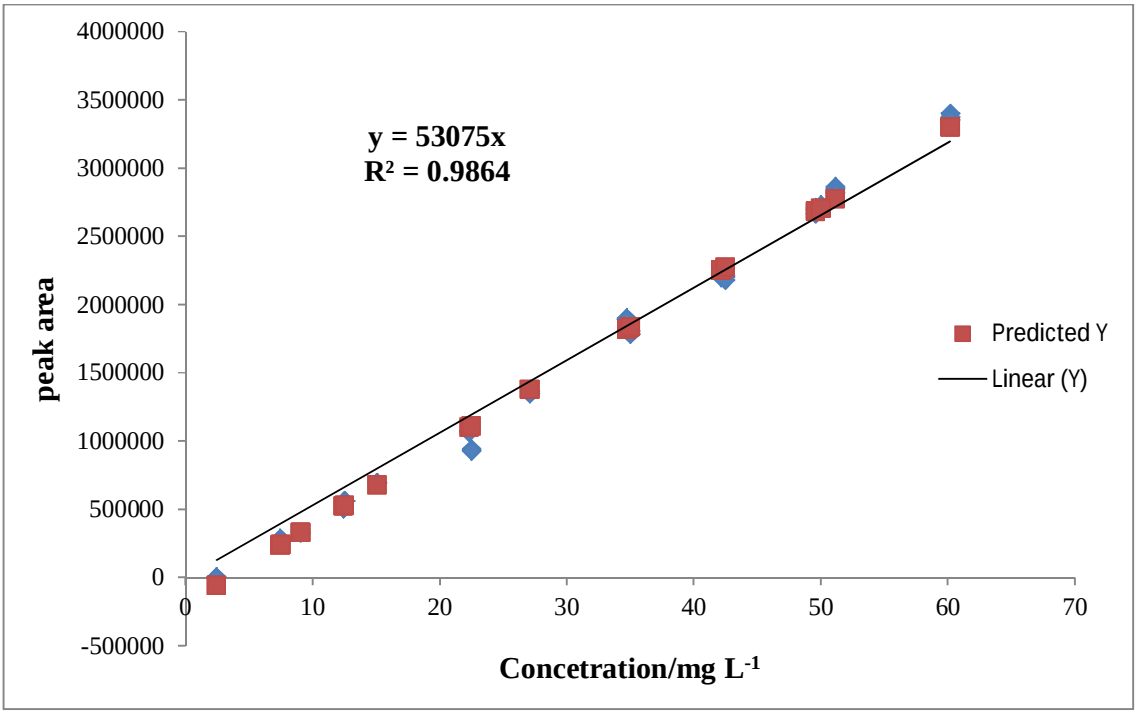

Figure C 7 Calibration curve for benzoquinone. The diamond points depict the experimental data and the solid line shows the linear regression model fitted.

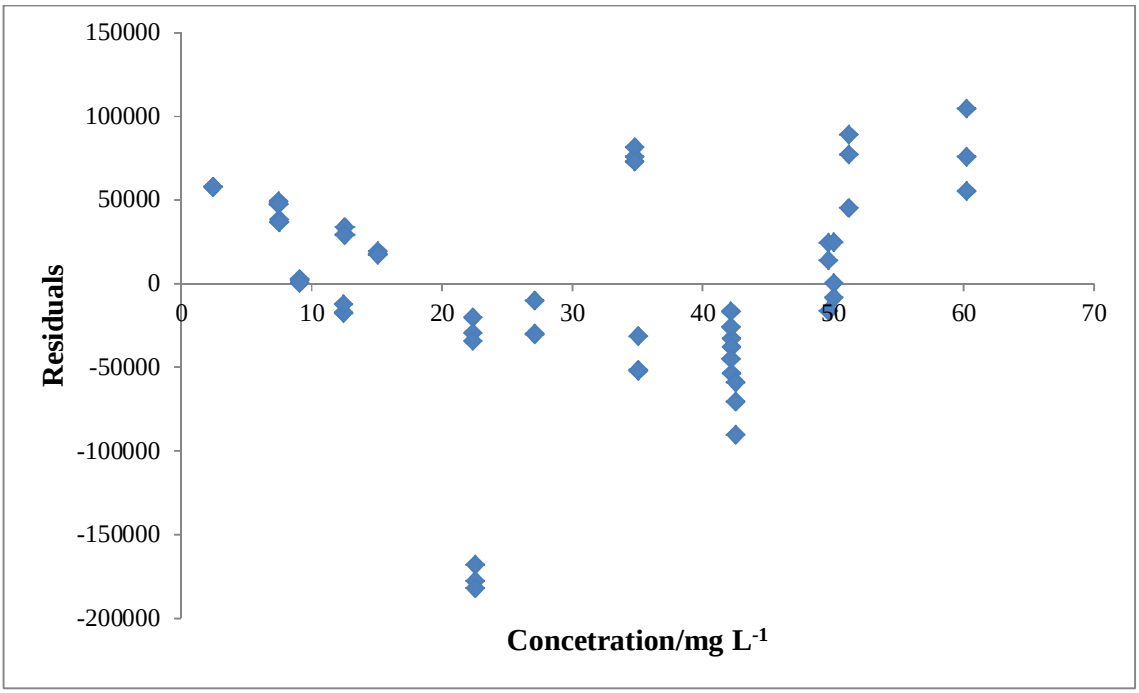

Figure C 8 Residual plot for benzoquinone.

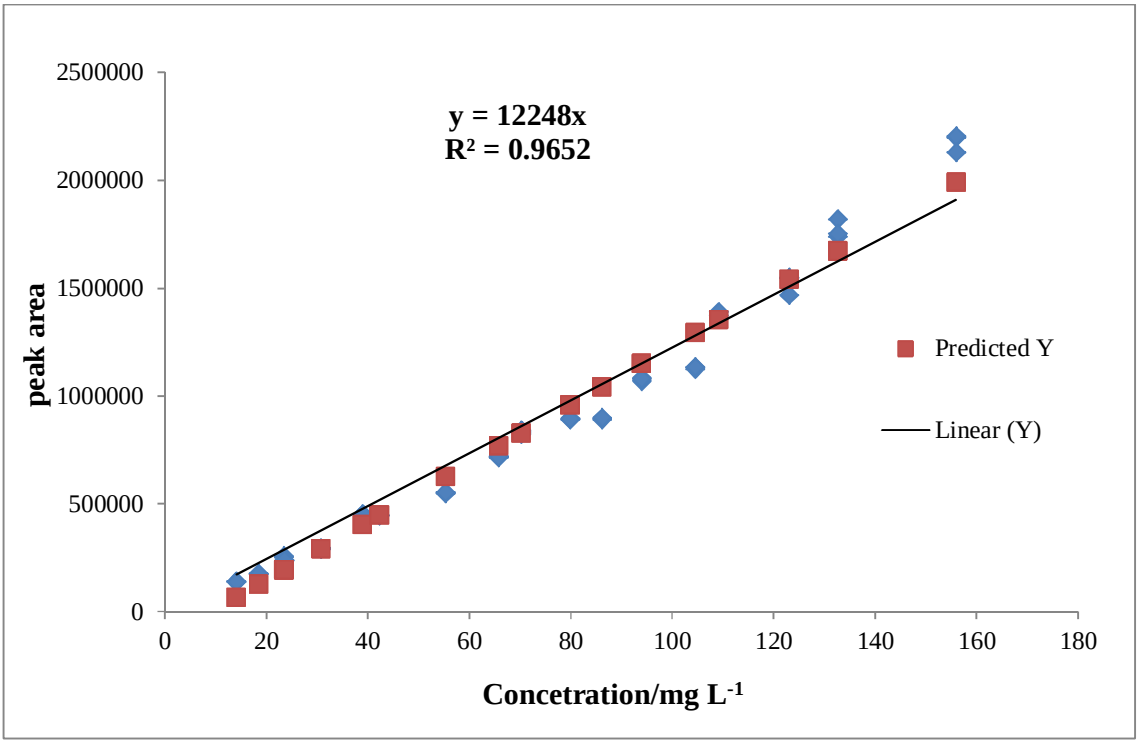

Figure C 9 Calibration curve for hydroquinone. The diamond points depict the experimental data and the solid line shows the linear regression model fitted.

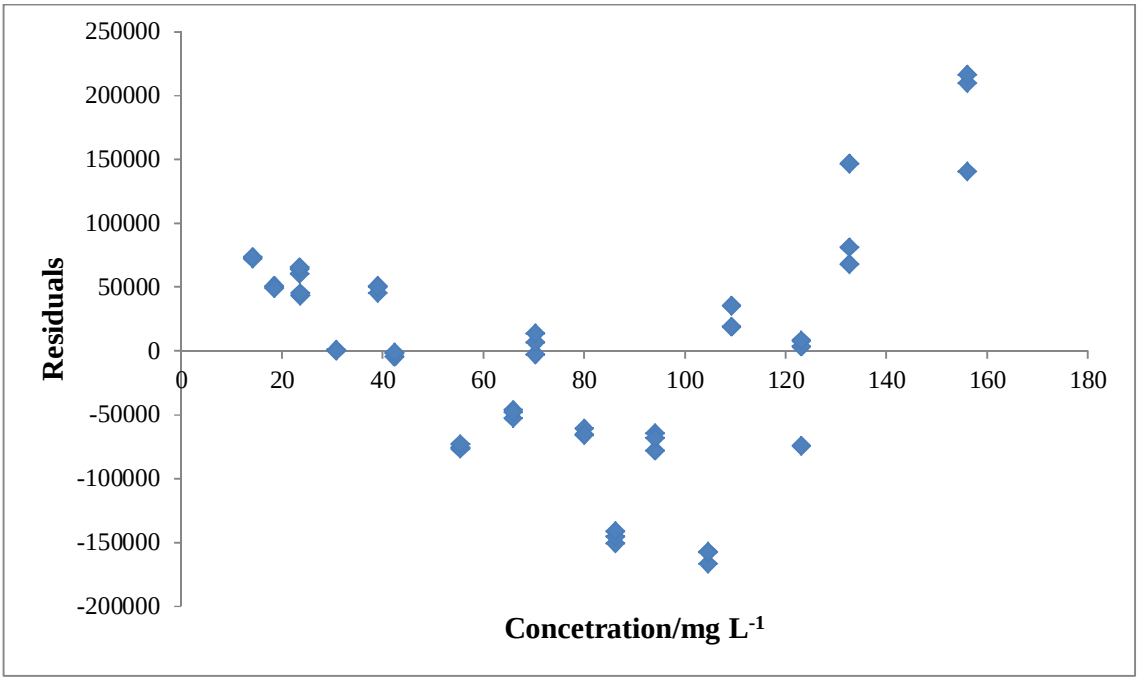

Figure C 10 Residual plot of hydroquinone.

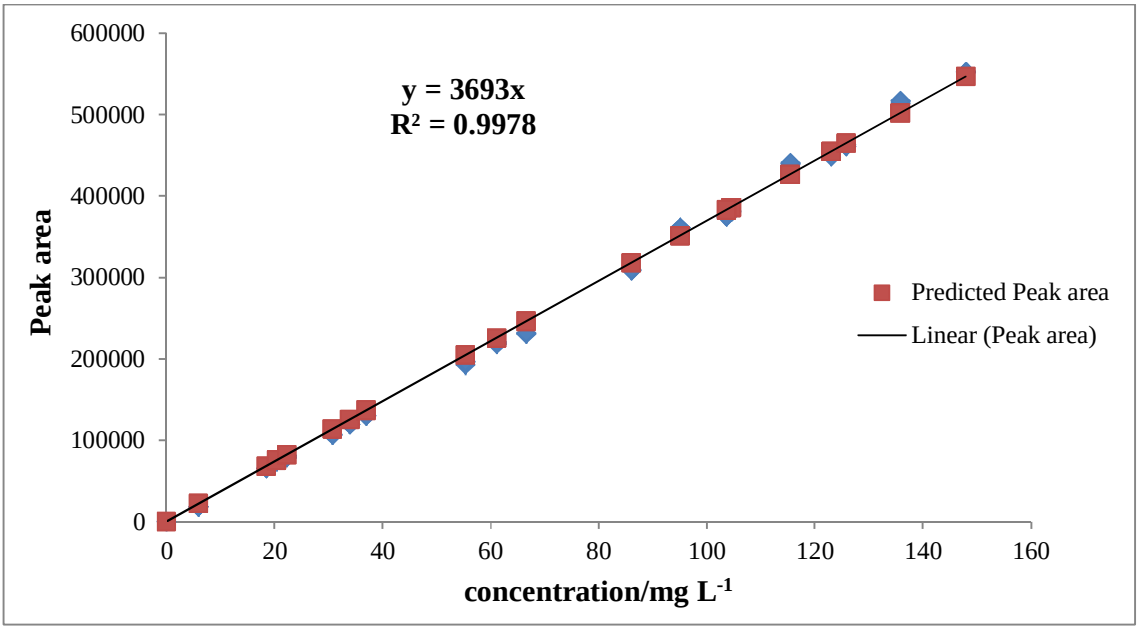

Figure C 11 Calibration curve for arbutin. The diamond points depict the experimental data and the solid line shows the linear regression model fitted.

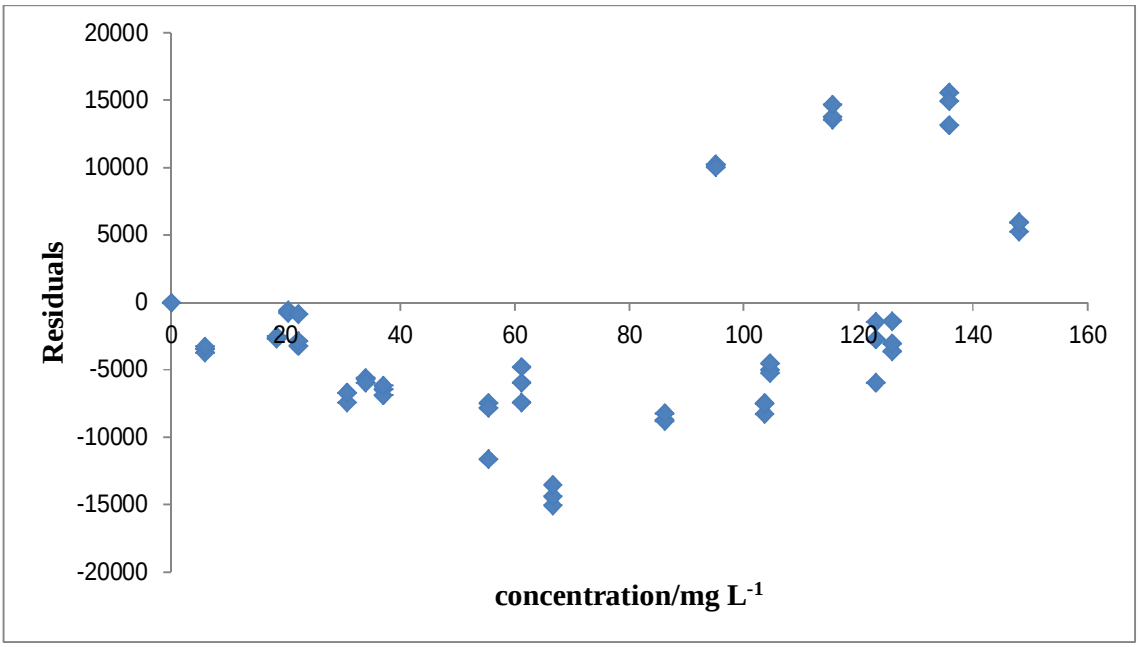

Figure C 12 Residual plot for arbutin.

Appendix D

HPLC chromatograms of samples

The HPLC chromatograms of all samples analysed are presented here.

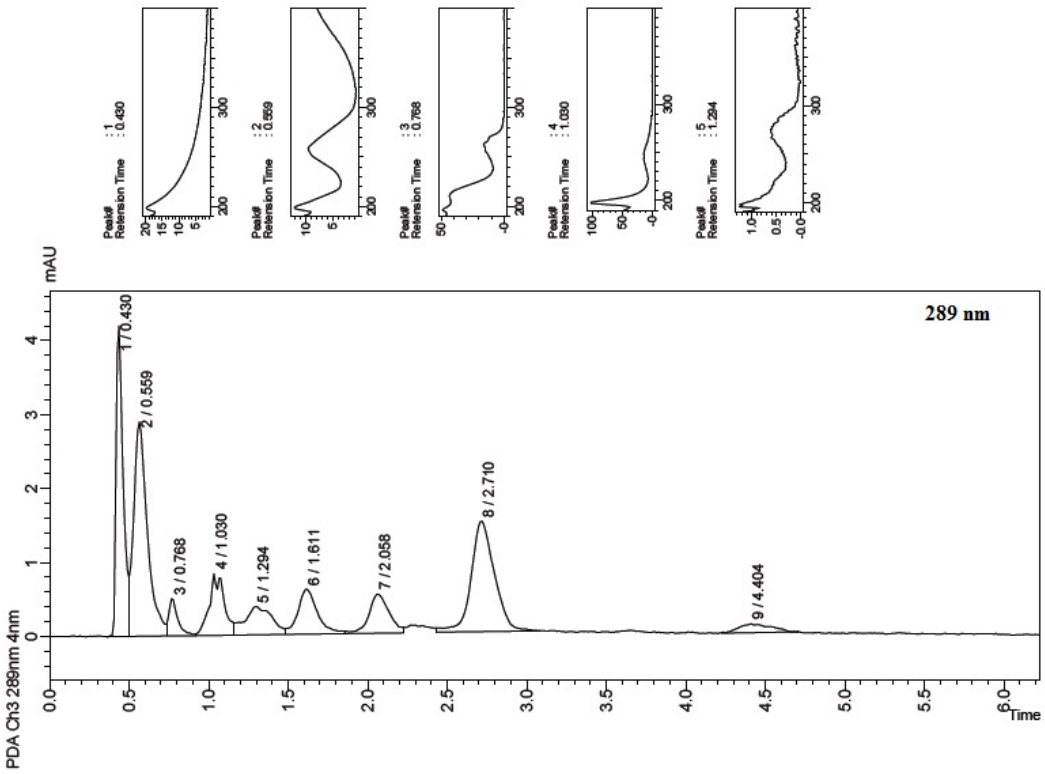

Figure D 1 Chromatogram of sample 1 showing the separation of components from the extracted sample. The chromatographic conditions used were: Brownlee C_{18} (100 × 4.6 mm I.D., 3 µm particle size) column, injection volume 10 µL, mobile phase methanol:water (50:50, v/v), flow rate of 1.0 mL min^{-1}, and detection wavelength of 289 nm.

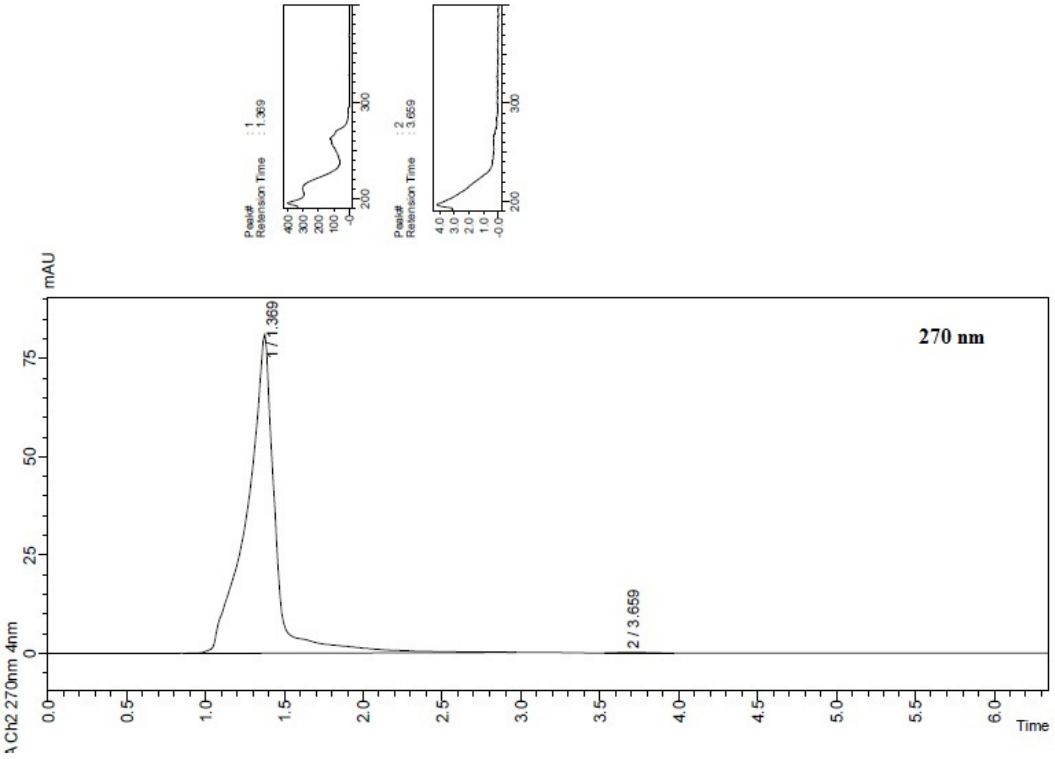

Figure D 2 Chromatogram of sample 2 showing the separation of components from the extracted sample. The chromatographic conditions used were: Brownlee C_{18} (100 × 4.6 mm I.D., 3 µm particle size) column, injection volume 10 µL, mobile phase methanol:water (50:50, v/v), flow rate of 1.0 mL min^{-1}, and detection wavelength of 270 nm.

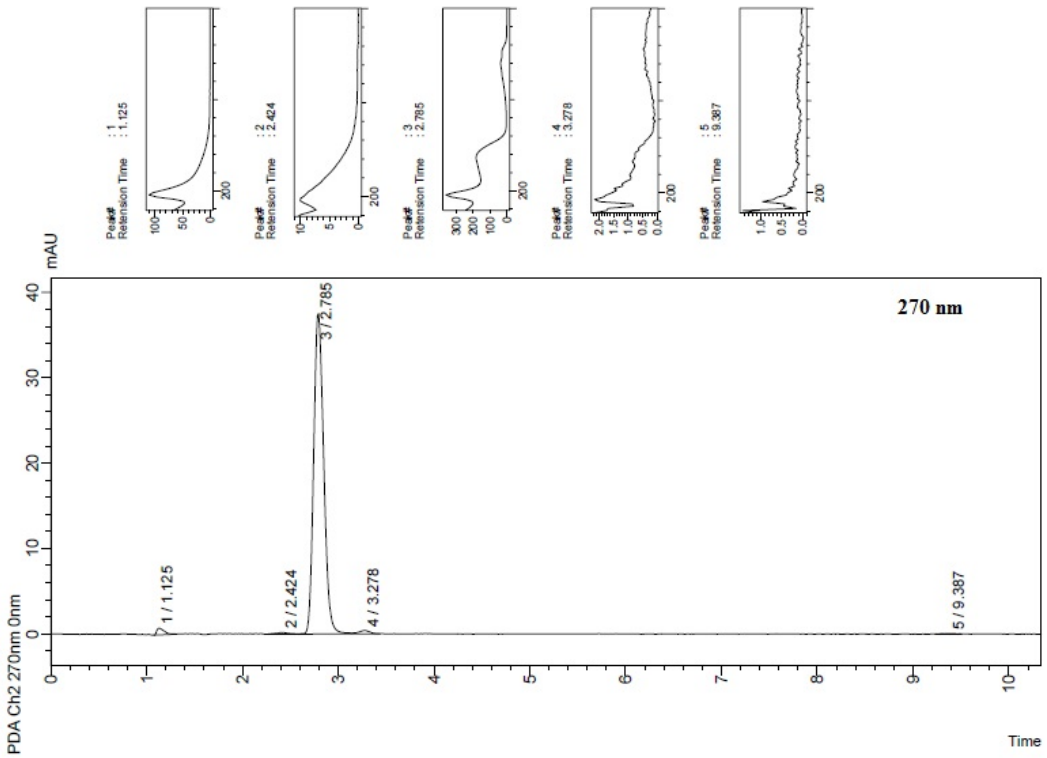

Figure D 3 Chromatogram of sample 3 showing the separation of components from the extracted sample. The chromatographic conditions used were: Brownlee C_{18} (100 × 4.6 mm I.D., 3 µm particle size) column, injection volume 10 µL, mobile phase methanol:water (50:50, v/v), flow rate of 1.0 mL min^{-1}, and detection wavelength of 270 nm.

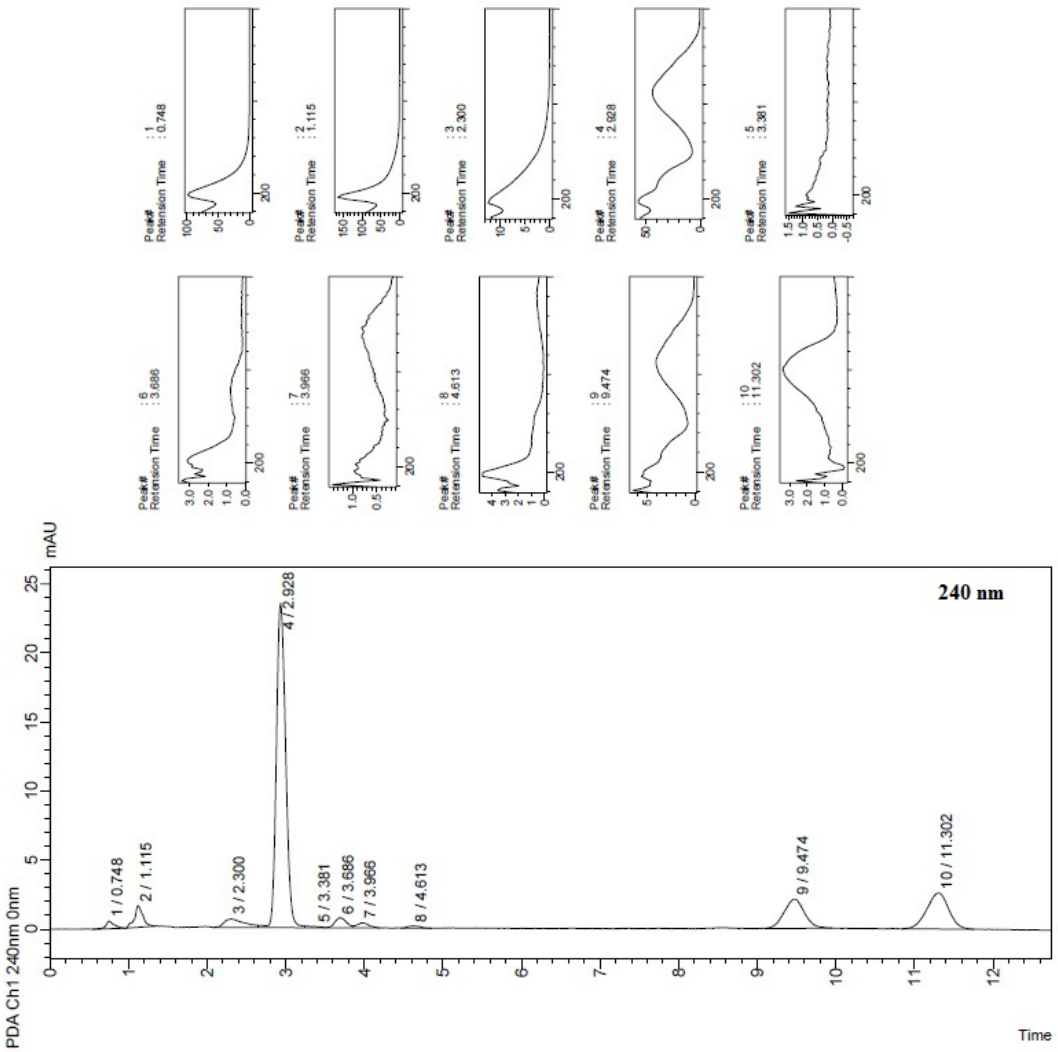

Figure D 4 Chromatogram of sample 4 showing the separation of components from the extracted sample. The chromatographic conditions used were: Brownlee C_{18} (100 × 4.6 mm I.D., 3 µm particle size) column, injection volume 10 µL, mobile phase methanol:water (50:50, v/v), flow rate of 1.0 mL min^{-1}, and detection wavelength of 240 nm.

