Chemical characterisation and *in vitro* permeation of *Siphonochilus aethiopicus* extracts

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It all starts here ™

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PREFACE

Proverbs 13:12 says that “hope deferred makes the heart sick”. What is “hope deferred”? I believe it is what we call disappointment. We are all disappointed when things do not work out the way we want them to. We become disappointed when we have a plan that fails, a hope that does not materialise, or a goal that is not reached. We are disappointed by everything that does not turn out as you would have expected it to. When things like this happen, for a certain period of time we experience a feeling of let down- one that can lead to depression if it is not handled properly. That is when we have to make a decision to adapt and adjust, to take a new approach despite our emotional feelings. That is when we must remember that we have the Greater One residing within us, so that no matter what happens or come your way, and how much it frustrates you, or even how long it may take for you to reach your dreams and goals, we are not going to give up and quit because of our emotions.

This is when you must remember that when you get disappointed, you can always make the decision to get reappointed

Disappointment often leads to discouragement, which is a worse form dragging us down. We have all experienced the depressing feeling that comes after we have tried our very best to do something and either nothing happens or it all falls totally apart. How disappointing and discouraging it is to see the things we love senselessly destroyed by others or, even worse, by our own neglect or failure. Regardless of how it may happen or who may be responsible, it is hard to go on when everything we have counted on falls down around us. That is when those of us who have the creative power of the Holy Spirit on the inside can get new vision, a new direction, and a new goal to help us overcome the frustrating, downward pull of disappointment. Hope deferred does make a heart sick, but hope can be rekindles, and our hearts can be made whole again by the power of the Holy Spirit.

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ABSTRACT

Studies have estimated that 80% of the people in southern Africa have an extensive history using plant materials for medicinal purposes. *Siphonochilus aethiopicus* (Schweinf.) B.L.Burtt (Zingiberaceae) is a critically endangered indigenous medicinal plant with a distinctive rhizome; the roots together with rhizomes are traditionally chewed for the relief of flu-like symptoms, as well as sinusitis, hysteria, dismennohria and other ailments. It is however known that for any biologically active compound to exert a pharmacological effect, a therapeutic concentration has to be reached first. The fresh rhizomes and roots of *S. aethiopicus* are traditionally chewed in no specific quantity; and very little is known concerning the active compounds present in *S. aethiopicus* as not all compounds have been isolated yet, characterised or studied.

Several techniques were used in this study to investigate the chemical composition of *S. aethiopicus*, since no chemical fingerprint is available for this plant species. High performance thin layer chromatography (HPTLC) was therefore employed to develop a distinctive fingerprint. Ultra Performance Liquid Chromatography coupled to a Time of Flight mass analyser (UPLC-Q-TOF/MS) was used to establish a general profile of the chemical composition of *S. aethiopicus*. Chemical marker molecules were identified and fractionated; their structures were identified using Nuclear Magnetic Resonance (NMR) spectroscopy. For the first time, the volatiles from *S. aethiopicus* were explored using gas chromatography coupled to a time of flight mass spectrometer (GCxGC-TOF/MS). Finally, sufficient quality control protocols were designed for the future identification and authentication of *S. aethiopicus*.

To establish if the isolated marker molecules of *S. aethiopicus* are indeed absorbed; at which site these molecules are absorbed; and to what extent absorption occurs; transport across porcine buccal and sublingual tissues as well as across Caco-2 cell monolayers were investigated. The results obtained indicated that poor absorption of the marker molecules across buccal and sublingual membranes transpires, whereas absorption of the marker molecules across Caco-2 cell monolayers indicated enhanced absorption. Additional studies investigating the chemical compounds absorbed and the gastro-intestinal tract as a site of absorption are therefore required.

UITREKSEL

Studies het beraam dat 80% van die mense in Suider-Afrika 'n uitgebreide geskiedenis aangaande die gebruik van plantmateriaal vir medisinale doeleindes het. *Siphonochilus aethiopicus* (Schweinf.) B.L.Burtt (Zingiberaceae) is 'n krities bedreigde, inheemse medisinale plant met 'n kenmerkende wortelstok; die wortels tesame met wortelstokke word tradisioneel gekou vir die verligting van griep-simptome, asook sinusitis, histerie, pyn tydens menstruasie en vele ander kwale. Dit is boonop bekend dat vir enige biologies aktiewe verbinding om 'n farmakologiese effek te lewer, 'n terapeutiese konsentrasie eers bereik moet word. Die vars wortelstokke en wortels van *S. aethiopicus* word tradisioneel gekou in geen spesifieke hoeveelheid nie; en baie min is bekend oor die aktiewe verbindings teenwoordig in hierdie plantspesie, aangesien alle verbindings nog nie geïsoleer, en geïdentifiseer is nie.

Verskeie tegnieke is in hierdie studie gebruik om die chemiese samestelling van *S. aethiopicus* te ondersoek. Aangesien daar geen chemiese vingerafdruk beskikbaar is vir *S. aethiopicus* nie, is daar dus van hoë werkverrigting dunlaagchromatografie (HPTLC) gebruik gemaak om 'n eiesoortige vingerafdruk te ontwikkel. Ultra werkverrigting vloeistofchromatografie gekoppel aan 'n tyd van vlug massa ontleder (UPLC-Q-TOF/MS) is gebruik om ’n algemene profiel van die chemiese samestelling van *S. aethiopicus* te vestig. Chemiese merker molekules is geïdentifiseer en gefraksioneer; hul strukture is geïdentifiseer met behulp van Kern Magnetiese Resonans (KMR) spektroskopie. Vir die eerste keer, is die vlugtige stowwe uit *S. aethiopicus* ondersoek met behulp van die gas chromatografie gekoppel aan n tyd van vlug massa analiseerder (GCxGC-TOF/MS). Voldoende gehaltebeheer protokolle is uiteindelik ontwerp vir die toekomstige identifikasie en verifikasie van *S. aethiopicus*.

Om te bepaal of die geïsoleerde merker molekules van *S. aethiopicus* wel geabsorbeer word; waar die plek van absorpsie vir hierdie molekules is; en in watter mate absorpsie plaasvind; is transport oor vark bukale en sublinguale weefsel, sowel as oor Caco-2 sel enkel lae getoets. Die resultate dui daarop dat daar swak opname van die merker molekules oor die bukale en sublinguale membrane plaasvind, terwyl die absorpsie van die merker molekules oor die Caco-2 sel enkel lae verhoogde absorpsie getoon het. Verdere studies word aanbeveel om ondersoek in te stel na watter chemiese merkers geabsorbeer word en of daar wel absorpsie in die gastroïntestinale kanaal plaasvind.

LIST OF ABBREVIATIONS

2/4/A1:	Rat fetal intestinal epithelial cells
AG 1:	African Ginger marker molecule 1
AG 2:	African Ginger marker molecule 2
AG 3:	African Ginger marker molecule 3
AG 4:	African Ginger marker molecule 4
AP:	Apical
BL:	Basolateral
BP:	British Pharmacopeia
BPI:	Base peak intensity
Caco-2:	Human colon adenocarcinoma cells
COX-1:	Cyclooxygenase-1
CYP 450:	Cytochrome P450
DAD:	Diode Array Detector
DMEM:	Dulbecco's Modified Eagle's Medium
DNA:	Deoxyribonucleic acid
ER:	Efflux ratio
FBS:	Foetal bovine serum
FC 1:	Fresh cone sample 1
FC 2:	Fresh cone sample 2
FC 3:	Fresh cone sample 3
FC 4:	Fresh cone sample 4
FC 5:	Fresh cone sample 5

FC 6:	Fresh cone sample 6
FC 7:	Fresh cone sample 7
FID:	Flame ionization detector
GCxGC-TOF/MS:	Gas chromatography coupled to a time of flight mass spectrometer
GIT:	Gastro-intestinal tract
HEPES:	n-(2-hydroxymethyl) piperazine-N-(2-ethanesulfonic acid)
HPTLC:	High performance thin layer chromatography
HT29:	Human colorectal adenocarcinoma cells
IR:	Infra-red spectrum
KRB:	Krebs Ringer Buffer
KZN:	KwaZulu Natal
LC/MS/MS:	Liquid chromatography coupled to tandem mass spectrometry
LLC-PK1:	Pig kidney epithelial cells
MDCK:	Dog kidney epithelial cells
MeOH:	Methanol
NEAA:	Non-essential amino acids
NIST:	National Institute of Standards and Technology
NMR:	Nuclear magnetic resonance spectroscopy
NW 1:	North West sample 1
NW 2:	North West sample 2
NW(C):	North West cultivated sample
OVA:	Ovalbumin
PAMPA:	Parallel membrane permeability assay

Papp:	Apparent permeability coefficient
PC:	Polycarbonate
PDA:	Photodiode array
Pen/Strep:	Penicillin/Streptomycin
Pgp:	P-Glycoprotein
Prep-HPLC:	Preparative high performance liquid chromatography
QC:	Quality control
RRI:	Retention indices
Rt:	Retention time
SANBI:	South African National Biodiversity Institute
TC7:	Caco-2 sub clone
TEER:	Transepithelial electrical resistance
TLC:	Thin layer chromatography
TUT(C):	Tshwane University of Technology cultivated sample
UPLC-Q-TOF/MS:	Ultra High pressure liquid chromatography coupled to a time of flight mass spectrometer
UV:	Ultra violet spectrum

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CHAPTER 1 – INTRODUCTION

1.1 Medicinal plants

The indigenous people of southern Africa have an extensive history of using plant materials for medicinal purposes (Mulholland & Drewes, 2004:769). These medicines form an intricate part of the culture and traditions of the African people (Fennell *et al*., 2004a:205). It was estimated that approximately 80% of the South African population or 27 million consumers have either used traditional remedies at some point in their life, or still make use of them (Mulholland & Drewes, 2004:775; Steenkamp, 2003:97). Apart from the cultural significance with respect to the use of traditional medicines, its broad use is also attributable to accessibility and affordability when compared to relatively inaccessible, expensive Western medications (Fennell *et al*., 2004a:205; Mander, 1998; Mander *et al*., 2007:195; Steenkamp, 2003:97; Zschocke *et al*., 2000:281). Furthermore, due to the cultural belief systems of some of the consumers regarding the use of traditional medicines and remedies, they rarely accept Western medication as the preferred treatment for ailments (Zschocke *et al*., 2000:282). Accordingly, demand has exceeded sustainable supply with several species such as *Siphonochilus aethiopicus* (Wild ginger) becoming locally extinct, especially outside of protected areas (Mander, 1998; Mulholland & Drewes, 2004:769; Steenkamp, 2003:97). The prescription and use of traditional medicine in South Africa is currently not well regulated; the result being the latent danger of miss-administration, especially of toxic plants. Potential genotoxic effects following prolonged use of some of the more popular herbal remedies, are also a cause for alarm (Fennell *et al*., 2004a:212; Fennell *et al*., 2004b:118). Since a large portion of the South African population still use traditional medicines, the local government decided in 1999 to promote more research into the country's natural resources and made more funds available for this purpose (Fouche *et al*., 2011:843; Mulholland & Drewes, 2004:769). The trade in medicinal plants is also a vital part of the regional economy with reports indicating that over 771 plant species are being traded as traditional herbal medicines (Mander *et al*., 2007:189). The value of trade in ethnomedicinal plants in South Africa was estimated to be worth R 2.9 billion per year in 2007 (Mander *et al.,* 2007:189). In South Africa alone there are at least 133 000 people deriving a main income from trading indigenous plants, with most of them being rural woman (Mander *et al*., 2007:189). As a consequence, there is an increasing trend worldwide to integrate traditional medicine with primary healthcare (Fennell *et al*., 2004b:114).

1.2 *Siphonochilus aethiopicus* (Schweinf.) B.L. Burtt

Siphonochilus aethiopicus, more commonly known as African or wild ginger, is a deciduous South African plant (Crouch *et al*., 2000:115; Van Wyk & Gericke, 2000:274). It has distinctive cone-shaped rhizomes, which together with the roots are used in the treatment of colds, coughs, influenza, hysteria and pain. The rhizomes of the wild ginger plant are usually chewed fresh (Van Wyk & Gericke, 2000:247). According to Ngwenya *et al.* (2010) the plant is being over harvested and is listed as critically endangered on the South African National Biodiversity Institute (SANBI) red list (Ngwenya *et al*., 2010:414). The possibility of this plant becoming completely extinct is therefore approaching reality, and could hold challenges for traditional healers in future. *S. aethiopicus* can only be found in nature with distribution in the Mpumalanga and Limpopo provinces (Smith, 1998:35; Van Wyk & Gericke, 2000:247), as studies have shown that the plant is already extinct in KwaZulu-Natal. It was brought to light by two studies that the plant can be cultivated on a small scale, thus these plants have been saved in the past from the verge of extinction (Manzini, 2005:5; Ngwenya *et al*., 2010:414). Extinction is, however, still a significant threat because of the plant roots and rhizomes that are being harvested and used for medicinal purposes. Many traditional uses have been studied, some of which include treatment of patients with mild asthma, sinusitis, allergies, wheezing, headaches or pain, and woman with dysmenorrhea (Fouche *et al*., 2011:843; Lategan *et al*., 2009:92;Van Wyk & Gericke, 2000:247; Van Wyk, 2008:350). Treatment against the fungal infection, *Candida albicans*, the bacterium, *Mycobacterium tuberculosis,* as well as treatment against the protozoan parasite, *Plasmodium falciparum,* has also been studied (Van Wyk & Gericke, 2000:247).

1.3 Research problem

It is known that for any biologically active compound to exert a pharmacological effect, a therapeutic concentration has to be reached first (Alqahtani *et al*., 2013:1; Le Ferrec, *et al*., 2001:650). The fresh rhizomes and roots of *S. aethiopicus* are traditionally chewed in no specific quantity; and very little is known concerning the active compounds present in *S. aethiopicus* as not all compounds have yet been isolated, characterised or studied. Furthermore, it is unclear where, and to what extent, absorption of the active compounds occurs. Despite these questions in hand, several products called “African Ginger” have been manufactured by pharmaceutical companies as either a capsule or a tablet containing dried *S. aethiopicus* plant material to be taken orally thrice daily against inflammation, colds, flu, sore throats, sinusitis or headaches, and Candida. Moreover, though various studies have been conducted on selected isolated compounds of *S. aethiopicus*, these studies only speculated on the pharmacological effects it may have on the human body; and none of

these studies tested the absorption of these compounds (Fouche *et al*., 2011:843; Lategan *et al*., 2009:92; Van Wyk, 2008:350). Therefore, questions still remain regarding the active compounds contained in the roots and rhizomes of *S. aethiopicus*; the concentrations and site of absorption of these compounds; the pharmacological effects these compounds may exert; potential metabolites that may form once the compounds are absorbed; the possible side effects that may occur; the potential interactions that may arise if synthetic drugs are taken together with the plant material; and whether or not this plant material is effective in a dosage form.

Consequently, it was important in this study to first characterise the active compounds present in *S. aethiopicus*; establish which compounds are absorbed and in which concentrations; as well as to determine the specific site and extent of oral absorption of these compounds in order to determine if this plant is indeed pharmacologically and pharmaceutically relevant.

1.4 Aims and objectives

The aims of this study were to isolate and identify some of the phytochemicals contained in *S. aethiopicus* and then to determine the permeability of these compounds across buccal, sublingual and intestinal tissue in order to establish which compounds are permeable across mucosal epithelia as well as where these compounds are best absorbed.

The specific objectives of this study were to:

- Prepare various crude extracts from *S. aethiopicus* in different solvents.
- Identify and isolate the active compounds of *S. aethiopicus* by means of preparative high performance liquid chromatography (HPLC, specifically UPLC-Q-TOF/MS).
- Chemically fingerprint the prepared *S.aethiopicus* extracts using automated thin layer chromatography (HPTLC), high pressure liquid chromatography (UPLC-Q-TOF/MS) and 3D gas chromatography, set in headspace analysis mode (GCxGC-TOF/MS).
- Obtain *S. aethiopicus* oil samples using hydro-distillation and chemically characterise the oil samples using gas chromatography (GC/FID).
- Conduct *in vitro* permeability experiments with crude extracts from *S. aethiopicus* across excised porcine sublingual and buccal tissues, using Sweetana-Grass diffusion chambers.
- Conduct *in vitro* permeability experiments with crude extracts from *S. aethiopicus* across Caco-2 cell monolayers in Transwell® plates.

CHAPTER 2 – LITERATURE OVERVIEW

2.1 Botanical classification and description

Siphonochilus aethiopicus (Schweinf.) B.L.Burtt (Zingiberaceae), more commonly known as wild or African ginger; or as Indungulo to the Zulu people, is a bisexual plant with large, hairless leaves, pink and white flowers and distinctive cone shaped rhizomes that develop each year (Figure 2.1). In Figure 2.1 D and E, the distinctive cone shape of the rhizomes can be seen. These plants develop small berry-like fruits that can be borne below or above the ground. The leaves and rhizomes are known to have a smell comparable to that of ginger (*Zingiber officinale*), but to a far less extent (Smith, 1998:274-275).

Figure 2.1: Photographs illustrating: A & B) *S. aethiopicus* flower and leaves, C) *S. aethiopicus* rhizomes and roots, D & E) *S. aethiopicus* distinctive cone shaped rhizomes, F) *S. aethiopicus* rhizomes cut into small pieces

It is, however, still not clear exactly where *S. aethiopicus* can be found naturally, but several researchers have reported that it grows naturally in small parts of Mpumalanga and the Limpopo Provinces; and it is said to be locally extinct in KwaZulu-Natal due to over

exploitation. Reports indicated that *S. aethiopicus* also grows in Zimbabwe, Malawi and Zambia (Fouche *et al.,* 2011:843; Holzapfel *et al.,* 2002:405; Street *et al.,* 2013:9). The green highlighted areas on the map in Figure 2.2 are where *S. aethiopicus* reportedly can be found in its natural environment, whereas the areas highlighted in red are where it is already extinct; and the areas highlighted in orange are where it is documented to be cultivated (Hartzell, 2011:8-10).

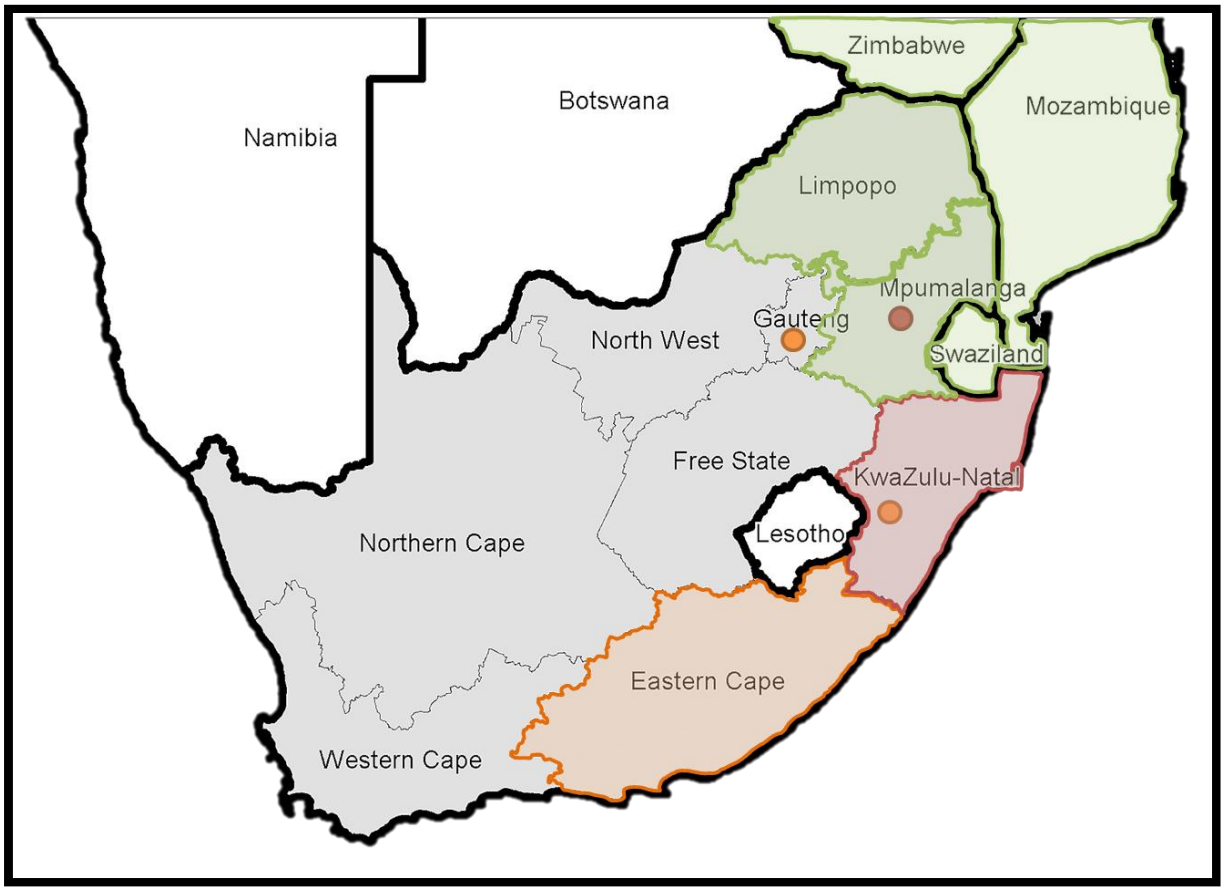


Figure 2.2: Map of southern Africa, where the areas highlighted in green are where *S. aethiopicus* can be found as a wild population, the areas highlighted in red are where the plant is extinct and areas highlighted in orange are where *S. aethiopicus* is being cultivated (Adapted from pix-hd, 2016)

2.2 Traditional and modern uses

Fresh roots and rhizomes of *S. aethiopicus* are used for medicinal purposes all over Africa for several aliments including colds, coughs, wheezing, sinus problems, influenza, hysteria and pain. It is also traditionally used to treat patients with asthma, headache, dysmenorrhoea, amenorrhoea, Candida, malaria; and more recently it was found to be effective in treating sleeping sickness. Several studies have been conducted in an attempt

to confirm the medicinal value of *S. aethiopicus*. Scientists are still testing the biological activity in order to establish whether the plant is truly effective in the treatment for all of the aliments mentioned above (Fouche *et al.,* 2011:843; Igoli *et al.,* 2012:88,92; Lategan *et al.,* 2009:92; Steenkamp, 2003:106). It has been reported by Smith (1998:37) that the highly aromatic roots are traditionally used by the Zulu people as protection against lightning, snakes as well as to treat hysteria. There are further reports of unspecified groups in South Africa that use an infusion of the rhizomes to treat epilepsy (Smith, 1998:37; Stafford *et al.,* 2008:523; Street *et al.,* 2013:9).