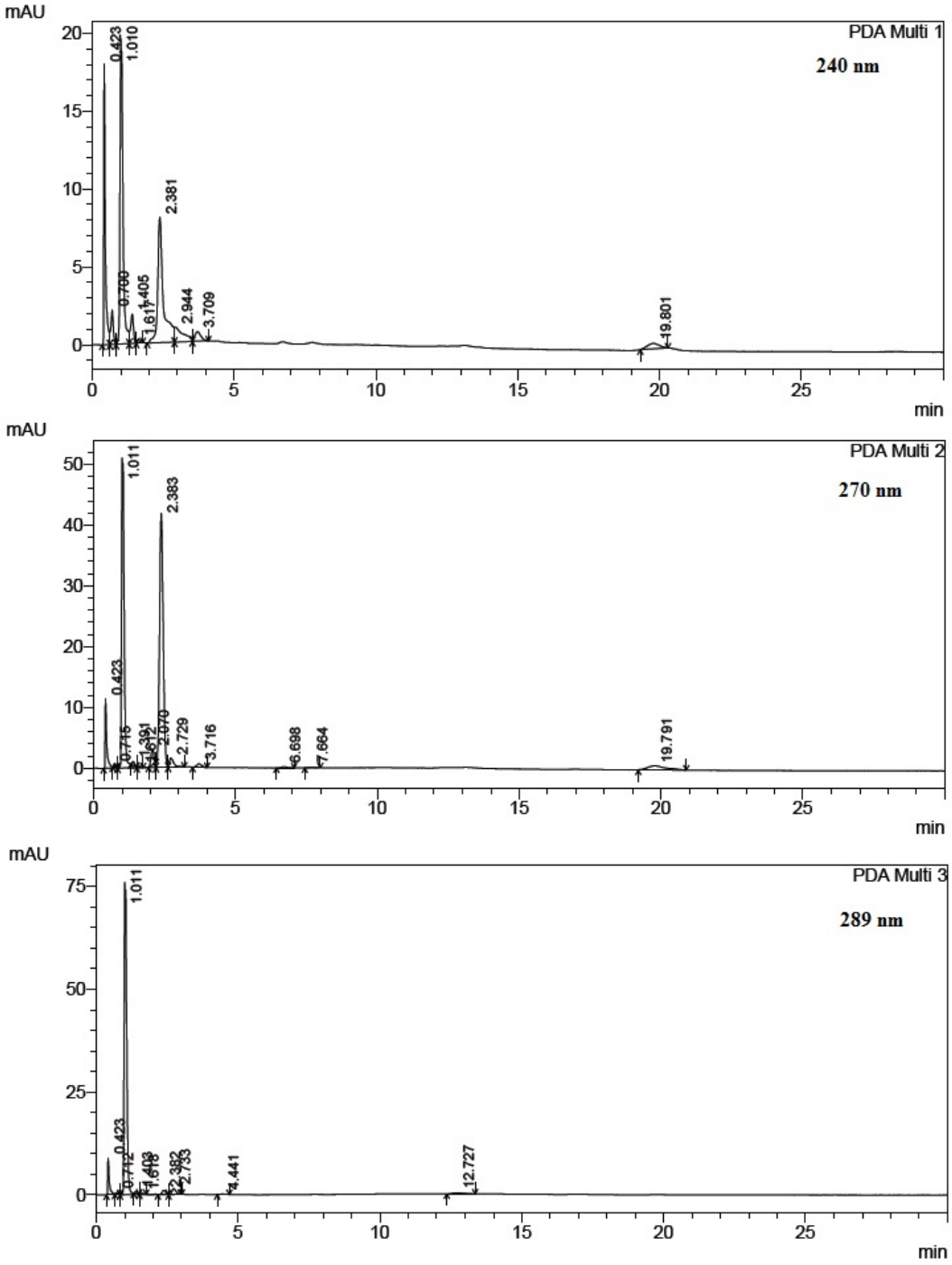

Figure D 5 Chromatogram of sample 5 showing the separation of components from the extracted sample. The chromatographic conditions used were: Brownlee C_{18} (100 × 4.6 mm I.D., 3 µm particle size) column, injection volume 10 µL, mobile phase methanol:water (50:50, v/v), flow rate of 1.0 mL min^{-1}, and detection wavelength of 240, 270 and 289 nm.

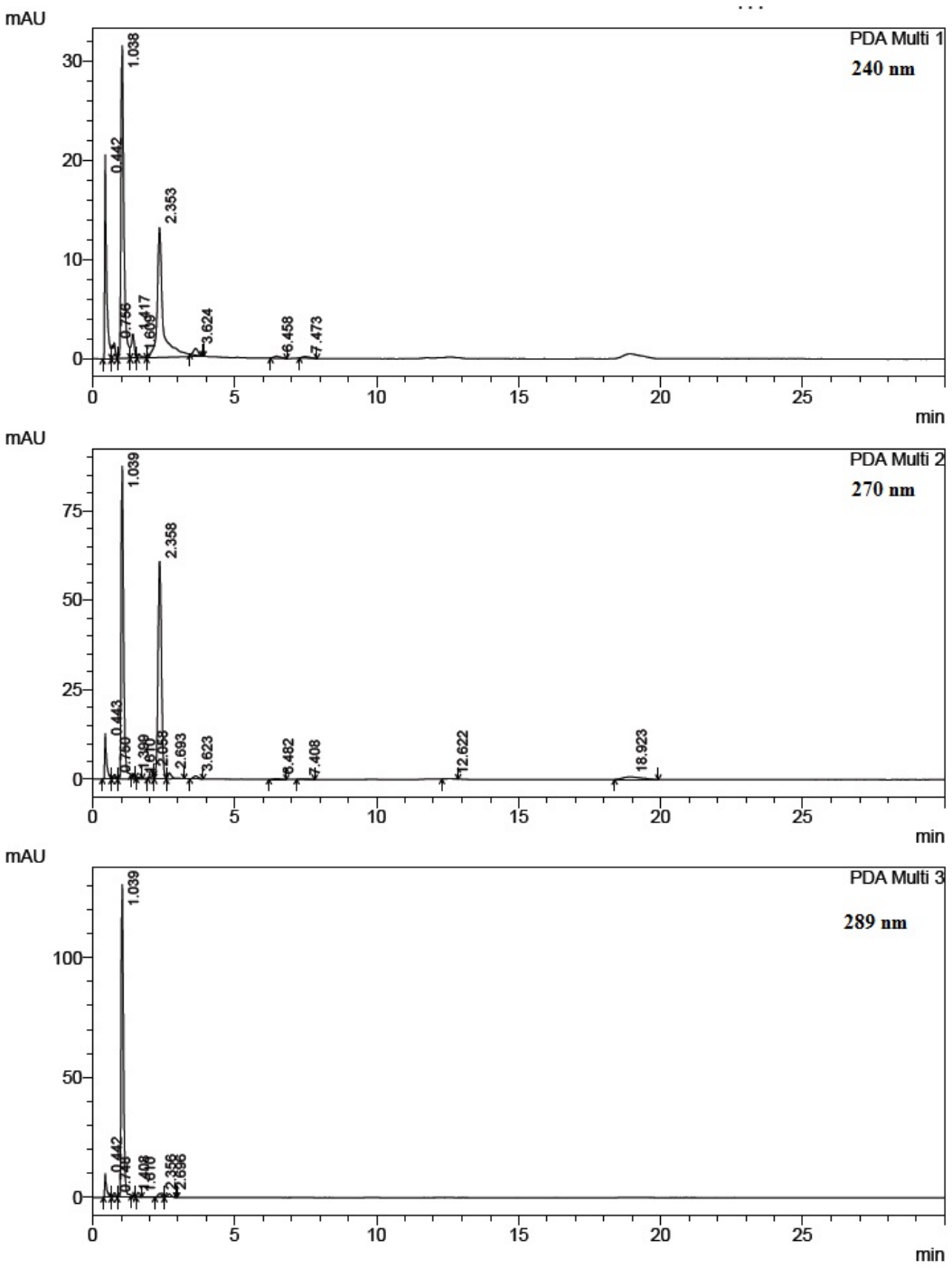

Figure D 6 Chromatogram of sample 6 showing the separation of components from the extracted sample. The chromatographic conditions used were: Brownlee C_{18} (100 × 4.6 mm I.D., 3 µm particle size) column, injection volume 10 µL, mobile phase methanol:water (50:50, v/v), flow rate of 1.0 mL min^{-1}, and detection wavelength of 240, 270 and 289 nm.

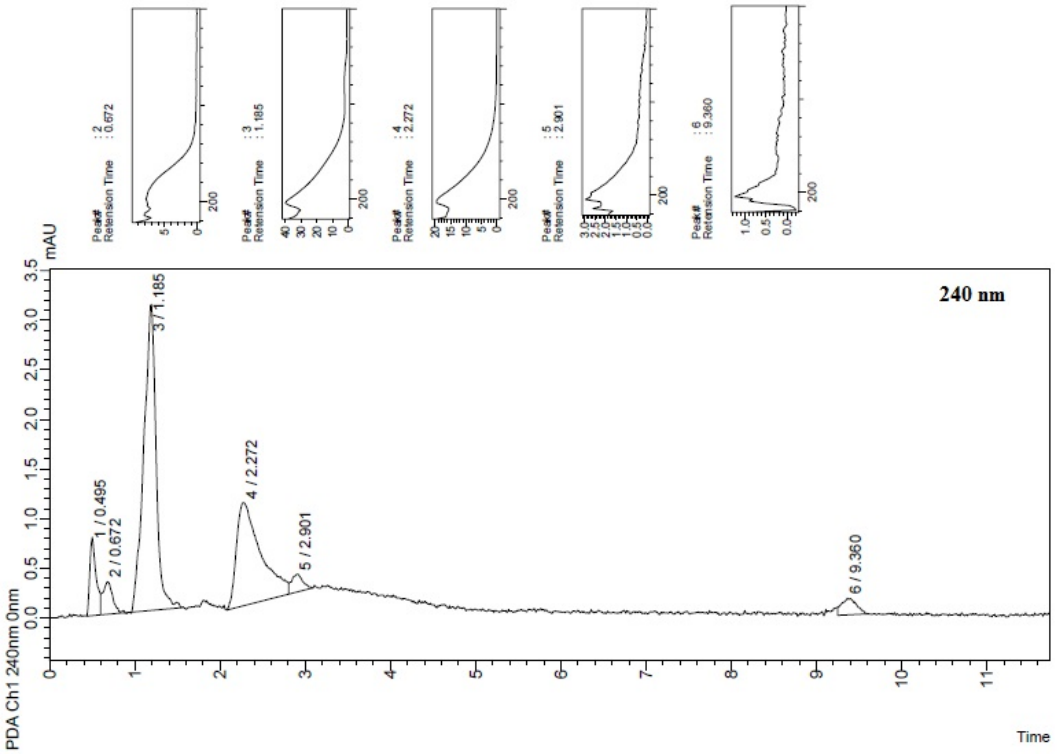

Figure D 7 Chromatogram of sample 7 showing the separation of components from the extracted sample. The chromatographic conditions used were: Brownlee C_{18} (100 × 4.6 mm I.D., 3 µm particle size) column, injection volume 10 µL, mobile phase methanol:water (50:50, v/v), flow rate of 1.0 mL min^{-1}, and detection wavelength of 240 nm.

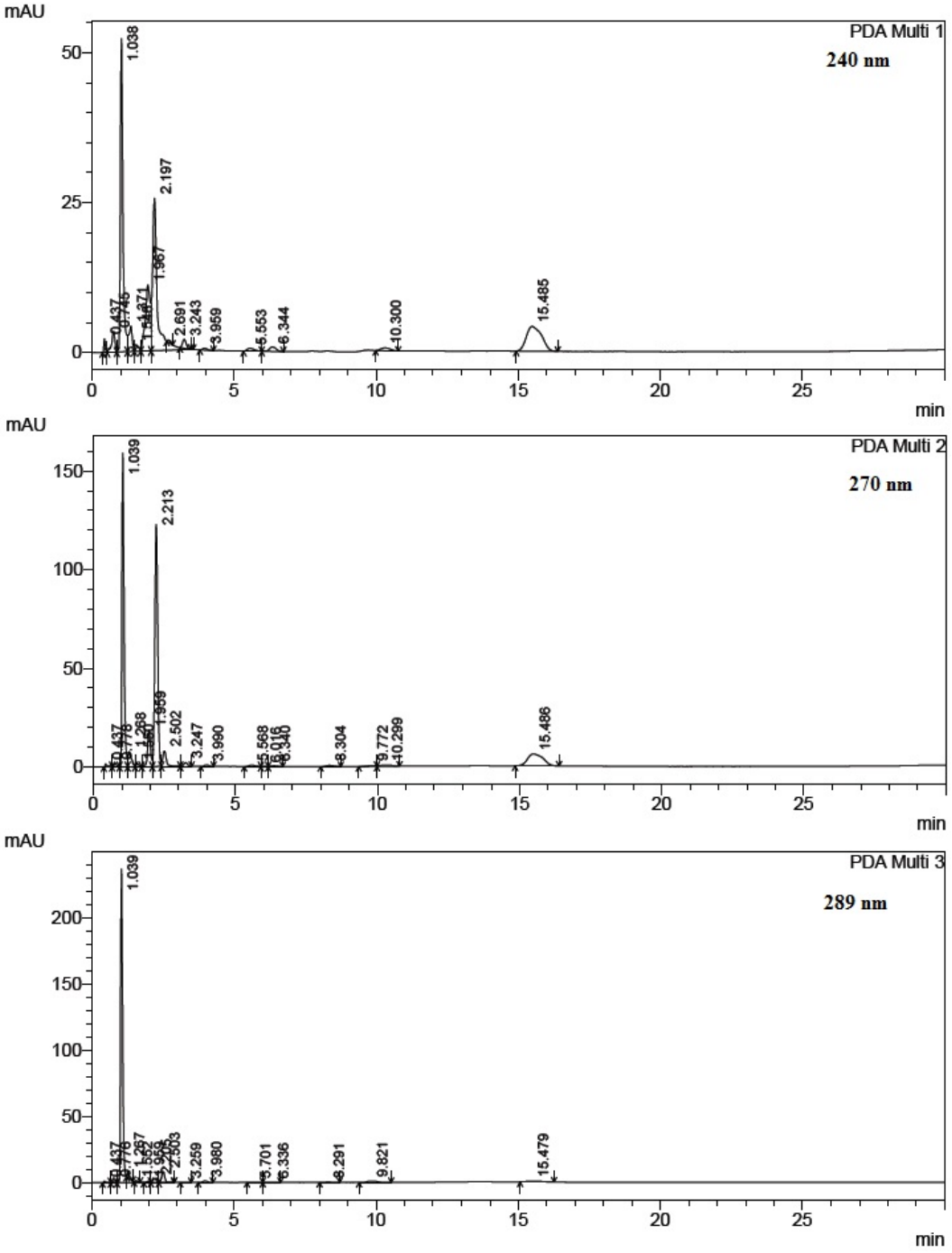

Figure D 8 Chromatogram of sample 8 showing the separation of components from the extracted sample. The chromatographic conditions used were: Brownlee C_{18} (100 × 4.6 mm I.D., 3 µm particle size) column, injection volume 10 µL, mobile phase methanol:water (50:50, v/v), flow rate of 1.0 mL min^{-1}, and detection wavelength of 240, 270 and 289 nm.

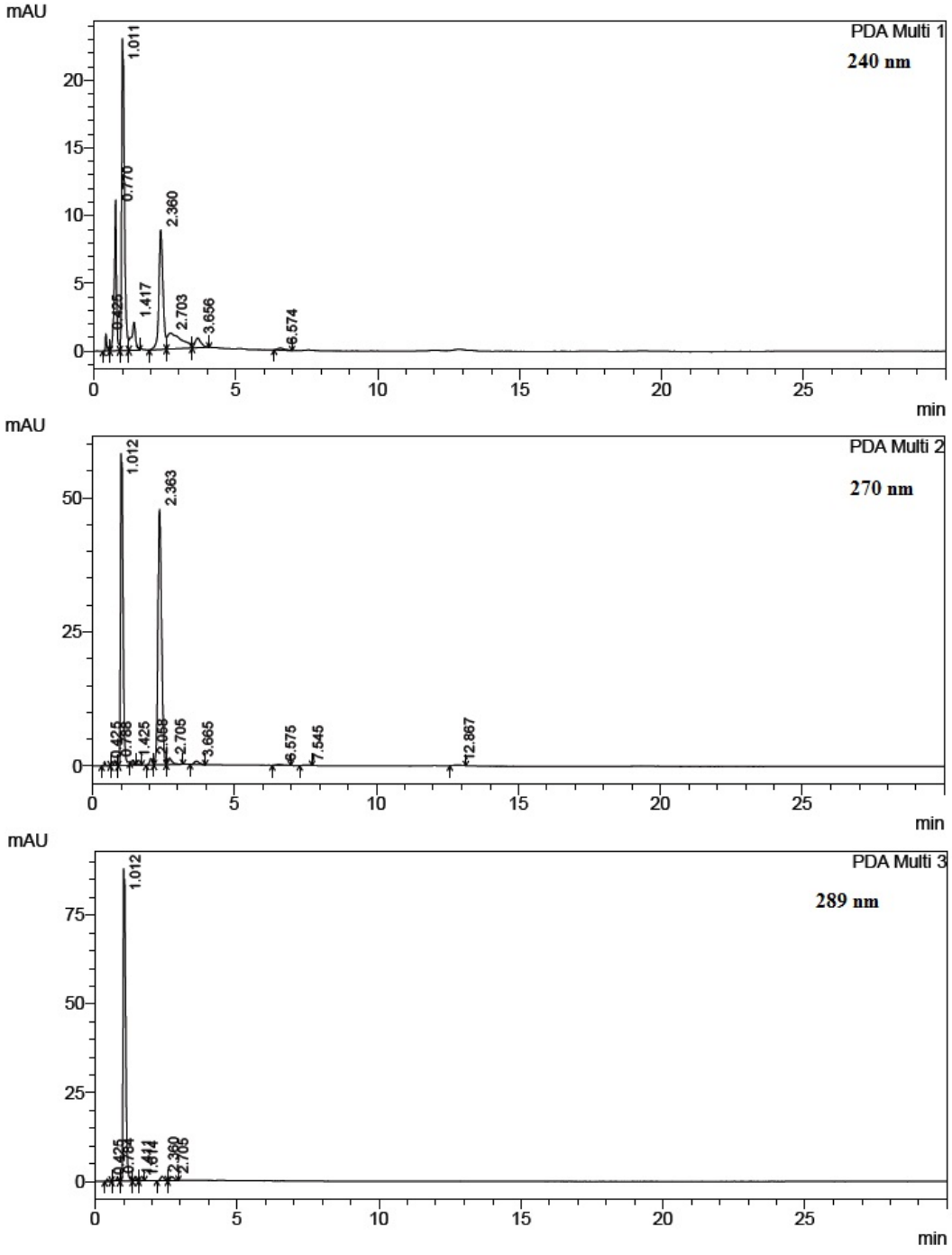

Figure D 9 Chromatogram of sample 9 showing the separation of components from the extracted sample. The chromatographic conditions used were: Brownlee C_{18} (100 × 4.6 mm I.D., 3 µm particle size) column, injection volume 10 µL, mobile phase methanol:water (50:50, v/v), flow rate of 1.0 mL min^{-1}, and detection wavelength of 240, 270 and 289 nm.

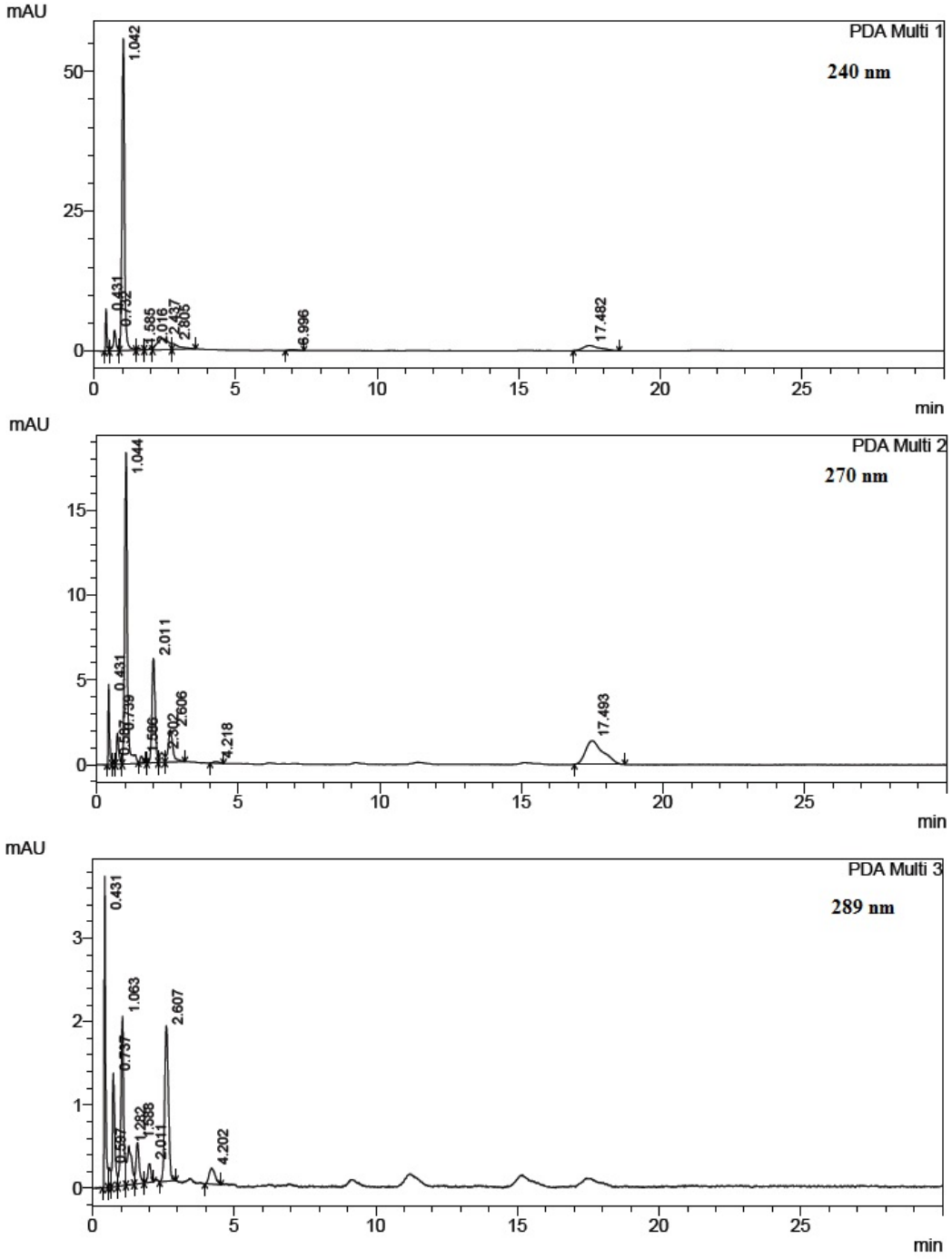

Figure D 10 Chromatogram of sample 10 showing the separation of components from the extracted sample. The chromatographic conditions used were: Brownlee C_{18} (100 × 4.6 mm I.D., 3 µm particle size) column, injection volume 10 µL, mobile phase methanol:water (50:50, v/v), flow rate of 1.0 mL min^{-1}, and detection wavelength of 240, 270 and 289 nm.

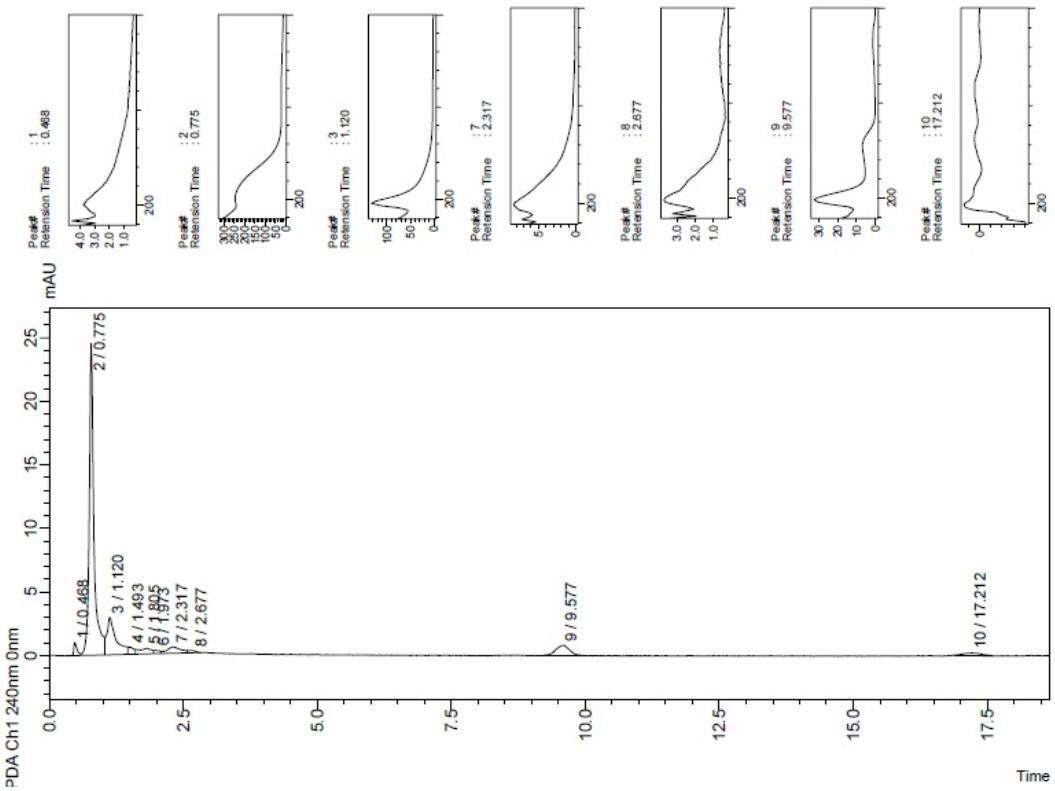

Figure D 11 Chromatogram of sample 11 showing the separation of components from the extracted sample. The chromatographic conditions used were: Brownlee C_{18} (100 × 4.6 mm I.D., 3 µm particle size) column, injection volume 10 µL, mobile phase methanol:water (50:50, v/v), flow rate of 1.0 mL min^{-1}, and detection wavelength of 240 nm.

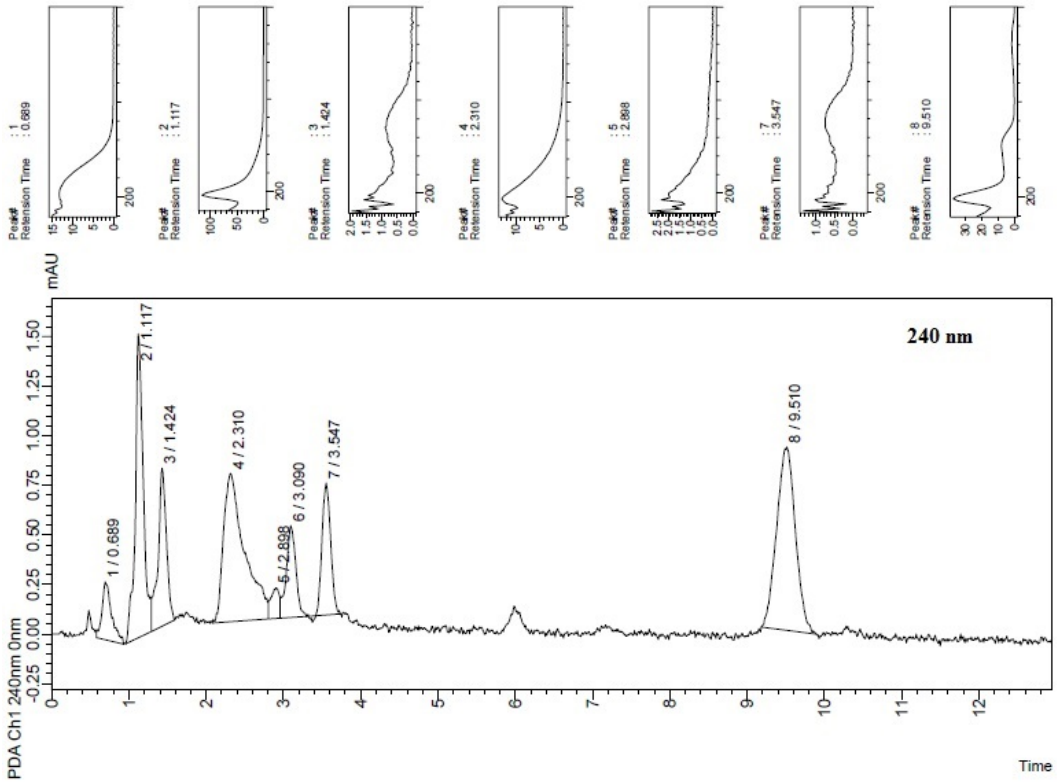

Figure D 12 Chromatogram of sample 12 showing the separation of components from the extracted sample. The chromatographic conditions used were: Brownlee C_{18} (100 × 4.6 mm I.D., 3 µm particle size) column, injection volume 10 µL, mobile phase methanol:water (50:50, v/v), flow rate of 1.0 mL min^{-1}, and detection wavelength of 240 nm.