Despite its high medicinal value, aromatic plants such as *S. aethiopicus* have also become of great interest in the food industry as sources of new flavours for foods and other confectioneries. The rhizomes of this plant have a delicious spicy taste and have considerable potential for the development of new functional foods (van Wyk*,* 2011b:864). Van Wyk (2011b:860, 864) reported that spices are a relatively rare finding in southern Africa and that plants similar to *S. aethiopicus* deserve more consideration to be used as a spice. Research has likewise shown that the Igede people of Nigeria already use the roots and rhizomes as spices in the flavouring of their dishes (Noudogbessi *et al.,* 2013:8489; van Wyk*,* 2011b:864). It is thus clear from the information stated above that the value of *S. aethiopicus* is not only limited to its medicinal properties.

2.3 Phytochemistry

As previously mentioned, little information with regards to the chemical composition of *S. aethiopicus* is available; and only a few scientists have conducted studies to attempt shedding more light on the complete chemical composition. The active components, which have been successfully isolated, and are known to be partly responsible for some of the medicinal effects or known biological activities, are mainly sesquiterpenoids of the furanoid type, one of the major classes present in *S. aethiopicus*, in addition to the diarylheptanoids (Holzapfel *et al.,* 2002:405). The essential oil of the rhizomes was investigated by Viljoen *et al.* (2002:116). They isolated high concentrations of the major compound Siphonochilus sesquiterpenoid or, as they suggested siphonochilone, whilst 1,8-cineole as well as (E)-β-ocimene were identified as minor compounds.

Research on the compound 1,8-cineole (cineole), also known as eucalyptol or cajeputol, revealed that it is a terpene oxide. This compound is often employed by the pharmaceutical industry in drug formulations as a percutaneous penetration enhancer as well as for its decongestive and antitussive effects. Cajeputol is also used during aromatherapy as a skin stimulant. Moreover, it is considered useful for the treatment of bronchitis, sinusitis and

rheumatism (Santos & Roo, 2000:240), which might suggest that it could possibly be one of the compounds of *S. aethiopicus* responsible for some of the reported medicinal effects.

In another study by Noudogbessi *et al.* (2013: 8489-8492) the focus was mainly on the determination of the chemical composition of the essential oils, the fatty acid content as well as the unsaponifiables of this plant. Results obtained indicated that the abundant chemical families in the rhizomes of *S. aethiopicus* are saponins, catechins, tannins, leucoanthocyans and mucilages. They furthermore identified that the most important fatty acids found in the rhizomes of these plants are palmitic acid ($C_{16:0}$), linoleic acid ($C_{18:2\ (9,12)}$) and oleic acid ($C_{18:1}$).

Lategan *et al.* (2009:92) isolated three novel furanoterpenoids by means of a bioassay guided fractionation; and concluded that these compounds all show signs of antiplasmodial activity. In a similar study by Igoli *et al.* (2012:88), whom also tested for antiparasitic activity, five new compounds that have not been previously reported, were isolated and identified. They found that these compounds exhibited anti-trypanosomal activity. Table 2.1 portrays a summary of all of the above stated compounds and their possible contribution to the biological activity noted for *S. aethiopicus*.

Table 2.1: **Major isolated chemical compounds from *S. aethiopicus* and their possible biological effects**

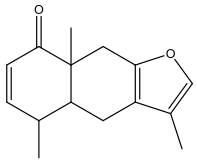
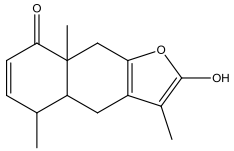
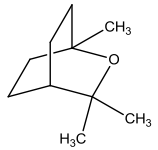
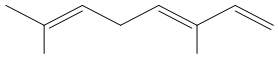
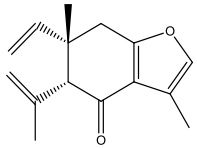
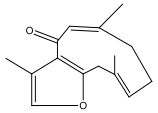
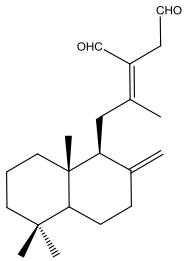
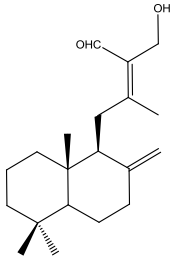
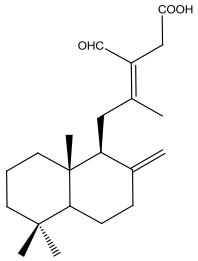
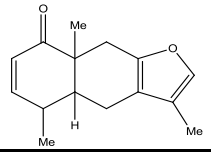
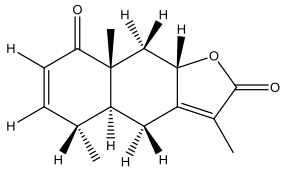
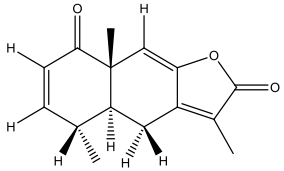
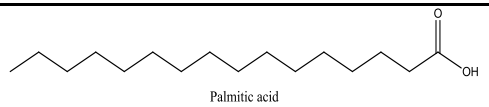
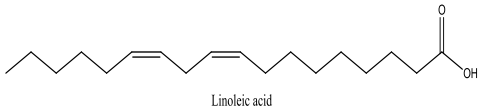
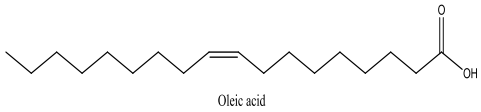
Major isolated chemical compound	Chemical structure	Biological activities	References
Furanoterpenoid 1 ***Siphonochilus sesquiterpenoid*** **siphonochilone** **or**		Anti-inflammatory, anti-allergic and bronchodilatory effects	(Fouche *et al.,* 2011:846; Holzapfel *et al.,* 2002:405; Viljoen *et al.,* 2002:116)
Furanoterpenoid 2		Anti-inflammatory, anti-allergic and bronchodilatory effects	(Fouche *et al.,* 2011:846; Holzapfel *et al.,* 2002:405)
1,8-cineole		Bronchitis, sinusitis and rheumatism, anti-inflammatory and pain	(Viljoen *et al.,* 2002:116; Santos & Roo, 2000:240)
(E)-β-ocimene		-	(Viljoen *et al.,* 2002:116)
Epi-curzerenone		-	(Igoli *et al.,* 2012:88-90)
Furanodienone		Antitrypanosomal activity	(Igoli *et al.,* 2012:88-90,92)

Table 2.1: **Major isolated chemical compounds from *S. aethiopicus* and their possible biological effects (continued)**

8(17),12E-labdadiene-15,16-dial		Antitrypanosomal activity	(Igoli *et al.,* 2012:88-90,92)
15-Hydroxy-8(17),12E-labdadiene-16-al		-	(Igoli *et al.,* 2012:88-90)
16-Oxo-8(17),12E-labdadiene-15-oic acid		-	(Igoli *et al.,* 2012:88-90)
Furanoterpenoid		Anti-asthmatic and anti-allergic activities	(Fouche *et al.,* 2011:846)

4aαH-3,5α,8aβ-trimethyl-4,4a,8a,9-tetrahydronaphtho-([2,3b]-dihydrofuran-2-one)-8-one		Antimalarial activity	(Lategan *et al.,* 2009:94)
4aαH-3,5α,8aβ-trimethyl-4,4a,8a-trihydronaphtho-([2,3b]-dihydrofuran-2-one)-8-one		Antimalarial activity	(Lategan *et al.,* 2009:94)
Saponins **Catechins** **Tannins, Leucoanthocyans and Mucilages**	-	-	(Noudogbessi *et al.,* 2013:8489-8492)
Palmitic acid ($C_{16:0}$), Linoleic acid ($C_{18:2\ (9,12)}$) Oleic acid ($C_{18:1}$)	  	Storage stability	(Noudogbessi *et al.,* 2013:8489-8492)

2.4 Biological activity

It was evident that ethanol extracts from both the rhizomes and the leaves of *S. aethiopicus* exhibited antibacterial and antifungal activities (Coopoosamy *et al.,* 2010:1230), contradicting as well as supporting the findings made by Lategan *et al.* (2009:96). It was, however, reported that the antimicrobial activities of the leaves are significantly lower than that of the rhizome extracts, but the leaves are still viable to use for the treatment of certain ailments. Thus, sustainable use of *S. aethiopicus* is ensured through conserving the rhizomes by means of plant part substitution, i.e. rhizome parts can be substituted by the leaves. Aqueous extracts of both the leaves and rhizomes were found to be non-effective against bacterial growth, but it was found to successfully inhibit the growth of fungal species (Coopoosamy *et al.,* 2010:1230).

The strains of bacteria as well as the specific compound or extract responsible for the biological activity can be observed in Table 2.2. Van Vuuren (2008:469) commented that even though a number of successful studies have proven that some plant species do indeed have *in vitro* antimicrobial activity, it is still necessary to subject these plants to animal models and human subjects in order to determine their efficacy in metabolic environments.

Table 2.2: Antibacterial activity of different *S. aethiopicus* parts and different strains of bacteria

Plant part	Strains of bacteria	Specific compound or extract	Reference
Rhizomes	*Mycobacterium aurum (M.aurum)*	Crude extract showed moderate activity	(Igoli *et al.,* 2012:88)
Rhizomes	*In vitro* activity tested against *Mycobacterium tuberculosis*, *Staphylococcus aureus*, *Klebsiella pneumoniae*	Three novel Furanoterpenoids showed no activity	(Lategan *et al.,* 2009:96)

Table 2.2: Antibacterial activity of different *S. aethiopicus* parts and different strains of bacteria (continued)

Plant part	Strains of bacteria	Specific compound or extract	Reference
Rhizomes and Leaves	*In vitro* activity tested against *Bacillus subtilis, Micrococcus kristinae, Bacillus cereus, Staphylococcus aereus, Staphylococcus epidermidis, Escherichia coli, Proteus vulgaris, Enterobacter aerogenes and Shigella sonnei*	Ethyl acetate and acetone extracts of leaves showed activity against *Bacillus subtilis, Micrococcus kristinae and Bacillus cereus* Ethyl acetate extract of the rhizomes showed activity against *Bacillus subtilis, Micrococcus kristinae, Bacillus cereus* and *Staphylococcus aereus* The acetone extracts of the rhizomes showed activity against *Bacillus subtilis, Micrococcus kristinae, Bacillus cereus, Staphylococcus aereus, Staphylococcus epidermidis, Escherichia coli* and *Proteus vulgaris,* Water extracts of leaves and rhizomes showed no activity against any strains	(Coopoosamy *et al.,* 2010:1229)

A study conducted by Motsei *et al.* (2003:239) showed that a water extract of *S. aethiopicus* had no significant anti-fungal activity (Table 2.3); however, the organic solvent extracts (i.e. ethanol, ethyl acetate and hexane) presented a definite anti-fungal activity against all three stains of *Candida albicans*. Thus, they concluded that *S. aethiopicus* contains anti-fungal compounds in the organic extracts (Motsei *et al.,* 2003:239).

Table 2.3: Antifungal activity of rhizomes and leaves of *S. aethiopicus*

Plant part	Fungal cultures	Specific compounds or extracts used in testing inhibition activity	Reference
Rhizome	*In vitro* testing against *Candida albicans*	Three novel Furanoterpenoids isolated showed no inhibitory activity	(Lategan *et al.,* 2009:96)
Rhizome and Leaves	*In vitro* testing against *Aspergillus flavus, Aspergillus glaucus, Candida albicans, Candida tropicalis, Trichophyton mentagrophytes* and *Trichophyton rubrum*	Ethanol and water extracts of leaves and rhizomes showed inhibitory activity	(Coopoosamy *et al.,* 2010:1230)
Roots and Rhizomes	*In vitro* testing against *Candida albicans*	Water extracts of leaves and rhizomes showed no significant anti-fungal activity	(Motsei *et al.,* 2003:239)
Roots and Rhizomes	*In vitro* testing against *Candida albicans*	Ethanol, ethyl acetate and hexane extracts of the leaves and rhizomes showed anti-fungal activity	(Motsei *et al.,* 2003:239)

Anti-trypanosomal activity against *Trypanosoma brucei brucei* was tested for the first time in 2012, where results obtained indicated that the crude extracts as well as the pure compounds, 8(17),12E-labdadiene-15,16-dial and furanodienone, showed significantly higher anti-trypanosomal activity compared to Suramin activity, which is used as the conventional anti-trypanosomal agent. This anti-parasitic effect could explain its traditional use as a febrifuge in treating sleeping sickness and malaria, which are both of parasitic origin and which are both major causes of fever in sub-Saharan Africa (Igoli *et al.,* 2012:88, 92).

The anti-malarial properties of *S. aethiopicus* have also been investigated *in vitro* and *in vivo* in a malaria mice-model. It was subsequently reported that *S. aethiopicus* contains anti-plasmodial compounds. The bioassay guided fractionation which was performed, led the researchers to isolate three novel furanoterpenoids (Table 2.4) (Lategan *et al.,* 2009:92). They also subjected the isolated compounds to further *in vitro* testing against several strains of bacterium and fungi, but no activity was established; suggesting that further bioassay

guided fractionation might reveal more active compounds that may be responsible for these activities as the approach that they used was not focused on antibacterial or antifungal activity, but was mainly focused on anti-plasmodial activity (Lategan *et al.,* 2009:96).

Table 2.4: Anti-parasitic activity of *S. aethiopicus* rhizomes

Plant part	Strains	Specific compound or extract	Reference
Rhizomes	*Trypanosoma brucei brucei*	Crude extract as well as pure compounds 8(17),12E-labdadiene-15,16-dial and Furanodienone showed activity	(Igoli *et al.,* 2012:88,92)
Rhizomes	*Plasmodium falciparum*	Three novel Furanoterpenoids and ethyl acetate extract showed activity	(Lategan *et al.,* 2009:92)

In a study conducted by Fouche *et al.* (2011:843), the potential anti-inflammatory and anti-allergic properties of *S. aethiopicus* were investigated *in vitro*; and the efficacy was tested in a mouse model for allergic asthma. The results from the biological assaying of the plant extracts and the isolated furanoterpenoid showed significant *in vitro* inhibition of glucocorticoid and histamine H_1 receptor binding; as well as inhibition of phosphodiesterase IV activity, which supports the possible anti-inflammatory, anti-allergic and bronchodilatory effects. Table 2.5 shows the results that have been found.

Table 2.5: Anti-inflammatory activity of *S. aethiopicus* using the COX-1 inhibition assay, anti-asthmatic and anti-allergic activity

Plant part used	Specific compound or extract	Reference
Roots and Rhizomes	Furanoterpenoid and plant extract showed anti-asthmatic and anti-allergic activity	(Fouche *et al.,* 2011:846)
Roots and Rhizomes	The furanoterpenoid showed anti-inflammatory activity	(Fouche *et al.,* 2011:846)
Stem and leaves	Extracts of the leaves and stems of young and mature plants showed the best anti-inflammatory activity when compared to the roots and rhizomes	(Zschocke *et al.,* 2000:288)
Roots and Rhizomes	The mature rhizomes exhibited higher anti-inflammatory activity than smaller and younger plants	(Zschocke *et al.,* 2000:288)

Administration of *S. aethiopicus* extracts to ovalbumin (OVA)-sensitised and challenged mice, significantly reduced lung inflammation and the percentage eosinophils in bronchoalveolar lavage fluid, but did not influence airway hyper reactivity. However, it could not be dismissed that a longer course of treatment, or even a higher dose, may potentially have an effect on the airway hyper-reactivity (Fouche *et al.,* 2011:847). Results acquired from this study demonstrated that cineole produced anti-inflammatory and anti-nociceptive effects. The mechanism through which cineole exerts its anti-inflammatory action is, nonetheless, still not clear. Moreover, only systemic administration provokes an anti-inflammatory effect. Furthermore, cineole was able to potentiate pentobarbital sleeping time in mice indicating a potential depressant action on the central nervous system. Furthermore, the formalin test is considered a valid model for clinical pain; and in this model cineole effectively inhibited the licking response in both early and late phases in a manner similar to morphine (Santos and Roo, 2000:243).

Stafford *et al.* (2005:112-113), on the other hand, tested the effects of storage on the biological activity of *S. aethiopicus* plant extracts. They found that after storing the water extracts for 90 days, the cyclooxygenase (COX-1) inhibition activity decreased. Contrary to this, it was found that the ethanol extracts depicted an increase in the COX-1 activity after the 90 day storage period, thereby increasing the percentage COX-1 inhibition from 86% to 93%. They concluded that there must be different active compounds in the water and ethanol extracts, which are responsible for the anti-inflammatory activity (Stafford *et al.,* 2005:113). The antibacterial activity of the ethanol extracts from *S. aethiopicus* also increased after storage for 90 days; this may be due to the stability of the fatty acids found in *S. aethiopicus* (Fennel *et al.,* 2004b:119).

2.5 Toxicity and cytotoxicity

Plants commonly used as traditional medicines are usually assumed to be safe. This safety is based on their long usage in the treatment of diseases according to knowledge accumulated over centuries by traditional healers and scientists. However, recent scientific research has shown that many plants used as food or in traditional medicines are potentially toxic, mutagenic and carcinogenic (Fennel *et al.,* 2004a:212). It was found using the comet assay that *S. aethiopicus* can cause DNA damage (Taylor *et al.,* 2003:144); this raised some concerns for the use of this plant as a medicine against several ailments. It was further evident that several plants used in South Africa as traditional medicines may cause damage to genetic material and therefore have the potential to cause long-term damage in patients when administered as medicinal preparations. It was also expressed by Fennel *et al.* (2004a) that prescription of this plant for the treatment of ailments should therefore be

considered with caution, and rigorous toxicological and clinical studies are necessary before they are widely prescribed as traditional medicine (Fennel *et al.,* 2004a:212; Street *et al.,* 2013:9).

It is important that the correct part of the plant is used, since a specific part may be toxic whilst another may have no harmful effect due to a difference in the concentration of the chemical components in different parts of the plant. The roots of *S. aethiopicus* are used in 57% of cases to prepare a remedy, whereas leaves are only used in 11% (Steenkamp, 2003:106). Steenkamp *et al.* (2005:40) conducted a study in 2005 to establish what the antioxidant and genotoxic properties of *S. aethiopicus* are; and the results indicated that *S. aethiopicus* methanol extracts depicted a higher ability to scavenge hydroxyl radicals when compared to the water extract. They found that the plant lacked anti-oxidant capacity, which might explain signs of toxicity, but the water extracts of *S. aethiopicus* showed substantial pro-oxidant capacities through its potentiation of the cellular membrane lipid peroxidation. Furthermore, it was suggested by Steenkamp *et al.* (2005:40) that the DNA damage induction could be one of the mechanisms for the observed antibacterial activity in the methanol and water extracts of this plant. In a different study by Igoli *et al.* (2012:88,92), cytotoxic studies in various cell lines where they used crude extracts as well as labdines, both showed specific cytotoxicity. This is an indication of possible anticancer potency; nonetheless further investigation is required to determine its possible use as an anti-cancer agent.

2.6 Commercialisation and conservation

Extensive research has revealed that medicinal plants and remedies have been used for centuries and that numerous cultures and people from rural areas still rely on these indigenous medicinal plants for their primary health care needs (Mulholland & Drewes, 2004:775; Steenkamp, 2003:97; Street *et al.,* 2013:1). Medicinal plants are now universally recognised as the basis for a number of critical human health, social and economic support systems and benefits. It was estimated that up to 700 000 tonnes of plant material is consumed annually to the value of about 150 million US dollars (Street *et al.,* 2013:1). It was reported that biologically active natural products from plants and their derivatives contributed up to 57% of the top selling prescription drugs in the US in 1997, and there seems to be a growing interest in the use of these natural products for the design and synthesis of new clinically useful agents since. Natural plant-based remedies are now being used as a major source in the discovery of new and approved drugs for the development of new commercial products (Igoli *et al.,* 2012:88; Street *et al.,* 2013:1).

Wild ginger is recorded as one of the most traded species in South Africa and was identified as one of the species with a high potential for commercial production. It is currently partially commercialised (Street *et al.,* 2013:9; van Wyk, 2011a:817). Several products containing *S. aethiopicus* as an active ingredient are commercially available (Figure 2.3), namely: Nature's health products African Ginger® capsules (Figure 2.3.A); Phyto nova African Ginger® tablets (Figure 2.3.B); Hot Toddy® effervescent tablets containing African ginger (Figure 2.3.C); Healing Earth products including body polish, butter, essential oil, body and bath oil and cream (Figure 2.3.D); Bioharmony bio-African ginger® tablets (Figure 2.3.E); Big Tree African ginger® tablets (Figure 2.3.F); and Hot Toddy® sachets containing African ginger (Figure 2.3.G).

Figure 2.3: A) Nature's health products African ginger® capsules, B) Phyto nova African ginger® tablets, 2C) Hot toddy® effervescent tablets containing African ginger, D) Healing Earth products including body polish, butter, essential oil, body and bath oil and cream, E) Bioharmony bio-African® ginger tablets, F) Big Tree® African ginger tablets, G) Hot toddy® sachets containing African ginger

S. aethiopicus is often referred to as locally extinct in some parts of South Africa as a result of over-exploitation, as this is a plant that is in high demand and short in supply (Mander *et*

al., 1998:2; Mulholland & Drewes, 2004:769; Steenkamp, 2003:97; Street *et al.,* 2013:9). In a study by Williams *et al.* (2013:24), the potential extinction risks posed to plants, including those threatened by harvesting for medicinal plant trade, were assessed by means of quantitative criteria, to determine their red list status. They listed *S. aethiopicus* as critically endangered. In a study by Mander (1998:47), it was estimated that 1.9 tonnes of African ginger, totalling a number of 52 000 plants are traded annually in KwaZulu-Natal only; and this would explain why it has become locally extinct (Street *et al.,* 2013:9). The need for genetic conservation of medicinal plants is thus of immense importance as more of the wild populations are now facing extinction. The existing circumstances necessitate an alternative supply of medicinal plant material to meet the high demand. For most plant species, the only option left is cultivation on a large scale to maintain wild species, but even with cultivation there are some setbacks as it was found in a study by Makhuvha *et al.* (1997:343) that extreme genetic diversity occurred within the natural population of *S. aethiopicus* making it more difficult to just cultivate randomly. Contradicting studies by van Wyk (2008:350) revealed that *S. aethiopicus* is easy to propagate and cultivate; and there is evidence that it was successfully cultivated in the warm parts of South Africa (Street *et al.,* 2013:9). Cultivation of *S. aethiopicus* should be a financially viable operation since there is always a high demand for this plant; and the income generated by traders justifies the effort. In a study by Mander (1998:88), it was estimated that street traders obtain around R 140/ kg while the shops and healers obtain a price of up to R 450/ kg (Mander, 1998:88; Street *et al.,* 2013:9) in the trading of wild ginger.

In another attempt to conserve *S. aethiopicus*, Zschocke *et al.* (2000:285) conducted a study where they determined the chemical composition of different plant parts of *S. aethiopicus* and during this study is was clear from the results that the chemical composition of the roots and rhizomes of this plant is similar, whereas the chemical composition of the leaves and stems is only fairly similar. They then went further to test the COX-1 activity and found that all of the plant parts exhibited COX-1 inhibitory activity. They did, however, report that the mature rhizomes have a significant higher degree of activity than that of the other plant parts tested. These results, and the fact that higher concentrations of active compounds are found in the stored plant parts, may shed some light on why the rhizomes are favoured by traditional healers (Zschocke *et al.,* 2000:288). In a similar study conducted by Lindsey *et al.* (1999:12), it was found that the leaves and stems of *S. aethiopicus* depicted a higher COX-1 inhibitory activity compared to both the roots and rhizomes, thus it was suggested that the leaves and the stems should be the preferred plant parts to use as treatment for people if an anti-inflammatory effect is required. The efforts made through these studies in effect contributed to the conservation of *S. aethiopicus* as more people can now be informed that

the leaves and stems will still deliver an effective treatment in the cases of certain ailments (Lindsey *et al.,* 1999:12; Zschocke *et al.,* 2000:288).

2.7 Pre-clinical discovery and development of new drug compounds

The pre-clinical discovery and development of a new pharmacologically active drug is very important to the pharmaceutical industry. Screening methods are improving constantly and this sector is rapidly expanding. There are mainly three phases in which new potential drugs are discovered and developed; i.e. the first phase being the discovery of the biologically active compound and the testing of this compound using *in vitro* and *in vivo* based models. During the second phase, the preclinical development, the different formulations are prepared and tested to determine all relevant pharmacokinetic and physiochemical properties, as well as testing the formulation to ensure that the dose is not toxic and that the formulation withstands all of the requirements set by the British Pharmacopeia (BP). The final phase is the clinical development of the drug, where it will undergo several tests to ensure that it is safe to use in humans (Zhang *et al*., 2012:556). Figure 2.4 is a summary of the phases of new drug discovery.

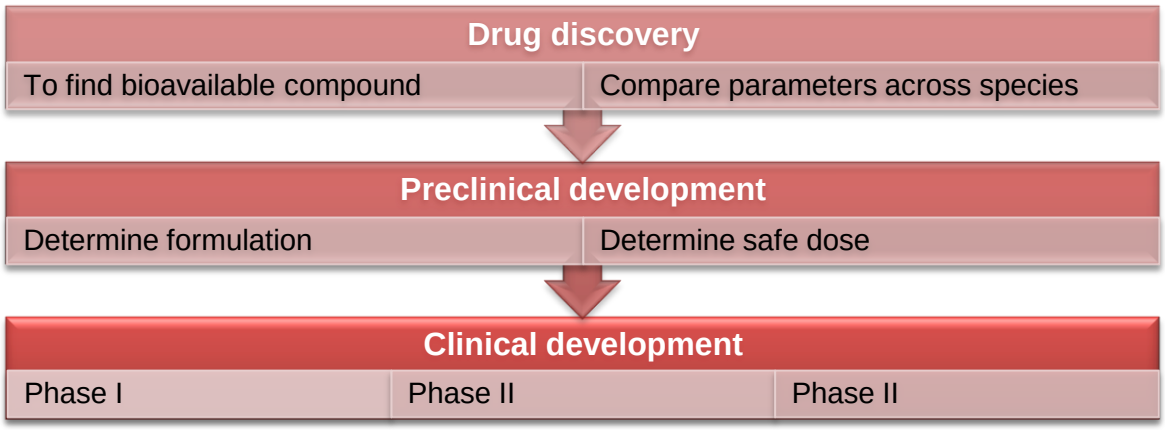


Figure 2.4: Phases of pre-clinical drug discovery and development adapted from (Zhang *et al*., 2012:556)

2.7.1 *Drug/herbal compound absorption*

Orally administered drugs are absorbed mainly from the gastro-intestinal tract, where most of the absorption usually occurs in both the duodenum and the small intestines as displayed in Figure 2.5 (Li., 2005:180). The oral cavity (Figure 2.6), which can be sub-divided into the buccal (inner lining of the cheeks) and sublingual (area beneath the tongue) cavities, is considered another possible site of absorption and have been used to achieve a rapid onset of action through either systemic or local action (Bhati & Nagrajan, 2012:659; Dodla &

Velmurugan, 2013:39). This rapid onset of action of the drug is achieved through its ability to bypass the first pass metabolism and the gastric enzymes of the gastrointestinal track when absorbed from the oral cavity (Bhati & Nagrajan, 2012:659).

To have sufficient knowledge of the absorption and metabolism of compounds is of great importance to the pharmaceutical industry, since the oral bioavailability of a drug is defined by the fraction of the oral dose that reaches the systemic circulation. It is a well-known fact that for the therapeutic effect to occur, the drug needs to become available at the site of action, in other words it needs to be bioavailable (Alqahtani *et al*., 2013:1; Le Ferrec, *et al*., 2001:650).

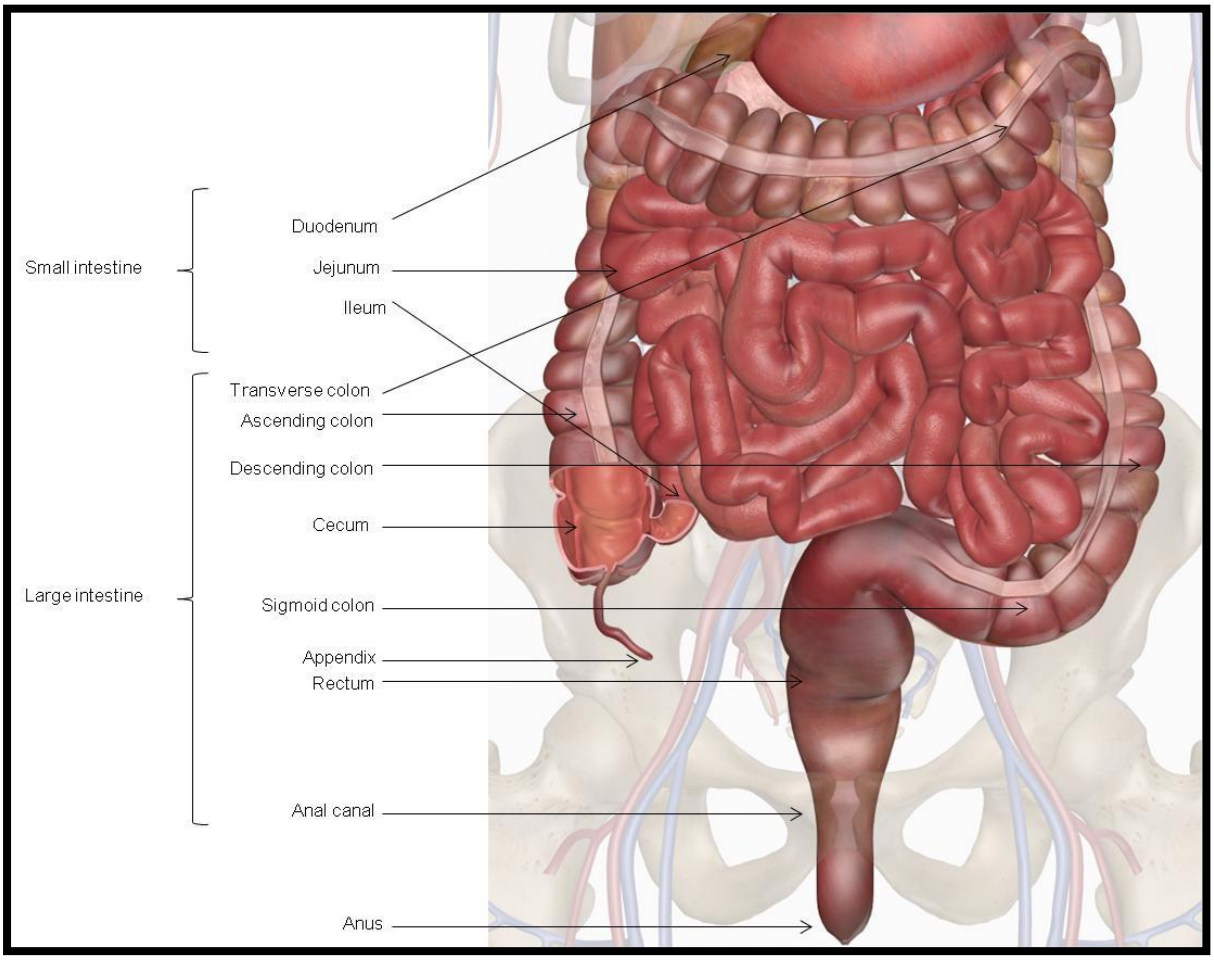


Figure 2.5: Human gastro-intestinal tract (http://www.innerbody.com/anatomy-images / intestines.png)

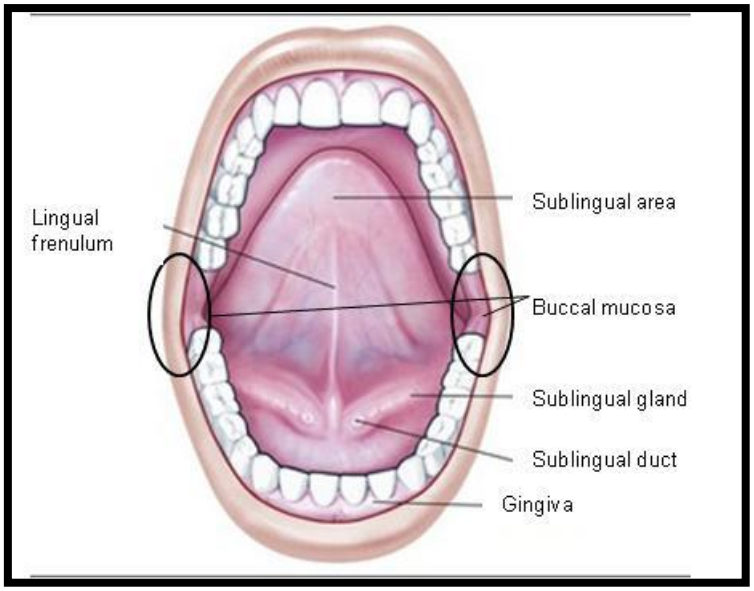


Figure 2.6: Human oral cavity; the buccal and sublingual areas was investigated during the in vitro permeability studies adapted from (http://www.cesarulla.com/wp-content/uploads/2017/01/diagram-of-the-oral-oral-cavity-definition-is-any-other-phrase-for-the-mouth-palate-is-the-roof-of-the-mouth-in-human-beings-and-other-mammals.jpg)

2.7.1.1 Absorption mechanisms within the intestinal epithelial

Oral administration of drugs are the most popular dosage form due to the convenience for patients to swallow the dosage form and cost effectiveness of the production thereof (El-Kattan & Varma, 2012:1). As mentioned previously, most of the drugs that are being administered orally are absorbed in the duodenum and other sections of the small intestines (Li, 2005:180). Absorption of the drug is the key component to the bioavailability of the specific drug or drug compounds. This ensures the drug reaching the systemic circulation and thereby target specific organs. In the small intestines the presence of villi and microvilli ensures a large absorption surface area that will increase the amount of drug being absorbed; and thus directly influence the bioavailability. There is, however, a disadvantage of drugs being absorbed in the gastro-intestinal tract. Prior to reaching the systemic circulation, the drug will pass through the hepatic portal system and undergo first pass metabolism, thereby hindering the amount of active drug components reaching the target organ. Drug absorption in the small intestine is also influenced by multiple factors that can mainly be divided into three groups, namely: physiological factors, physiochemical factors and biopharmaceutical factors. Furthermore, the absorption of drugs will also be influenced by the efflux transporter mechanisms found in the intestinal epithelia (El-Kattan & Varma, 2012:1).

After oral administration, drug molecules can cross the luminal membrane using various mechanisms involving passive diffusion or active transport. Passive diffusion is divided into two pathways; transcellular, where the molecules are required to move through the lipid membrane of the cell, and paracellular where the molecules diffuse through aqueous pores and tight junctions between the intestinal cells. Active transport on the other hand, requires transporter proteins and is divided into active influx and efflux transport (El-Kattan & Varma, 2012:3). The flow diagram in Figure 2.7 illustrates the major pathways of absorption as well as the efflux of compounds from the gastro-intestinal tract after oral administration (Li, 2005:180). The route of absorption followed by the drug will depend on the physiochemical properties of the drug as well as the affinity of the drug for certain transporter proteins (El-Kattan & Varma, 2012:3).

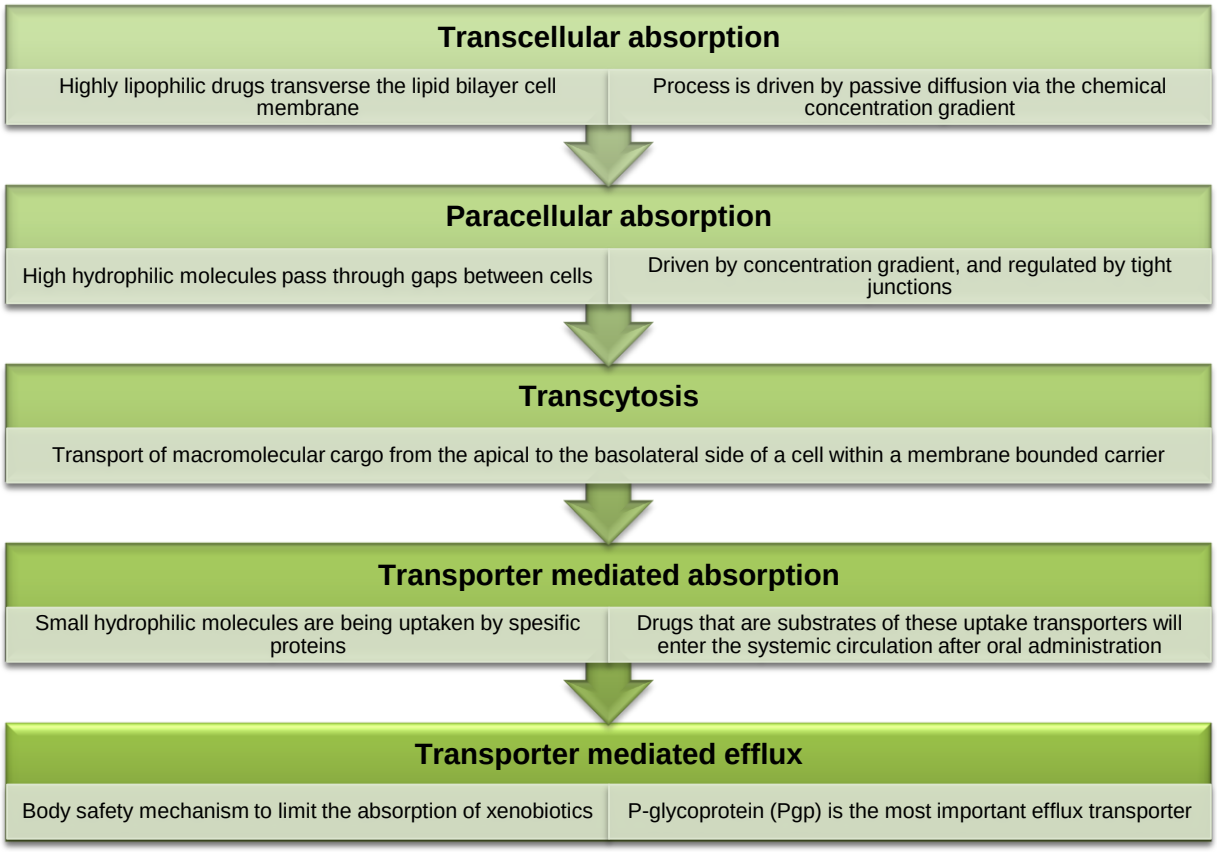


Figure 2.7: Flow diagram indicating all the possible mechanism of drug absorption (Li, 2005:180; Tuma & Hubbard., 2003:871)

There are four mechanisms by which drug components can be absorbed in the intestinal epithelia; the first being the transcellular absorption of drugs through passive diffusion. This route is mainly followed by drugs that are highly lipophilic, and can easily move through the lipid bilayer of the cell membrane. The second mechanism is the paracellular route of

absorption which is driven by concentration gradients and regulated by the opening and closing of tight junctions found in between the epithelia cells. Drugs that are highly hydrophilic will pass through these gaps located between the cells. The third route of absorption is through transcytosis that involves a membrane bound carrier that will transport the molecule from the apical to the basolateral side. Finally, the fourth route of absorption is transporter mediated absorption where small hydrophilic molecules are taken up by specific transporter proteins and moved through the cells by means of active transport (Li, 2005:180; Tuma & Hubbard., 2003:871). Figure 2.8 is a schematic representation of the different absorption pathways found across human intestinal epithelial cells.

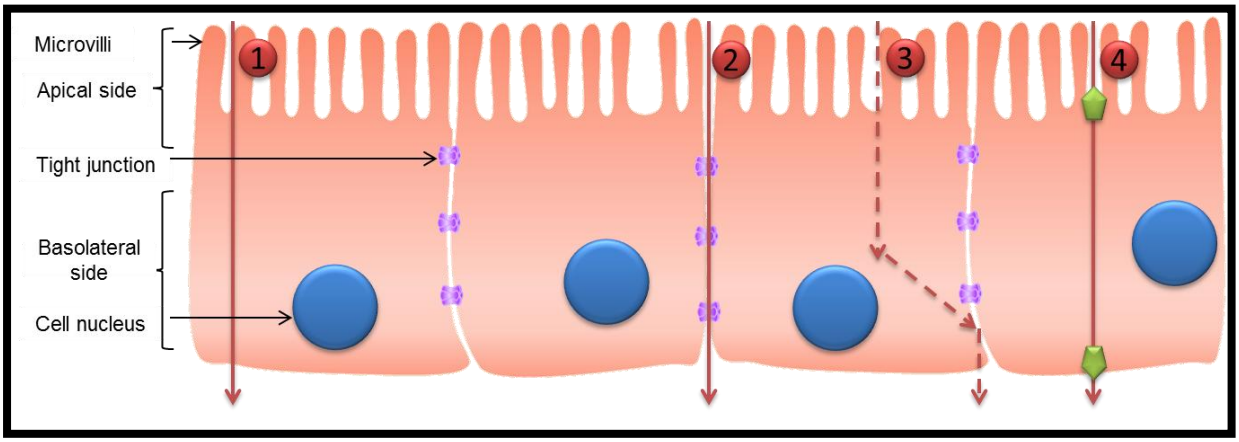


Figure 2.8: Schematic illustration of the Intestinal epithelia indicating 1) Transcellular absorption, 2) Paracellular absorption, 3) Transcytosis and 4) Transport mediated transcellular absorption adapted from (Alqahtani *et al*., 2013:3; Le Ferrec, *et al*., 2001:650)

2.7.1.2 Absorption mechanisms within the oral cavity

Absorption within the oral cavity has several advantages when compared to absorption in the gastro-intestinal tract; the major advantage being the ability of drugs to bypass the gastro-intestinal tract and hepatic portal system, thus not undergoing first pass metabolism, causing the bioavailability of the drug to potentially increase (Bhati & Nagrajan, 2012:659; Dodla & Velmurugan, 2013:39; Shojaei *et al*., 2001:70). Drugs used in oral formulations can either be used for sustained release or rapid onset of action, depending on the desired effect that is required, however, it has been reported that the buccal area does not show the rapid absorption seen in sublingual administration; and is therefore rather used during sustained release and not for rapid release as with sublingual formulations. The buccal and sublingual areas are both well vascularised and drugs are usually easily administered. Thus, patient compliance is enhanced compared to other routes of drug administration. As with all things, oral absorption also has a few disadvantages which include faster drug elimination due to

the constant flushing of the saliva, and subsequently more frequent dosages are required. Patient compliance is of extreme importance during administration; and patients might not appreciate the taste, or the colouring of their teeth that can be caused by a drug. Patients can also become irritated by the frequent dosage that is required to maintain a sustainable drug bioavailability in their system. Non-uniform drug distribution has also been reported in the use of this type of administration. Another key disadvantage is that the oral cavity is impermeable to large hydrophilic compounds and can only absorb pH-stable drugs that are absorbed through passive diffusion (Bhati & Nagrajan, 2012:659-660; Dodla & Velmurugan, 2013:39-40; Shojaei *et al*., 2001:70). The buccal mucosa consist of both hydrophilic and lipophilic regions and have a low permeability ability when compared to that of the sublingual area (Nicolazzo *et al*., 2003:2399; Shojaei *et al*., 2001:70). There are mainly two routes through which drugs are being absorbed through the oral cavity, i.e. paracellular for more hydrophilic compounds; and transcellular for lipophilic compounds (Bhati & Nagrajan, 2012:662; Nicolazzo *et al*., 2003:2399). Figure 2.9 illustrates the routes of absorption across the buccal mucous membrane. There are a few factors that could influence the absorption of drugs across buccal and sublingual mucosa. These factors include lipid solubility, degree of ionisation, the pKa value of the drug, pH of the drug solution, molecular weight and size of the drug, membrane characteristics, the presence of saliva, physicochemical properties of the formulation, and the presence or absence of permeation enhancers (Bhati & Nagrajan, 2012:663). Oral mucosae of pigs have been used in this study due to the similarities in the anatomy of the buccal and sublingual tissue when considering the non-keratinisation of oral epithelium (Lee *et al*., 2002:546).