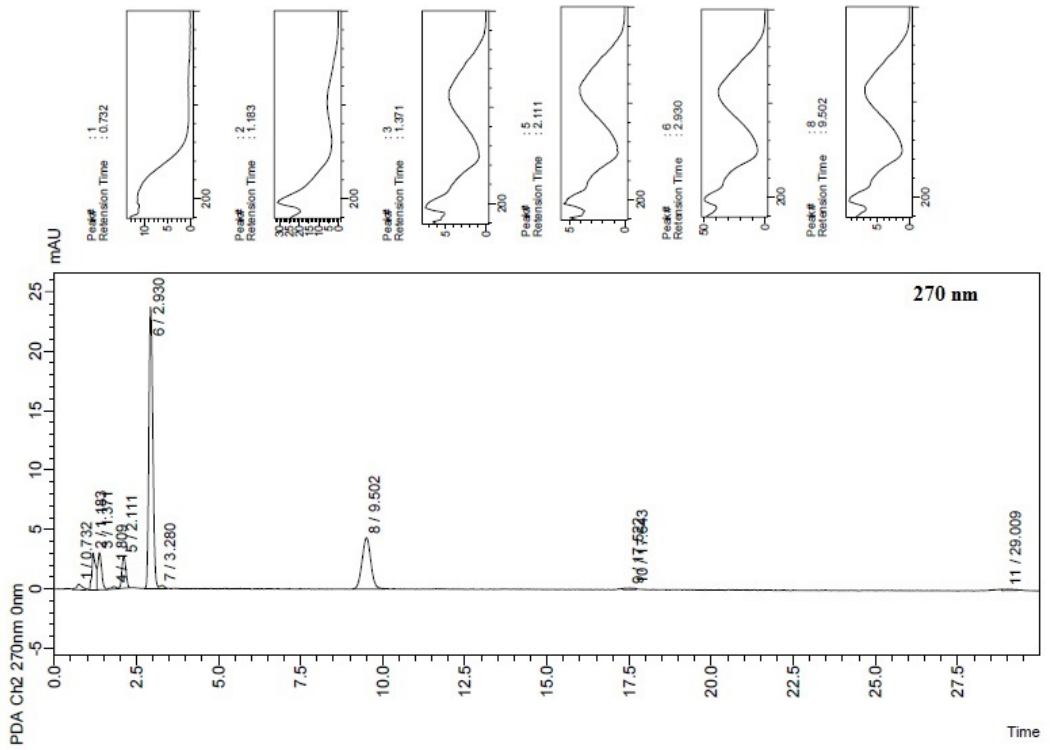

Figure D 13 Chromatogram of sample 13 showing the separation of components from the extracted sample. The chromatographic conditions used were: Brownlee C_{18} (100 × 4.6 mm I.D., 3 µm particle size) column, injection volume 10 µL, mobile phase methanol:water (50:50, v/v), flow rate of 1.0 mL min^{-1}, and detection wavelength of 270 nm.

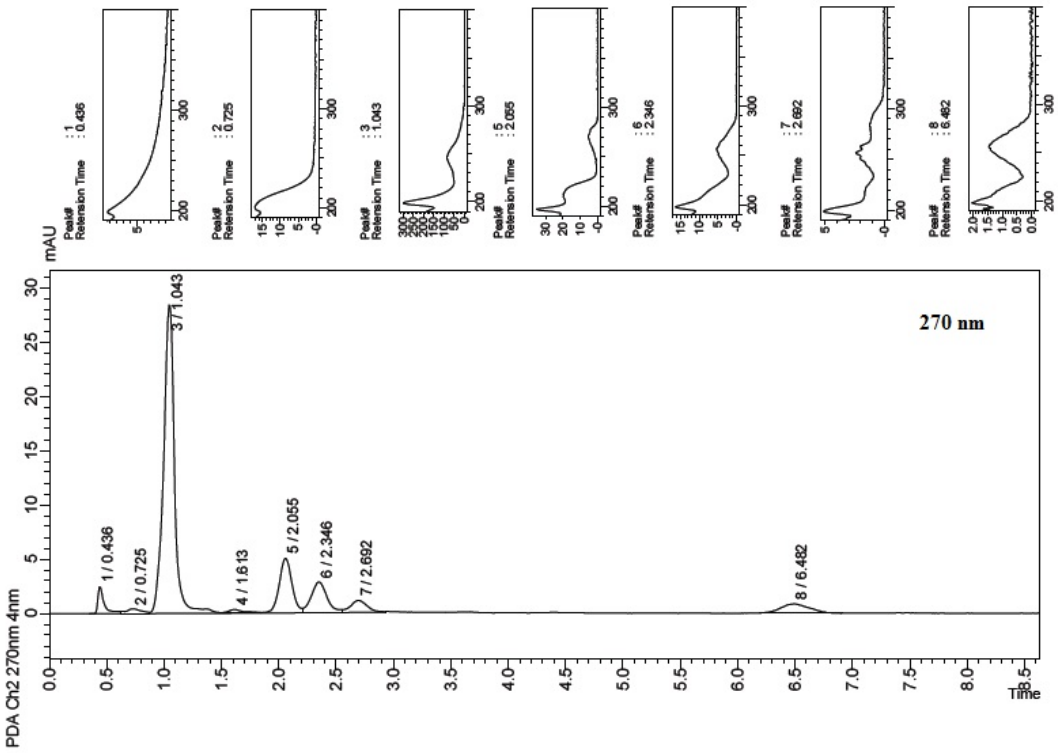

Figure D 14 Chromatogram of sample 14 showing the separation of components from the extracted sample. The chromatographic conditions used were: Brownlee C_{18} (100 × 4.6 mm I.D., 3 µm particle size) column, injection volume 10 µL, mobile phase methanol:water (50:50, v/v), flow rate of 1.0 mL min^{-1}, and detection wavelength of 270 nm.

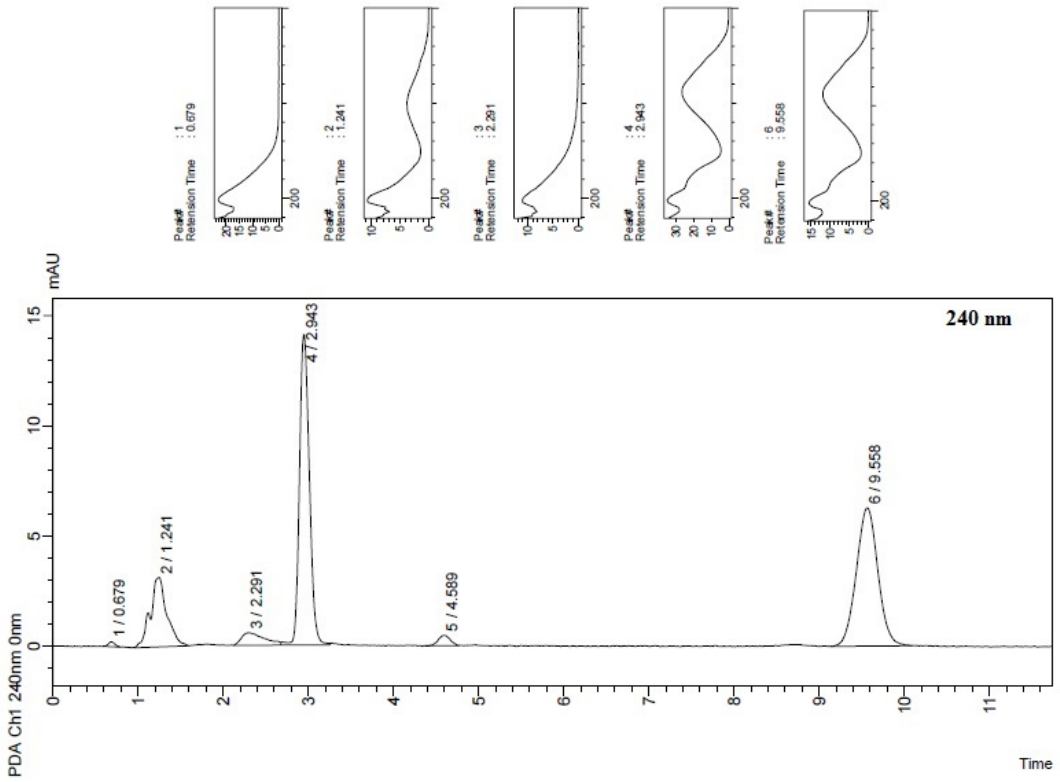

Figure D 15 Chromatogram of sample 15 showing the separation of components from the extracted sample. The chromatographic conditions used were: Brownlee C_{18} (100 × 4.6 mm I.D., 3 µm particle size) column, injection volume 10 µL, mobile phase methanol:water (50:50, v/v), flow rate of 1.0 mL min^{-1}, and detection wavelength of 240 nm.

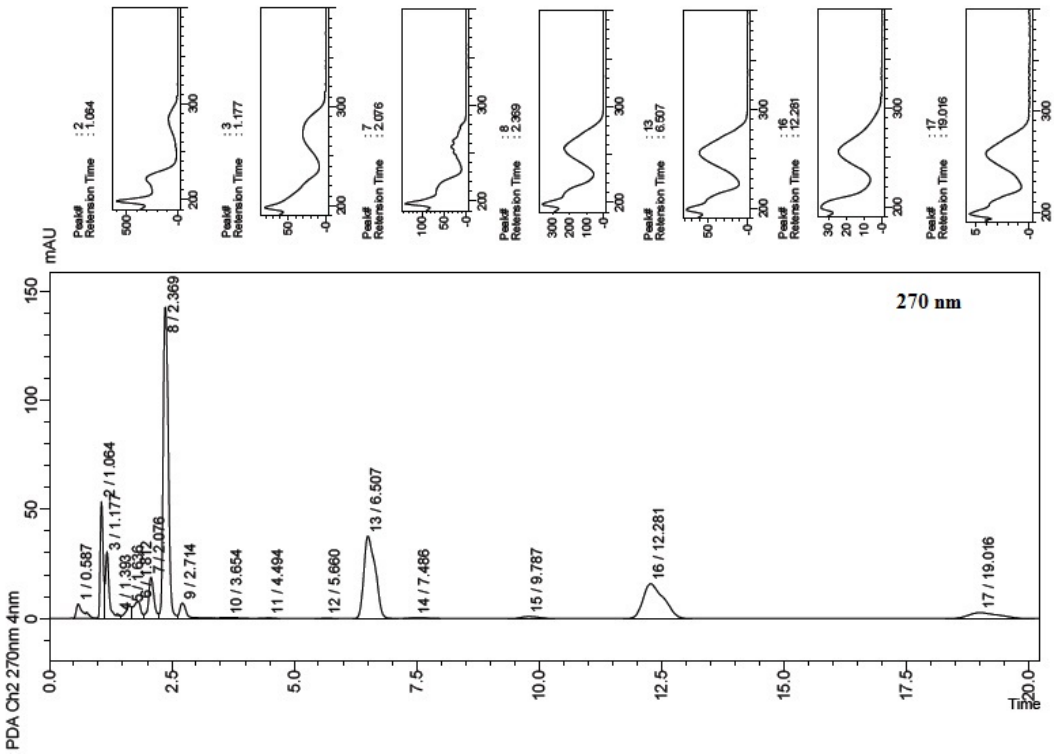

Figure D 16 Chromatogram of sample 16 showing the separation of components from the extracted sample. The chromatographic conditions used were: Brownlee C_{18} (100 × 4.6 mm I.D., 3 µm particle size) column, injection volume 10 µL, mobile phase methanol:water (50:50, v/v), flow rate of 1.0 mL min^{-1}, and detection wavelength of 270 nm.

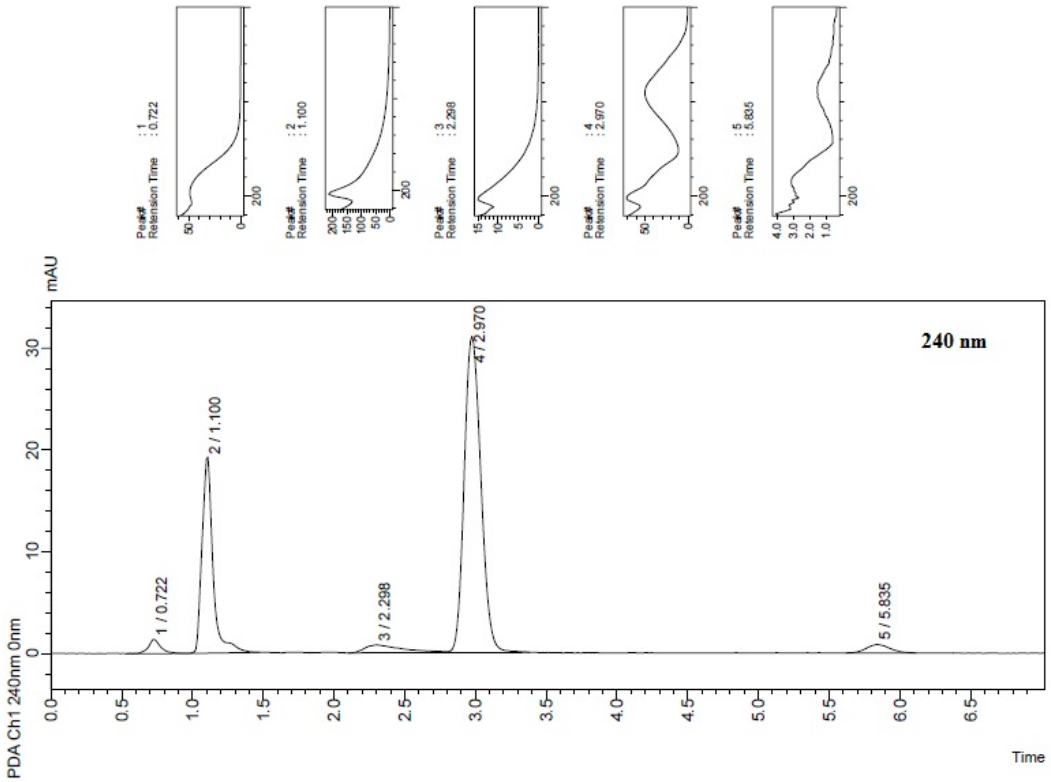

Figure D 17 Chromatogram of sample 17 showing the separation of components from the extracted sample. The chromatographic conditions used were: Brownlee C_{18} (100 × 4.6 mm I.D., 3 µm particle size) column, injection volume 10 µL, mobile phase methanol:water (50:50, v/v), flow rate of 1.0 mL min^{-1}, and detection wavelength of 240 nm.

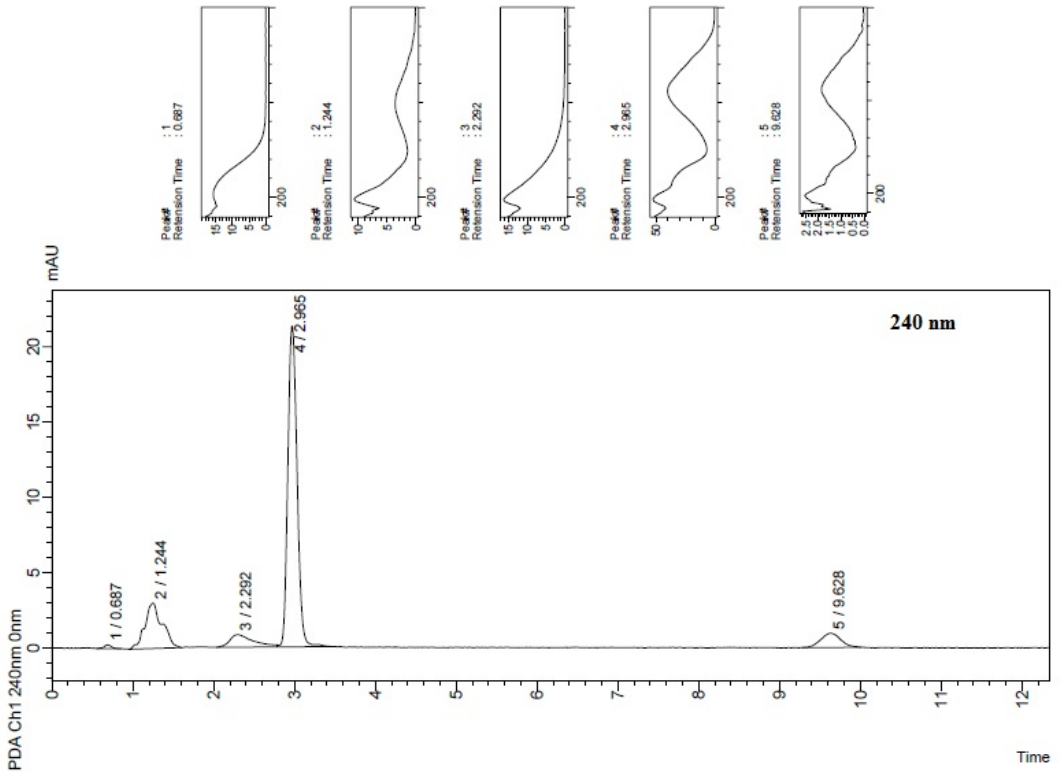

Figure D 18 Chromatogram of sample 18 showing the separation of components from the extracted sample. The chromatographic conditions used were: Brownlee C_{18} (100 × 4.6 mm I.D., 3 µm particle size) column, injection volume 10 µL, mobile phase methanol:water (50:50, v/v), flow rate of 1.0 mL min^{-1}, and detection wavelength of 240 nm.

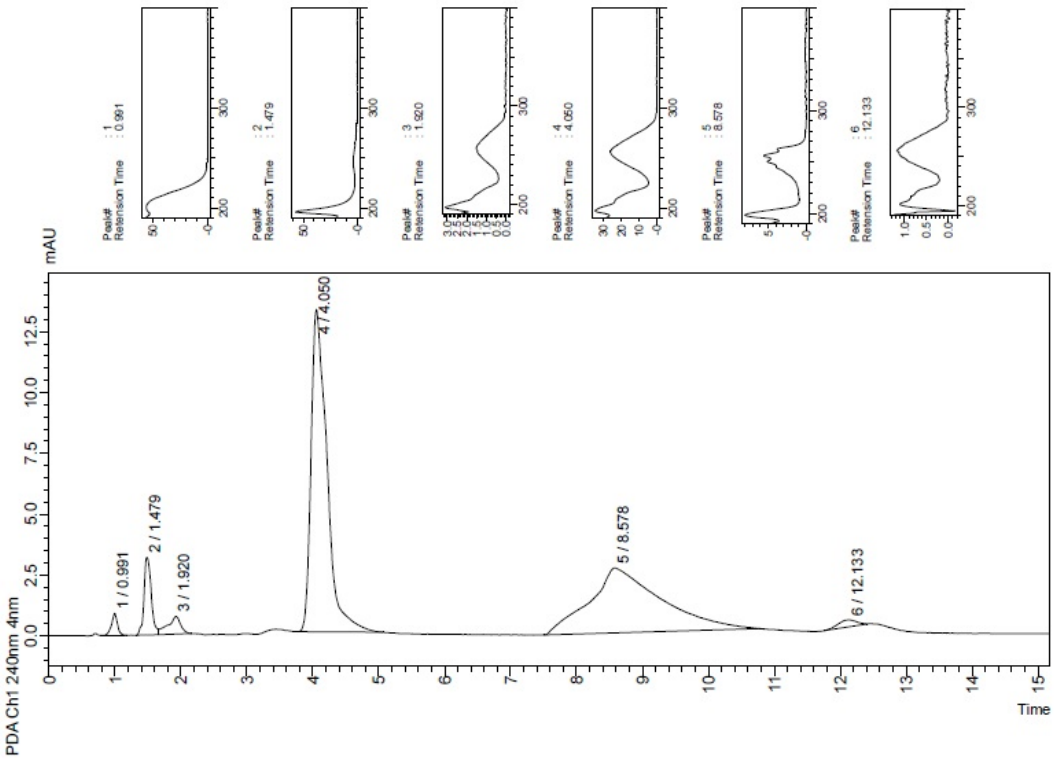

Figure D 19 Chromatogram of sample 19 showing the separation of components from the extracted sample. The chromatographic conditions used were: Brownlee C_{18} (100 × 4.6 mm I.D., 3 µm particle size) column, injection volume 10 µL, mobile phase methanol:water (50:50, v/v), flow rate of 1.0 mL min^{-1}, and detection wavelength of 240 nm.

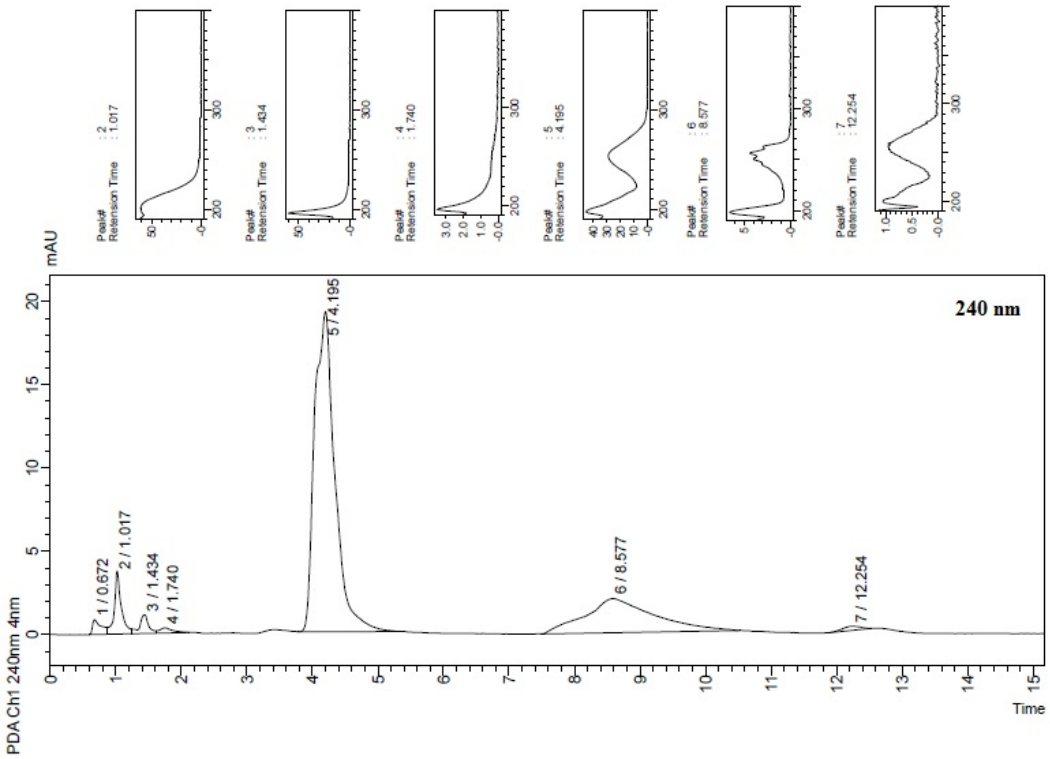

Figure D 20 Chromatogram of sample 20 showing the separation of components from the extracted sample. The chromatographic conditions used were: Brownlee C_{18} (100 × 4.6 mm I.D., 3 µm particle size) column, injection volume 10 µL, mobile phase methanol:water (50:50, v/v), flow rate of 1.0 mL min^{-1}, and detection wavelength of 240 nm.

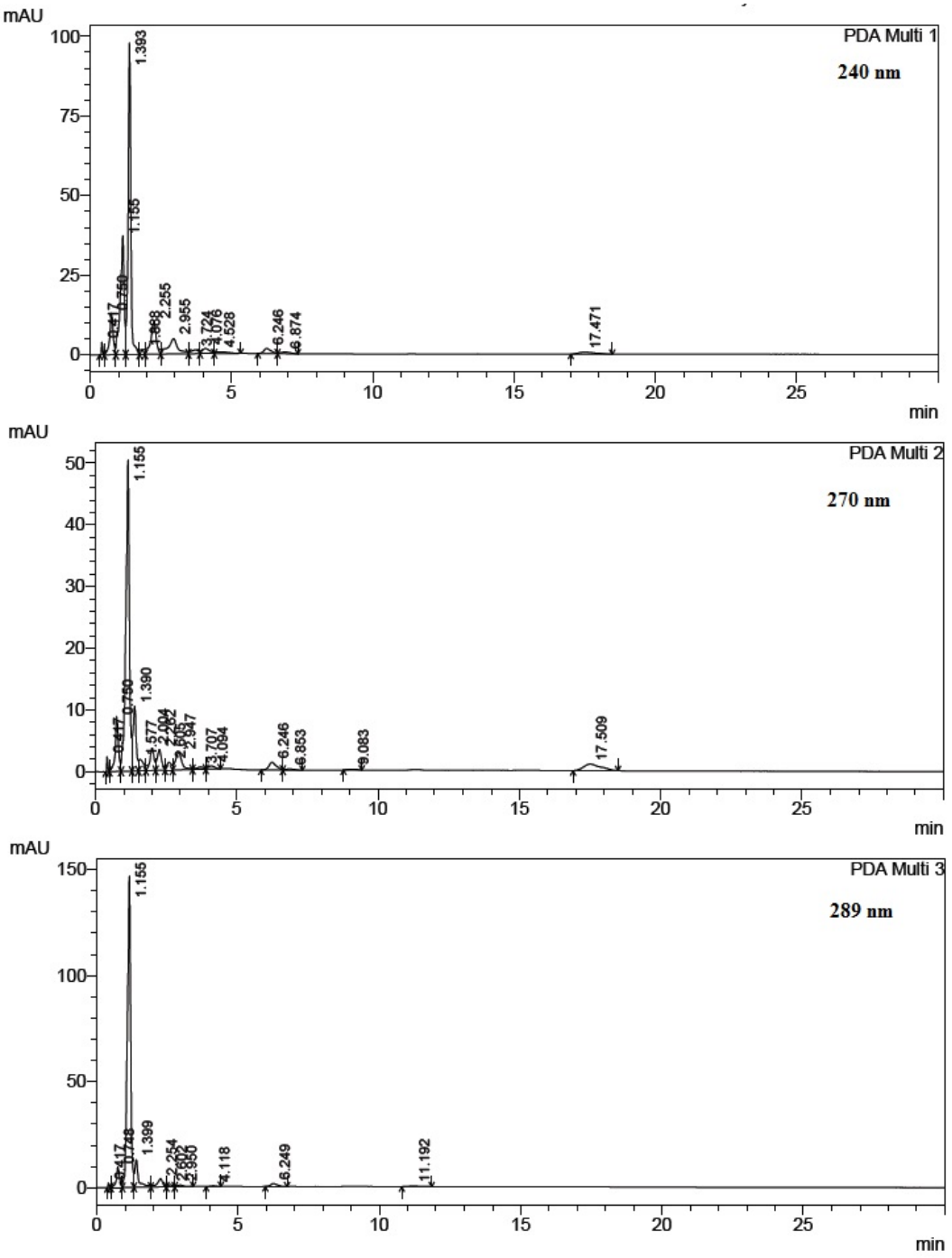

Figure D 21 Chromatogram of sample 21 showing the separation of components from the extracted sample. The chromatographic conditions used were: Brownlee C_{18} (100 × 4.6 mm I.D., 3 µm particle size) column, injection volume 10 µL, mobile phase methanol:water (50:50, v/v), flow rate of 1.0 mL min^{-1}, and detection wavelength of 240, 270 and 289 nm.

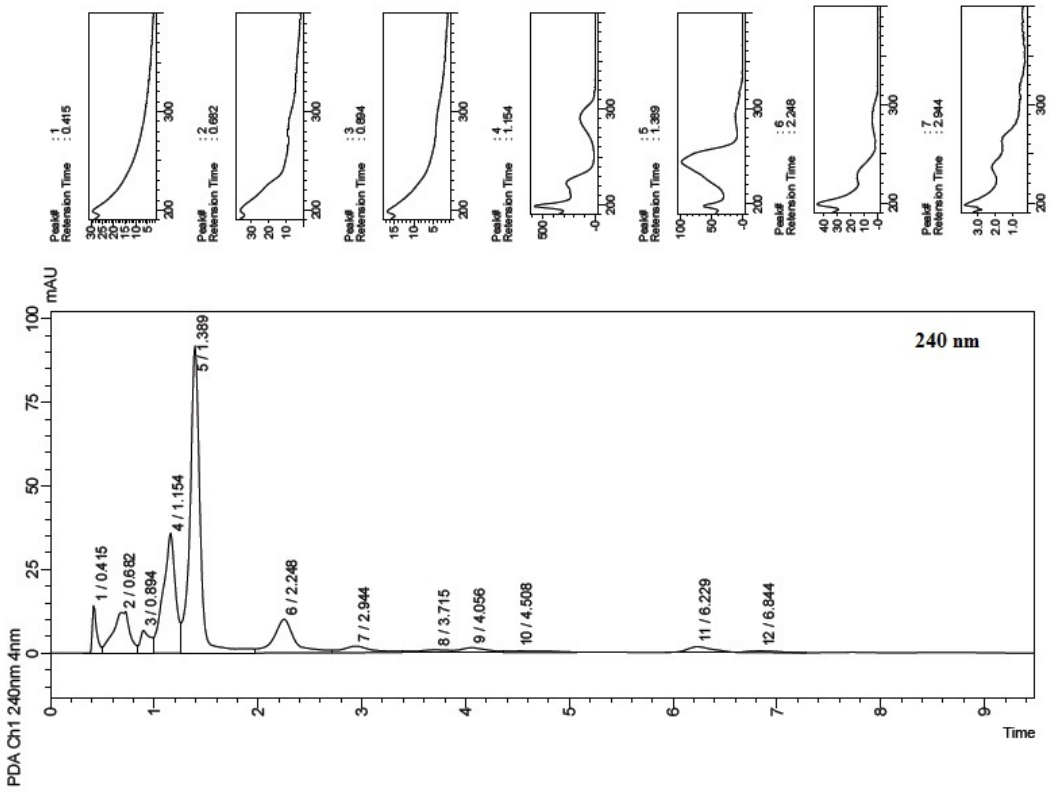

Figure D 22 Chromatogram of sample 22 showing the separation of components from the extracted sample. The chromatographic conditions used were: Brownlee C_{18} (100 × 4.6 mm I.D., 3 µm particle size) column, injection volume 10 µL, mobile phase methanol:water (50:50, v/v), flow rate of 1.0 mL min^{-1}, and detection wavelength of 240 nm.