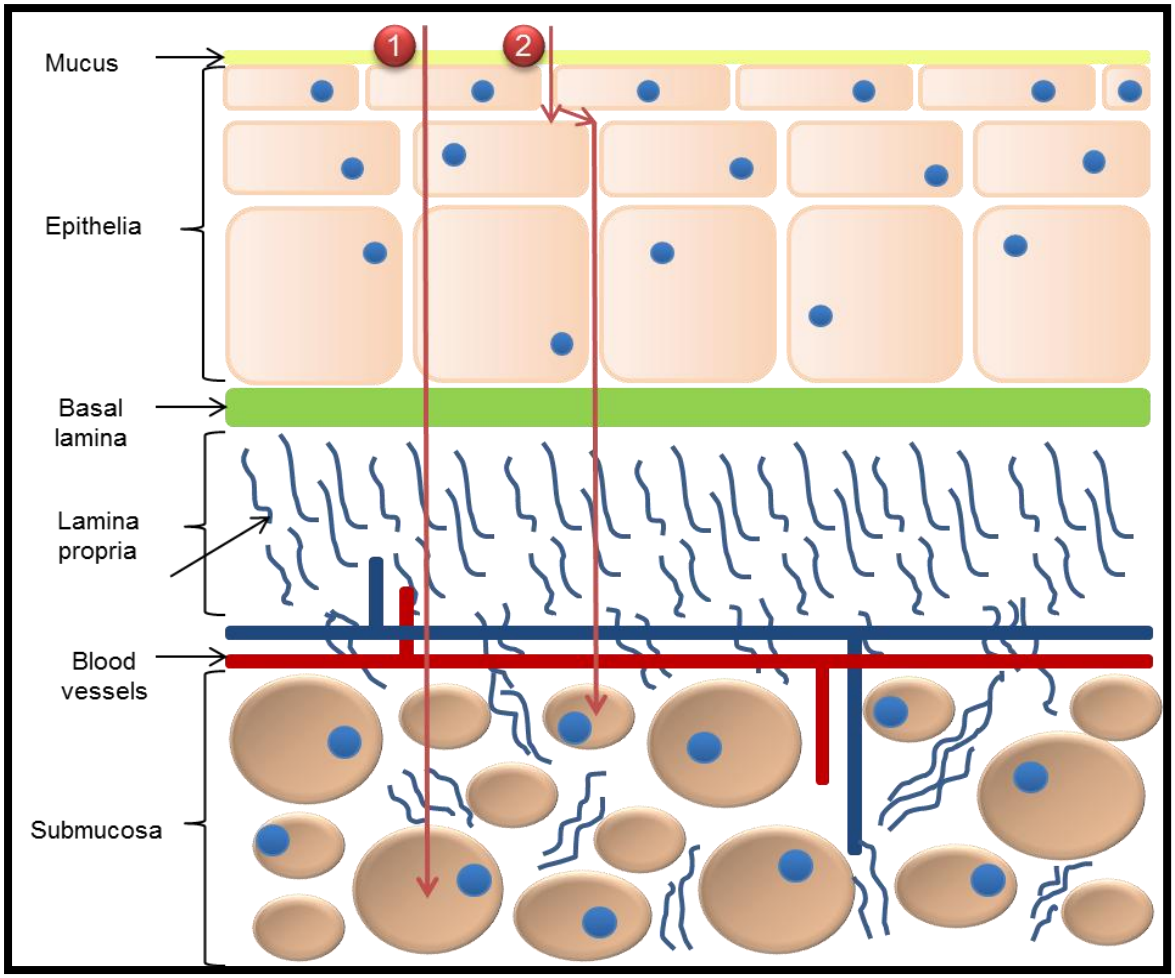


Figure 2.9: Schematic illustration of the Buccal mucus membrane indicating: 1) transcellular absorption through passive diffusion and 2) paracellular absorption through passive diffusion (Adapted from Bhati & Nagrajan, 2012:665)

2.8 Models to investigate membrane permeability

Several animal and cell culture models have been well established and are used to study the permeation of drugs across biological membranes (Alqahtani *et al*., 2013:1). The main reasons why scientists are constantly trying to find alternative methods and models to use in screening studies are to reduce the use of animal models, refine the experiments and/or to replace the use of animals with an alternative model that can provide the same viable results in the specific study (Le Ferrec, *et al*., 2001:649). Figure 2.10 displays a general overview of these models and in Table 2.7, the advantages and disadvantages of each model are listed. During this study the two *in vitro* models, namely Ussing type diffusion chambers containing excised animal tissues and cell cultures (Caco-2), were used and therefore these two models will be discussed in more detail.

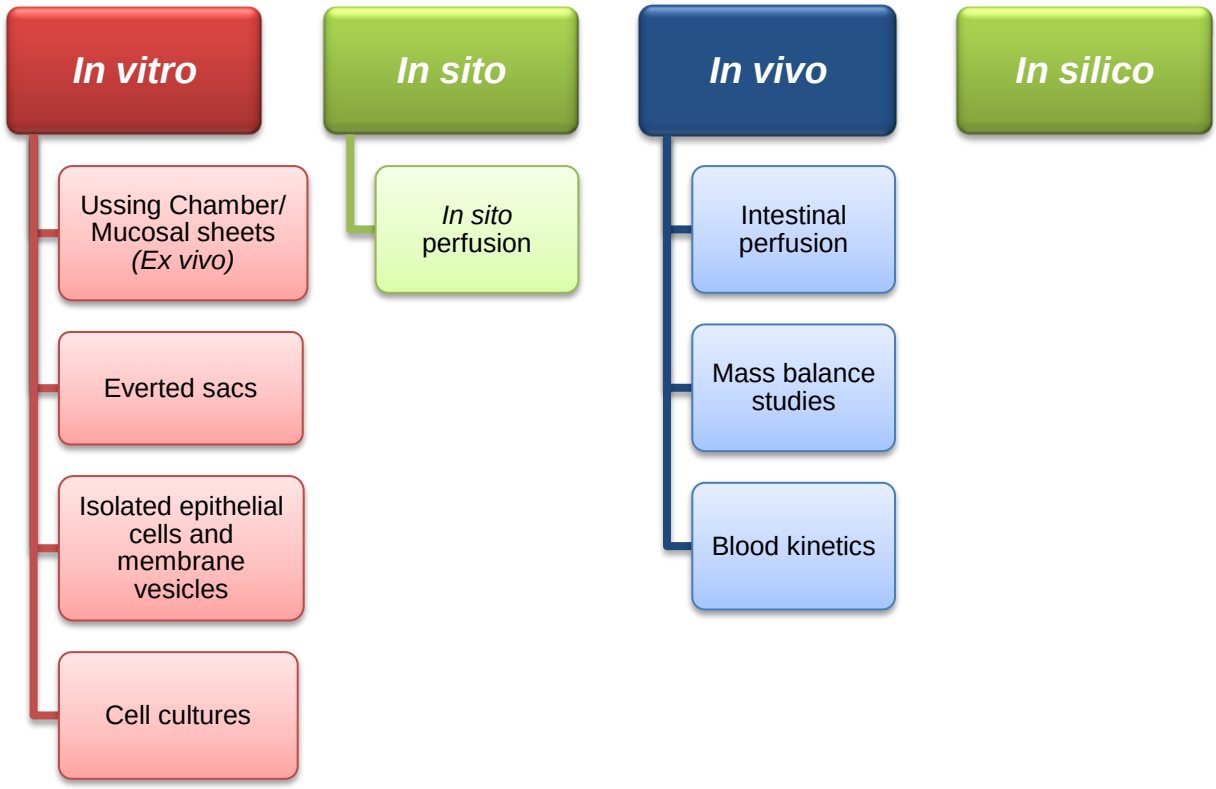


Figure 2.10: Schematic illustration indicating all available models to study permeation of molecules across the intestinal membrane (Adapted from Alqahtani *et al*., 2013:4)

2.8.1 *In vitro models for studying membrane permeability*

2.8.1.1 Ussing chamber/Mucosal sheets

The Ussing chamber is a popular technique used by scientist to study drug permeation across the epithelium of different sections of the intestine. This enables prediction of the intestinal absorption of a specific compound (Versantvoort *et al.,* 2000:25). The mechanism of absorption can also be studied using this technique and could be very useful for interspecies comparison. The effectiveness of the data is determined by how closely the model mimics the *in vivo* situation and thus the choice for a suitable animal model is critical. During this technique, excised tissue from an animal (e.g. rat, mouse, dog, pig, rabbit or monkey) or human is mounted between two diffusion cell compartments, exposing the apical and basolateral sides of the tissue to a liquid medium in the compartments. The compartments are filled with KRB in addition to carbon gas (95% O_2: 5% CO_2) to maintain the tissue viability. The test compound can then be added on either side of the membrane depending on the results required for the study. If a compound is applied to the mucosal side (apical), absorptive permeation can be studied, whereas if the compound is applied to

the basolateral compartment, secretory permeation can be observed (Alqahtani *et al*., 2013:3; Le Ferrec *et al*., 2001:654).

This technique is relatively quick and delivers reproducible intestinal absorption data. As with all other *ex vivo* techniques, the major setback of using the Ussing chamber is that the tissue used in the experiment should be fresh due to the relatively rapid decrease in the viability of the tissue (Versantvoort *et al.,* 2000:25-27). However, integrity of the membrane could be monitored using a non-absorbable compound simultaneously during the experiment. This is also a simple, cost effective technique (Versantvoort *et al.,* 2000:27).

2.8.1.2 Everted sac

The everted sac system offers a simple and inexpensive technique to investigate drug membrane permeation without specialised equipment. It can provide information on the mechanism of absorption and affords to investigate differences in absorption of compounds along the length of the gastro intestinal tract and on interspecies comparison. Everted sacs are prepared from freshly isolated animal intestine. After isolation the sacs are rinsed with solutions to clean the sacs, everted with a glass rod, and then filled with a pre-oxygenated medium. The everted sacs are usually 2-4 cm in length. They are then submerged in medium containing the drug or compound of interest for a desired time. The drug concentration is measured inside each sac (Alqahtani *et al*., 2013:4,5; Le Ferrec *et al*., 2001:654). Similarly to the use of mucosal sheets, fresh intestinal tissues should be used. Another major disadvantage is that the serosal compartment is a closed compartment with a small volume. Therefore, the concentration of compounds can rapidly increase inside the sac and back flux of the compound will occur due to a concentration gradient that occurs. This will distort the kinetics of rapidly absorbed compounds. Furthermore, many sacs are needed to obtain information on the kinetics of compounds since for each time point a sac is required (Versantvoort *et al.,* 2000:28).

2.8.1.3 Membrane vesicles

Membrane vesicles can be prepared from either intestinal tissue, for example, the liver, kidney and intestines; or from transfected cell lines, e.g. the MDCK and LLC-PK1 cell lines. These tissues have high levels of expression for certain transporters (Zhang *et al*., 2012:554). No specialised equipment is needed for this technique. Position specific transport and interspecies comparison can easily be studied. Viability of the tissue is prolonged and the tissue can still be used after several hours for the preparations of membrane vesicles compared to the use of mucosal sheets in Ussing chambers and everted

intestinal sacs that are only viable for a short amount of time before the membrane integrity is lost. Carrier mediated transport processes can be studied in detail by using membrane vesicles, since the apical and basolateral membranes are separated from each other. However, the consequences are that the use of membrane vesicles is restricted to carrier mediated transport processes only; and that this technique is not suitable to estimate the intestinal absorption of compounds *in vivo* (Versantvoort *et al.,* 2000:30).

2.8.1.4 Cell cultures

2.8.1.4.1 Caco-2 (Human colon adenocarcinoma cells)

There are various cell culture models as summarised in Table 2.6. This study will only use Caco-2 cells. Mammalina cell cultures, especially Caco-2 monolayers, have proven their value in various types of transport studies of compounds. Caco-2 cells are usually cultured for 21-24 days as a monolayer on a semi-permeable membrane at specified conditions (37°C, 5% CO_2: 95% O_2) prior to performing the experiment. When Caco-2 cell monolayers are confluent, they display similar morphological and functional characteristics as the intestinal epithelium enterocytes. The test solution or compounds of interest can be applied on either the apical or basolateral side of the monolayer depending on desired study results. Once the method has been established in a cell culture laboratory, experiments are easy to perform, relatively inexpensive, rapid to perform, and very reproducible compared to other absorption models. A pitfall may be that the expression level and substrate specificity of the carriers and efflux systems, as well as the metabolising enzymes found in Caco-2 monolayers, tend to be variable in comparison to the *in vivo* situation (Alqahtani *et al*., 2013:2,3; Le Ferrec *et al*., 2001:655,662; Versantvoort *et al.,* 2000:34).

2.8.2 *In situ perfusion*

The *in situ* perfusion method represents the closest model when compared to the *in vivo* situation as the lymphatic and blood circulation systems are still intact. Part of the intestine is exposed to a drug solution that is perfused through the lumen. The disappearance of drug molecules from the perfusate is then used as an indication of drug absorption. Perfusion experiments can be used for kinetic studies as well as regional absorption studies without the hepatic and renal influences. Furthermore, the effects of drug absorption enhancers and absorption mechanisms can be observed. It is also used to study carrier mediated transport, efflux transport and CYP450 metabolism (Alqahtani *et al*., 2013:5; Le Ferrec *et al*., 2001:653; Li, 2005:181; Versantvoort *et al.,* 2000:36).

2.8.3 *In vivo techniques*

In vivo techniques are widely applied and provide combined information regarding the absorption, distribution, metabolism and excretion of drugs. This method also provides valuable information with regards to the bioavailability of a certain drug or drug compounds (Zhang *et al*., 2012:555). Information regarding the pharmacokinetic parameters as well as toxicology is also available when using *in vivo* techniques. The basic principle involves the administration of a drug to an animal model; withdrawing samples on selected time intervals; and determining the drug concentration at those specific time intervals. From the results the bioavailability of the drug can be determined. There are several animal models available for these studies including; pigs, dogs, monkeys and rats, depending on the system that is studied. This technique resembles the *in vivo* situation the best, but is time consuming and significantly expensive. The main advantage of this technique, however. is the ability to study drug transport and metabolism within an intact system; and thus the results obtained from this study closely resembles what could be expected in humans. Still, there are anatomical and physiological differences between humans and animal models that cause pronounced difficulty when data is extrapolated from animals to humans. Another huge limitation includes the labour intensive studying of individual mechanisms that will require a large amount of resources (Alqahtani *et al*., 2013:5-6; Versantvoort *et al.,* 2000:41).

2.8.4 *In silico*

In silico is a computer program based approach, and there has been a rapid increase in the use of *in silico* methods to study the CYP's, phase II detoxification enzymes, P-gp and the interactions that they have with xenobiotics and herbal drug compounds. The continues development of improved technology and programming has led to an advantage in the prediction of intestinal drug absorption based on the physicochemical properties of the studied drug or dug compound (Spalding *et al*., 2000:70; Stenberg *et al*., 1999:234; Zhou *et al*., 2007:669). This method provides a high throughput screening of several new compounds that are still in the early phases of the drug discovery process, and is a much more cost effective way to predict drug permeability. Some of the more important advantages of this method are that no animal models are required, and no experiments need to be performed, thus making this the most time effective manner in preliminary drug discovery. This method also provides fast results without the need of a physical sample (Spalding *et al*., 2000:70-72).

Table 2.6: The advantages and disadvantages of different drug permeation models

Technique		Advantage	Disadvantage	References
In vitro	**Ussing Chamber/Mucosal sheets (*Ex vivo*)**	• Useful for interspecies comparison • Fast and delivers reproducible data • Simple and cost effective • Good screening model • Comparison of data with these of the *in vivo* situation indicates a good correlation	• Tissue used should be fresh, the tissue is only viable for two to three hours after isolation thereafter the integrity of the intestinal cells will decrease over time • Difficult to use human material	(Alqahtani *et al*., 2013:4; Deferme *et al*., 8:189; Le Ferrec *et al*., 2001:654; Versantvoort *et al*., 2000:25-27)
	Everted sacs (*Ex vivo)*	• Simple, and inexpensive • No need for specialised equipment • High reliability and reproducibility	• Tissue used should be fresh • Difficult to use human material • Small closed serosal compartment • Back flux of compounds occur • Back flux of compounds distort the kinetic data • Many sacs are needed • Presence of the muscularis mucosa, and therefore this model does not reflect the actual intestinal barrier	(Alqahtani *et al*., 2013:5; Le Ferrec *et al*., 2001:654; Versantvoort *et al*., 2000:28)
	Isolated epithelial cells and membrane vesicles			
	Isolated intestinal cells	• Animal or human origin • When method is established relatively easy to perform	• Not yet widely applied because of limitations • Limited viability of the primary cells • Radiolabeled or fluorescent compounds are needed • Differences in preparation of cells causes noteworthy intervariability between cells	(Deferme *et al*., 8:188; Versantvoort *et al*., 2000:29)

	Membrane vesicles	• Less strict regulations on the use of fresh tissue • Human and animal tissue can be used • Apical and basolateral membranes are separate from one another • Useful for studies regarding mechanisms	• Restricted to carrier mediated transport • Non-specific binding • Can only predict a part of the absorption process • Not suitable for intestinal absorption of compounds *in vivo*	(Deferme *et al*., 8:188; Versantvoort et al., 2000:30)
	Cell cultures			
	Caco-2 (Human colon adenocarcinoma cells)	• Experiments are easy to perform • Relatively cheap and rapid • Very reproducible when compared to other absorption models • Most popular model being used • Derived from human cells	• Expression level and substrate specificity of the carriers and efflux systems as well as the metabolising enzymes tend to vary in comparison to the *in vivo* situation, therefore the interpretation of data proof to be difficult • Low expression of CYP3A • Physiological factors contributing to transport is absent • Cells have a tumoral origin and is a static model • Model contains only one cell type • The influence of P-gp is difficult to establish	(Alqahtani *et al*., 2013:2,3; Le Ferrec *et al*., 2001:655,662; Versantvoort *et al*., 2000:34)
	MDCK (Dog kidney epithelial cells)	• Polarised cells with low intrinsic expression of transporters • Do not need three weeks of culturing before they can be used but only 3-5 days • Fast and simple method	• Not an intestinal model • It is derived from canine cells, thus an animal model	(Alqahtani *et al*., 2013:3; Deferme *et al*., 8:193; Irvine *et al*., 1998:28; Le Ferrec *et al*., 2001:655,662)
	LLC-PK1 (Pig kidney epithelial cells)	• Cell line used to study binding, endocytosis and transcellular transport of proteins and protein bound molecules in the proximal tubule kidney cells • Expresses the multiligand receptor megalin • Stability over a number of passages	• Not an intestinal model	(Nielsen *et al*., 1998:1767)

	2/4/A1 *(Rat fetal intestinal epithelial cells)*	• Mimics the permeability of the human small intestine • Used to study passive drug transport • Paracellular permeability similar to that found in the human small intestine • Only cultured for 3- 8 days before transport studies can be performed • Appear more *in vivo* like than Caco-2 cells • Experimental time is shorter than Caco-2 transport experiments	• Temperature sensitive • Less and underdeveloped microvilli when compared to *in vivo* • Efflux systems not functional or expressed	(Tavelin *et al*., 1999:1212)
	TC7 *(Caco-2 sub clone)*	• Express CYP3A • Grows faster than Caco-2 cells • Needs less glucose that Caco-2 cells		(Le Ferrec *et al*., 2001:662)
	HT29 *(Human colon)*	• Exhibit mucus secretary properties		(Deferme *et al*., 8:188; Le Ferrec *et al*., 2001:662)
	PAMPA (parallel membrane permeability assay)	• High throughput • Not too expensive • Different lipid compositions readily available • Predictability is good	• Can only predict a part of the absorption process • Membranes tend to retain some of the lipophilic compounds • pH and lipid composition dependant	
In situ	***In Situ* perfusion** *(Rat small intestine/ Human intestine)*	• Stomach can be bypassed • Asses formulation independent breakdown in the stomach under acidic conditions • Mesenteric blood flow is intact • Moving the sampling location from the portal vein to the hepatic vein can provide further information regarding the liver first pass effect	• Need to use live animals • Time and labour intensive experiments • Anaesthesia can have severe effects on the intestinal drug absorption • The disappearance of drugs from the perfusate does not always equate with absorption • Not practical for high throughput screening	(Alqahtani *et al*., 2013:5; Le Ferrec *et al*., 2001:653; Li., 2005:181; Versantvoort *et al*., 2000:29)
In vivo	**Intestinal perfusion**	• In combination with blood studies, the absorption as well as the first pass	• Too complex to use as a routine study • High perfusion rates	(Versantvoort *et al*., 2000:38)

		metabolism through the liver and gut can be studied • Delivers good permeability values • Good correlation between permeability and absorption values • Valuable pharmacokinetic data		
	Mass balance studies	• Non-invasive method, that can be used on humans and animals • Used to study the bioavailability of a certain compound	• Difficult to collect urine and faeces separately • Special housing of animals can cause animal distress that will affect the absorption and bioavailability of the drug tested • Time consuming • Studies are expensive due to hospitalisation of the test subjects or animals	(Versantvoort *et al*., 2000:39)
	Blood kinetics	• Used to determine oral bioavailability	• Analytical methods used to detect compound • Long term blood sampling of humans or animals • Possible toxic effects from compound, so cannot always be tested in human subjects • Expensive	(Versantvoort *et al*., 2000:40,41)
In silico		• No animals required • Prediction of intestinal absorption before synthesis of compound	• Difficult to present active processes • Difficult to present complex interplay of all the contributing factors	(Deferme *et al*., 8:188)

2.9 Summary

The botanical classification of *S. aethiopicus* is well established. All of the traditional uses of this plant as well as the exact location where it naturally occurs, is however still unclear. Several studies have attempted to elucidate the phytochemistry and biological activity of *S. aethiopicus*, but it still remains incomplete. The plant is partially conserved in botanical areas, and even though it is locally extinct and listed as critically endangered, the trade demand of *S. aethiopicus* remains high. Moreover, several commercial products containing *S. aethiopicus* have become available, despite the lack of scientific evidence regarding the safety and efficacy of the plant material.

During this study extensive characterisation of the plant material was performed, utilising several analytical techniques in order to establish a chemical fingerprint for *S. aethiopicus*. No biological activity is possible without the active components becoming bioavailable. Therefore, several *in vitro* techniques were investigated and employed to study the transport of selected marker molecules in an effort to clarify whether absorption of the marker molecules occur, and where they are being absorbed. The following two chapters deal with the characterisation and transport results obtained.

CHAPTER 3 – CHEMICAL PROFILING OF *SIPHONOCHILUS AETHIOPICUS*

3.1 Introduction

The standardisation and quality control of herbal drugs is rather complex due to the inherent complexity and rampant variation of herbal raw materials and formulated commercial products. Often, phytochemical variation is found within the same plant, between different parts of the same plant or may be due to chemotypic variation, within and between natural populations. The variability may also be introduced due to harvest techniques and post-harvest handling of the plant material. Herbal drugs are composed of a complex suite of chemical compounds; including active, inactive and unknown compounds which may be dietary rather than therapeutic. Therefore, methodologies that produce a chemical fingerprint for herbal extracts in a large collection of samples are preferable (Seasotiya *et al*., 2014:605). The chemical characterisation of plant material can reveal important detail regarding the active constituents responsible for pharmacological effects observed in the medicinal plant material consumed.

Several techniques were used to investigate the chemical composition of *S. aethiopicus*. Since there is no chemical fingerprint available for this ethnomedicinal plant, high performance thin layer chromatography (HPTLC) was employed in order to create a chemical fingerprint for *S. aethiopicus*. A major advantage of the HPTLC analysis is that it facilitates repeated detection of chromatograms with the same or different parameters, small quantities of the sample is needed and it enables the analysis of several samples simultaneously (Seasotiya *et al*., 2014:605). The fingerprints produced through this technique can be used for identification, authentication and characterisation of *S. aethiopicus*. Analysis of different plant samples was employed to detect any variation within the samples (wild versus cultivated). Ultra performance liquid chromatography coupled to a time of flight mass detector was used (UPLC-Q-TOF/MS) to generate a characteristic profile for *S. aethiopicus* extracts. Biomarkers were identified and selected based on their relative abundance in the methanol extract of *S. aethiopicus*. These newly identified compounds were fractionated and their structures identified using NMR through a collaborative initiative. For the first time, the volatiles from *S. aethiopicus* were investigated using the GCxGC-TOF/MS, this technique provides separation with higher separation ability than what is offered by conventional one-dimensional GC system. Separation is achieved by using two phases with different separation mechanisms; this offers access to different chemical compounds or classes present in the sample (Gao *et al*., 2011:1159).

3.2 Plant material and reagents

A total of 11 different *Siphonochilus aethiopicus* (African Ginger) rhizomes were studied. Nine of these samples were obtained from the local “muti” market in Potchefstroom and one sample was purchased from Kirstenbosch. A sample which was sourced from a cultivation site in KwaZulu-Natal was kindly donated by Prof. A. Viljoen from the Department of Pharmaceutical Sciences at the Tshwane University of Technology. Table 3.1 summarises all the relevant information and characteristics of the plant materials that were investigated during this study. Analytical grade methanol, ethanol, ethyl acetate, sulphuric acid and acetic acid were purchased from Merck (Darmstadt, Germany).

Quantitative and qualitative variation of biomarkers was observed between samples. Based on this observation upon these differences, a decision was made to continue the biopharmaceutical analyses with only one of the samples (NW 2); thereby ensuring that inter-individual variation was excluded as a variable during the biopharmaceutical part of this study. This sample was chosen due to the large amount of plant material that was commercially available in the North West Provence where the experiments were conducted.

Table 3.1: Information on the *Siphonochilus aethiopicus* plant materials investigated in this study

Sample name	Purchase location	Sample origin	Dry/ fresh	Wild/ cultivated	Powder/ Course material	Combined/ individual rhizomes	Extract/ oil	Analysis performed on sample
North West 1 (NW1)	Potchefstroom muti market	KwaZulu-Natal	Dry	Wild	Powder	Combined rhizomes	MeOH extract and oil	Fingerprinting of oil using GC/MS HPTLC LCMS
North West 2 (NW2)	Potchefstroom muti market	KwaZulu-Natal	Dry	Wild	Powder	Combined rhizomes	MeOH extract, H_2O extract and oil	Fingerprinting of oil using GC/MS HPTLC LCMS Bidirectional transport
North West (Cultivated)	Bulk suppliers	Kirstenbosh	Dry	Cultivated	Powder	Combined rhizomes	MeOH extract	HPTLC
TUT (Cultivated)	Muti Futhi (KZN)	KwaZulu-Natal	Dry	Cultivated	Powder	Combined rhizomes	MeOH extract and H_2O extract	Isolated 4 pure compounds to use as biomarkers for *Siphonochilus aethiopicus in vitro* studies HPTLC LCMS
Fresh Cone 1 (FC1)	Potchefstroom muti market	KwaZulu-Natal	Fresh	Wild	Powder	Individual rhizome	MeOH extract	Headspace analysis using GC/GC/TOF HPTLC LCMS
Fresh Cone 2 (FC2)	Potchefstroom muti market	KwaZulu-Natal	Fresh	Wild	Powder	Individual rhizome	MeOH extract	Headspace analysis using GC/GC/TOF HPTLC LCMS

Table 3.1: Plant material information (Continuing)

Sample name	Location bought	Sample origin	Dry/ Fresh	Wild/ Cultivated	Powder/ Course	Combined rhizomes/ Individual	Extract/ Oil	Analysis performed on sample
Fresh Cone 3 (FC3)	Potchefstroom muti market	KwaZulu-Natal	Fresh	Wild	Powder	Individual rhizome	MeOH extract	Headspace analysis using GC/GC/TOF HPTLC LCMS
Fresh Cone 4 (FC4)	Potchefstroom muti market	KwaZulu-Natal	Fresh	Wild	Powder	Individual rhizome	MeOH extract	Headspace analysis using GC/GC/TOF HPTLC LCMS
Fresh Cone 5 (FC5)	Potchefstroom muti market	KwaZulu-Natal	Fresh	Wild	Powder	Individual rhizome	MeOH extract	Headspace analysis using GC/GC/TOF HPTLC LCMS
Fresh Cone 6 (FC6)	Potchefstroom muti market	KwaZulu-Natal	Fresh	Wild	Powder	Individual rhizome	MeOH extract	Headspace analysis using GC/GC/TOF HPTLC LCMS
Fresh Cone 7 (FC7)	Potchefstroom muti market	KwaZulu-Natal	Fresh	Wild	Powder	Individual rhizome	MeOH extract	Headspace analysis using GC/GC/TOF HPTLC LCMS

3.3 Preparation of plant material

The roots and rhizomes of *S. aethiopicus* were cut into small pieces and dried in a conventional oven at 55-60°C for 48 h. The dried plant material was subjected to grinding using a stainless steel grinder (Russell Hobbs, South Africa) until the material became a fine, uniform powder. All the plant materials were stored in separate air tight glass containers at ambient temperatures prior to the preparation of the various extracts (Fouche *et al.*, 2011:844).

3.4 Preparation of crude extracts

3.4.1 *Preparation of a methanol (MeOH) extract*

During preparation of the methanol extracts, each sample (1 g) of ground *S. aethiopicus* plant material was weighed and the content extracted with 10 ml methanol:water (70:30 v/v) solvent by means of sonication for 30 min at 37°C with the sonicator set to full power. The extracts were filtered through Munktell Ahlstrom folded filters (Lasec, Johannesburg, South Africa) and the filtrate collected. The extraction process was repeated three times with fresh solvent to maximise the yield. All of the subsequent extracts were combined and dried overnight *in vacuo* at 40°C under 0.2 bar pressure using the Genevac EZ-2 plus vacuum oven (United Scientific (Pty) Ltd, Cape Town, South Africa). The weight of each dried extract was subsequently recorded. Solutions of 5 mg/ml and 1 mg/ml were prepared in LC/MS grade methanol; and were used for HPTLC and LC/MS analysis, respectively. All of the samples were pre-filtered prior to analysis using a 0.22 µm syringe filter (Sigma-Aldrich Pty. Ltd., Johannesburg, South Africa) (Shikanga *et al*., 2011:261).

3.4.2 *Preparation of an aqueous extract*

An aqueous extract was prepared by means of the addition of 100 ml boiling water to 20 g of the dry *S. aethiopicus* plant material. This pulp was sonicated for 30 min at 37°C using a sonicator set to full power, followed by stirring for 30 min with a magnetic stirrer on a heated plate with the temperature set to 37°C. The extract was centrifuged for 10 min at 5000 rpm, followed by filtering through Whatman no. 1 filter paper (Macherey-Nagel, Separations, Randburg, South Africa). This extraction process was repeated three times and the subsequent filtrates were combined and lyophilised for 5 days with a Virtis SP Scientific Sentry 2.0 freeze-dryer (United Scientific Pty. Ltd., Johannesburg, South Africa) (Fouche *et al*., 2011:844; Shikanga *et al*., 2011:261).

3.5 Isolation of marker molecules from *Siphonochilus aethiopicus* using preparative High Performance Liquid Chromatography (prep-HPLC)

The four marker molecules, i.e. AG 1–AG 4, were isolated using prep–HPLC coupled with Waters PDA (2998) and MS detectors (Waters, Milford, MA, USA). To achieve chromatograms with improved resolution in short analysis time, the chromatographic conditions were optimised. Separation was achieved on an XBridge Prep C18–column (19 x 250 mm, i.d., 1.7 µm particle size, Waters), maintained at 40°C. The mobile phase consisted of 0.1% formic acid in water (solvent A) and methanol (solvent B) at a flow rate of 20 ml/min; and a gradient elution was applied as follows: The initial ratio was 80% A: 20% B, which was maintained for 1 min, changed to 50% A:50% B within 2 min, changed to 20% A: 80% B within 10 min, then to 5% A:95% B within 1 min, which was maintained for 0.5 min and changed back to the initial ratio in 0.5 min. The total running time was 15 min. The injection volume was 200 µL. Data were collected by chromatographic software MassLynx 4.1^{TM} (Waters, USA). The prep–HPLC system was interfaced with a QDa mass spectrometer and negative ion mode was selected. The probe temperature was set at 500 °C while the source temperature was 120°C. The capillary and cone voltages were set to 800 and 15 V, respectively. Data were captured between 100 and 650 *m/z*.

3.6 Separation and chemical characterisation of marker molecules from a methanol extract of *Siphonochilus aethiopicus*

The methanol extract of *S. aethiopicus* was chemically characterised by Ultra Performance Liquid Chromatography (UPLC) linked to mass spectroscopy (MS), as well as nuclear magnetic resonance spectroscopy (^{1}H-NMR). A chemical profile of *S. aethiopicus* was subsequently established through analysis of different samples by means of HPTLC and headspace analysis using the GCxGC-TOF/MS system to develop a qualitative profile of the plant.

3.6.1 *^{1}H-NMR structure elucidation*

Proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy was used to elucidate the chemical structures of the four isolated marker molecules (AG 1–AG 2) from the *S. aethiopicus* methanol extract. Spectra were recorded on a Bruker 600 Avance II NMR (Bruker, Bellerica, MA, USA) at 600 MHz for ^{1}H-NMR and 150 MHz for ^{13}C-NMR. Two dimensional (2D) NMR experiments were performed using standard Bruker microprograms. Measurements were made in CD_3OD and the solvent signals were used for calibration. The

purities and identities of isolated compounds were confirmed by comparing ^{1}H- and ^{13}C-NMR signals; UPLC-MS data; IR signals; and UV absorption wavelengths, with literature values.

3.6.2 *Ultra Performance Liquid Chromatography (UPLC) linked to mass spectroscopy (MS)*

As described previously, 11 different samples (Table 3.1) were analysed using UPLC coupled to MS to obtain a chromatographic profile of each of the individual samples. These chromatographic profiles were then used to compare the samples to one another. UPLC analysis was performed on a Waters Acquity Ultra Performance Liquid Chromatographic system with a photodiode array (PDA) detector (Waters, Milford, USA). Chromatographic separation was achieved on an Acquity UPLC Ethylene Bridged Hybrid (BEH) C_{18}–column (150 mm × 2.1 mm, i.d., 1.7 µm particle size, Waters, Milford, USA) thermostatted at 40°C. The mobile phase consisted of 0.1% formic acid (solvent A) and acetonitrile (solvent B) at a flow rate of 0.3 ml/min. The gradient profile was optimised as follows: initial ratio of 85% A and 15% B was changed to 65% A and 35% B in 10 min. It was then changed to 50% A and 50% B in 0.5 min, where it was maintained for 1 min before it was changed back to the initial ratio in 0.5 min. The total running time was 15 min and the injection volume was set at 2.0 µl (full-loop injection).

The UPLC system was interfaced with a Xevo G2QTof mass spectrometer (Waters, Milford, USA). A positive ion mode was selected. Nitrogen (N_2) was used as the desolvation gas and the following parameters were set: capillary voltages, 3000 V; sampling cone voltage, 30 V; extraction cone, 4; source temperature, 100°C; desolvation temperature, 400°C; and desolvation gas flow rate, 500 L/Hr. The mass scan was set between 100 and 1000 *m/z*. All raw chromatographic data were recorded and processed using chromatographic software (Masslynx 4.1, Waters, Milford, USA). The four marker molecules, i.e. AG 1–AG 4 (Table 3.3), were fractionated and the various fractions obtained were concentrated *in vacuo*.

3.6.3 *High Performance Thin Layer Chromatography (HPTLC) analysis of Siphonochilus aethiopicus*

Using a CAMAG automatic TLC Sampler 4 (CAMAG, Muttenz, Switzerland), 2 µl rhizome extract (5 mg/ml) from each individual sample, as well as the reference marker molecules, i.e. AG 1–AG 4 (1 mg/ml), were spray–applied onto a glass HPTLC plate, silica gel 60 F_{254}, 20 x 10 cm, (Merck, Darmstadt, Germany) with a 25 µl Hamilton syringe, connected to nitrogen gas. The samples were applied as 6–7 mm bands. All of the samples on the plates

were applied 8 mm from the lower edge, and 15 mm from the left and right edges of the plate. The plate was developed for 30 min over a distance of 70 mm in an ADC2 development chamber that was pre-saturated for 20 min with toluene and ethyl acetate (93:7 v/v) as the solvent. The derivatising reagent was prepared fresh by mixing 20 ml acetic acid (99%), 170 ml methanol, 10 ml sulphuric acid (98%), and 1 ml anisaldehyde (Wagner & Bladt, 1996). After the 30 min development, the plate immersion device was used to immerse the plate in the derivatising agent for 2 s. Excess reagent was removed keeping the plate upright and left to air dry. The plate was heated at 110–115°C for 45 s on a TLC plate heater. An electronic image acquisition Digistore Reprostar system (CAMAG, Muttenz, Switzerland) was used to photograph the derivatised plate under several different wavelengths of light.

3.6.4 *Gas chromatography linked to a Time of Flight mass spectrometer set in headspace analysis mode (GCxGC-TOF/MS) of Siphonochilus aethiopicus volatiles*

Headspace analysis was performed on the individual samples to obtain information regarding the composition of the volatile components in each sample. The GCxGC system consisted of a Multi-Purpose Sampler (Gerstel, Mülheim an der Ruhr, Germany), which was operating in headspace mode. This system was equipped with a 1 000 µl gas syringe and a tray for 20 ml vials (Gerstel, Mülheim an der Ruhr, Germany). Samples were placed in headspace vials and analysed using a LECO Pegasus© 4 GCxGC–TOF/MS system (LECO Africa (Pty.) Ltd., Johannesburg, South Africa). Each sample (0.5 g) was heated and agitated concurrently for 3 min at 80°C in the pre-heating module. From each vial, 1 000 µl headspace was collected and injected into the system using a 1:20 split ratio. The system consisted of an Agilent© 7890 Gas Chromatograph (Chemetrix (PTY) Ltd., Johannesburg, South Africa) with a cryogenic thermal modulator and a secondary oven. A Stabilwax® capillary column (30 m, 0.25 mm internal diameter, 0.25 µm film thickness) served as the primary column and an Rxi-5Sil–MS® (0.790 m, 0.25 mm internal diameter, 0.25 µm film thickness) capillary column was used as the second column. Helium was utilised as the carrier gas at a constant flow rate of 1.50 ml/min. The front inlet septum purge flow was set to 3 ml/min; and the purge valve time was set at 60 s after initiation of the run. The inlet temperature was maintained at 200°C throughout the analysis and the primary column was programmed with an initial oven temperature of 40°C for 1 min; after which the temperature was increased at a rate of 7°C/min to 220°C; and maintained for 3 min. The secondary column temperature program was set to an initial temperature of 60°C for 30 s, after which it was increased at 7°C/min to 240°C and conserved for 2 min. Both the front inlet and

transfer line temperatures were maintained at 200°C and 225°C, respectively. Operation of the time of flight mass spectrometer (TOF/MS) was set at an acquisition rate of 100 spectra/s for data processing; and an ion source temperature of 230°C was used. A mass acquisition range of 45- 550 *m/z* was utilised, with the hot pulse time and modulation period of 0.60 s and 1.5 s, respectively.

3.6.5 *Hydro-distillation of Siphonochilus aethiopicus*

A Clevenger apparatus (Figure 3.1) was used for the hydro–distillation of two (NW 1 and NW 2) samples. Samples (50 g) of the selected dry plant material (rhizomes) were weighed and added to a flask, to which 600 ml distilled water was added. The flasks were heated by immersion in an oil bath at 140°C for 2 h, counted from the time after condensation of the first drop of oil in the calibrated tube was observed. Oil samples were collected in separate glass vials and stored at 4°C (Arabhosseini *et al*., 2007:562). The essential oil composition of *S. aethiopicus* was analysed using the one dimensional GC system.

Figure 3.1: Clevenger apparatus used for the hydro-distillation of *S. aethiopicus* (Samples: NW 1 and NW 2)

3.6.6 *Analysis of Siphonochilus aethiopicus oil using gas chromatography (GC/FID)*

The essential oils obtained from the hydro-distillation process were analysed using an Agilent 7683B gas chromatograph, coupled to mass spectrometry (Agilent 5973) and a flame ionisation detector (FID). One microliter of each essential oil (20%) diluted with hexane, was injected into the GC. An HP-Innowax polyethylene glycol column, 60 m x 250 µm x 0.25 µm film thickness was used and was operating under the column oven temperature, which was increased from 60°C to 220°C at a rate of 4°C/min; held for 10 min. It was then increased to 240°C at a rate of 1°C/min. Helium was used as the carrier gas at a constant flow rate of 1.2 ml/min. Mass spectra were obtained for the range 35– 550 *m/z*, following electron impact ionisation at 70 eV. The identification of the compounds was carried out using various mass spectrum libraries such as National institute of standards and technology (NIST), Mass Finder, and retention indices (RRI) (Kamatou *et al*., 2010).

3.7 Introduction to results and discussion

Siphonochilus aethiopicus is a deciduous South African plant, listed as critically endangered by the SANBI. Relatively little is known about the chemical composition and the active constituents responsible for the pharmacological effects obtained when consuming the distinctive cone-shaped rhizomes, together with the roots for the treatment of several ailments. Despite being critically endangered and the lack of quality control (QC) protocols available, a number of commercial products containing Wild ginger are currently available. The phytochemical investigation was conducted to develop adequate QC protocols for *S. aethiopicus* by means of various chromatographic techniques such as High Performance Thin Layer Chromatography (HPTLC), Liquid Chromatography coupled to Mass Spectrometry (LC/MS), 2D Gas Chromatography coupled to Time of Flight, Mass Spectrometry set in headspace mode to analyse the volatiles (GCxGC–TOF/MS), and Gas Chromatography coupled to Mass spectrometry (GC/MS) for analysis of the essential oil composition.