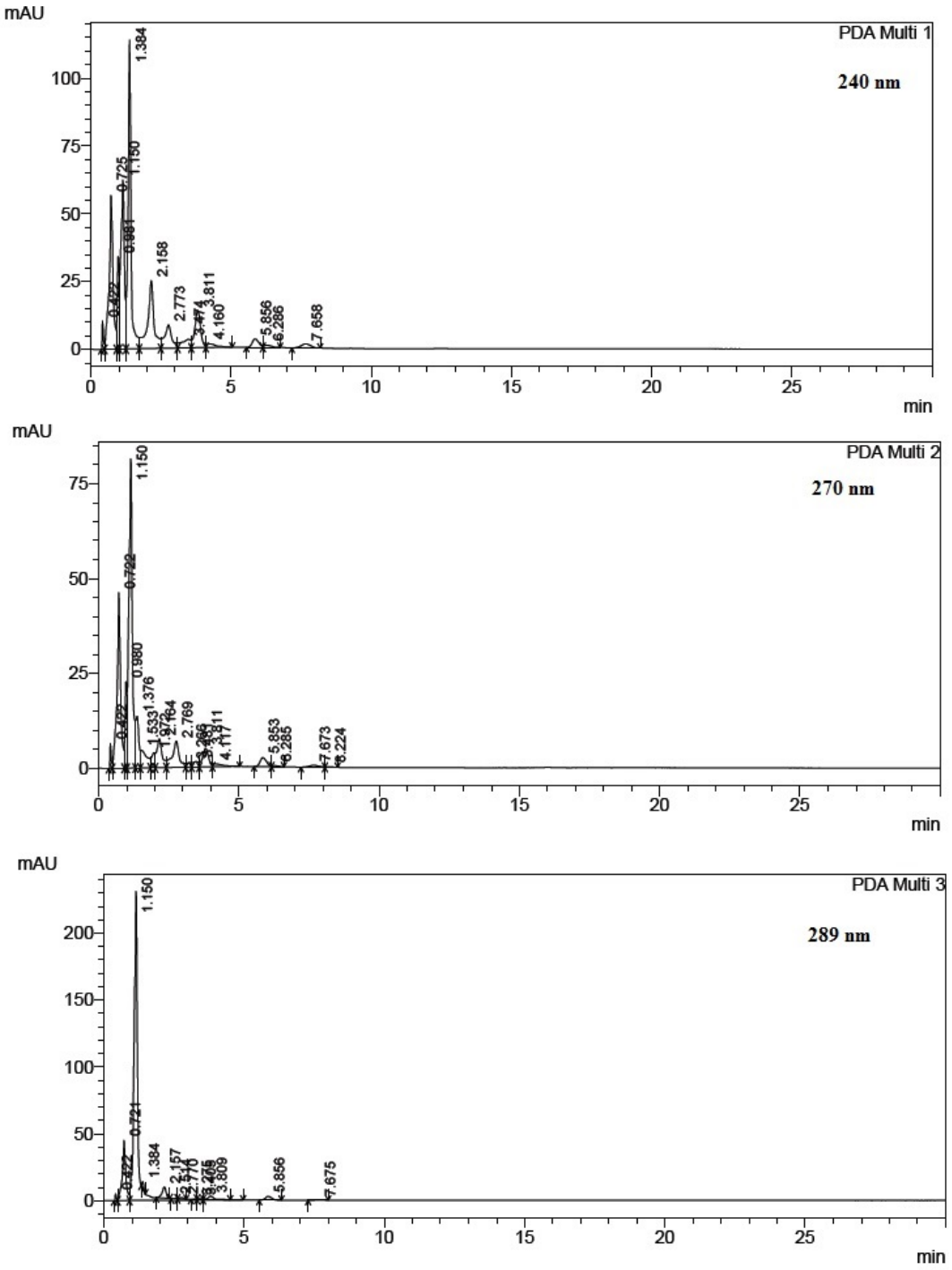

Figure D 23 Chromatogram of sample 23 showing the separation of components from the extracted sample. The chromatographic conditions used were: Brownlee C_{18} (100 × 4.6 mm I.D., 3 µm particle size) column, injection volume 10 µL, mobile phase methanol:water (50:50, v/v), flow rate of 1.0 mL min^{-1}, and detection wavelength of 240, 270 and 289 nm.

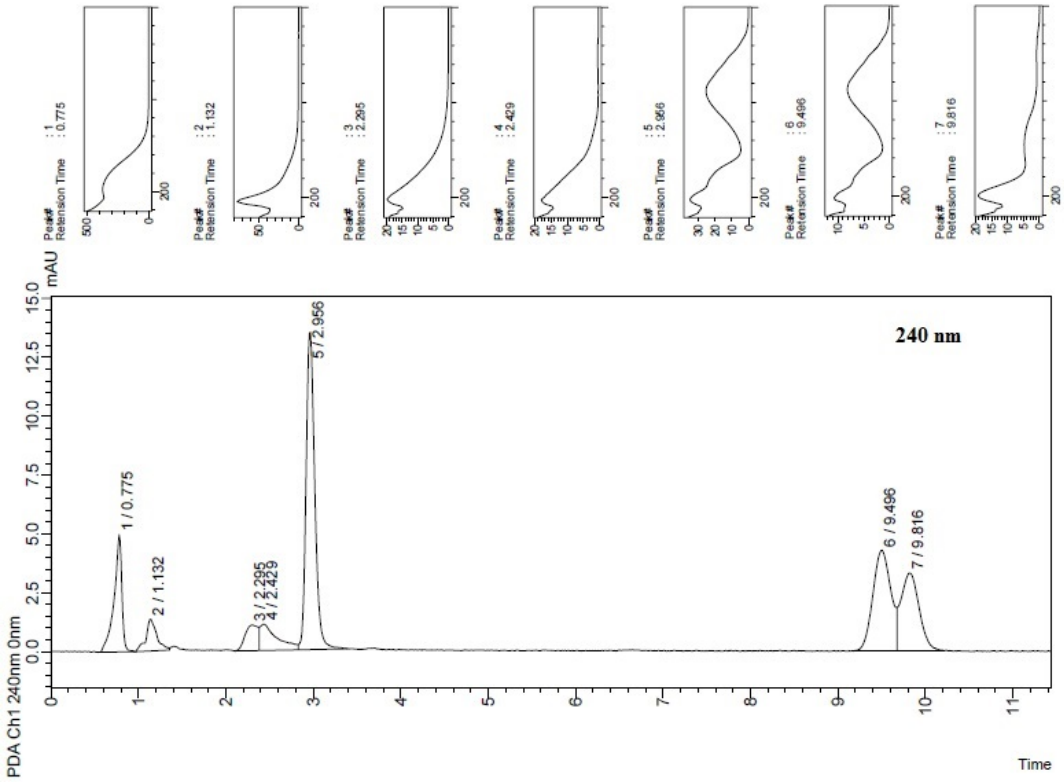

Figure D 24 Chromatogram of sample 24 showing the separation of components from the extracted sample. The chromatographic conditions used were: Brownlee C_{18} (100 × 4.6 mm I.D., 3 µm particle size) column, injection volume 10 µL, mobile phase methanol:water (50:50, v/v), flow rate of 1.0 mL min^{-1}, and detection wavelength of 240 nm.

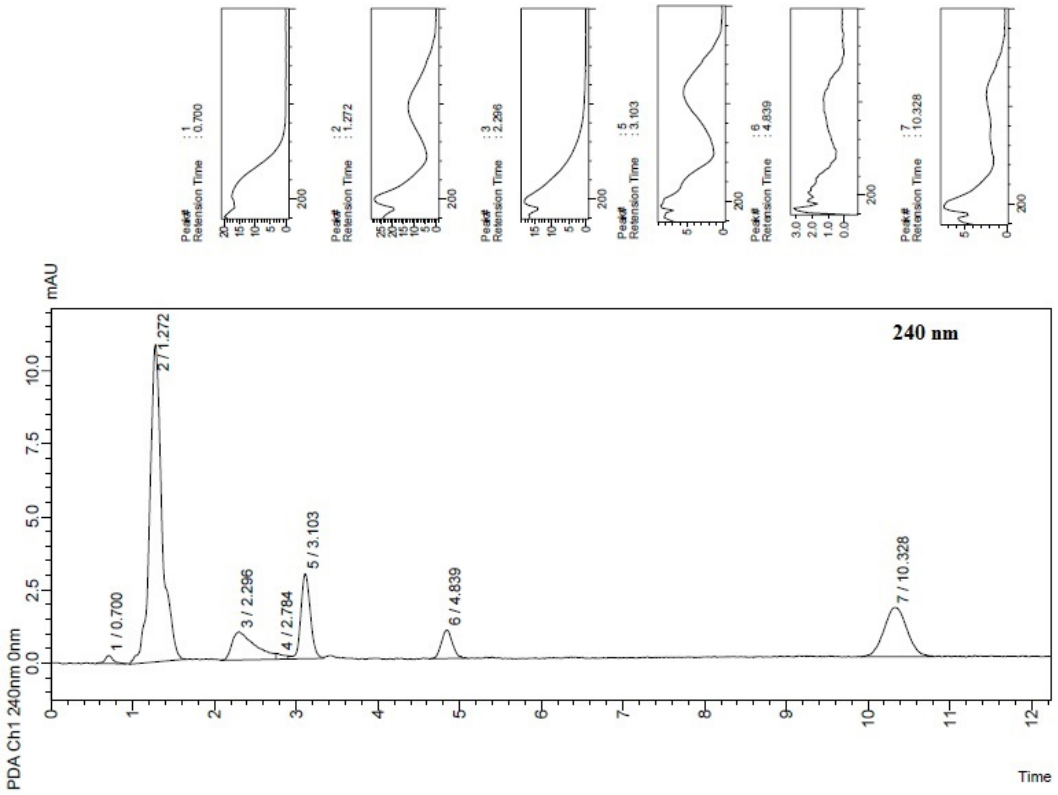

Figure D 25 Chromatogram of sample 25 showing the separation of components from the extracted sample. The chromatographic conditions used were: Brownlee C_{18} (100 × 4.6 mm I.D., 3 µm particle size) column, injection volume 10 µL, mobile phase methanol:water (50:50, v/v), flow rate of 1.0 mL min^{-1}, and detection wavelength of 240 nm.

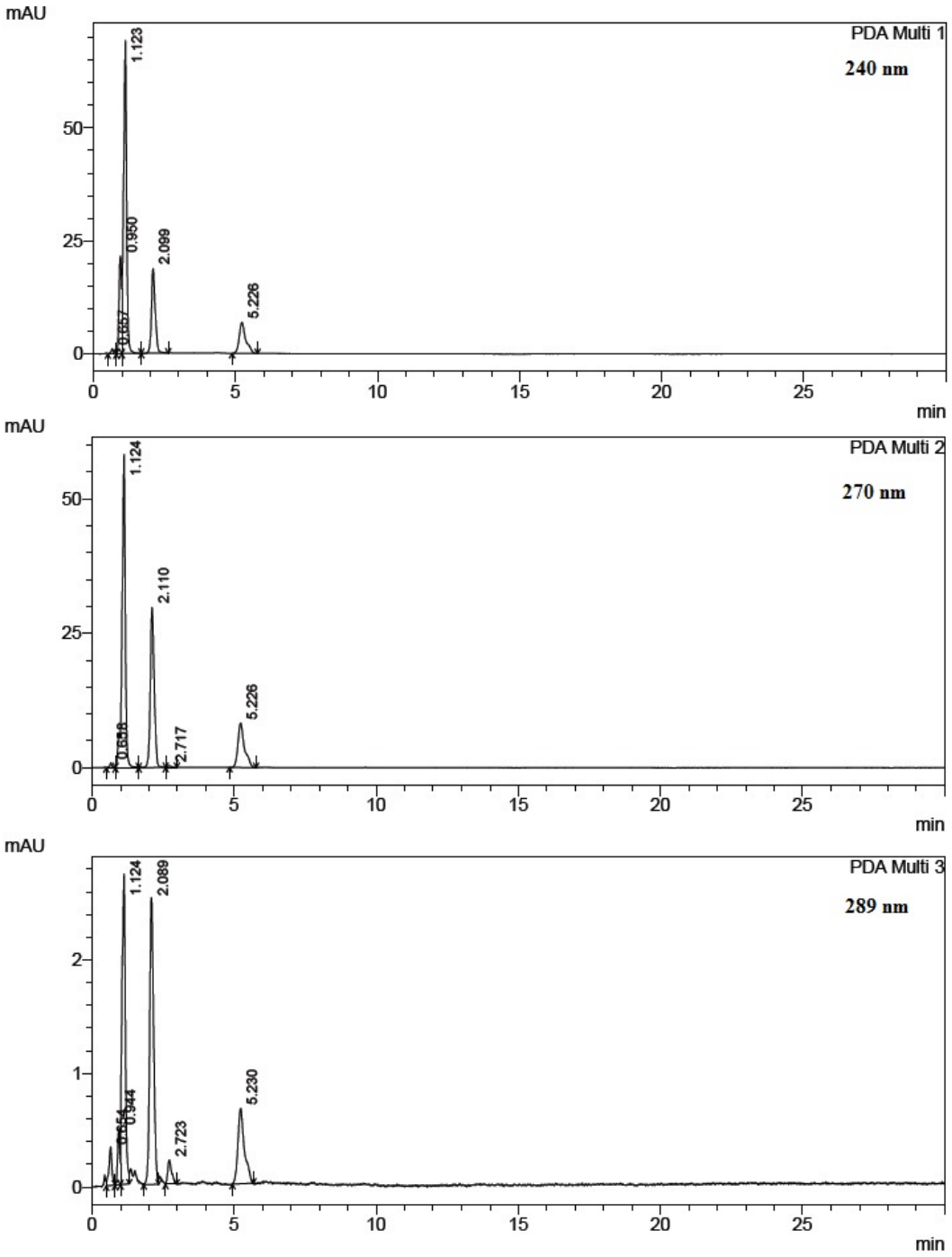

Figure D 26 Chromatogram of sample 26 showing the separation of components from the extracted sample. The chromatographic conditions used were: Brownlee C_{18} (100 × 4.6 mm I.D., 3 µm particle size) column, injection volume 10 µL, mobile phase methanol:water (50:50, v/v), flow rate of 1.0 mL min^{-1}, and detection wavelength of 240, 270 and 289 nm.

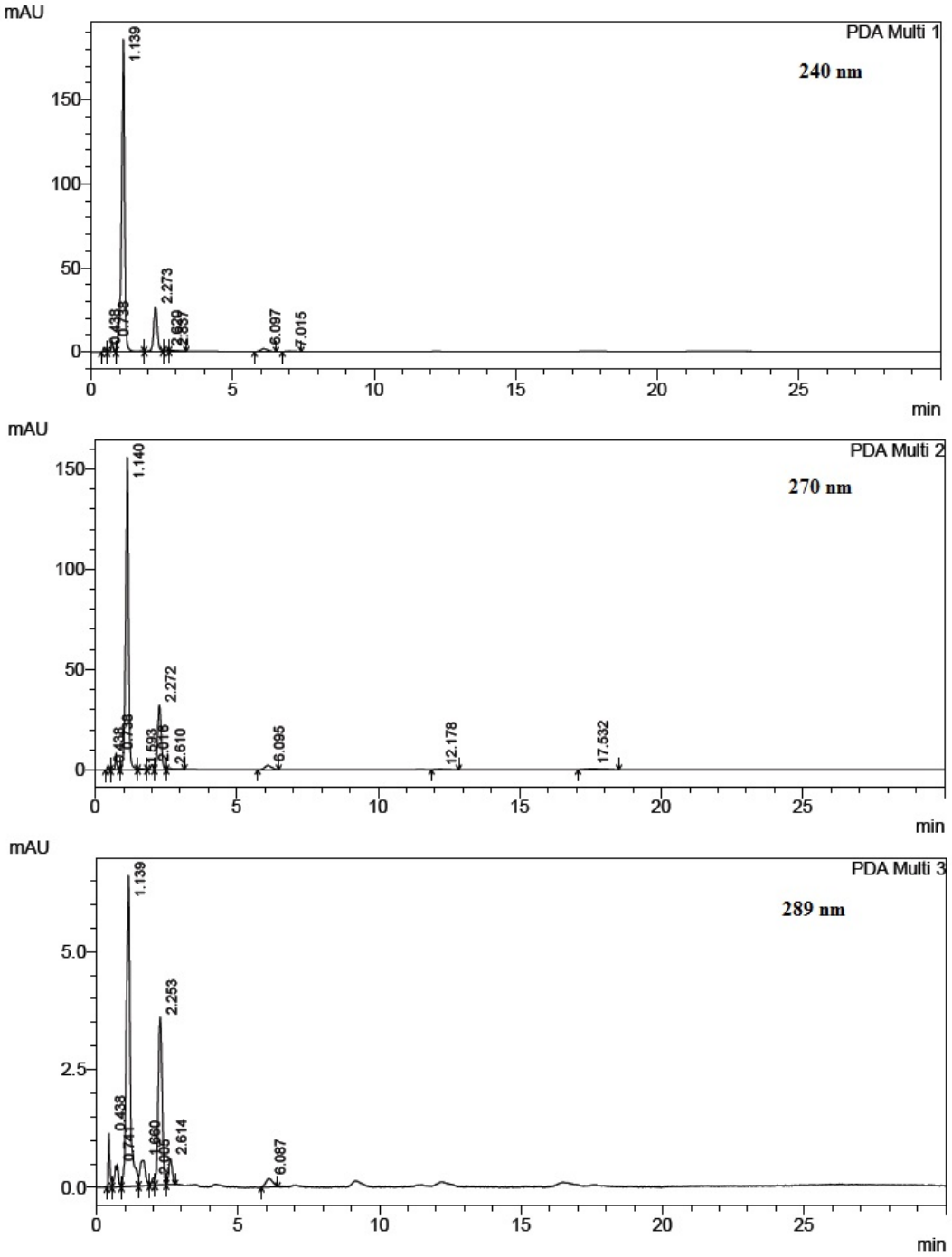

Figure D 27 Chromatogram of sample 27 showing the separation of components from the extracted sample. The chromatographic conditions used were: Brownlee C_{18} (100 × 4.6 mm I.D., 3 µm particle size) column, injection volume 10 µL, mobile phase methanol:water (50:50, v/v), flow rate of 1.0 mL min^{-1}, and detection wavelength of 240, 270 and 289 nm.

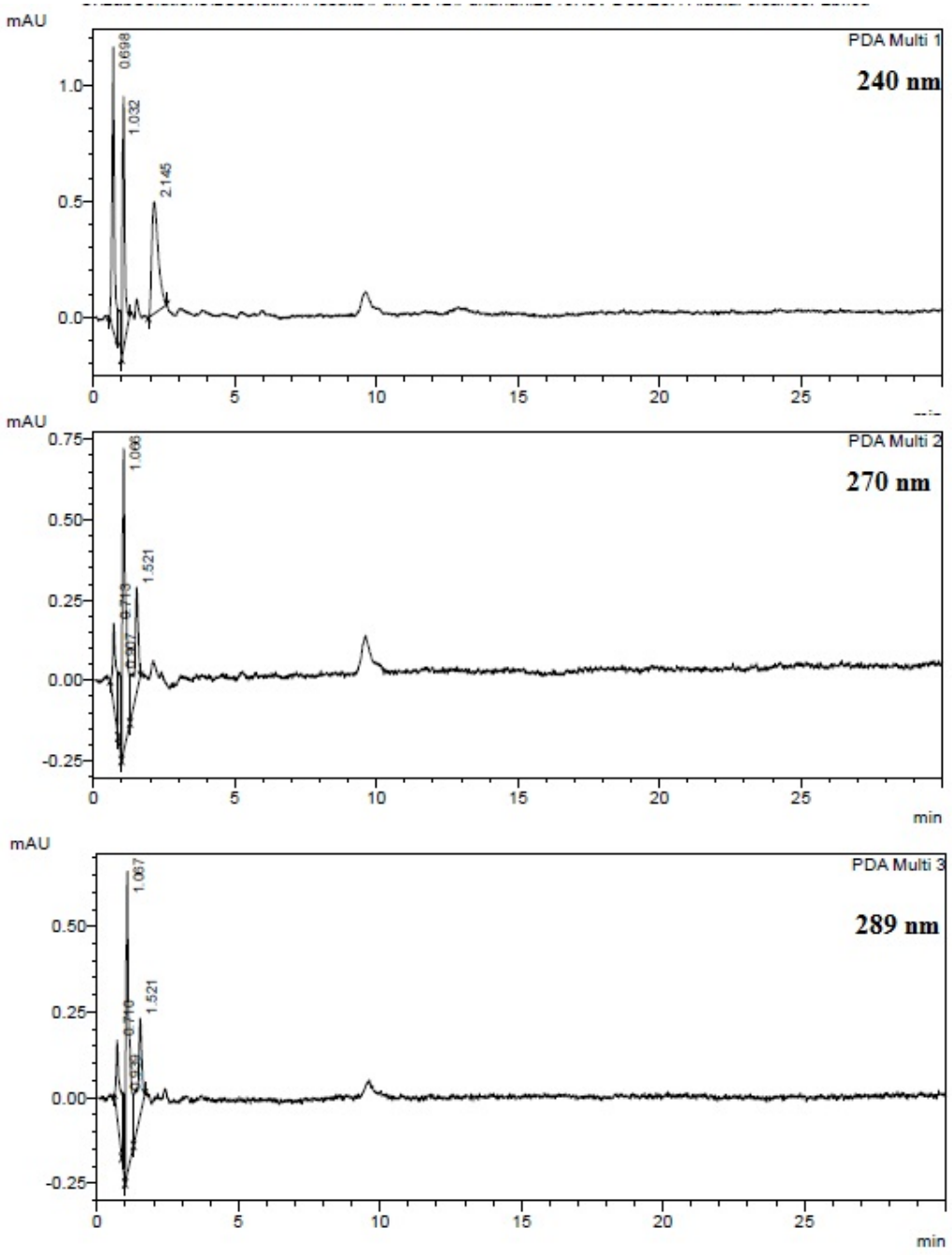

Figure D 28 Chromatogram of sample 28 showing the separation of components from the extracted sample. The chromatographic conditions used were: Brownlee C_{18} (100 × 4.6 mm I.D., 3 µm particle size) column, injection volume 10 µL, mobile phase methanol:water (50:50, v/v), flow rate of 1.0 mL min^{-1}, and detection wavelength of 240, 270 and 289 nm.

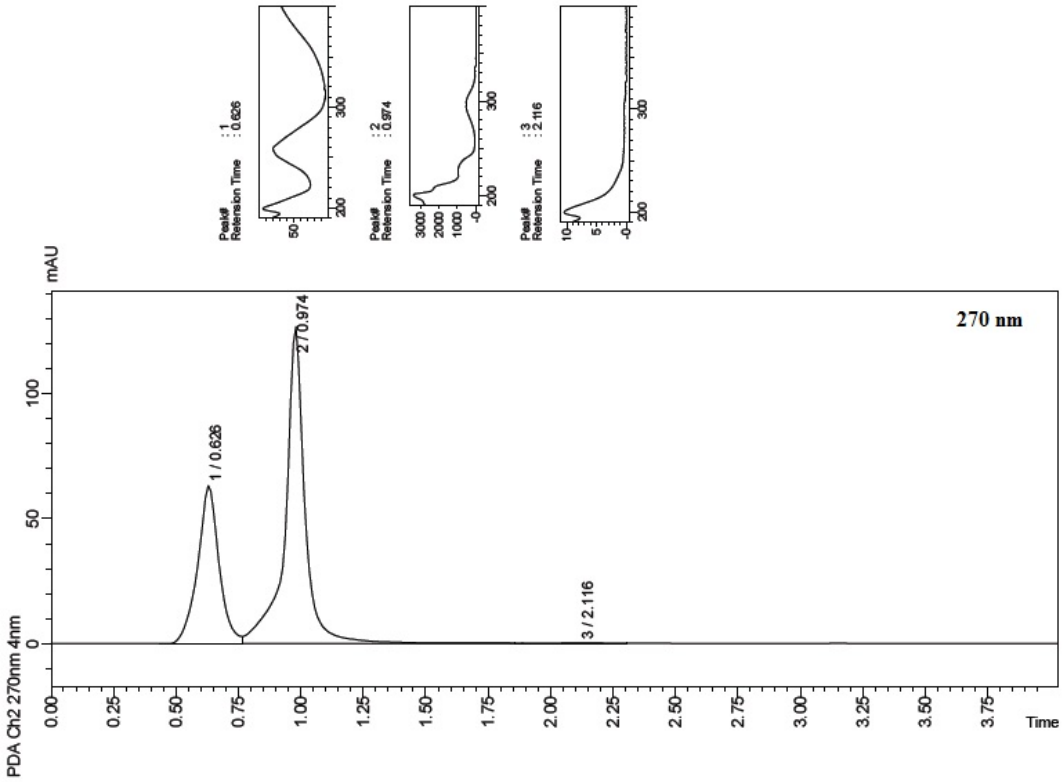

Figure D 29 Chromatogram of sample 29 showing the separation of components from the extracted sample. The chromatographic conditions used were: Brownlee C_{18} (100 × 4.6 mm I.D., 3 µm particle size) column, injection volume 10 µL, mobile phase methanol:water (50:50, v/v), flow rate of 1.0 mL min^{-1}, and detection wavelength of 270 nm.

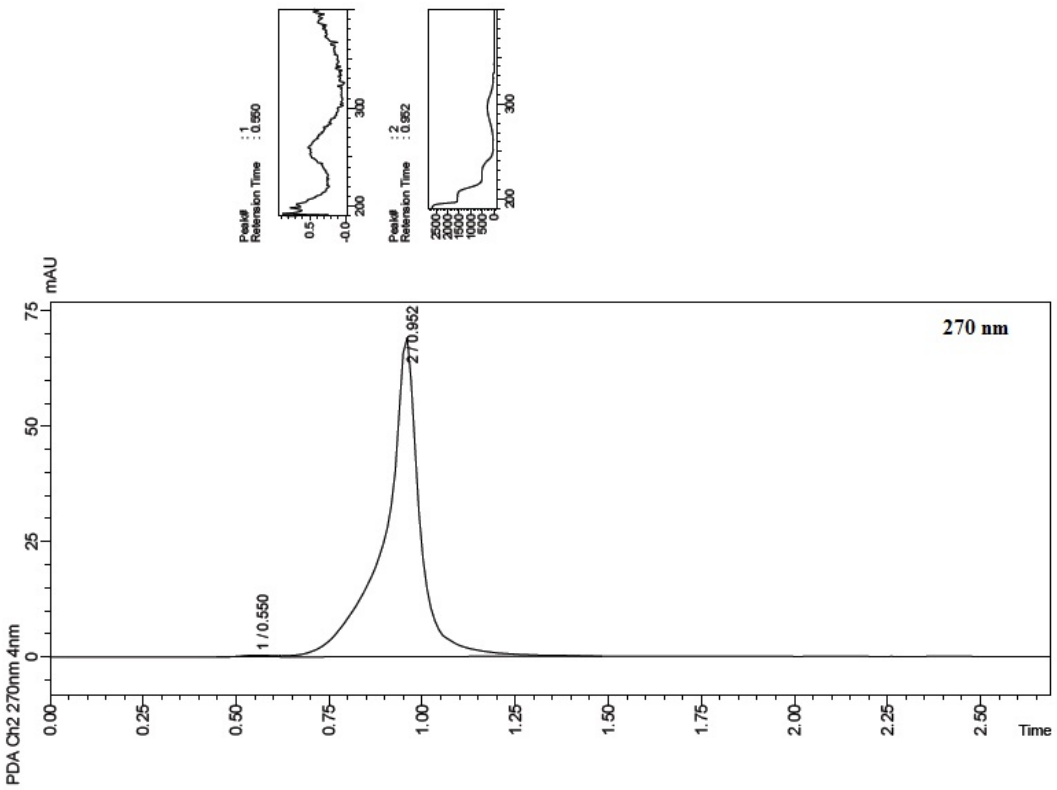

Figure D 30 Chromatogram of sample 30 showing the separation of components from the extracted sample. The chromatographic conditions used were: Brownlee C_{18} (100 × 4.6 mm I.D., 3 µm particle size) column, injection volume 10 µL, mobile phase methanol:water (50:50, v/v), flow rate of 1.0 mL min^{-1}, and detection wavelength of 270 nm.

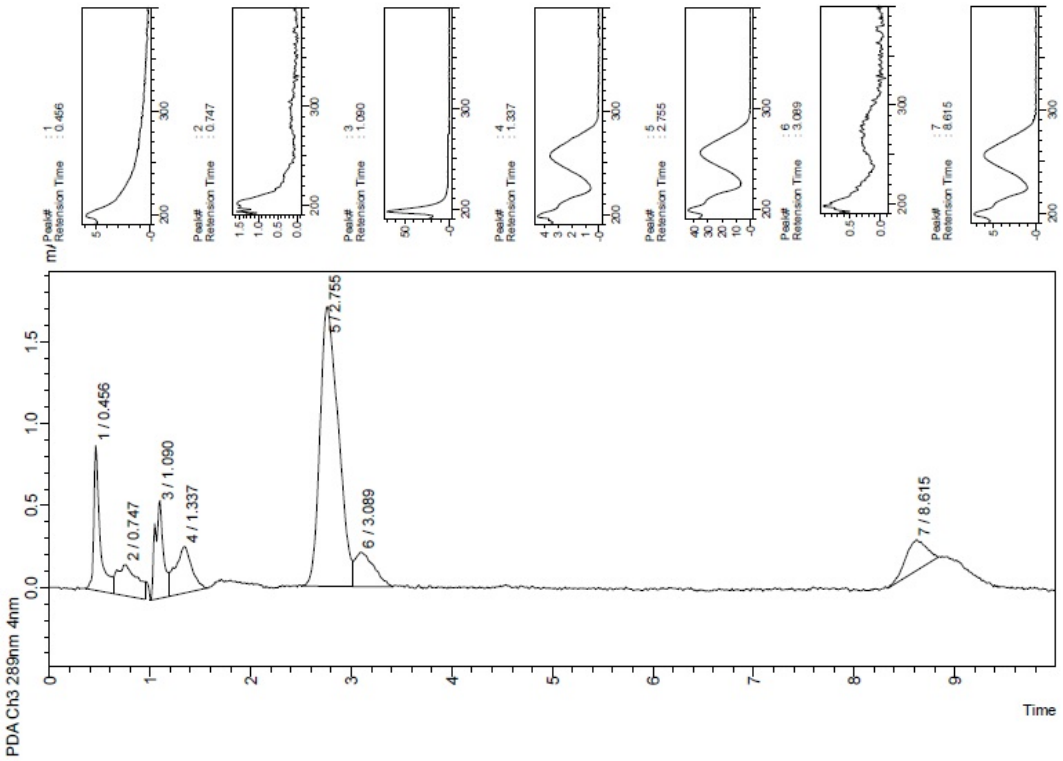

Figure D 31 Chromatogram of sample 31 showing the separation of components from the extracted sample. The chromatographic conditions used were: Brownlee C_{18} (100 × 4.6 mm I.D., 3 µm particle size) column, injection volume 10 µL, mobile phase methanol:water (50:50, v/v), flow rate of 1.0 mL min^{-1}, and detection wavelength of 289 nm.

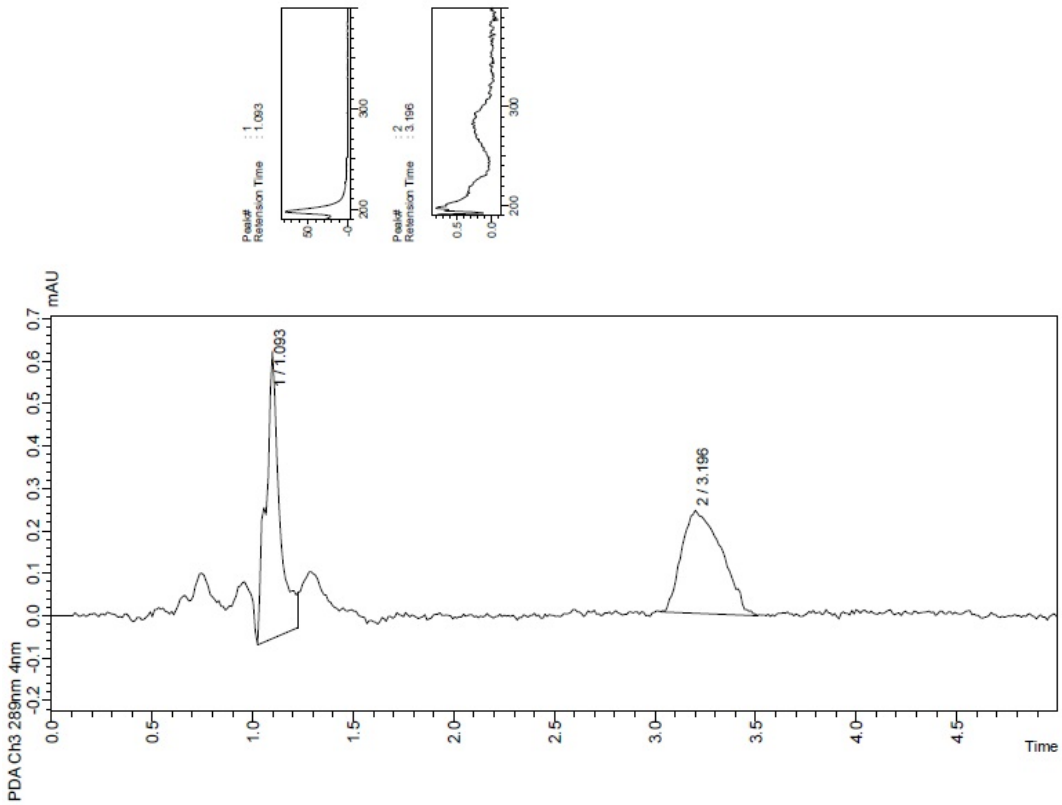

Figure D 32 Chromatogram of sample 32 showing the separation of components from the extracted sample. The chromatographic conditions used were: Brownlee C_{18} (100 × 4.6 mm I.D., 3 µm particle size) column, injection volume 10 µL, mobile phase methanol:water (50:50, v/v), flow rate of 1.0 mL min^{-1}, and detection wavelength of 289 nm.

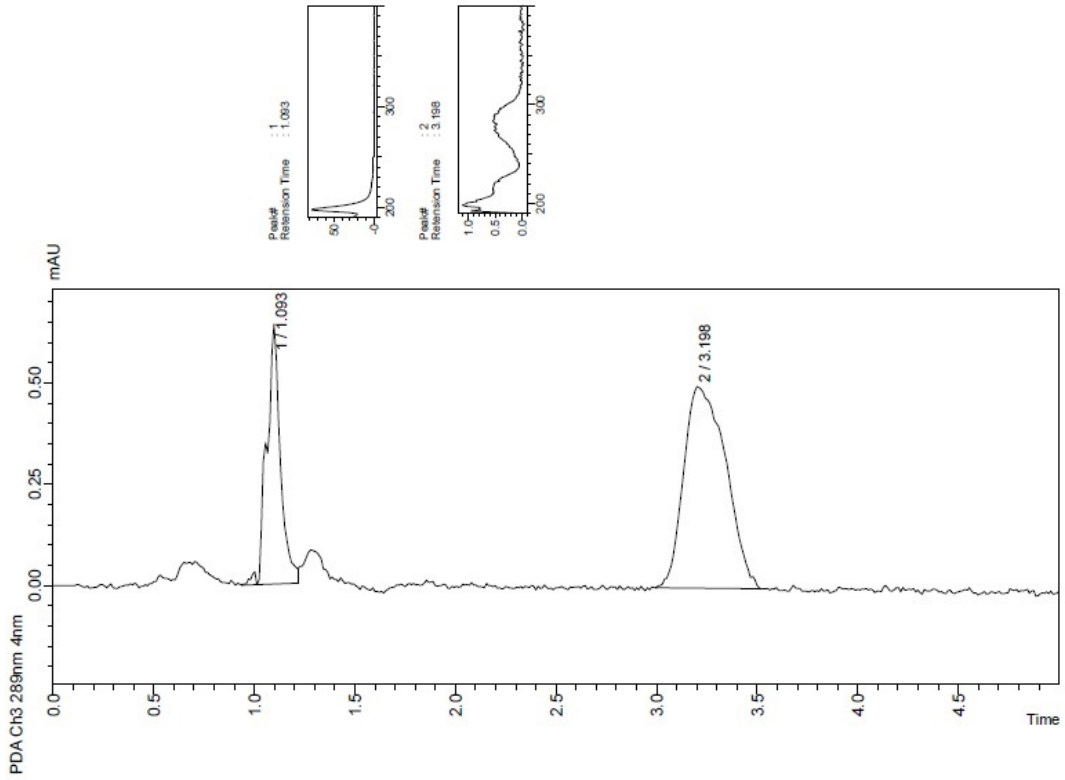

Figure D 33 Chromatogram of sample 33 showing the separation of components from the extracted sample. The chromatographic conditions used were: Brownlee C_{18} (100 × 4.6 mm I.D., 3 µm particle size) column, injection volume 10 µL, mobile phase methanol:water (50:50, v/v), flow rate of 1.0 mL min^{-1}, and detection wavelength of 289 nm.

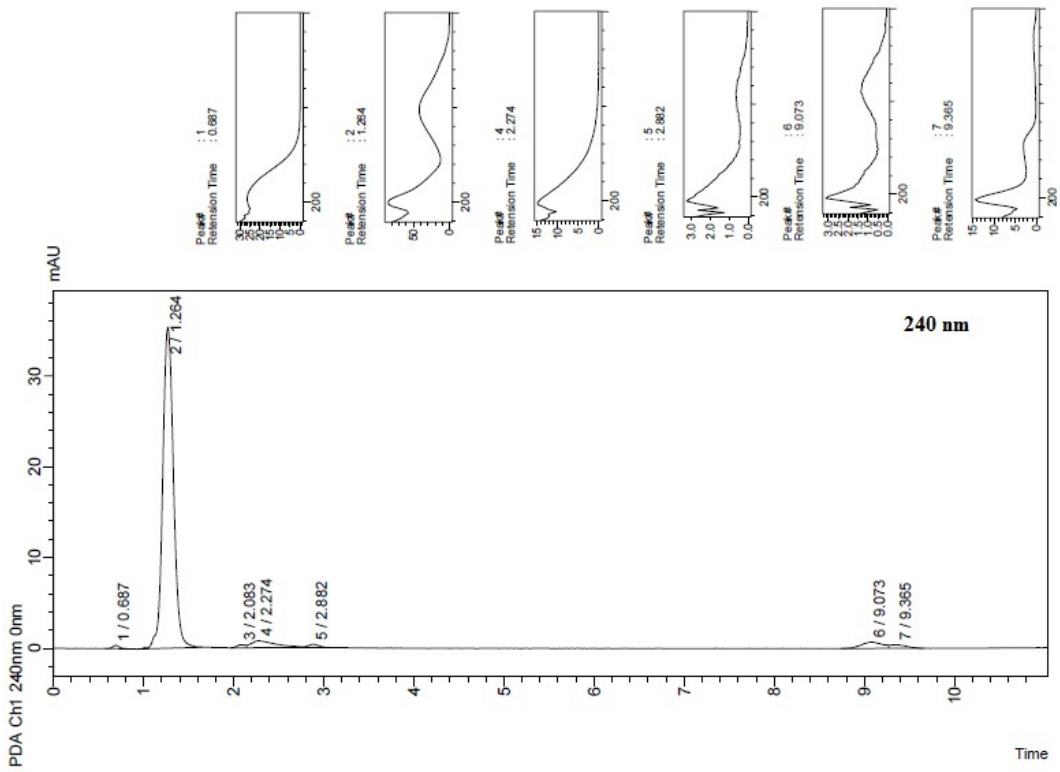

Figure D 34 Chromatogram of sample 34 showing the separation of components from the extracted sample. The chromatographic conditions used were: Brownlee C_{18} (100 × 4.6 mm I.D., 3 µm particle size) column, injection volume 10 µL, mobile phase methanol:water (50:50, v/v), flow rate of 1.0 mL min^{-1}, and detection wavelength of 240 nm.

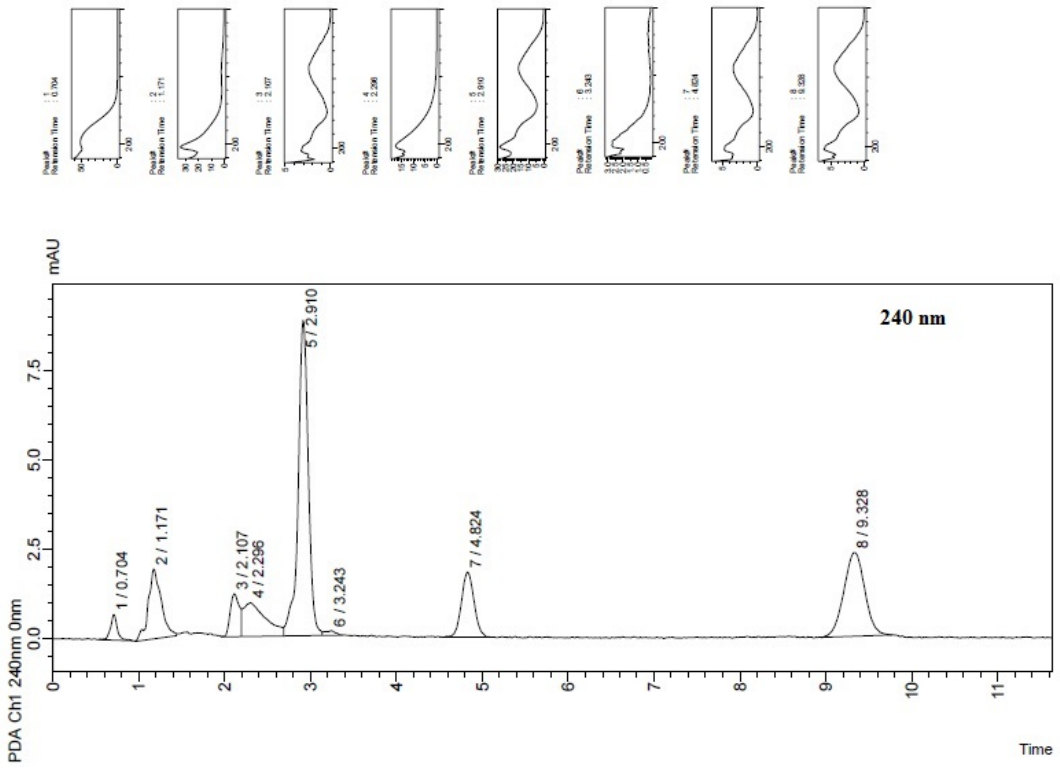

Figure D 35 Chromatogram of sample 35 showing the separation of components from the extracted sample. The chromatographic conditions used were: Brownlee C_{18} (100 × 4.6 mm I.D., 3 µm particle size) column, injection volume 10 µL, mobile phase methanol:water (50:50, v/v), flow rate of 1.0 mL min^{-1}, and detection wavelength of 240 nm.

Appendix E

GC-MS

The GC-MS chromatograms were obtained using the GC-MS method reported. The temperature programme was set as, 50 °C held for 2 minutes, then an increase of 20 °C min^{-1} to 295 °C then held there for 10 minutes. The split ratio was set at 1:50. The injection volume used was 1 µL. The column used was DB5 (30 metres).

E.1 Standards chromatograms

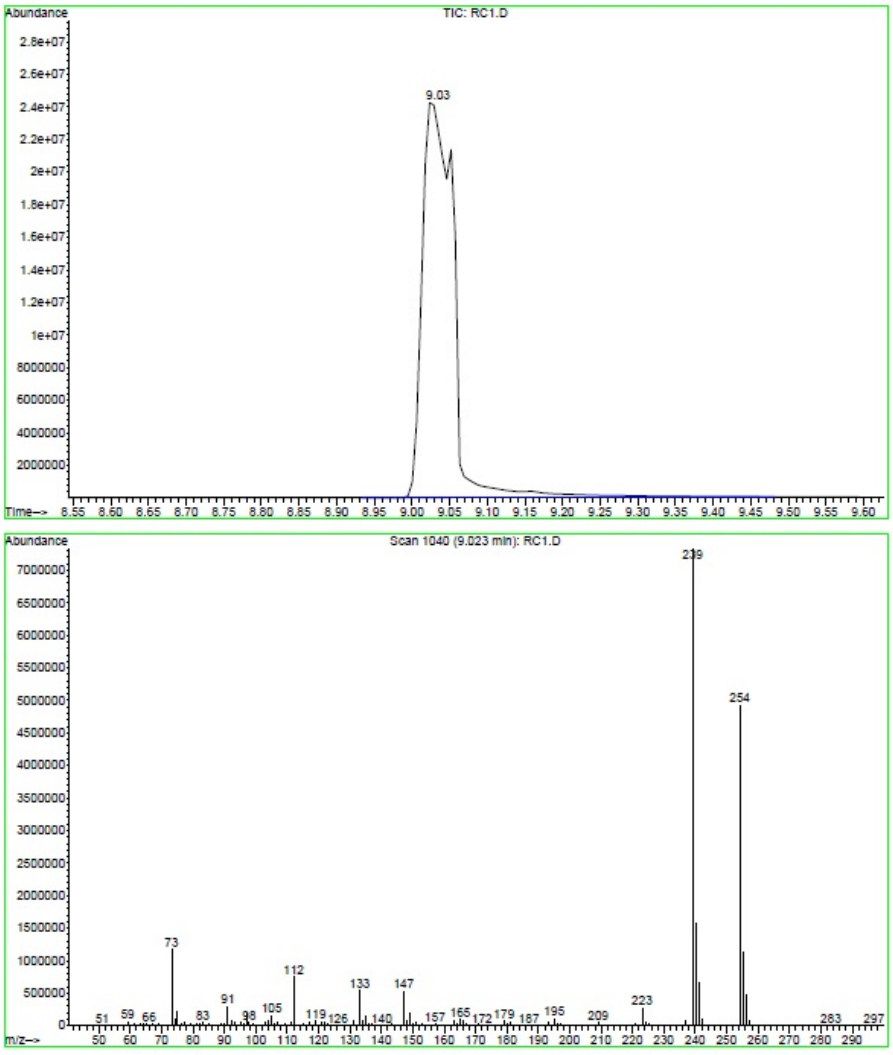

Figure E 1 Total ion chromatogram and mass spectrum of a derivatised resorcinol standard.

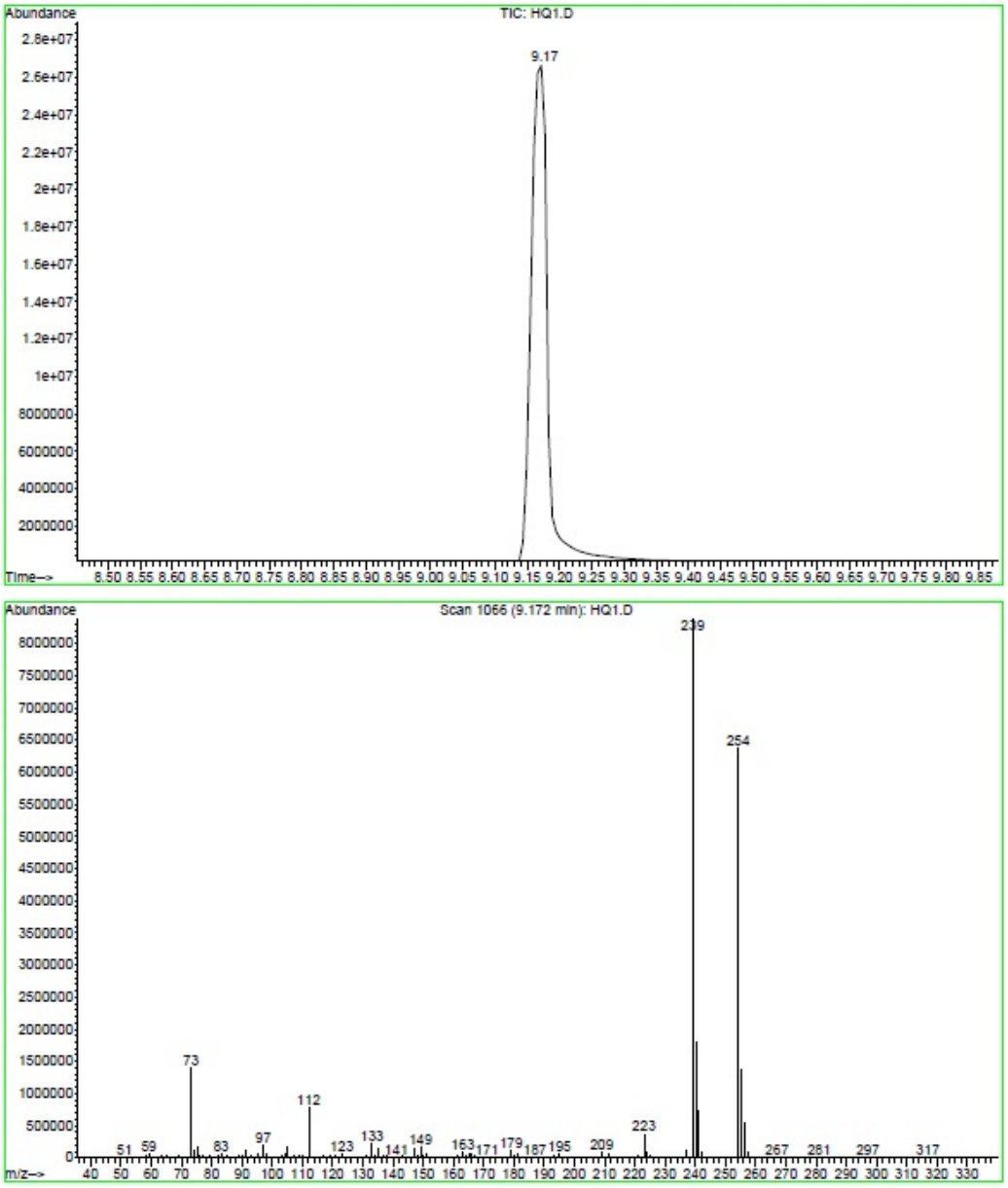

Figure E 2 Total ion chromatogram and mass spectrum of derivatised hydroquinone standard.

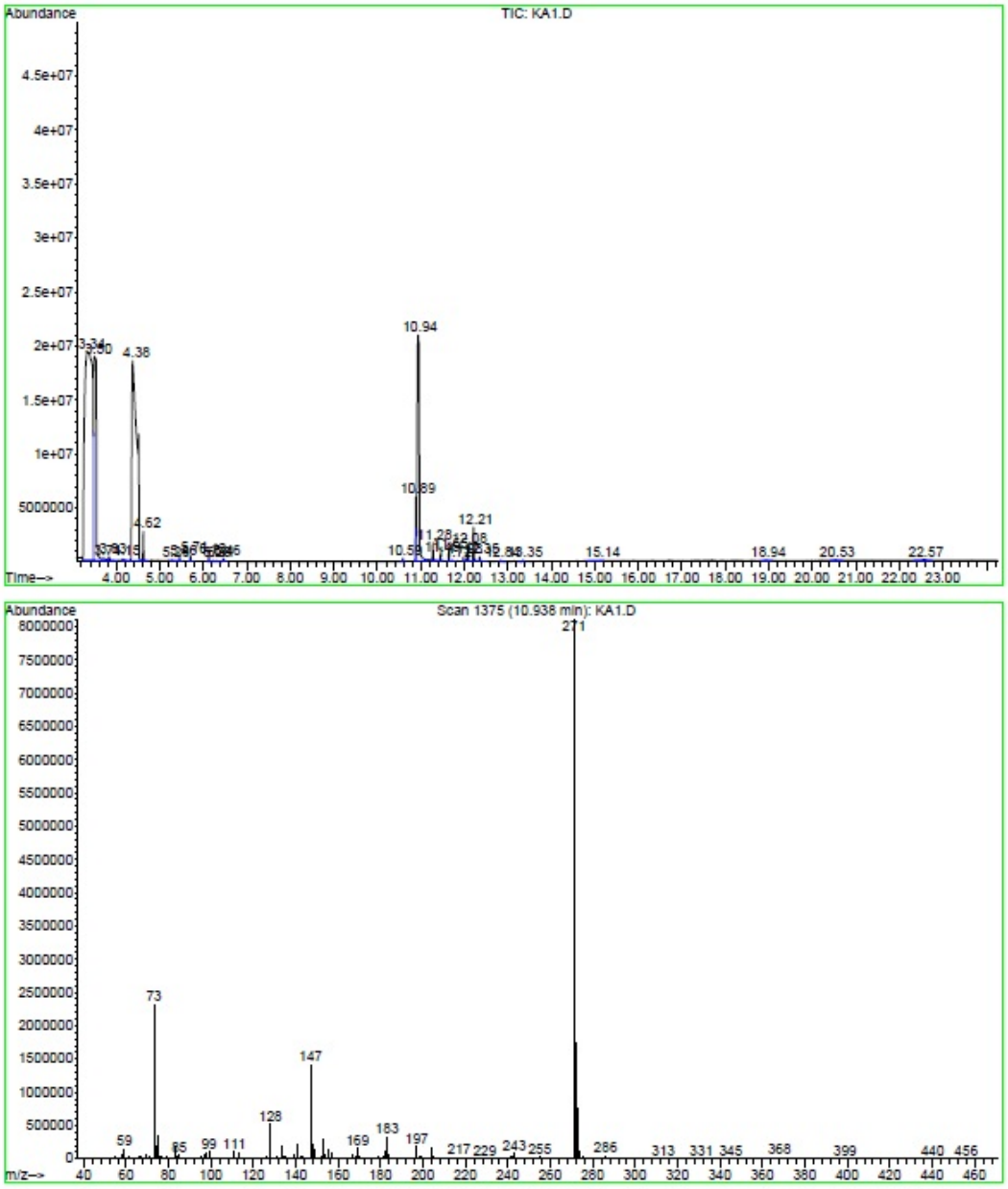

Figure E 3 Total ion chromatogram and mass spectrum of derivatised kojic acid standard.

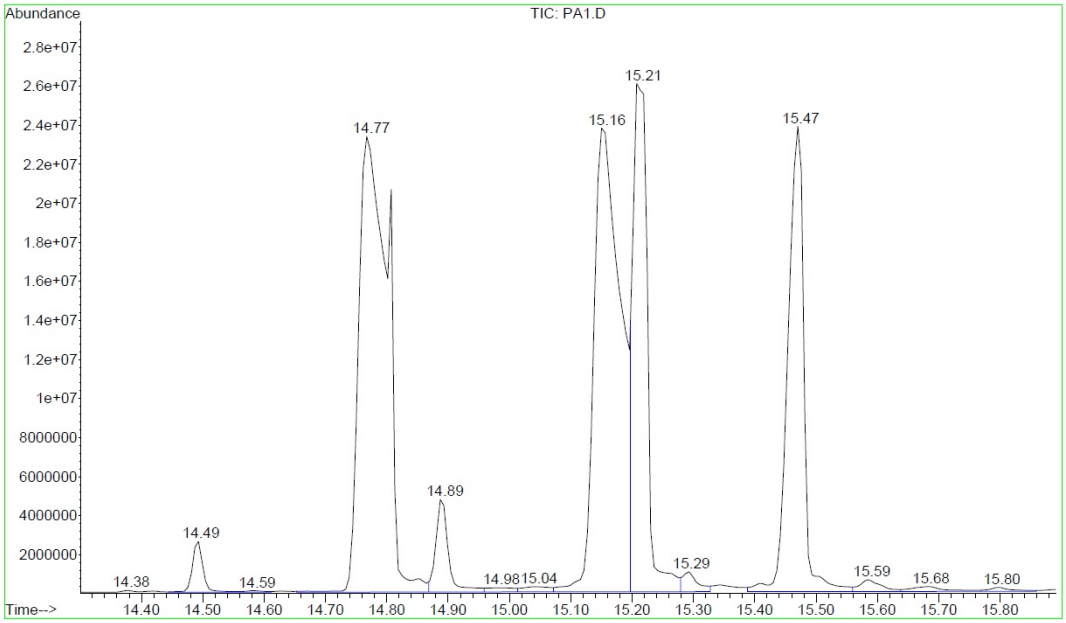

Figure E 4 Total ion chromatogram of derivatised arbutin.

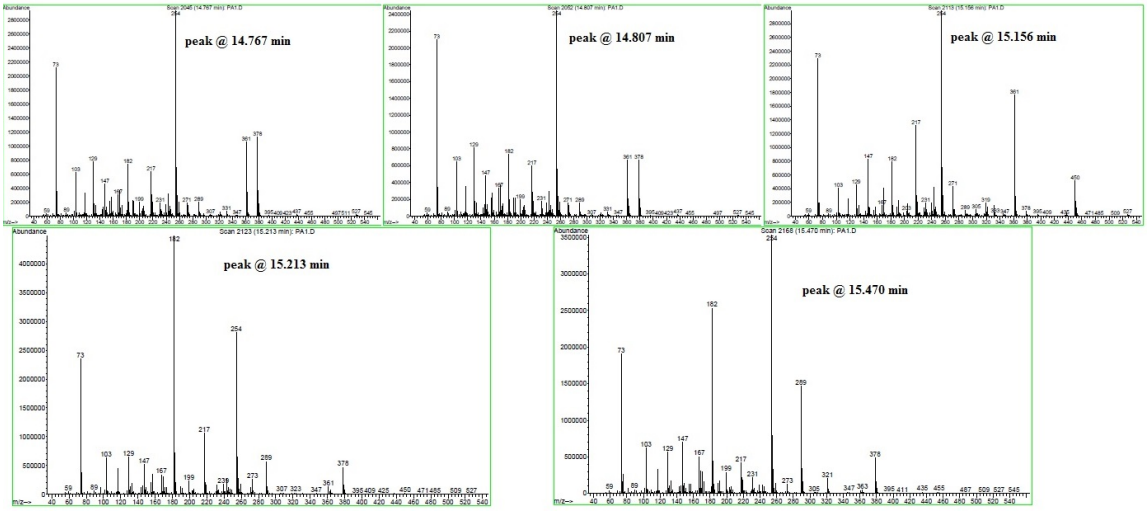

Figure E 5 Mass spectra of the derivatised arbutin peaks.