3.8 Preparation of plant material

During the process where the samples were dried, it was observed that some of the samples dried faster than others; and the drying time in the conventional oven varied among the different samples. Subsequently, the mass of the wet plant material and dry plant material were compared. There seemed to be a relatively high loss in the net weight before and after drying the plant material; with a net water loss of ±85%. Results regarding weight loss during the drying process can be seen in Annexure A.

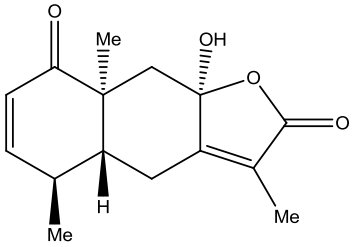
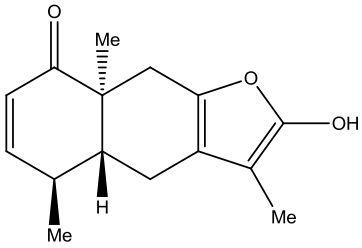
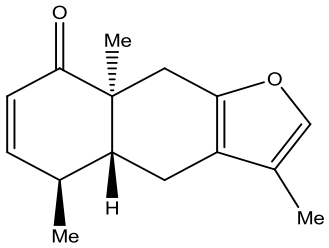
During the preparation of the crude methanol extracts, variations within the different sample groups in terms of percentage yield were obtained, which ranged from 8.73% to 28.59% (Annexure A, Table A-2). Variation in the percentage yield was furthermore observed for the water extracts, and the average yield (6.64%) seemed to be less when compared to the yield of the methanol extracts. Calculations for the percentage yield of all the individual samples are summarised in Annexure A.

3.9 Separation and chemical characterisation of marker molecules from a *Siphonochilus aethiopicus* methanol extract

3.9.1 ^{1}*H-NMR structure elucidation*

Nuclear Magnetic Resonance was used to identify and elucidate the structures of the isolated marker molecules as shown in Table 3.2 (Gottlieb *et al*., 1997:7512-7515).

Table 3.2: Chemical structures and names of marker molecules isolated from *Siphonochilus aethiopicus,* as determined with NMR

Marker molecule	Chemical structure	Name	Stability
AG 1		(4aR,5S,8aS,9aR)-9a-hydroxy-3,5,8a-trimethyl-4a,5,9,9a-tetrahydronaphtho[2,3-b]furan-2,8(4H,8aH)-dione	Stable
AG 2		(4aR,5S,8aS)-2-hydroxy-3,5,8a-trimethyl-4a,5,8a,9-tetrahydronaphtho[2,3-b]furan-8(4H)-one	Stable
AG 3	To be established	To be established	Unknown
AG 4		(4aR,5S,8aS)-3,5,8a-trimethyl-4a,5,8a,9-tetrahydronaphtho[2,3-b]furan-8(4H)-one	Unstable

3.9.2 *Ultra Performance Liquid Chromatography (UPLC) linked to mass spectroscopy (MS)*

The representative UPLC-MS chromatogram (i.e. Diode Array detector (DAD) and Base Peak Intensity (BPI) chromatograms) of the *S. aethiopicus* methanol extract is presented in Figure 3.2.

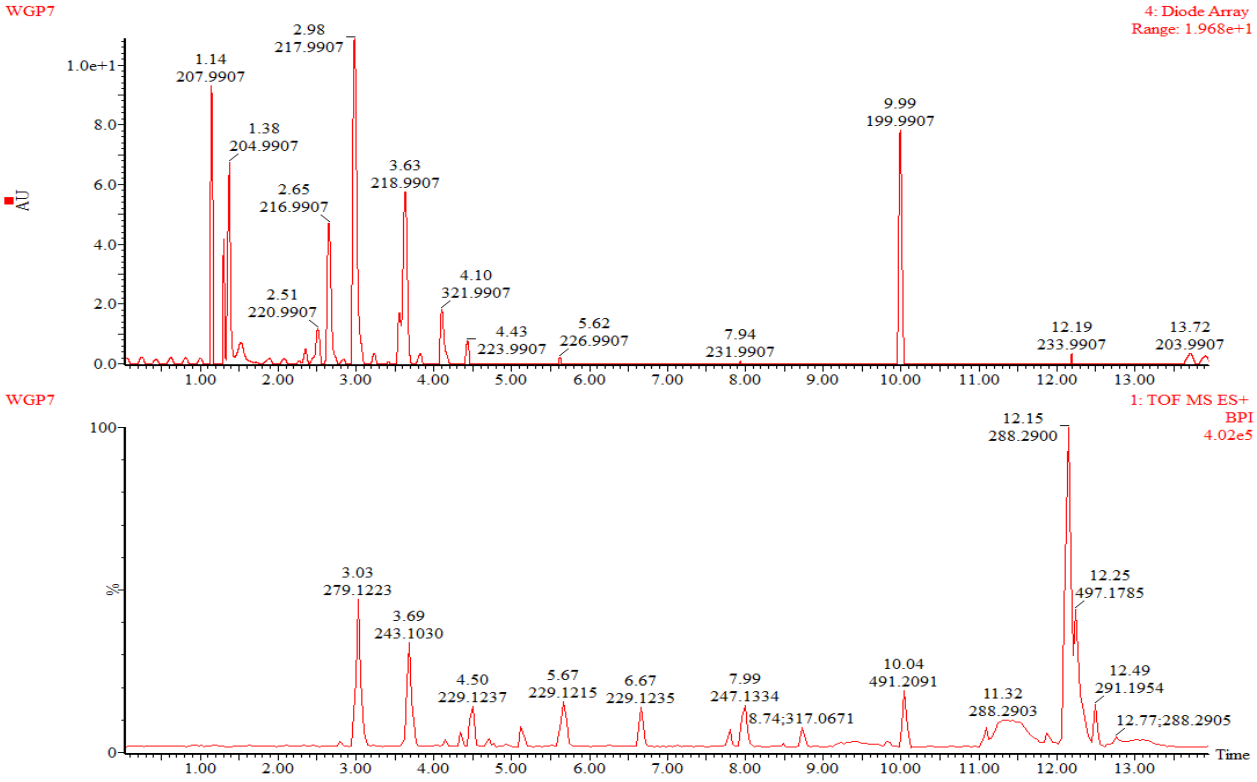


Figure 3.2: UPLC-MS chromatogram (DAD and BPI) of the methanol extract of *Siphonochilus aethiopicus*

The four selected marker molecules, AG 1 – AG 4 (Table 3.3), were fractionated from the cultivated *S. aethiopicus* plant material (TUT material), and the various fractions obtained were concentrated *in vacuo*. The TUT plant material was donated by Prof. A. Viljoen, all of the chemical analysis were performed at TUT, and therefore the marker compounds were isolated from this sample. Individual LC/MS/MS spectra for each of the four marker molecules are reported in Figures 3.3 – 3.6. The four marker molecules were selected based on the most abundant peaks found over the chromatographic spectrum (Figure 3.2) of *S. aethiopicus*. The isolated marker molecules were used as chemical identification markers in the different plant materials (Table 3.1) that were analysed during the quality control experiments of the plant material. Table 3.3 shows the different abbreviations allocated to the four marker molecules, as well as the retention time of these individual compounds. Based on the retention times of the individual marker molecules, the peak areas at these specific retention times were used to calculate the area percentage of each marker molecule within each different plant material extract, individually.

Table 3.3: Isolated marker compound name, sample origin and individual retention times of the four selected marker molecules

Marker molecule	Sample origin	Extract	M+1	Time compound appeared on the MS
African Ginger 1 (AG 1)	KwaZulu-Natal	MeOH	245.1185	6.90
African Ginger 2 (AG 2)	KwaZulu-Natal	MeOH	247.1343	9.10
African Ginger 3 (AG 3)	KwaZulu-Natal	MeOH	415.1762	12.32
African Ginger 4 (AG 4)	KwaZulu-Natal	MeOH	231.1390	14.32

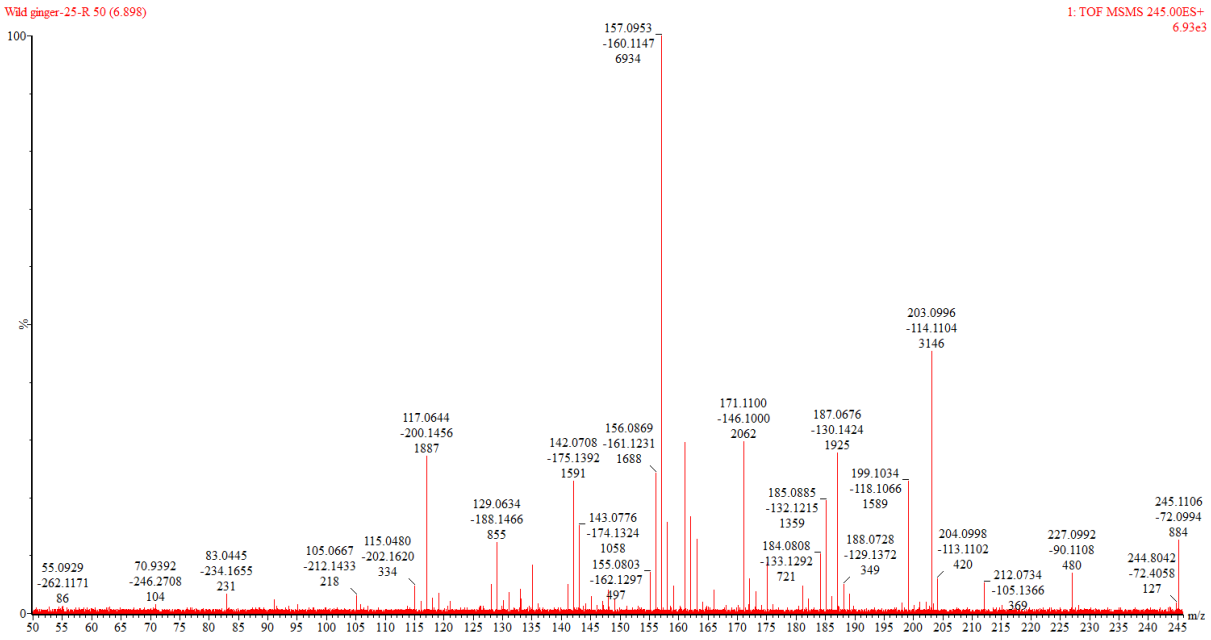


Figure 3.3: LC-MS/MS spectrum of marker molecule AG 1 (*m/z* =245).

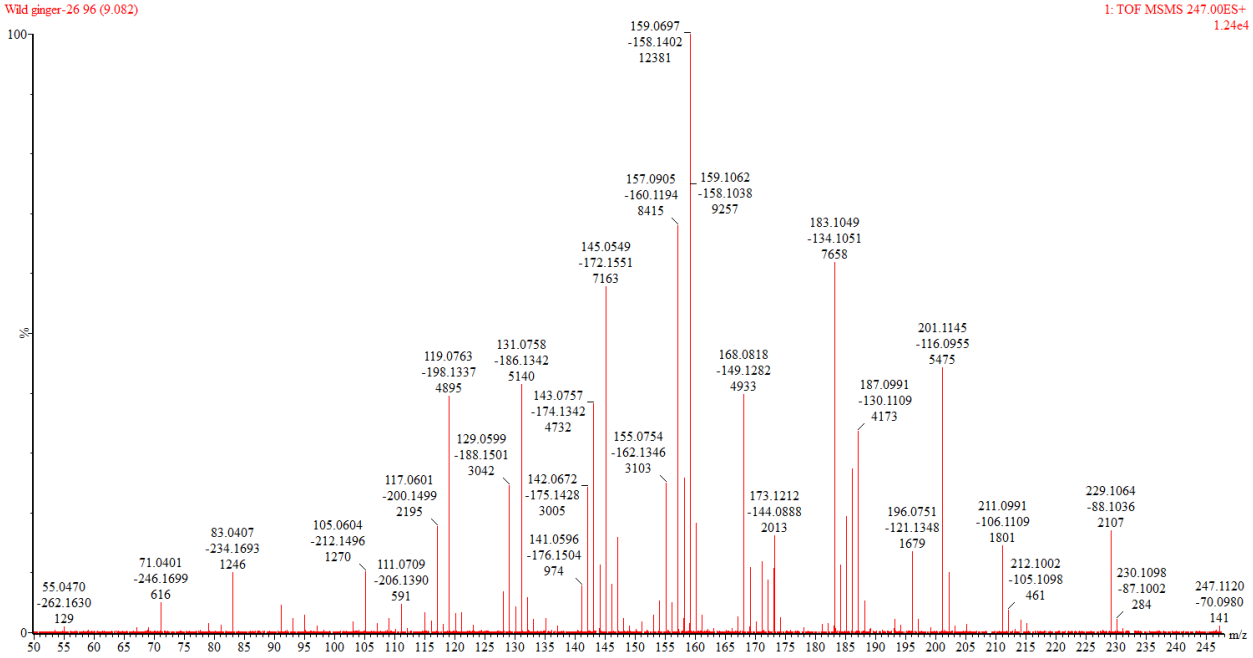


Figure 3.4: LC-MS/MS spectrum of marker molecule AG 2 (*m/z* =247).

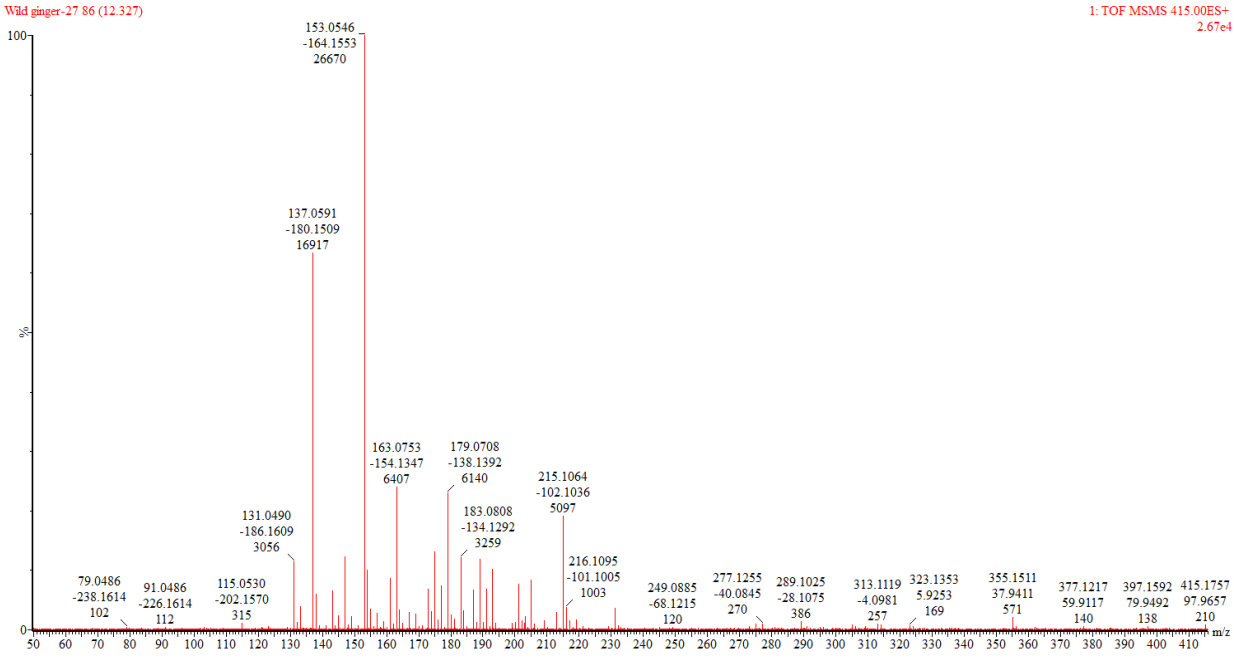


Figure 3.5: LC-MS/MS spectrum of marker molecule AG 3 (*m/z* =415).

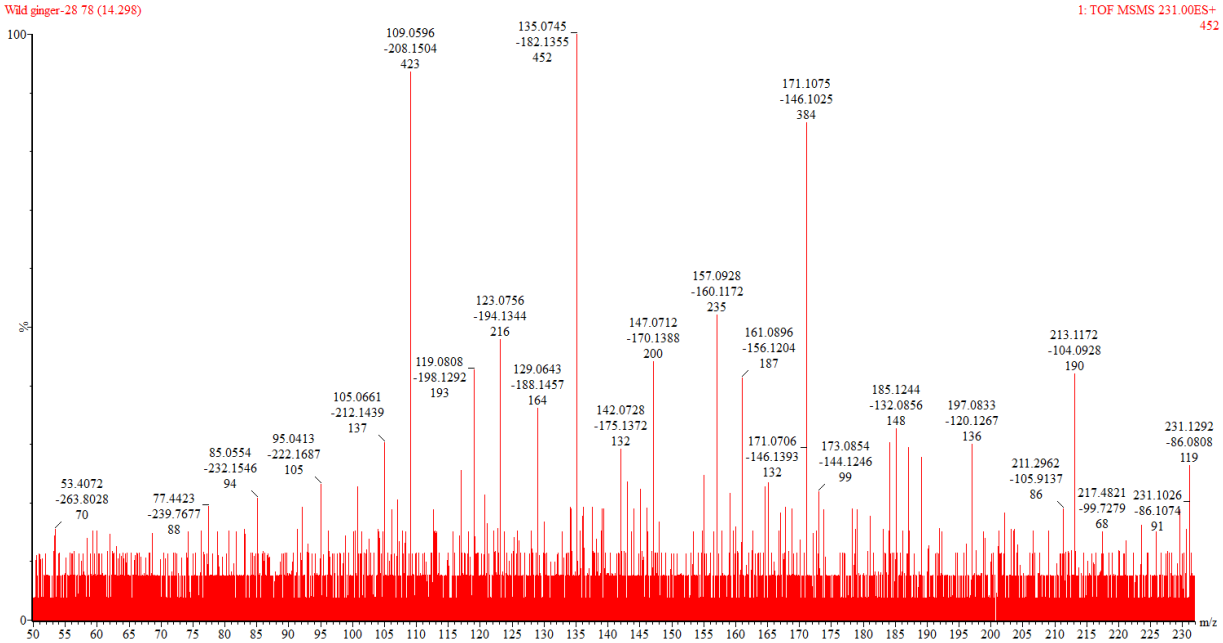


Figure 3.6: LC-MS/MS spectrum of marker molecule AG 4 (*m/z* =231).

The LC-MS/MS spectra (Figures 3.3 – 3.6) were used to determine and confirm whether the presence of the correct marker molecule was identified in the chromatograms from each of the individual samples that were analysed using UPLC-Q-TOF/MS analysis.

The four marker molecules were identified within each of the individual samples using the retention time (R_t) of the different marker molecules and their mass fragmentation patterns (Figure 3.7). The area percentage was calculated for each of the four marker molecules indicating their relative abundance in the methanol extract. The marker molecules (AG 1, AG 2, AG 3 and AG 4) depicted retention times of 6.89 min, 9.08 min, 12.33 min and 14.30 min, respectively (Table 3.4).

Table 3.4: Percentage area of the four marker molecules in the different *Siphonochilus aethiopicus* plant materials

	LC-MS analysis results										
Compound	**R_t (min)**	**Samples analysed (Area %)**									
		NW1	**NW2**	**TUT**	**FC1**	**FC2**	**FC3**	**FC4**	**FC5**	**FC6**	**FC7**
AG 1	6.90	0.88	0.42	23.43	2.77	1.6	0.83	2.68	1.63	-	3.06
AG 2	9.06	3.04	0.83	10.60	12.08	6.9	5.39	8.02	6.08	7.43	4.68
AG 3	12.32	17.30	10.51	15.12	14.90	25.6	22.98	14.93	15.25	-	14.40
AG 4	14.32	47.44	62.11	5.11	63.00	48.01	16.92	64.69	51.87	49.77	59.38

All of the UPLC-Q-TOF/MS chromatograms of the different *S. aethiopicus* plant materials were considered similar, as all four of the chemical marker molecules were depicted in the individual chromatograms of the various samples, with the exception of sample FC6. The different MS peaks of the four marker molecules in each of the *S. aethiopicus* sources are encircled in Figure 3.7. The presence of the four marker molecules (quantitative) in each sample is clearly visible in this figure. Individual chromatograms with a higher resolution can be seen in Annexure C.

In conclusion, even though all four of the marker molecules were present in the majority of the samples, the quantity of the molecules varied, indicating quantitative differences between these samples. The dissimilarity observed could be attributed to inter-individual variation among the different samples. In the NW 1, NW 2, as well as FC 1–FC 7 *S. aethiopicus* plant material, the relative area percentage as indication of quantity of AG 1 and AG 2 present in the methanol extract is less compared to the area percentage obtained in the TUT plant material. On the other hand, the area percentage of the marker compounds, AG 3 and AG 4 are lower in the TUT sample compared to the other samples, indicating that there is a significant difference between the level of phytochemicals found in the cultivated *versus* the wild plant material.