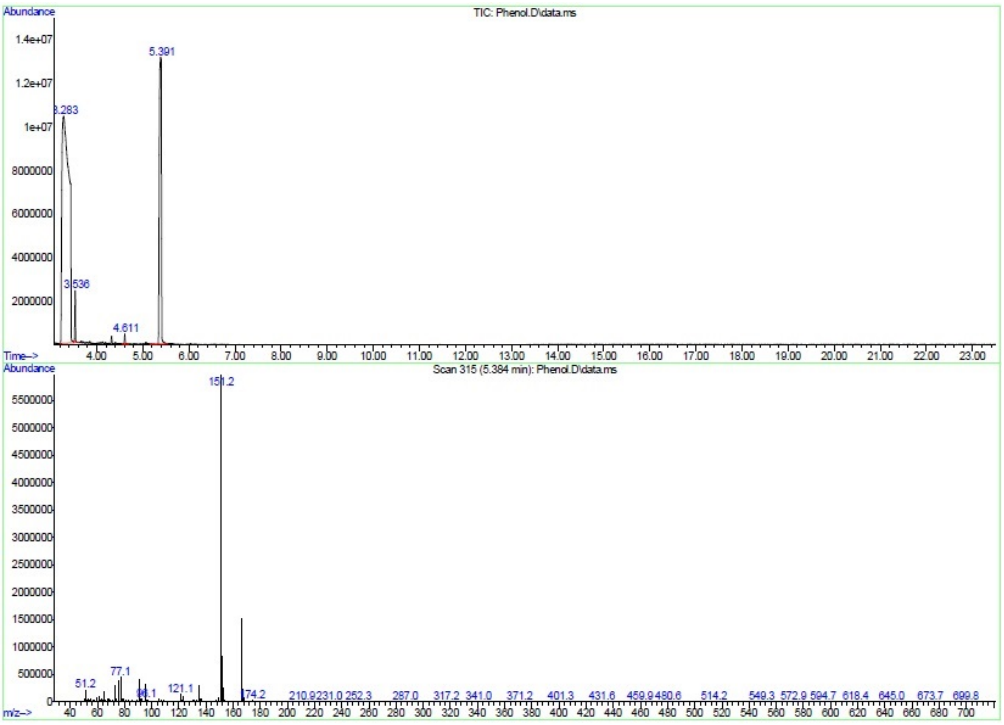

Figure E 6 Total ion chromatogram and mass spectrum of derivatised phenol standard.

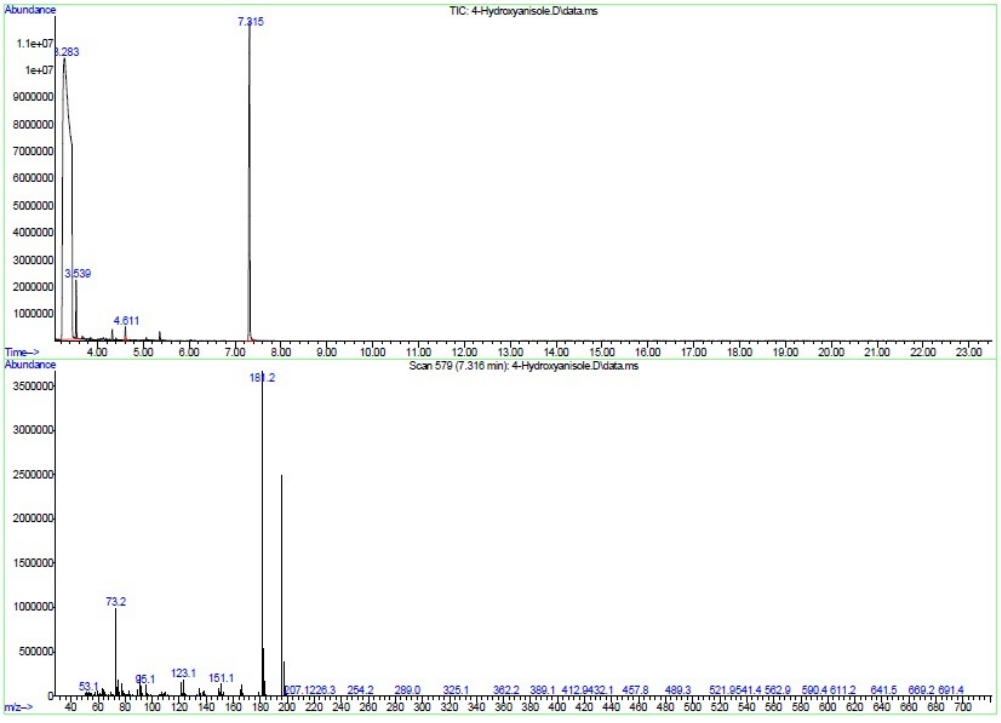

Figure E 7 Total ion chromatogram and mass spectrum of derivatised 4-hydroxyanisole standard.

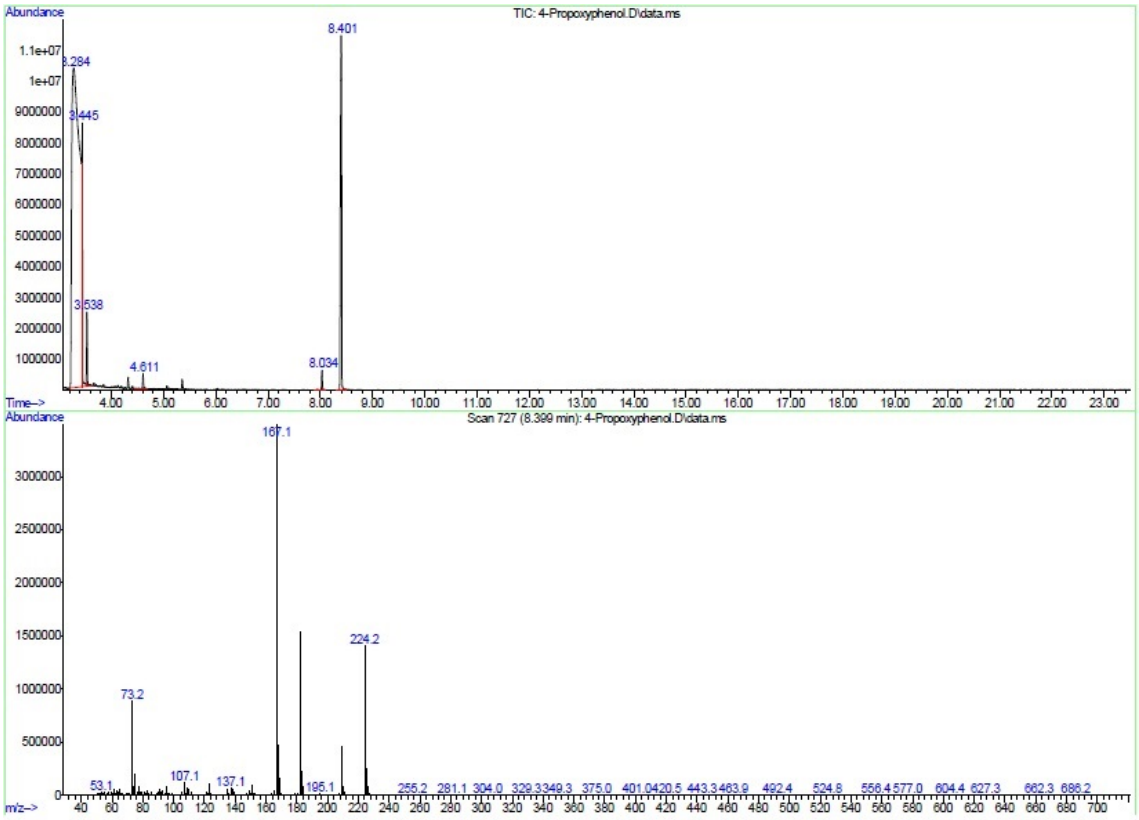

Figure E 8 Total ion chromatogram and mass spectrum of derivatised 4-propoxyphenol standard.

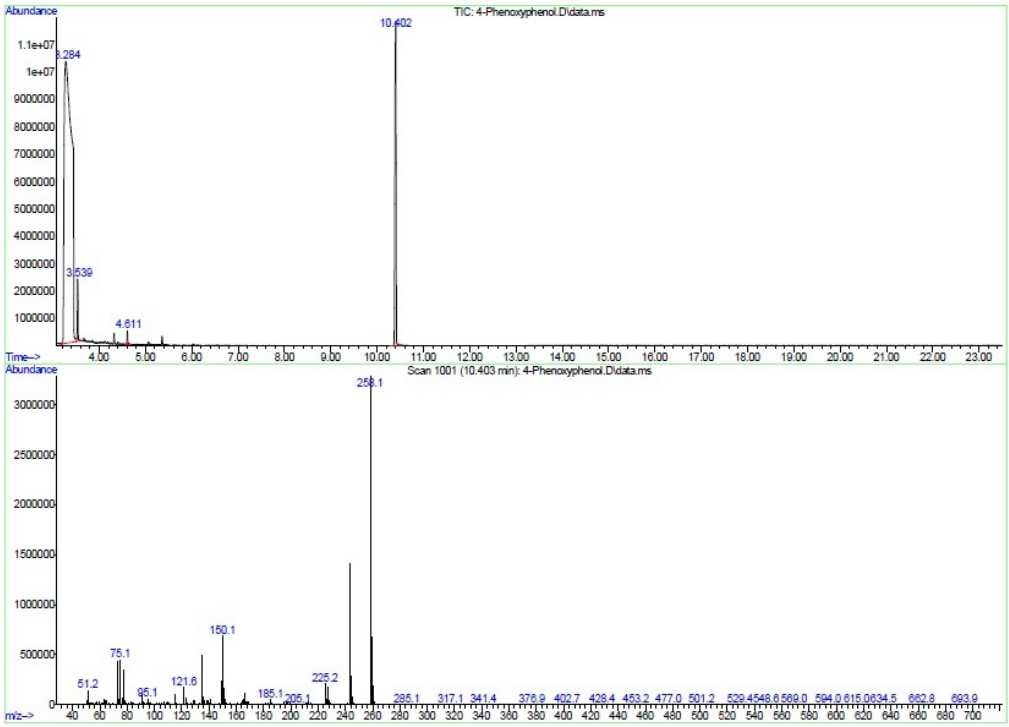

Figure E 9 Total ion chromatogram and mass spectrum of derivatised 4-phenoxyphenol standard.

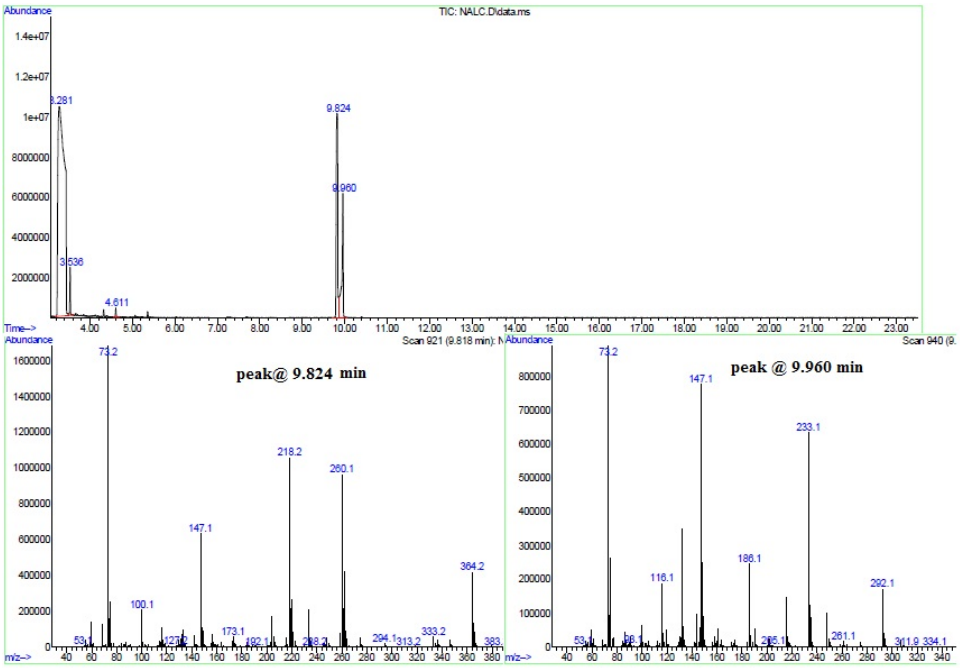

Figure E 10 Total ion chromatogram and mass spectrum of derivatised N-acetly-L-cysteine standard.

E.2 Sample GC chromatograms

The chromatograms of the samples analysed will be displayed here.

Table E 1 commercial skin-lightening creams analysed by means of GC-MS.

Sample number	Sample name
1	BCO original skin repair cream
2	Gentle Magic lotion
3	unknown
4	Beta cream
6	Pure perfect parfait (7)
7	Pure perfect parfait (8)
8	Pure perfect parfait (24)
9	Pure perfect parfait (25)
10	Quick and clear blemish cream
11	Bioclear vanishing cream
12	Bioclear nourishing cream
14	Skin spor G.S.
15	Fair and white whitening cream
16	Dabur Uveda complete fairness cream
17	Fem bleach cream
18	Fem post bleach
19	Fem pre-bleach
20	Magic amabala lotion
23	Hypoderm cream with moisturiser
26	Ponds perfect colour beauty cream very oily skin

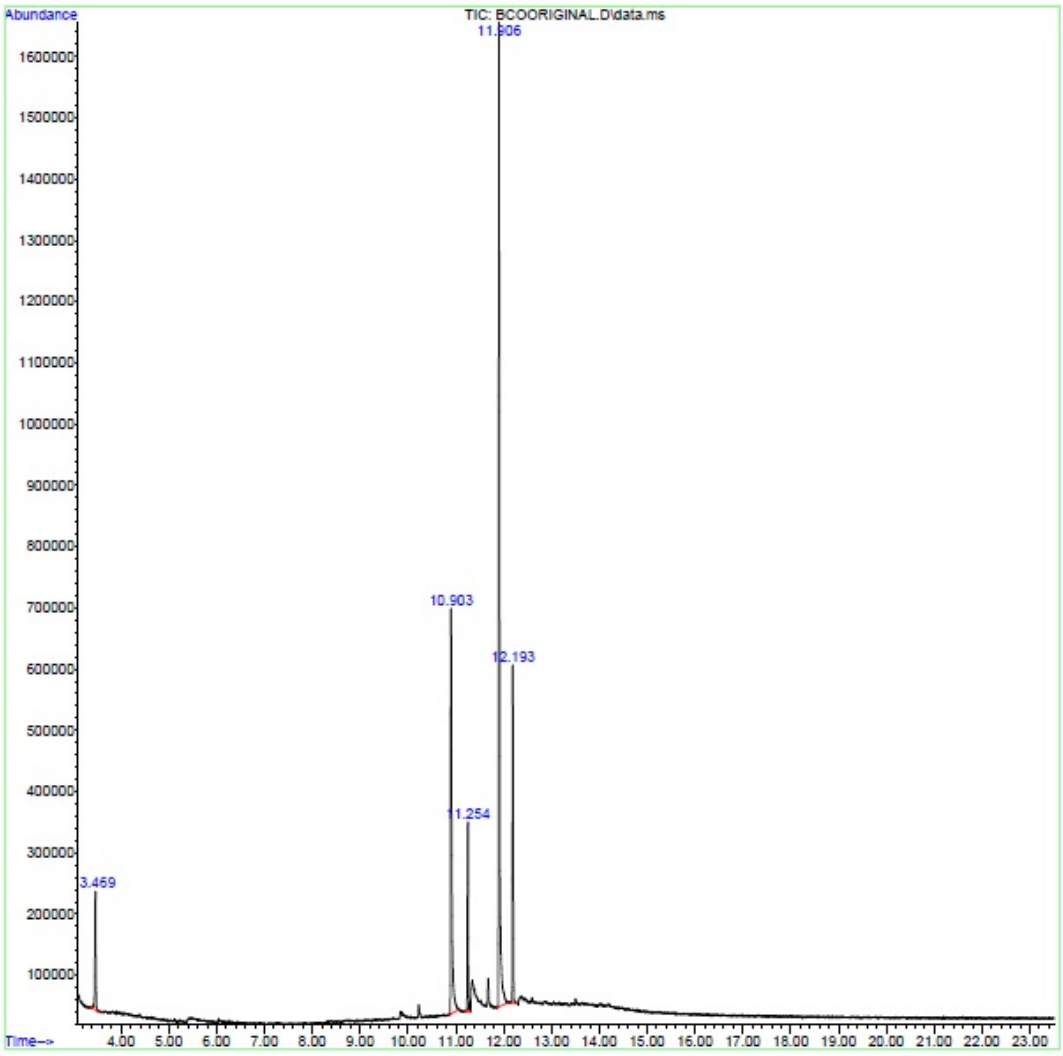

Figure E 11 Total ion chromatogram of derivatised SLC sample 1.

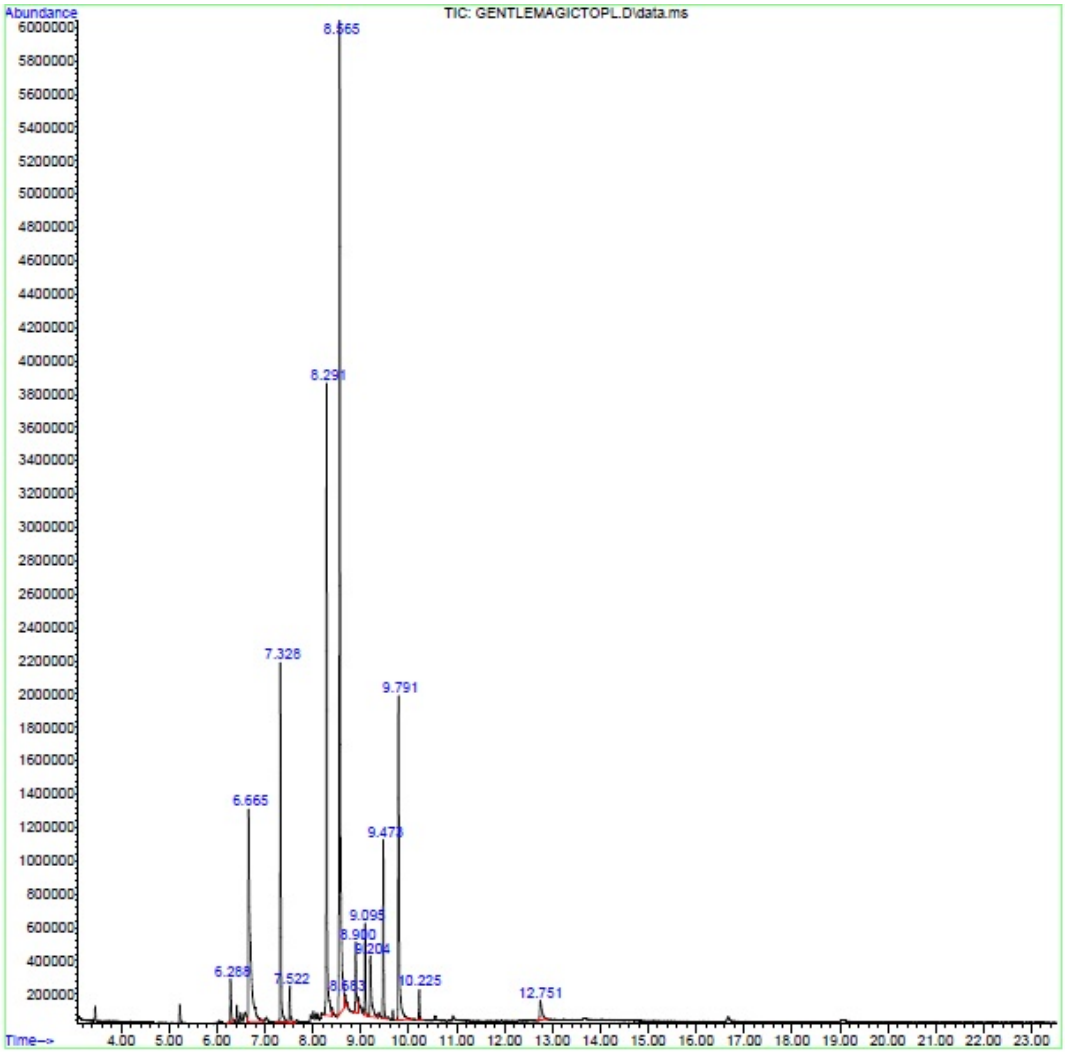

Figure E 12 Total ion chromatogram of derivatised SLC sample 2.

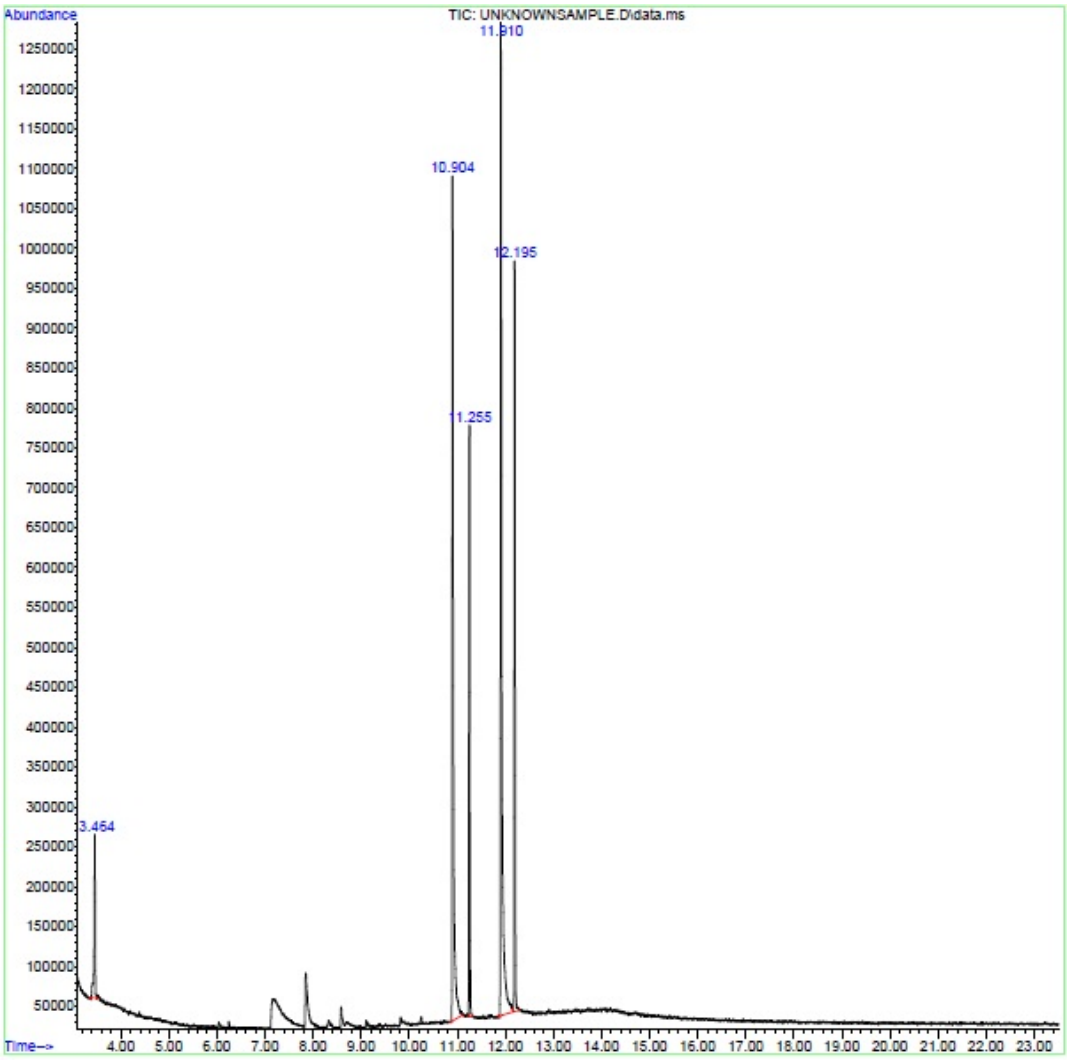

Figure E 13 Total ion chromatogram of derivatised SLC sample 3.

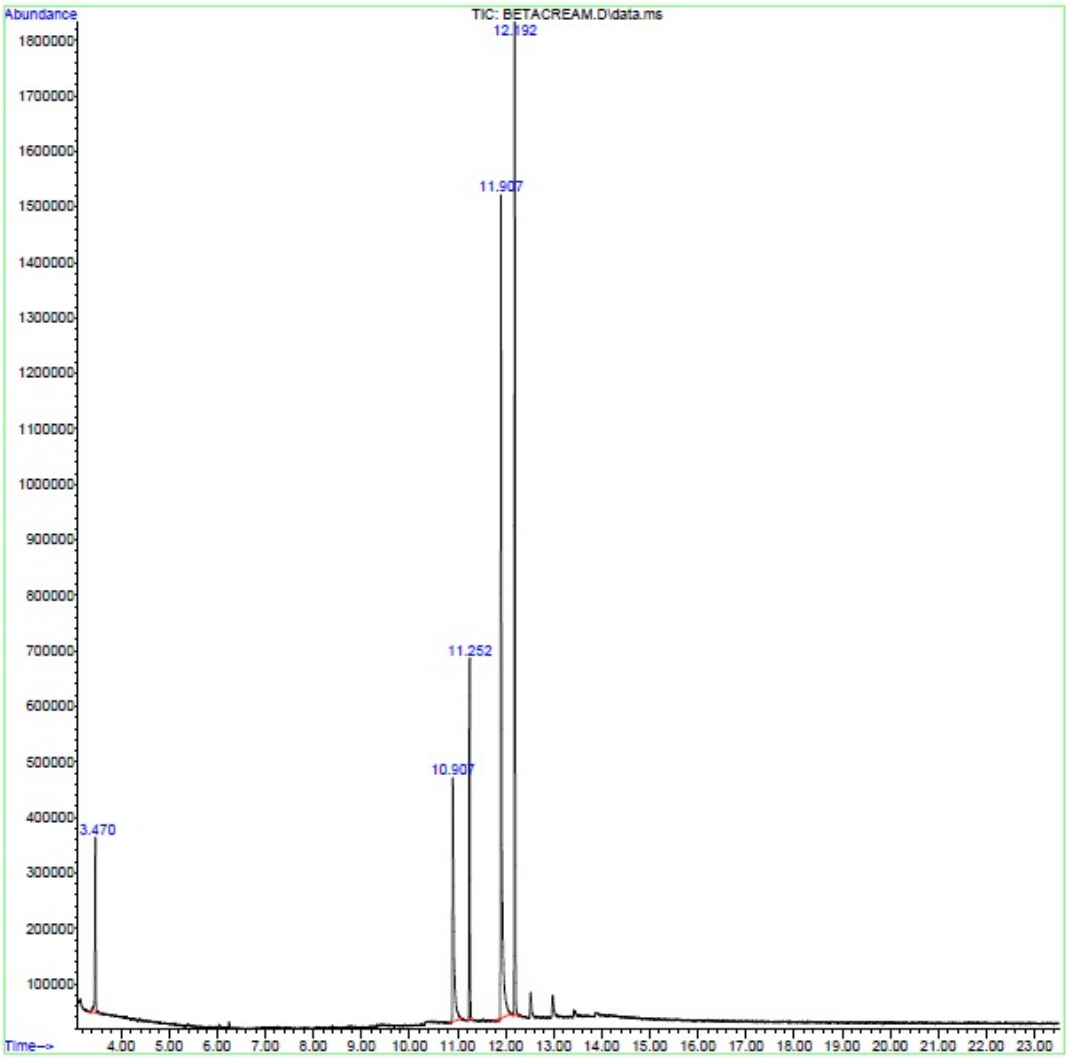

Figure E 14 Total ion chromatogram of derivatised SLC sample 4.

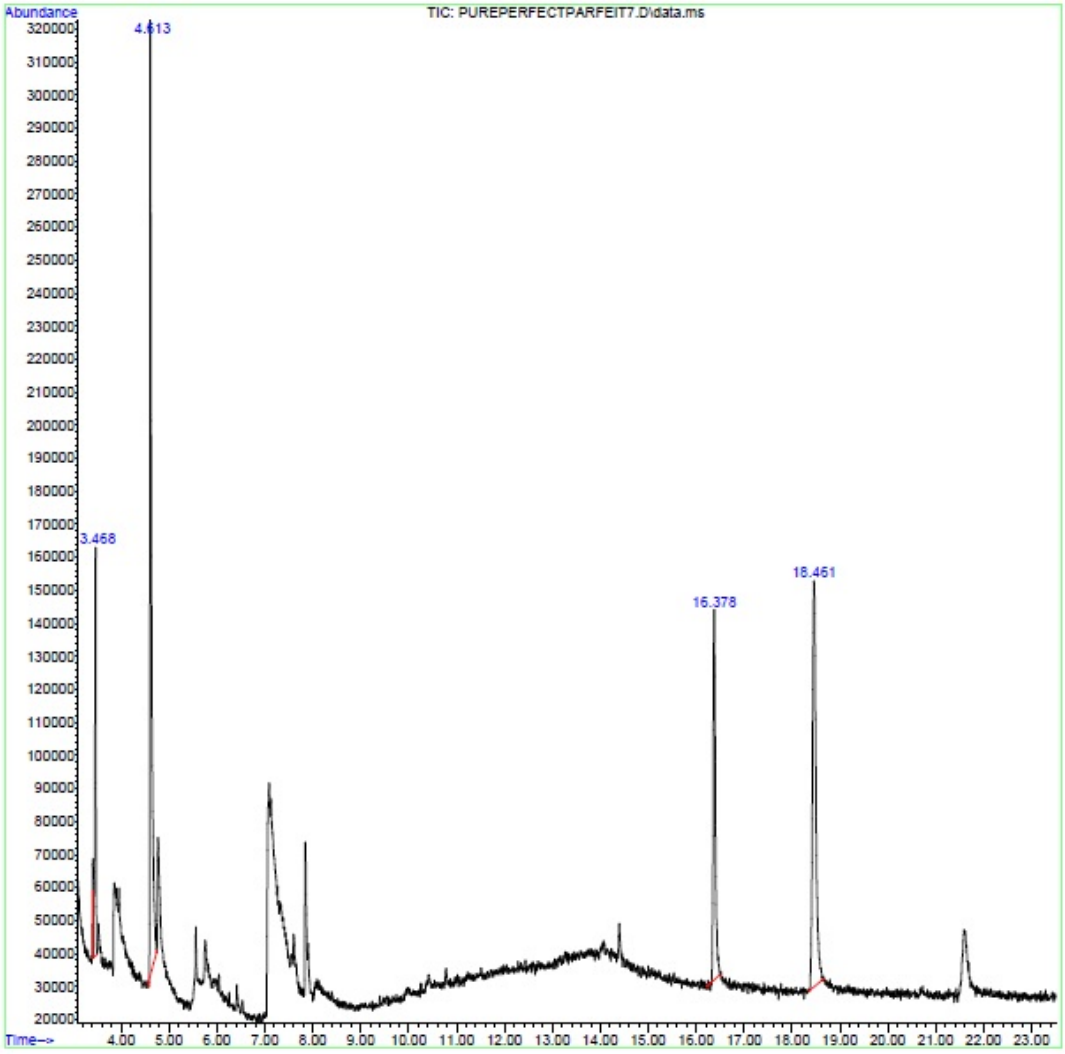

Figure E 15 Total ion chromatogram of derivatised SLC sample 6.

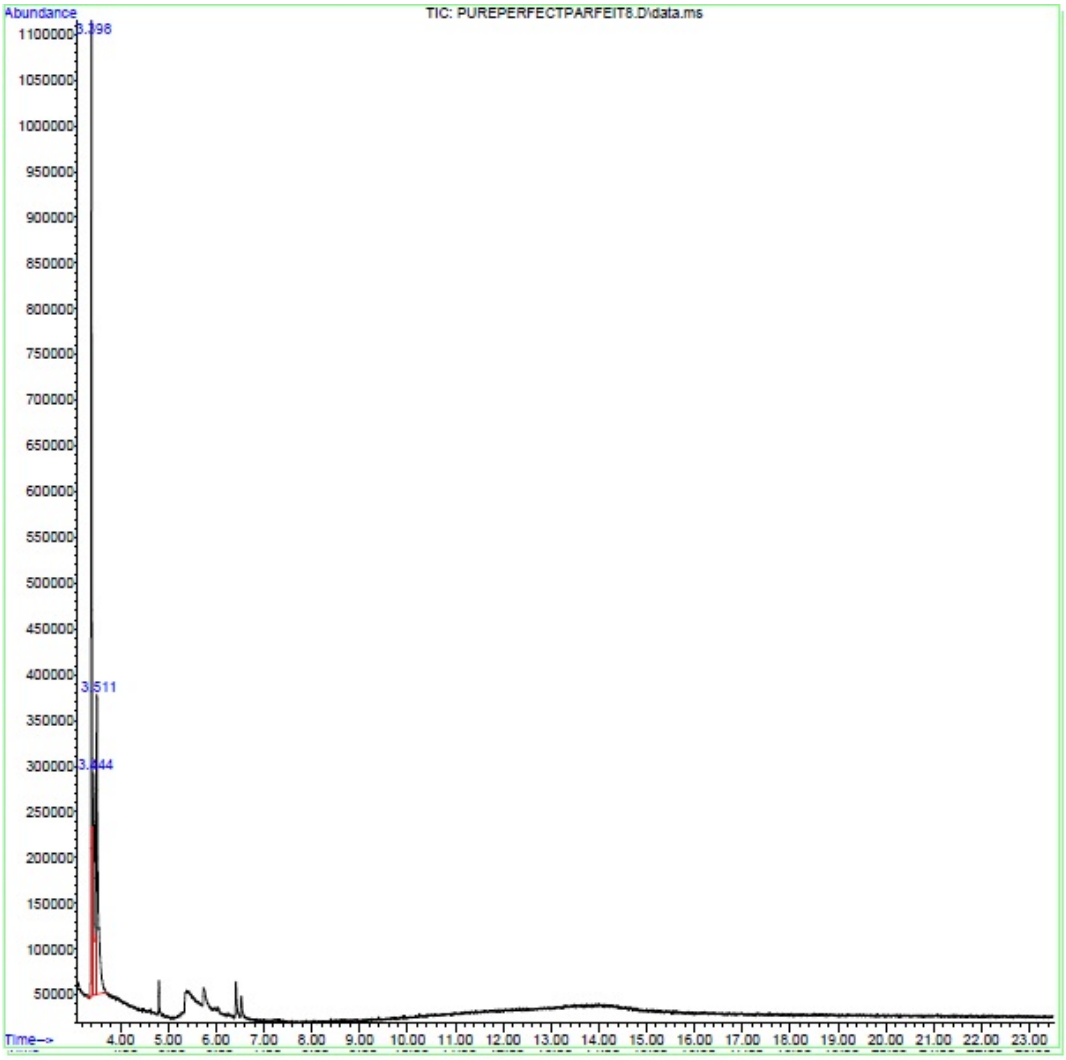

Figure E 16 Total ion chromatogram of derivatised SLC sample 7.

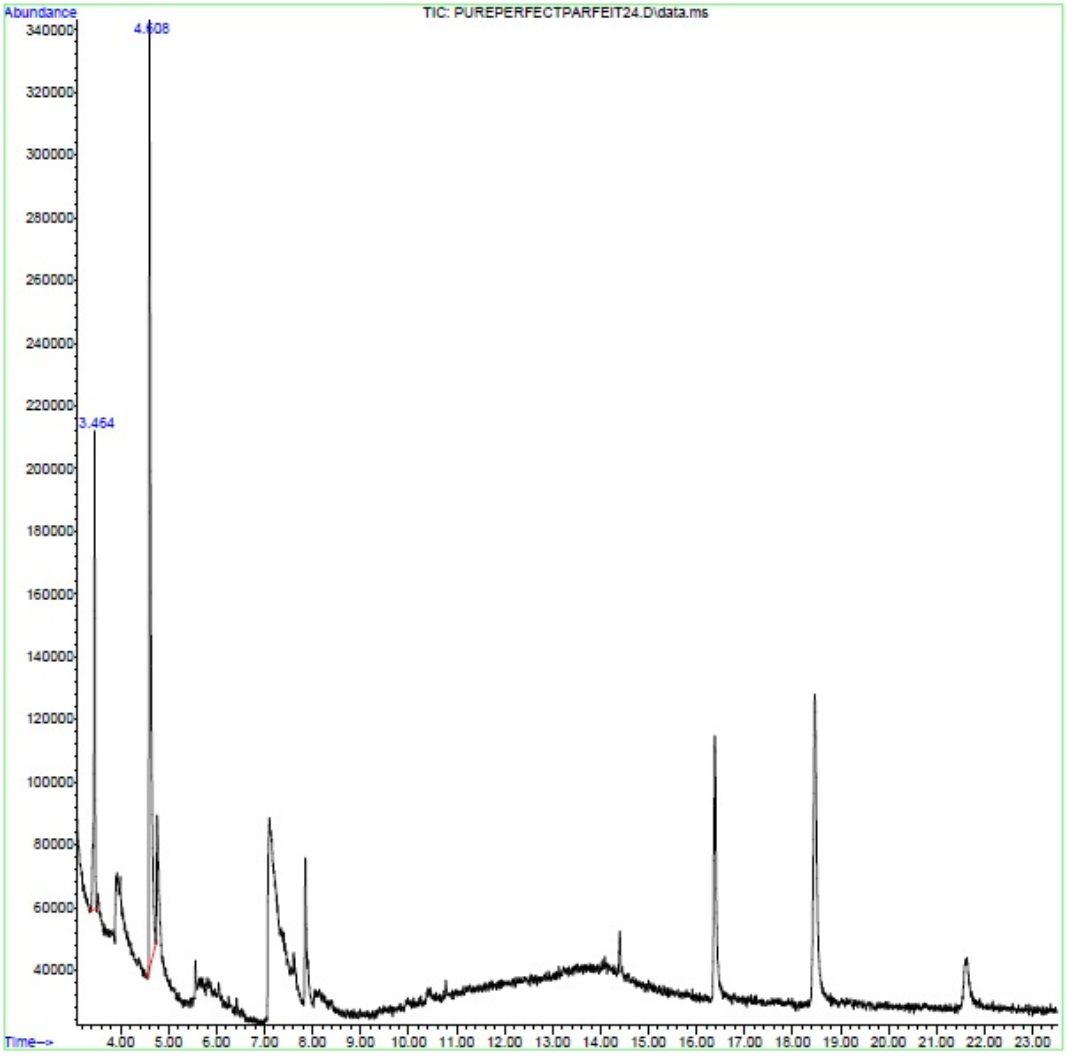

Figure E 17 Total ion chromatogram of derivatised SLC sample 8.

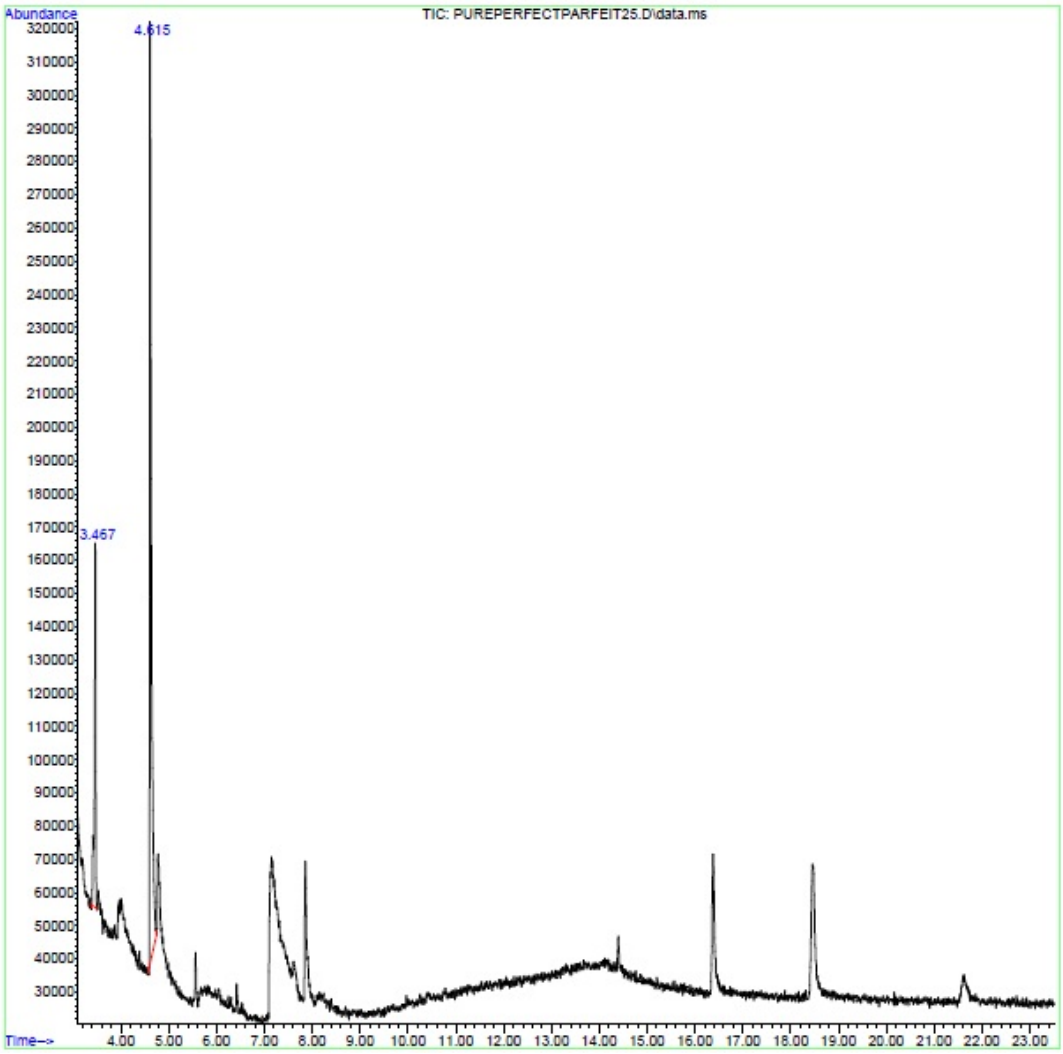

Figure E 18 Total ion chromatogram of derivatised SLC sample 9.

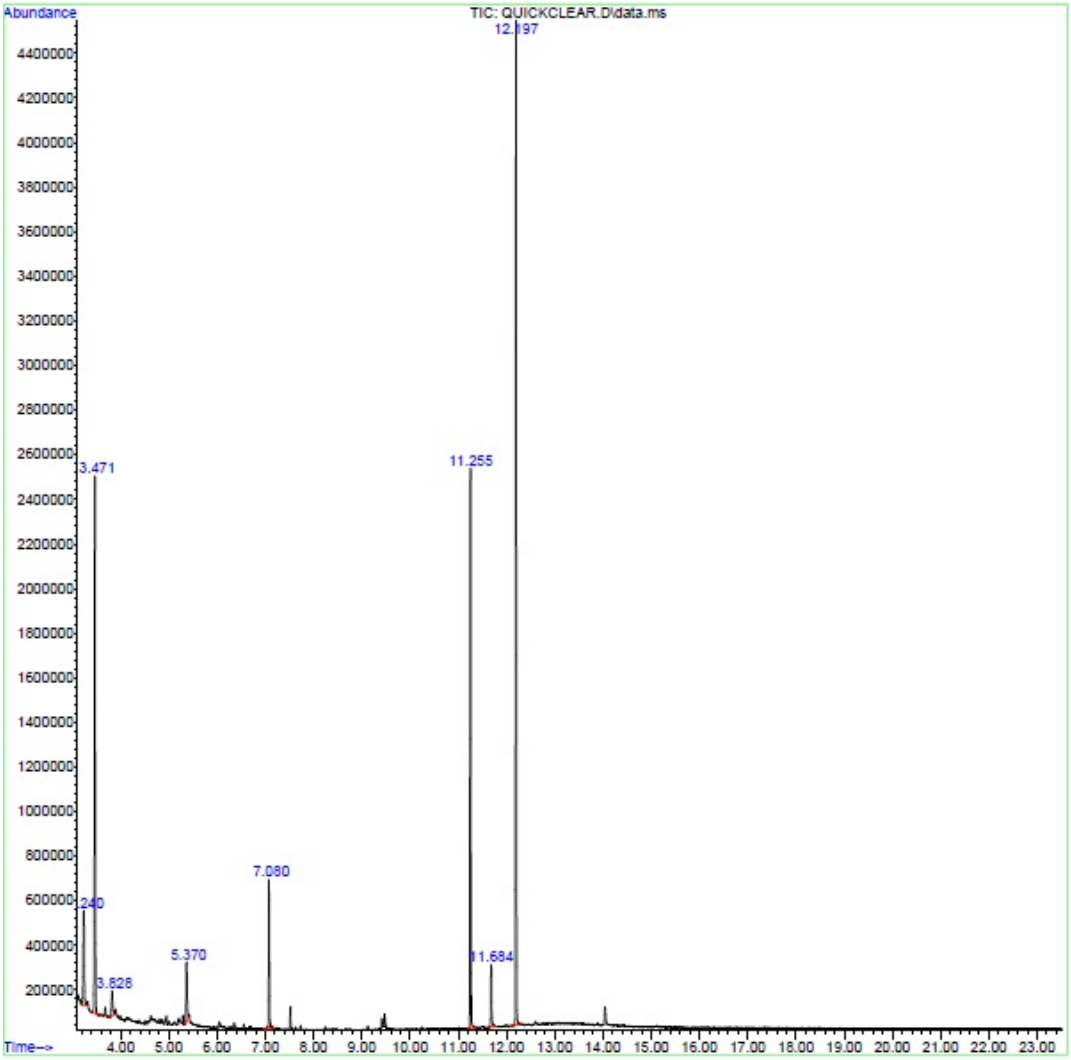

Figure E 19 Total ion chromatogram of derivatised SLC sample 10.

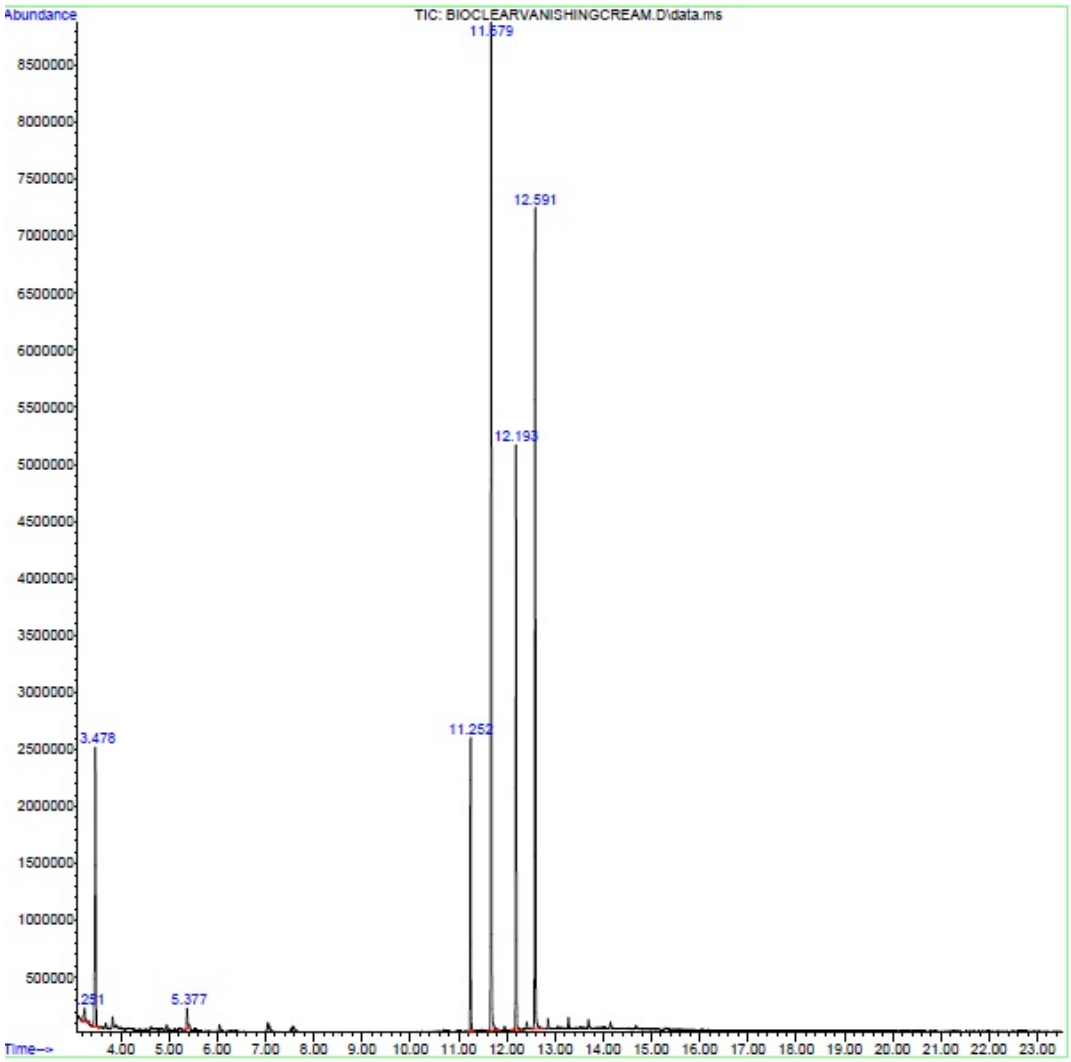

Figure E 20 Total ion chromatogram of derivatised SLC sample 11.

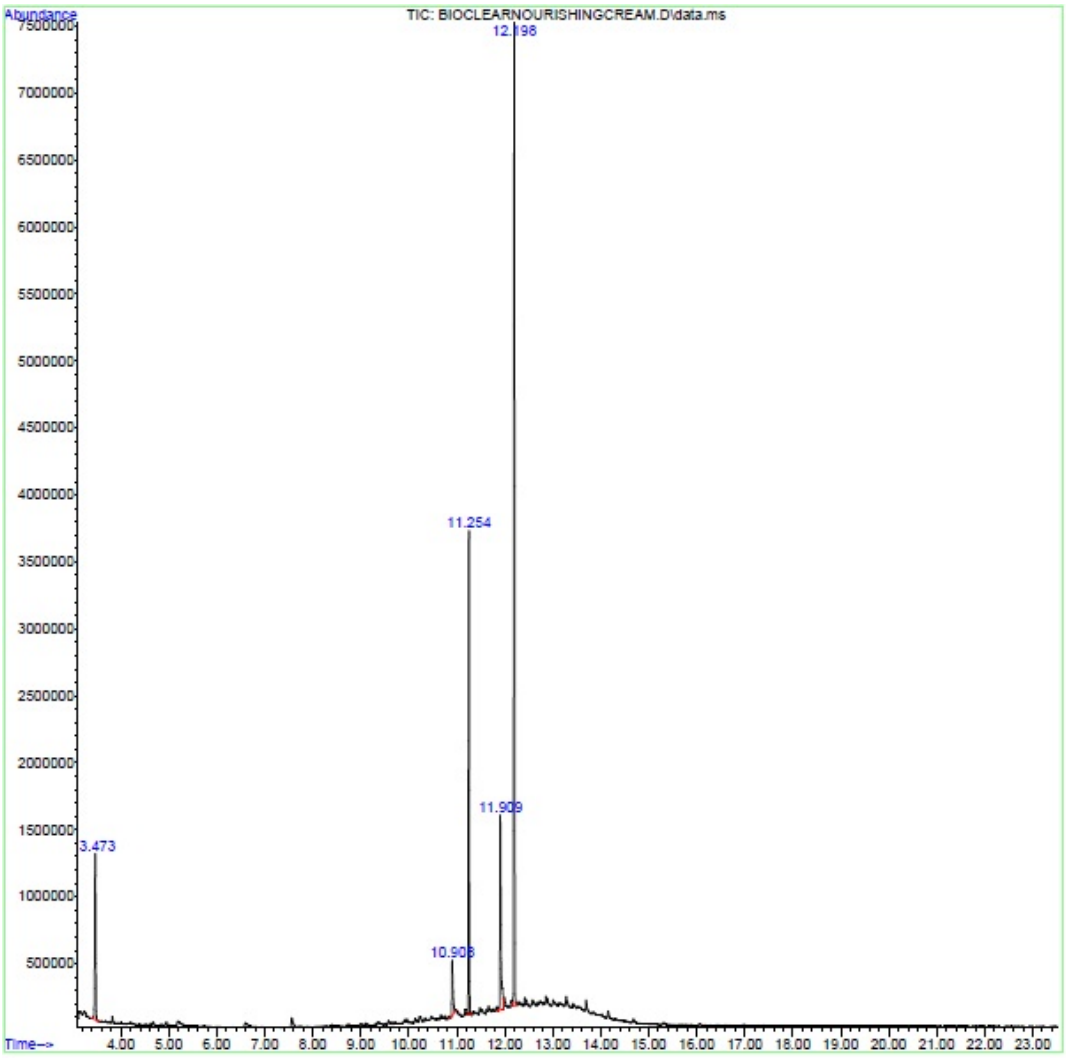

Figure E 21 Total ion chromatogram of derivatised SLC sample 12.

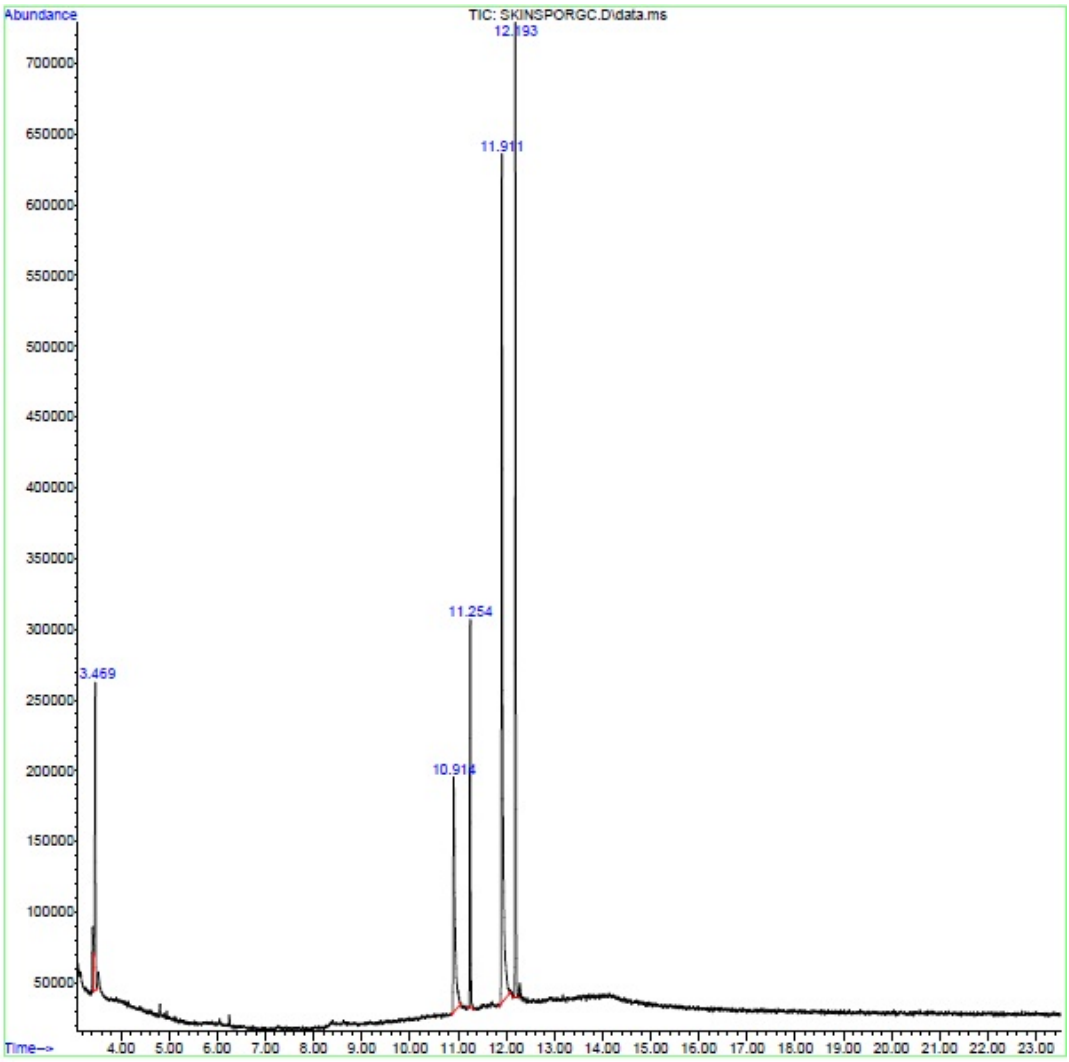

Figure E 22 Total ion chromatogram of derivatised SLC sample 14.