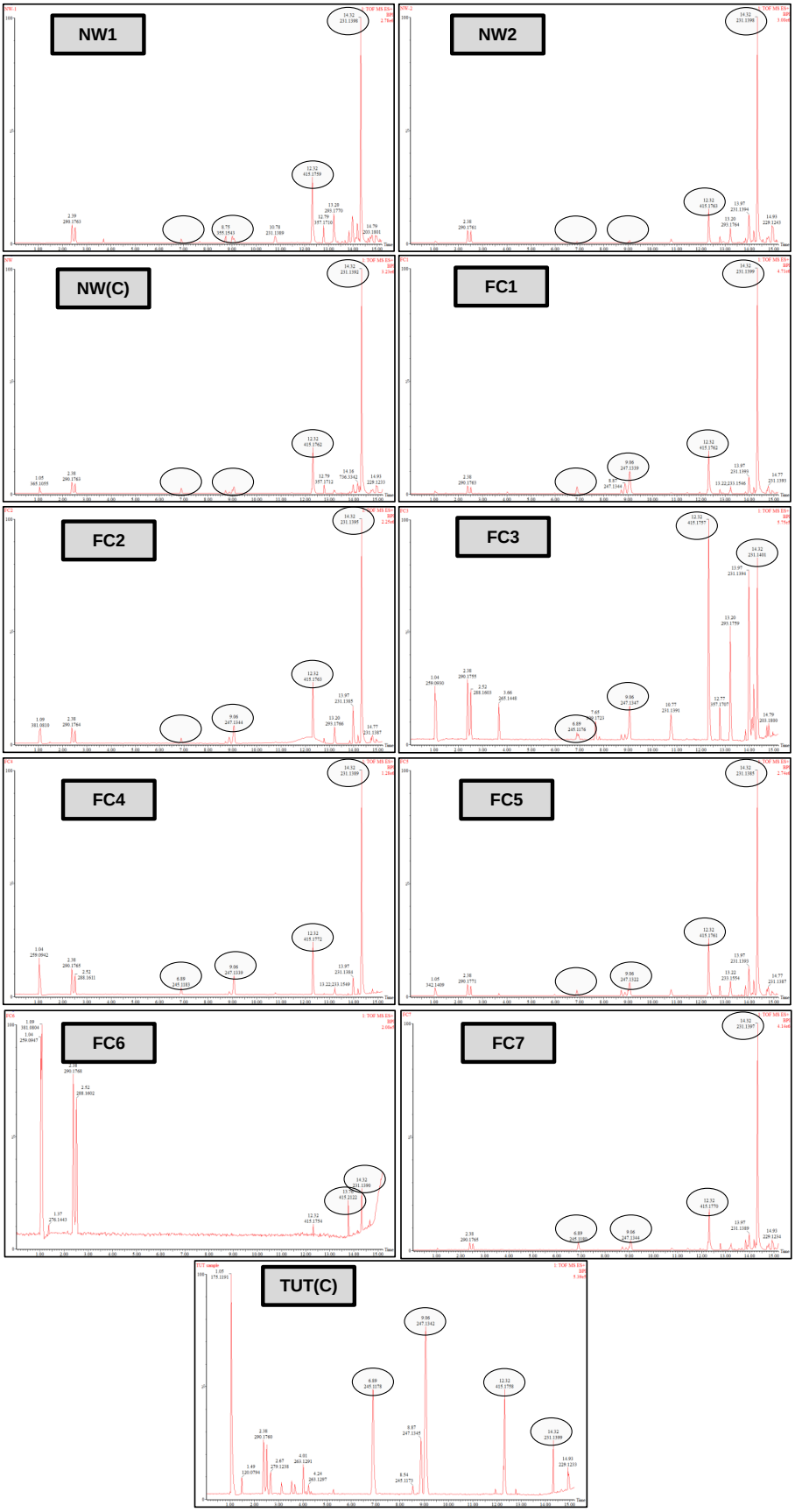


Figure 3.7: LC/MS chromatograms of methanol extracts of the various *Siphonochilus aethiopicus* plant materials

3.9.3 *High Performance Thin Layer Chromatography (HPTLC) analysis*

Several different mobile phase systems with different polarities were investigated in order to establish the optimal mobile phase for the HPTLC analysis of the *S. aethiopicus* methanol extracts. The mobile phase consisting of toluene and ethyl acetate (93:7, v/v) revealed the most optimum separation of the individual components and was employed for the HPTLC analyses. All the HPTLC results are displayed in Annexure B. Furthermore, two different derivatising agents were investigated during the study; the first being anisaldehyde sulphuric acid dipping reagent, and the second vanillin sulphuric acid dipping reagent. Both of the derivatising agents showed similar results, however, the anisaldehyde sulphuric acid dipping reagent was preferred due to a higher resolution that was obtained and was used for all the HPTLC plates prepared thereafter. The HPTLC fingerprints from the methanol extracts of *S. aethiopicus* are shown in Figures 3.8 and 3.9.

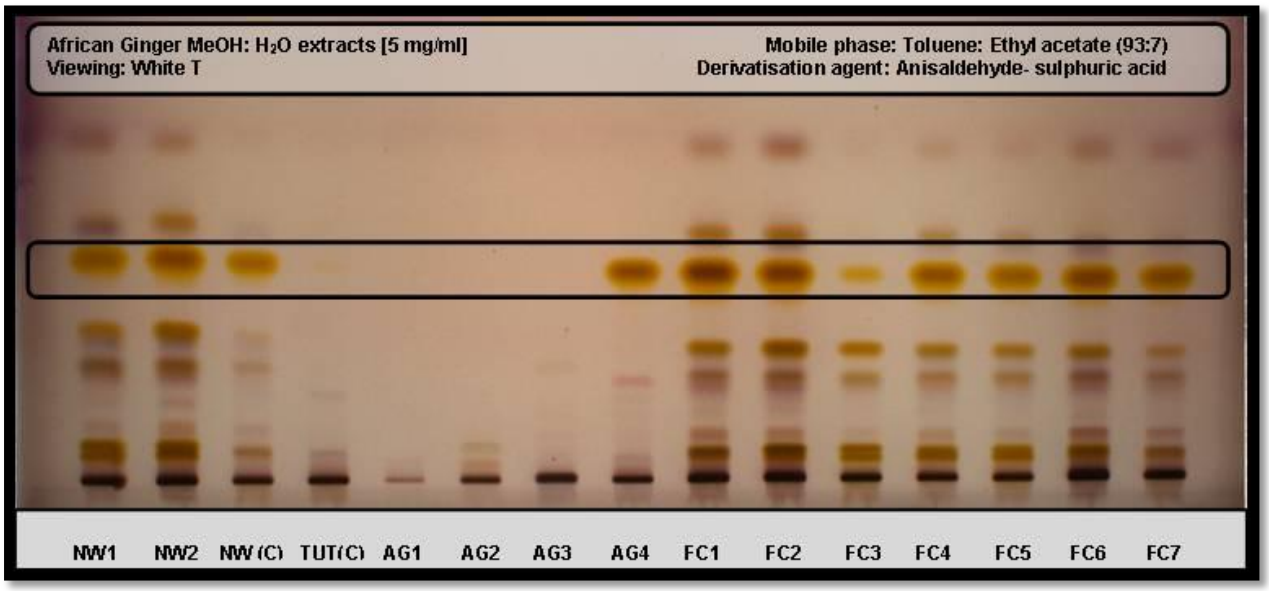


Figure 3.8: HPTLC plate 1 demonstrating the chemical fingerprints for methanol extracts from the selected *Siphonochilus aethiopicus* plant materials viewed with White T light.

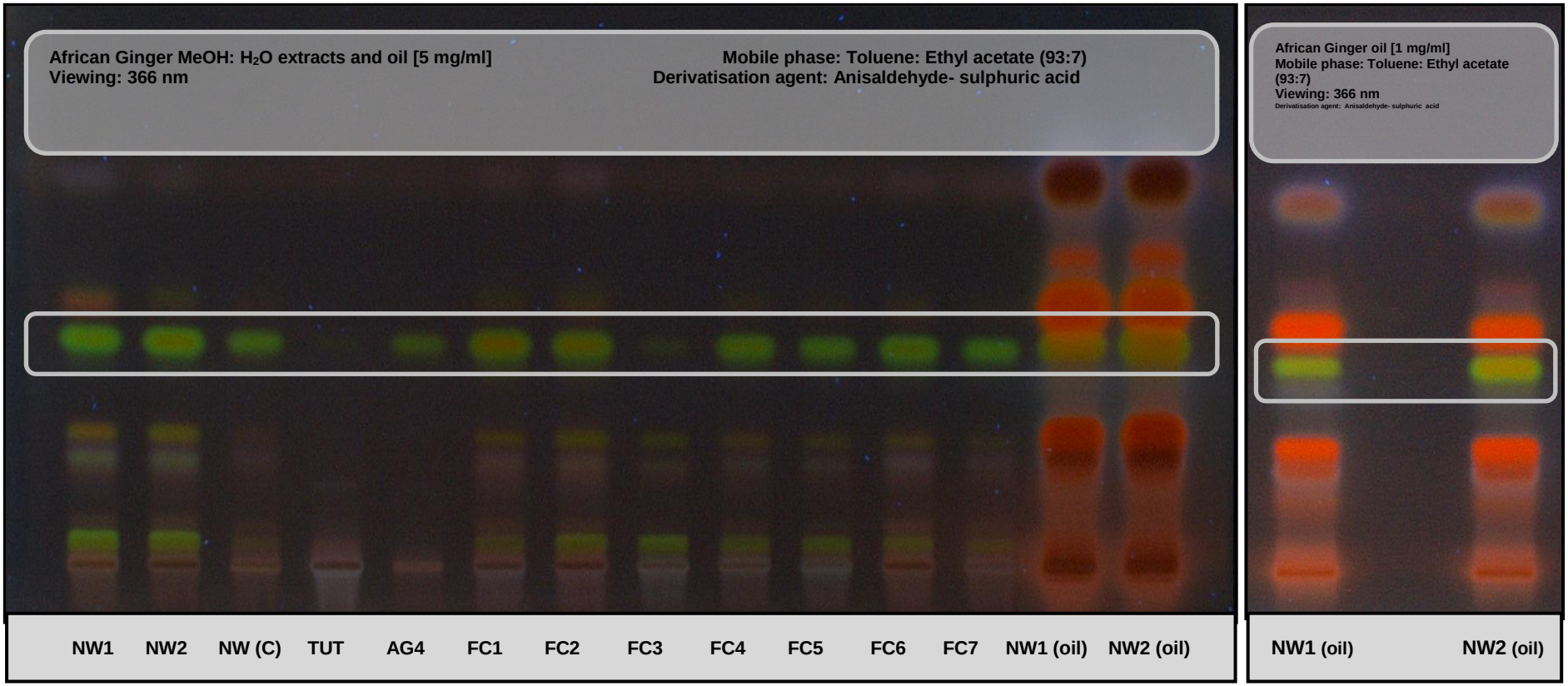


Figure 3.9: HPTLC plate 2 demonstrating chemical fingerprints for methanol extracts from the selected *Siphonochilus aethiopicus* plant materials at a wavelength of 366 nm.

The individual R_f values calculated for each marker molecule was as follows: AG 1 (R_f value of 0.026), AG 2 (R_f value of 0.065), AG 3 (R_f value of 0.091) and AG 4 (R_f value of 0.442). All of the samples evaluated contained all four of the marker molecules, although there was a distinct indication of quantitative variation among the different samples. No mobile phase could be developed to show complete separation for all four of the isolated molecules, and thus AG 4 (R_f value of 0.442), the most prominent band visible in all of the samples investigated (thick yellow band); was selected to further investigate the profiles of the different samples. Figure 3.9 indicates the presence of the marker, AG 4, in the essential oil, confirming that this marker molecule can be used in future to identify any *S. aethiopicus* sample. Results from the experiments presented in these two figures (Figures 3.8 and 3.9), indicated that the HPTLC profiles of the different samples have minimal qualitative variation among the samples, indicating that the different *S. aethiopicus* plant materials used in the study are relatively similar.

3.9.4 *Gas chromatography linked to a Time of Flight Mass Spectrometery set in headspace analysis mode (GCxGC-TOF/MS) of Siphonochilus aethiopicus volatiles*

The volatile constituents of the methanol extracts from eleven *S. aethiopicus* plant material samples were analysed by GCxGC–TOF/MS headspace analysis for the first time. The total ion chromatogram of FC 2 is given in Figure 3.10. This figure represents the intensities of all the mass spectral peaks belonging to the FC 2 material. Quantitative rather than qualitative variation of chemical compounds was obtained when comparing all of the individual samples (Annexure D, Figure D 1- D 9). Since no significant qualitative variation was observed, one sample (FC 2) was arbitrarily selected as a representative sample for comprehensive headspace analysis of *S. aethiopicus* by means of 2D GC (Figure 3.11). Annexure D contains the data of all the individual samples.

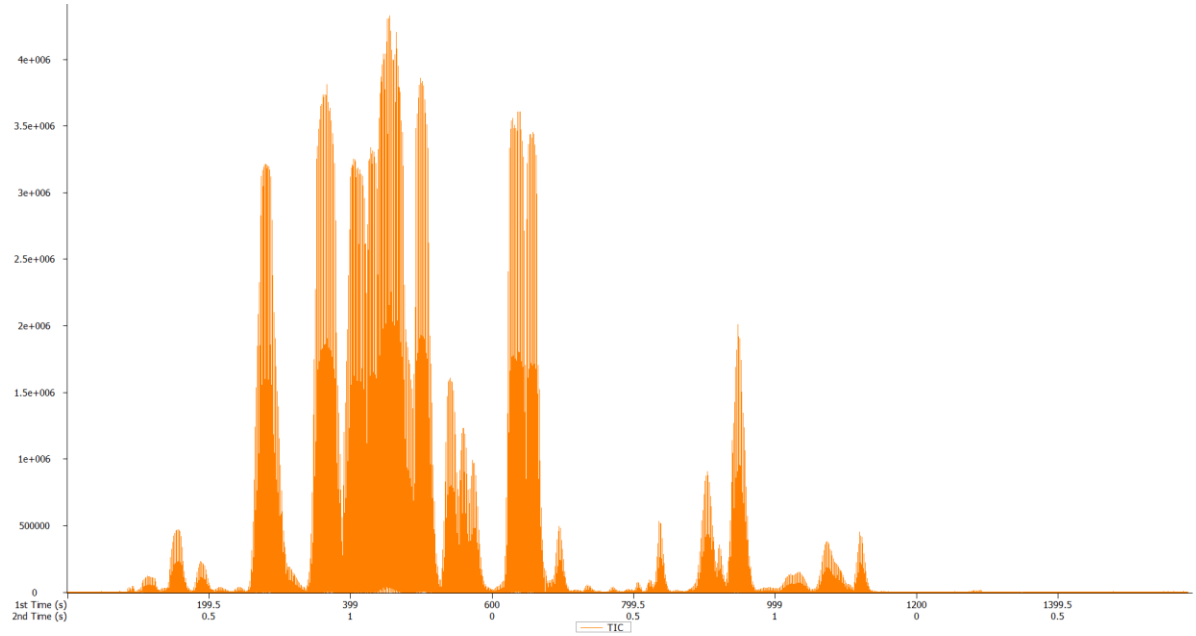


Figure 3.10: TIC chromatogram of *Siphonochilus aethiopicus* FC 2 material

In Figure 3.11 the 2D surface plot for FC 2 *Siphonochilus aethiopicus* are given. The data was used to identify all peaks in the GC x GC chromatogram contour plots (Figure 3.12) with an *S/N* threshold >100, and a similarity match >800. The resulting peak table for each individual sample can be viewed in Annexure D.

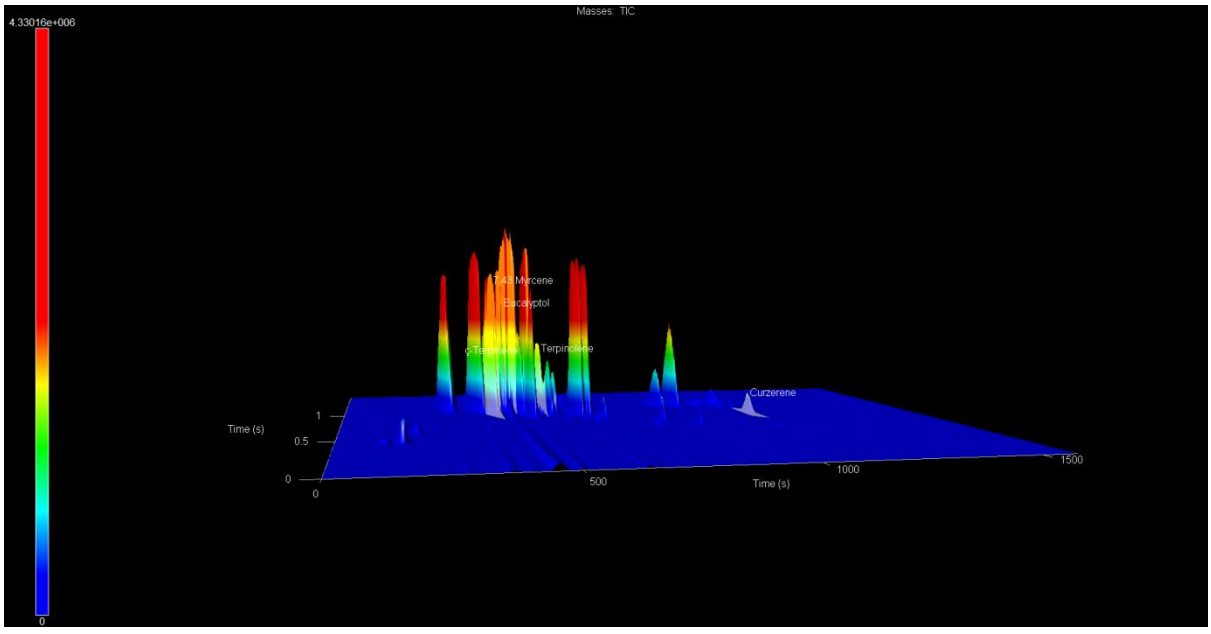


Figure 3.11: 2D surface plot of *Siphonochilus aethiopicus* FC 2 material

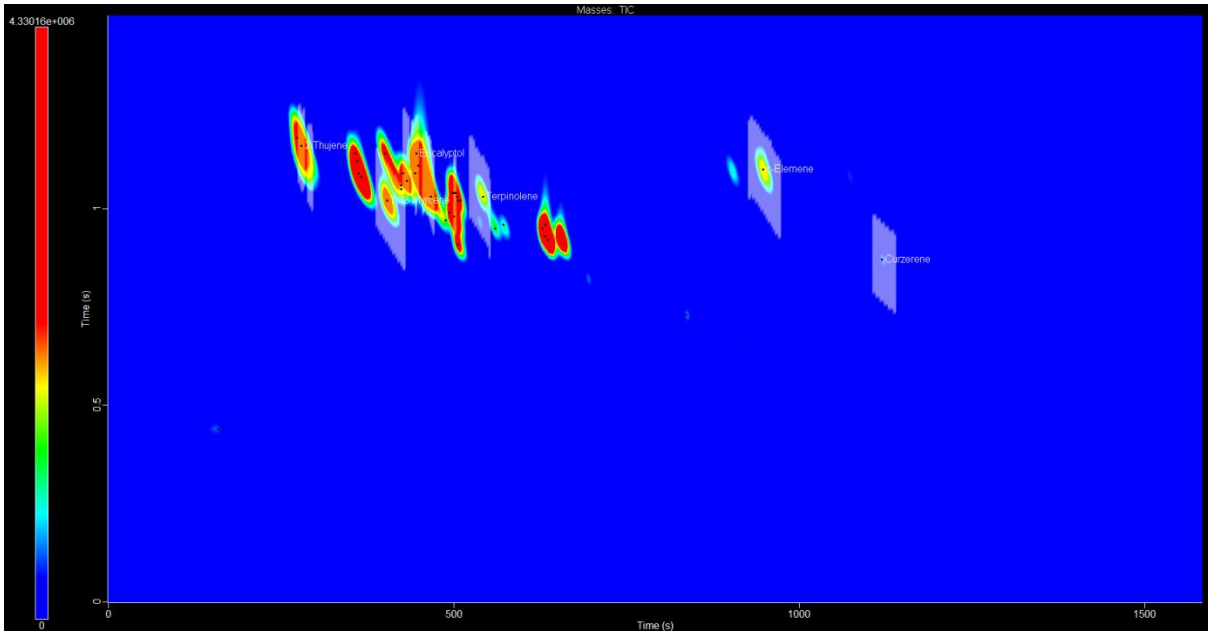


Figure 3.12: Counter 2D plot of *Siphonochilus aethiopicus* FC 2 material

The volatile chemical constituents identified in the *S. aethiopicus* samples, as well as the % area as indication of quantity, are presented in Table 3.5.