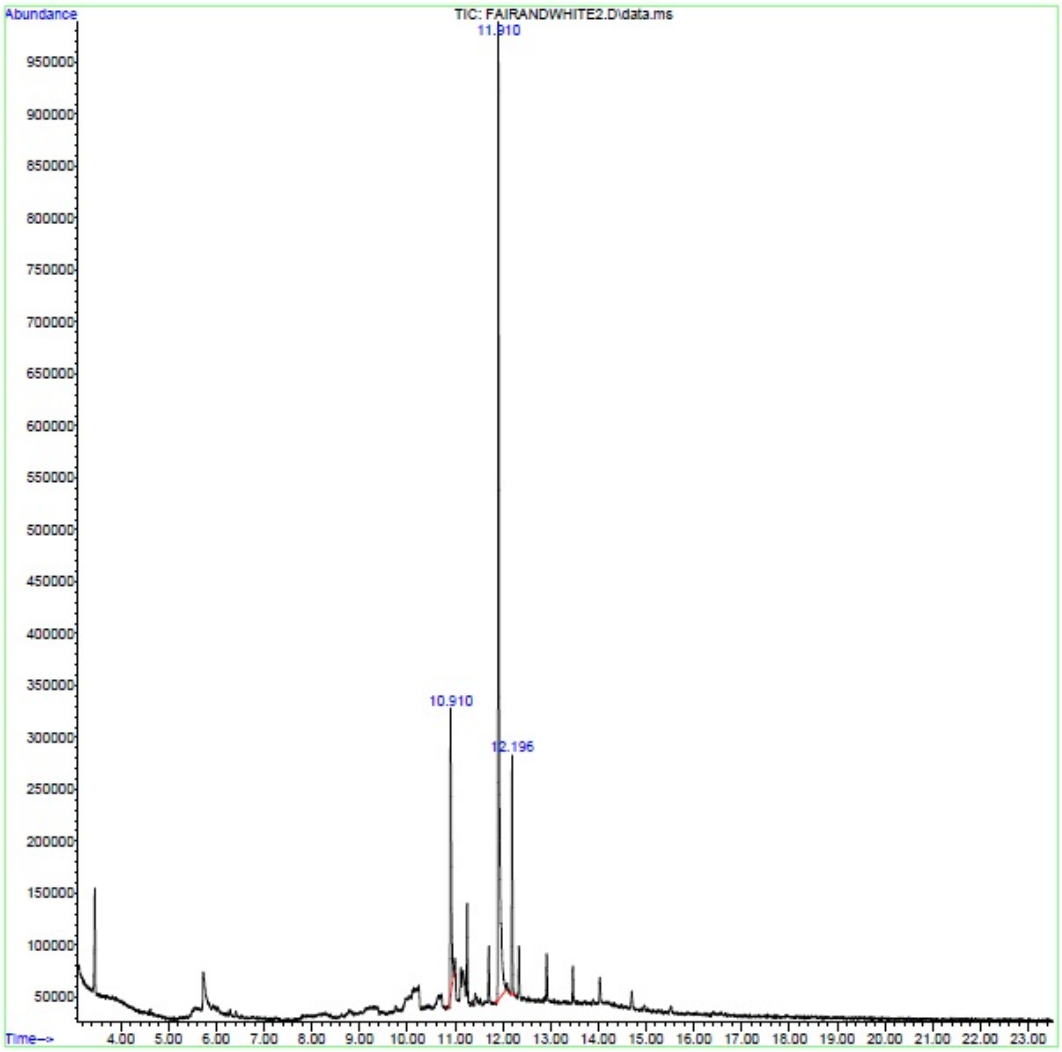

Figure E 23 Total ion chromatogram of derivatised SLC sample 15.

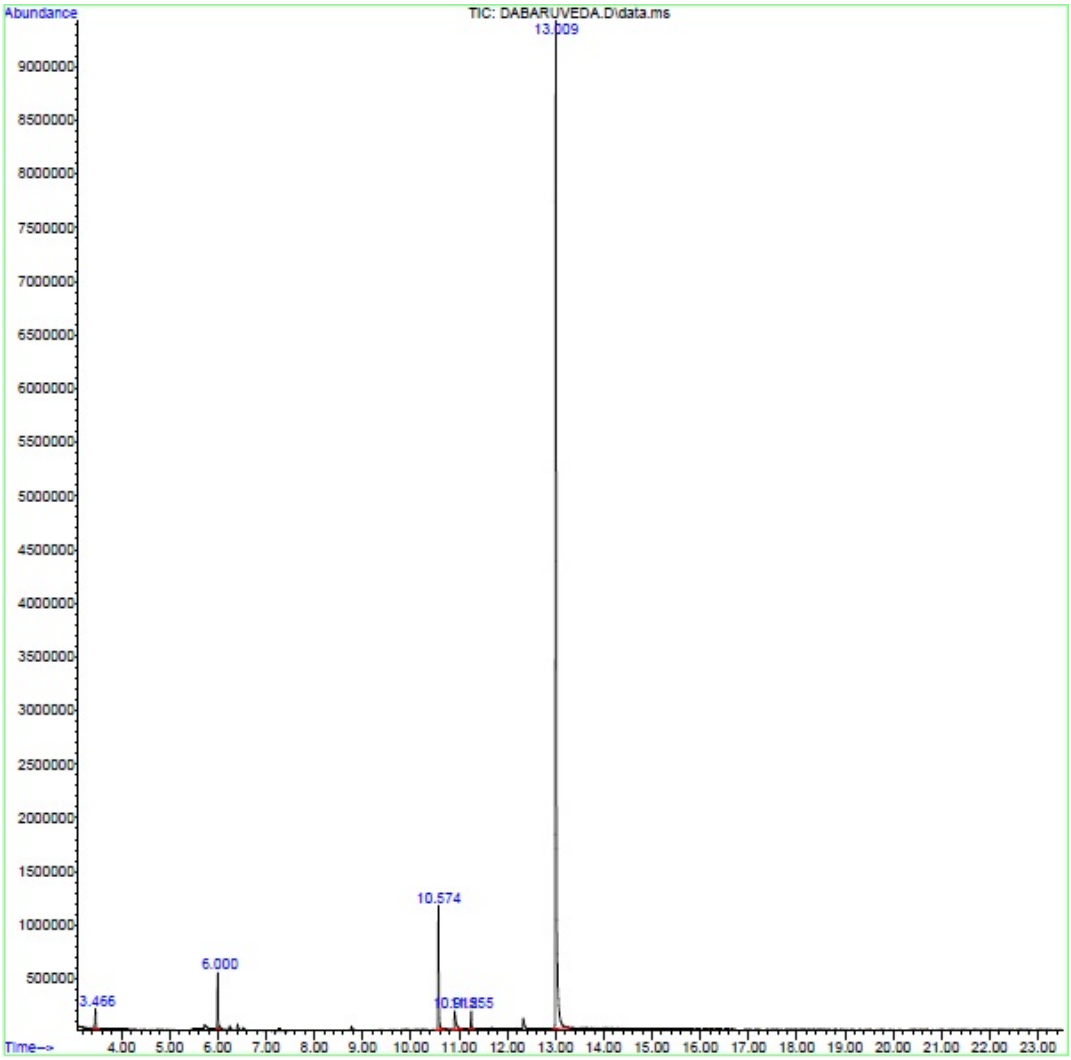

Figure E 24 Total ion chromatogram of derivatised SLC sample 16.

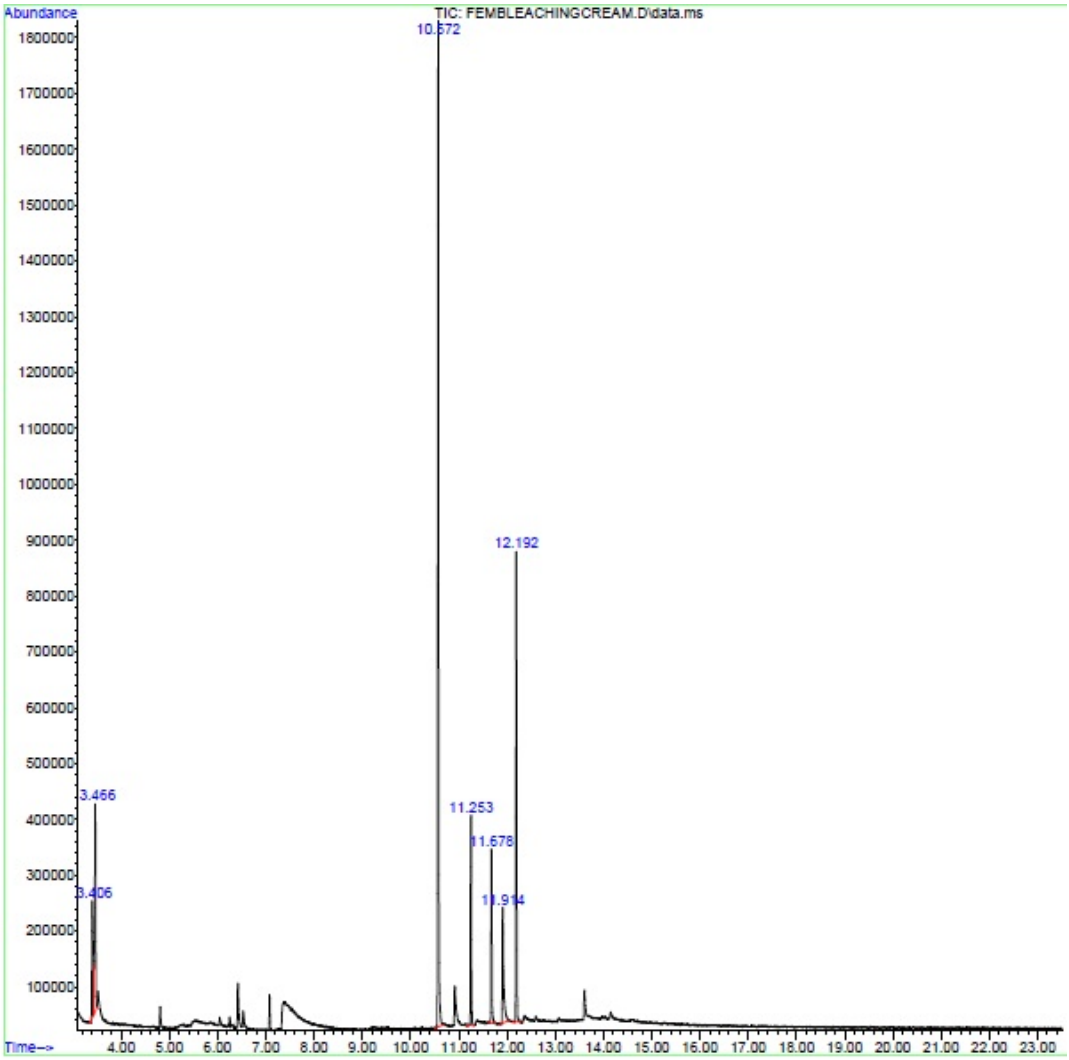

Figure E 25 Total ion chromatogram of derivatised SLC sample 17.

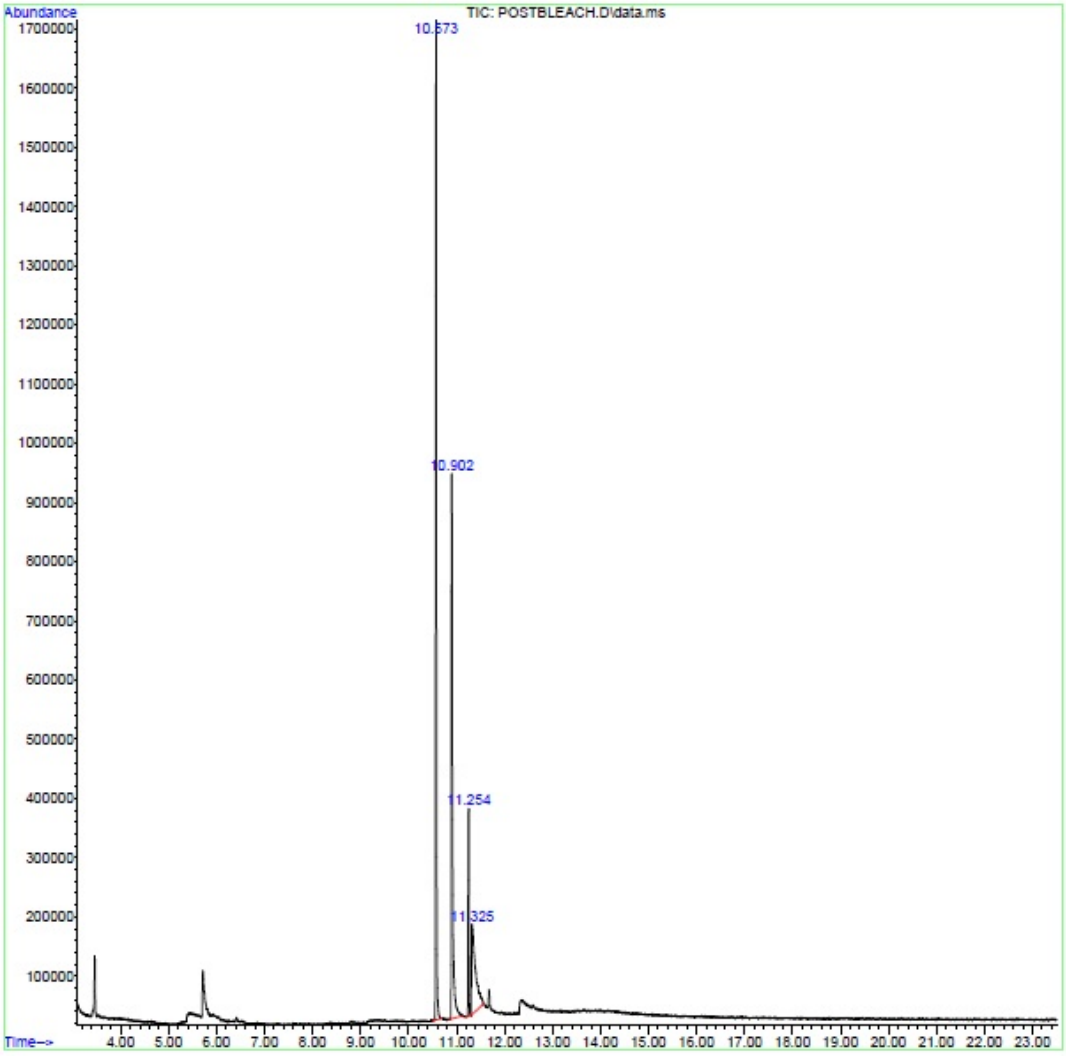

Figure E 26 Total ion chromatogram of derivatised SLC sample 18.

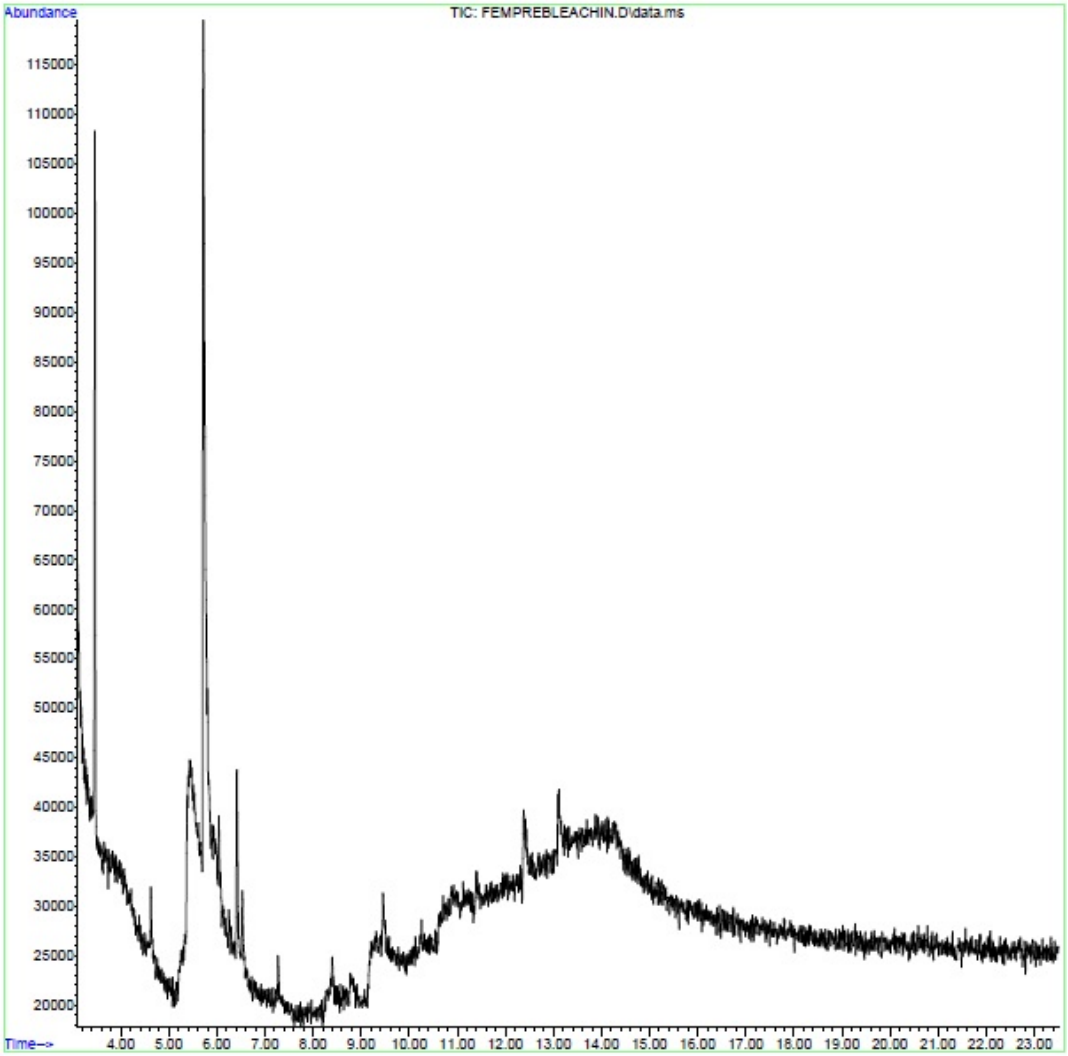

Figure E 27 Total ion chromatogram of derivatised SLC sample 19.

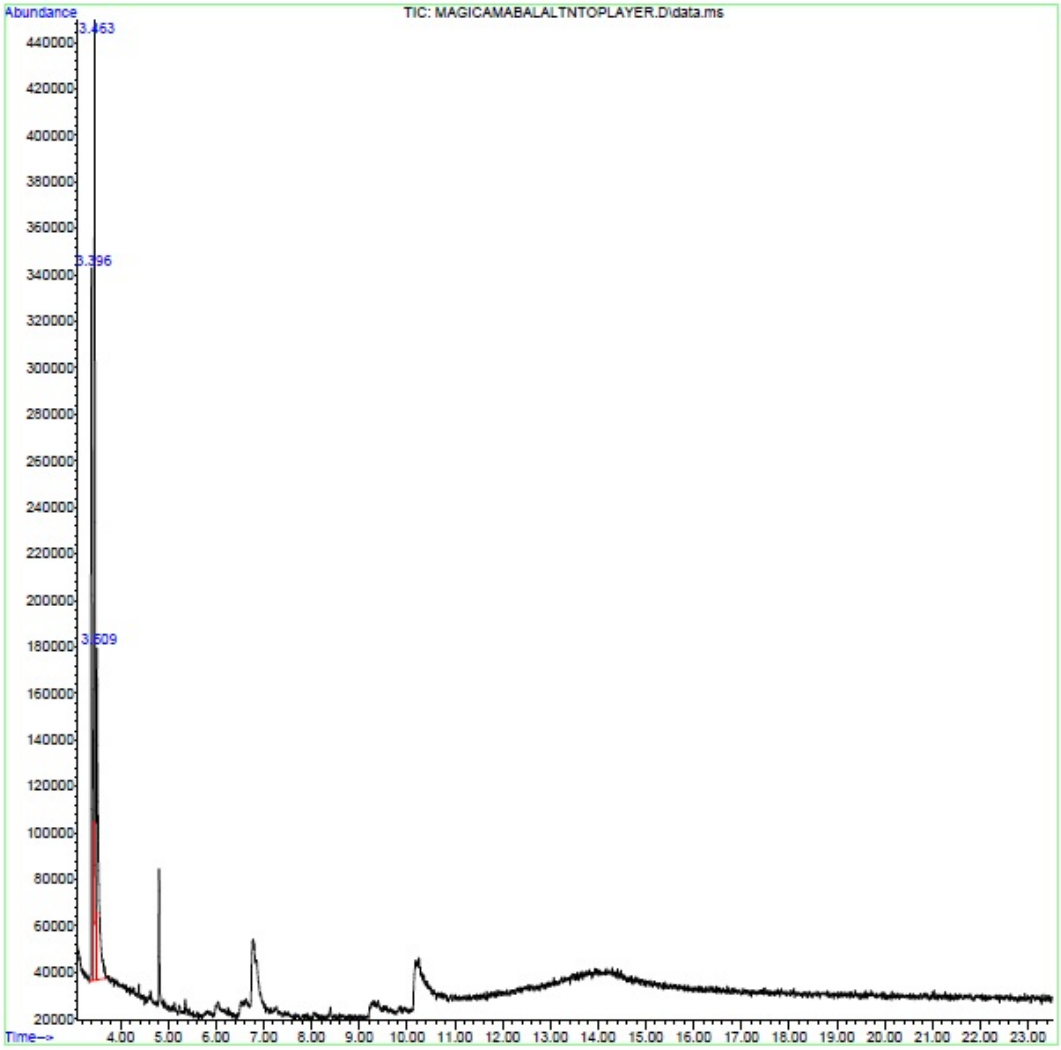

Figure E 28 Total ion chromatogram of derivatised SLC sample 20.

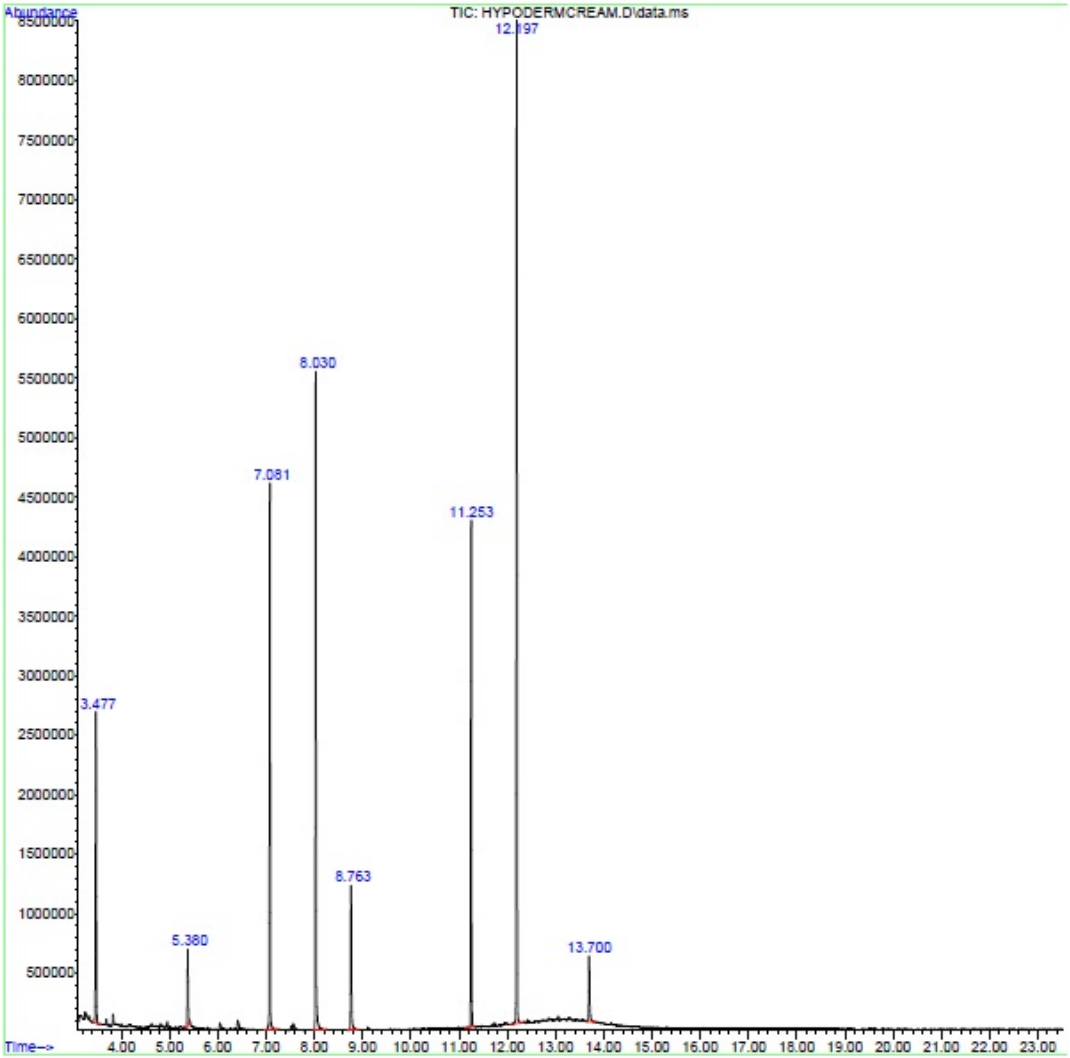

Figure E 29 Total ion chromatogram of derivatised SLC sample 23.

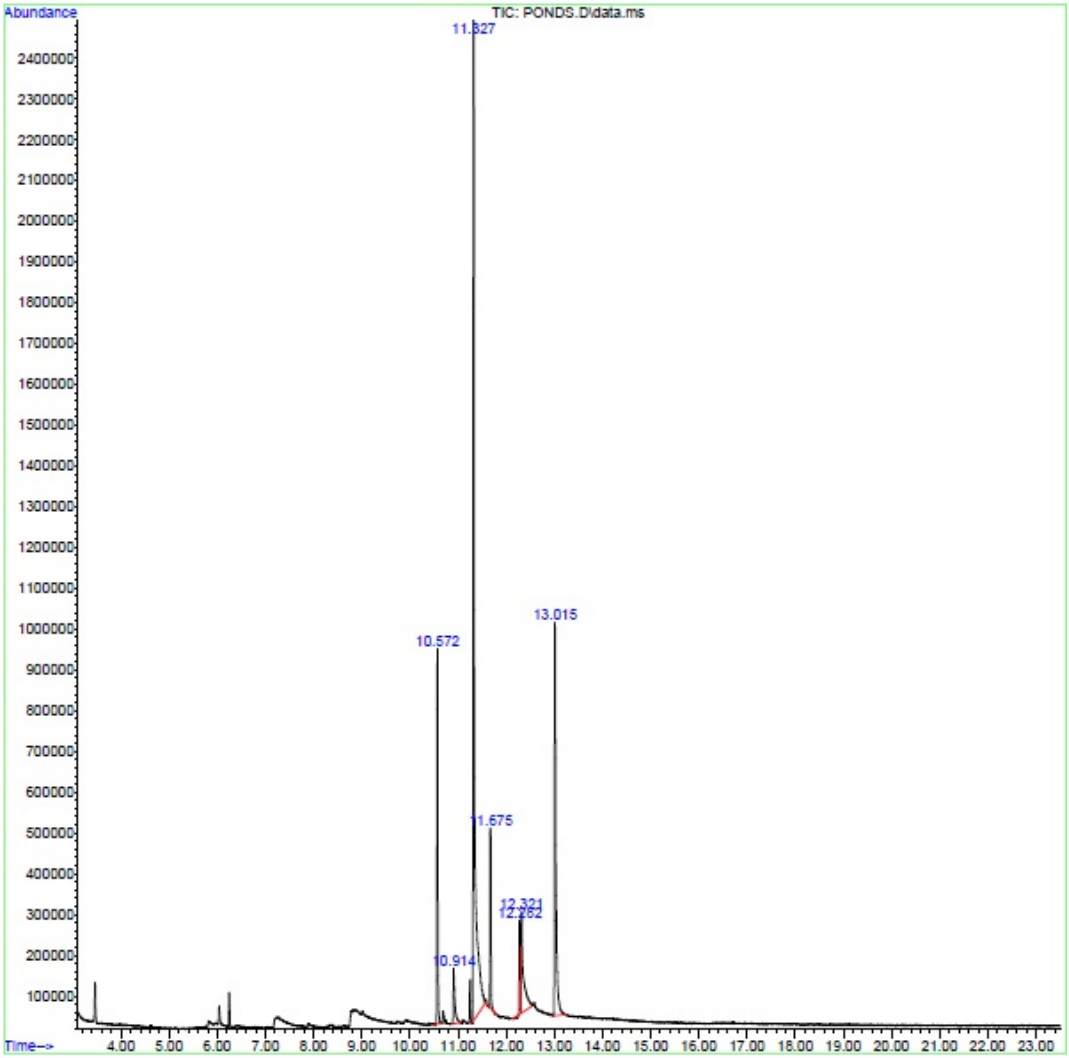

Figure E 30 Total ion chromatogram of derivatised SLC sample 26.