Table 3.5: Headspace volatiles identified in *Siphonochilus aethiopicus* plant material FC 2 by means of GCxGC-TOF/MS

Compounds		RT (1st and 2nd dimension)	% Area
	Name		**Headspace**
1	**α-Pinene**	**277.5; 1.170**	**5.3**
2	β-Pinene	349.5; 1.110	0.3
3	**Sabinene**	**363; 1.090**	**4.4**
4	δ-3-Carene	400.5; 1.140	2.1
5	Myrcene	403.5; 1.020	3.2
6	α-Terpinene	429; 1.080	4.9
7	Sylvestrene	438; 1.120	0.2
8	Acetoin	448.5; 0.480	0.1
9	**1,8-Cineole**	**448.5; 1.110**	**15.1**
10	**β-Phellandrene**	**463.5; 1.040**	**10.6**

11	*cis*-Sabinene hydrate	463.5; 1.080	0.9
12	***E*-β-Ocimene**	**496.5; 0.99**	**5.7**
13	γ-Terpinene	501; 1.040	1.0
14	*p*-Cymene	507; 0.910	1.3
15	*trans*-β-Ocimene	510; 1.041	1.5
16	Terpinolene	540; 1.050	3.2
17	1-Methyl-4-1 (1-methylethylidene)-cyclohexene	553.5; 1.0	1.9
18	5,5,-dimethyl-2-propyl-1,3-cyclopentadiene	573; 0.950	1.2
19	***cis allo* Ocimene (isomer 1)**	**621; 0.960**	**3.7**
20	***trans allo* Ocimene (isomer 2)**	**634.5; 0.940**	**13.0**
21	Ethylanisol	636; 0.940	0.8
22	1-Ethyl-4-methoxy benzene	642; 0.910	1.7
23	3,6-Dimethylene, 1-7-octadiene	642; 0.940	1.4
24	4,4,6,6-Tetramethyl bicyclo hexene	663; 0.910	1.3
25	*p*-Cymenene	672; 0.78	0.4
26	Terpinen-4-ol	837; 0.730	0.7
27	λ-Elemene	901.5; 1.100	0.8
28	α-Terpineol	921; 0.660	0.9
29	δ-Elemene	946; 1.10	0.9
30	Curzerene	1120.5; 0.870	0.2

The main compounds detected in all of the samples (indicated by bold text in the table) were 1,8-cineole (15.1%), *trans-allo* ocimene (12.3%), β-phellandrene (10.6%), *E*-β-ocimene (5.7%), α-pinene (5.3%), α-terpienene (4.9%) and sabinene (4.4%). Some of the major headspace volatiles (1,8-cineole, β-phellandrene, *trans-allo* ocimene, and sabinene) were also main constituents of the essential oil of *S. aethiopicus*. It has been reported that curzerenone, furanodiene and β-phellandrene present in the *S. aethiopicus* distillate, were important pertaining to the characteristic odour of the plant (Igoli & Obanu, 2011:543-544).

3.9.5 *Analysis of Siphonochilus aethiopicus oil using gas chromatography (GC/FID)*

The essential oil was distilled using the Clevenger apparatus setup, and it was analysed using GC/FID, and Figure 3.13 illustrates the volume of oil disstilled from NW 1 and NW 2.

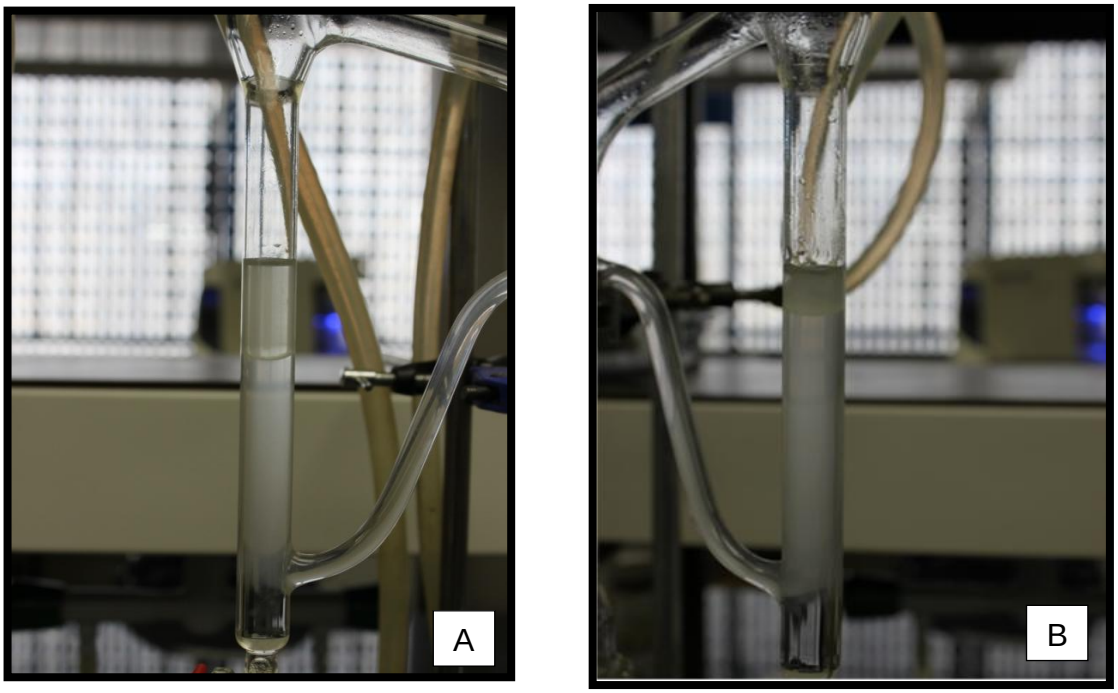


Figure 3.13: Oil in collection tube of NW 1 and NW 2

As can be observed in Figure 3.13, there was a relatively large difference in the amount of oil that was produced with the hydro-distillation from the two samples (Sample A: 3.10 ml, Sample B: 1.55 ml). These two oil samples (NW 1 and NW 2) were analysed via GC/FID, and the GC total ion chromatograms (GC- TIC) of the two samples are shown in Figure 3.14.

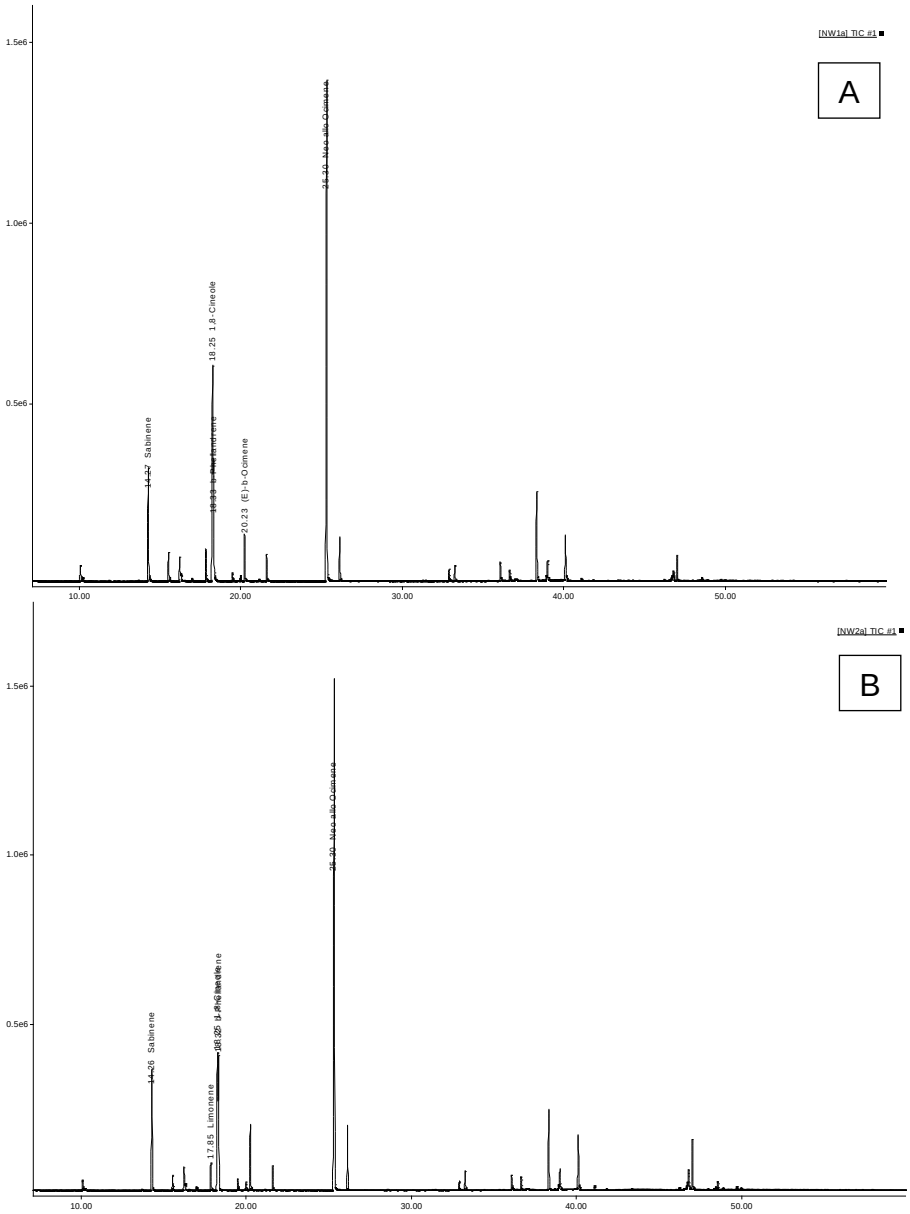


Figure 3.14: GC chromatograms of the volatile oil from selected *Siphonochilus aethiopicus* samples (A: NW 1 and B: NW 2 Oil)

Some of the main compounds found in both of the *S. aethiopicus* oil samples included sabinene, β-phellandrene, 1,8-cineole, and *cis*-alloocimene. The rest of the foremost compounds found in the oil samples (Compounds with an area percentage higher than 1%) are reported in Table 3.6.

Table 3.6: Comparison of essential oil composition of NW 1 and NW 2 samples as determined through GC/MS analysis.

GC/MS analysis data of the 2 oil extracts			
Compounds		**Samples (Area %)**	
	Name	NW 1 (Muthi) % of total	NW 2 (Muthi) % of total
1	α - Pinene	1.047	0.745
2	Sabinene	6.742	7.179
3	δ -3- Carene	1.808	1.109
4	Myrcene	1.852	1.763
5	Limonene	2.403	1.998
6	Eucalyptol	13.806	7.433
7	β - Phellandrene	4.896	7.118
8	1,3,6-Octatriene,3,7-dimethyl-,(2)-	3.286	4.292
9	Terpinolene	1.700	1.489
10	*Cis allo* ocimene	31.129	31.201
11	*Allo* Ocimene (isomer)	2.900	3.669
12	α - Terpineol	1.648	3.876
13	Benzene,1-metyl-3-(1-methylethyl)	1.624	1.818
14	Unknown 1	6.181	-
15	Unknown 2	3.409	0.261
16	α - Pathoulene	1.363	5.315
17	β - Pathoulene	1.222	3.478

Sabinene, β-phellandrene, 1,8-cineole, and *cis*-alloocimene have previously been reported as the major constituents of the *S. aethiopicus* plant oil (Igoli & Obanu, 2011:543). However, siphonochilone, a main constituent previously found in the rhizome and the root of the plant (Viljoen *et al.*, 2002:116), was not detected in either of the oil samples in this study. This highlights the inter-individual variation observed between the plant material samples.

3.10 Conclusion

Four chemical compounds (AG 1- (4aR,5S,8aS,9aR)-9a-hydroxy-3,5,8a-trimethyl-4a,5,9,9a-tetrahydronaphtho[2,3-b]furan-2,8(4H,8aH)-dione, AG 2- (4aR,5S,8aS)-2-hydroxy-3,5,8a-trimethyl-4a,5,8a,9-tetrahydronaphtho[2,3-b]furan-8(4H)-one, AG 3 and AG 4- (4aR,5S,8aS)-

3,5,8a-trimethyl-4a,5,8a,9-tetrahydronaphtho[2,3-b]furan-8(4H)-one) were isolated. Development of the HPTLC fingerprints of methanol extracts from *S. aethiopicus* rhizomes can be employed in the identification, authentication and characterisation of this plant and commercial products. Chromatographic profiles obtained using HPTLC, UPLC/TOF/MS; GCxGC-TOF/MS; and GC/MS–FID analysis indicated a strong qualitative resemblance between the 11 *S. aethiopicus* plant material samples studied. However, UPLC/TOF/MS chromatograms showed quantitative variation between the muti market and cultivated samples. Headspace volatiles of the plant showed 1,8-cineole (15.1%), *cis*-β-ocimene (12.3%) and β-phellandrene (10.6%) as major constituents, whereas the essential oil constituent was dominated by *cis allo*ocimene (31.2%).

CHAPTER 4 – *IN VITRO* PERMEABILITY STUDIES

4.1 Introduction to *in vitro* permeability studies

Various *in vitro*, *in situ*, and *ex vivo* models are available to study and predict pre-clinical drug transport (Alqahtani *et al*., 2013:2). Two models have been employed to determine the permeability of *S. aethiopicus* marker molecules; which include an *ex vivo* model, namely excised porcine tissue and the *in vitro* Caco-2 mammalian cell culture model. The use of excised porcine tissues; in the form of either buccal, sublingual or intestinal mucosa have regularly been utilised in drug transport studies due to the morphological and physiological resemblance to human tissue (Shikanga *et al*., 2011:261).

The Caco-2 cell line is one of the most frequently used epithelial cell lines in the prediction of pre-clinical drug permeability, offering various advantages (Artursson *et al*., 2001:27, 28; Tavelin *et al*., 2002:233,234). This cell line is easily obtainable; and due to its human origin, various morphological and functional similarities exist, such as the expression of brush border enzymes and the formation of tight junctions, resulting in behaviour similar to its *in vivo* counterpart (Tavelin *et al*., 2002:233,234). The transport of two of the four identified marker molecules in the methanol and aqueous extracts of *S. aethiopicus* was investigated across sublingual and buccal porcine mucosal tissues in the apical-to-basolateral (AP-BL) direction, as well as bi-directional transport across Caco-2 cell monolayers (i.e. apical-to-basolateral (AP-BL) and basolateral-to-apical (BL-AP) directions). All permeability experiments were conducted in triplicate.

4.2 Preparation of test solutions

Methanol and aqueous crude extract test solutions at concentrations of 1.9 mg/ml and 40 mg/ml were prepared in Krebs Ringer bicarbonate buffer (KRB) for the buccal and sublingual permeability experiments; and in high-glucose Dulbecco's Modified Eagle's Medium (DMEM) for the Caco-2 permeability experiments. These concentrations were based on the dose of a commercial product as well as previous *in vitro* permeation studies of medicinal plant extracts across buccal and sublingual tissues (Shikanga *et al*., 2011:261).

4.3 Collection and preparation of porcine tissue for transport studies

Porcine sublingual- and buccal tissues were obtained from a local abattoir in Potchefstroom (Potch Abattoir, Potchefstroom, South Africa) directly after routine slaughter of pigs for meat production (NWU-00025-15-A5). Once the tongue, mouth floor and cheek were excised from the pig, the tissues were rinsed with ice cold KRB and placed into cold KRB (pH 7.4) in a cooler box to adhere to the cold chain during transport to the laboratory.

4.3.1 *Preparation of porcine buccal tissue*

The excessive connective and adipose tissue were trimmed away from the buccal mucosa until an approximately 1.0 ± 0.4 mm thick mucosal membrane was obtained. The buccal mucosa was placed on a piece of heavy duty filter paper with the apical side facing upwards, followed by placement onto a Perspex plate positioned on ice. The buccal mucosa was cut into smaller 2 cm segments, and throughout the procedure the tissue was kept cold and moist with ice cold KRB. The tissue segments, with the apical sides facing downwards and the filter paper (basolateral side) facing upwards, were mounted onto the Sweetana Grass diffusion chamber half-cells (Easy Mount Diffusion Chamber, Physiologic Instruments, San Diego, USA) with a transport surface area of 1.78 cm^2. The filter paper was subsequently removed and the half-cells assembled and clamped together with metal rings. These combined chambers were placed onto a heating block (Easy Mount Diffusion Chamber, Physiologic Instruments, San Diego, USA) and filled with 7 ml pre-heated (37°C) KRB. The half-cells were linked to parallel gas flow (5% CO_2, 95% O_2) with a flow rate of 15-20 ml/min. All of the assembled cells were allowed 20 min to reach a state of equilibrium prior to commencement of the transport studies.

4.3.2 *Preparation of porcine sublingual tissue*

During preparation of the porcine sublingual tissue, care was taken to only remove the sublingual mucosa from the pig tongue and mouth floor. The excessive connective and adipose tissue were trimmed away from the sublingual mucosa in order to obtain a 0.7 ± 0.3 mm thick membrane. All of the excised sublingual mucosal tissue pieces were consequently placed on a piece of heavy duty filter paper (apical side facing upwards) and positioned onto a Perspex plate on ice. Each sublingual mucosal piece on the filter paper was cut into ± 2 cm segments while ensuring that the tissue was kept cold and moist with ice cold KRB throughout the process. The sublingual segments were placed onto Sweetana Grass diffusion half-cells (Easy Mount Diffusion Chamber, Physiologic Instruments, San Diego, USA) in the same manner as described in section 4.3.1.

4.4 Permeability across buccal and sublingual mucosal tissue

Permeability of the test compounds was determined in the AP-BL direction across the porcine buccal and sublingual mucosal tissues, respectively. A schematic representation of the transport setup for the *ex vivo* experiment in the Sweetana-Grass diffusion apparatus is provided in Figure 4.1. The tissue integrity was evaluated by means of transepithelial electrical resistance (TEER) measurements. The TEER measurements were obtained using a Dual Channel voltage clamp (Warner instruments, Hamden, Connecticut, USA) after equilibrium was established in KRB buffered at pH 7.4. The apical buffer solution for each cell was removed and subsequently replaced with 7 ml of the pre-warmed *S. aethiopicus* crude extract solutions. Samples (200 µl) were withdrawn from the basolateral chambers at pre-determined time intervals (20, 40, 60, 80, 100, 120 min) which were replaced with pre-heated KRB after each withdrawal. A final TEER measurement was taken after 120 min to confirm the integrity of the membranes after exposure to the test solutions. All experiments during the buccal and sublingual permeability studies were conducted at 37ºC, while bubbling a mixture of 95% oxygen and 5% CO_2 through the transport medium. All samples were stored in HPLC vials at -80°C until these samples were analysed by means of LC/MS.

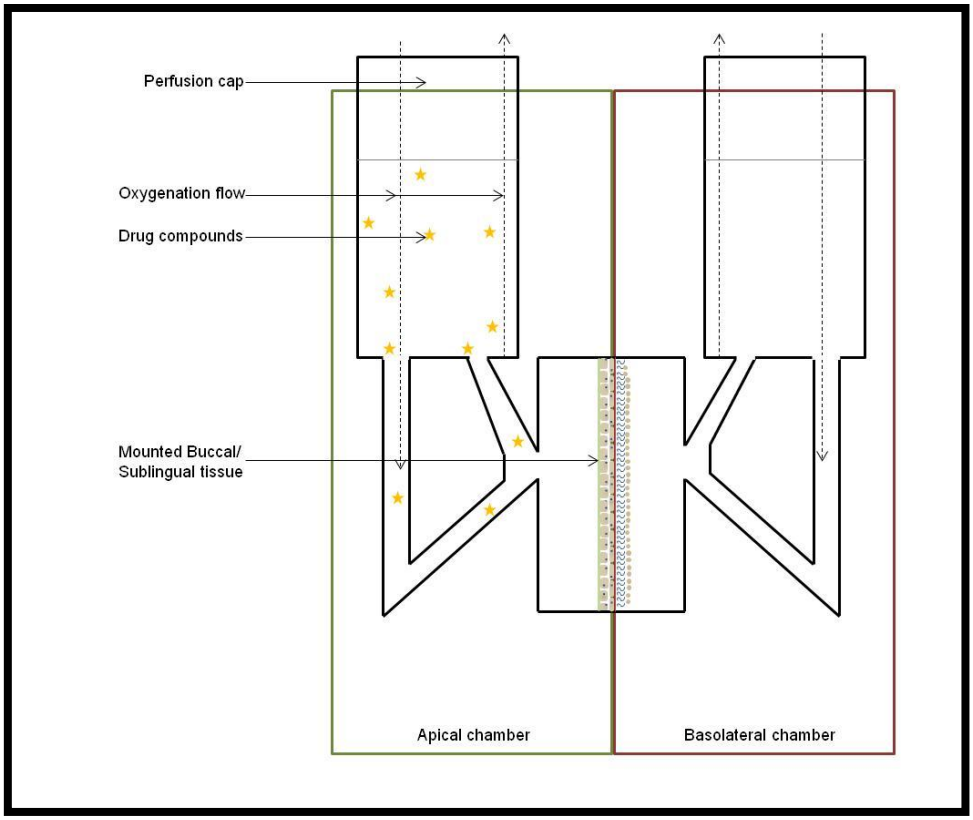


Figure 4.1: Schematic illustration of the Sweetana-Grass diffusion chamber setup for measurement of the permeation of *S. aethiopicus* marker molecules across excised buccal and sublingual pig tissues adapted from (https://warneronline.com/img_lg/660008_Navicyte-Vertical_gr.jpg)

4.5 Culturing and seeding of Caco-2 cell monolayers for bi-directional transport

4.5.1 *Culturing of Caco-2 cell monolayers*

The human colorectal adenocarcinoma cell line (Caco-2 cells; ECACC) was utilised for *in vitro* transport studies and was obtained from the European Collection of Cell Cultures (ECACC) by Sigma Aldrich, Johannesburg, South Africa. The cells were cultured in high-glucose Dulbecco's Modified Eagle's Medium (DMEM; Separations, Randburg, South Africa) supplemented with 10% foetal bovine serum (The Scientific Group, Johannesburg, South Africa); 1% non-essential amino acids (Whitehead Scientific, Cape Town, South Africa); 1% penicillin/streptomycin (10 000 u/ml) (Separations, Johannesburg, South Africa); 2 mM L-glutamine (Whitehead Scientific, Cape Town, South Africa); and 1% amphotericin B (250 µg/ml) (The Scientific Group, Randburg, South Africa).

The Caco-2 cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 and 95% O_2 in a Galaxy 170R incubator (Eppendorf Company, Stevenage, UK). The culture medium was changed every 2-3 days. Once the cells were sub-confluent (50%) they were either sub-cultured by means of trypsinisation or used for seeding onto Transwell® membrane inserts.

4.5.2 *Seeding of Caco-2 cells onto Transwell® membrane filters*

Prior to seeding, the Caco-2 cells were counted with a haemocytometer using a microscope (Nikon Eclipse TS100/TS100F, Nikon Instruments, Tokyo, Japan) and Trypan blue staining (Sigma Aldrich). A cell suspension was attained through trypsinisation with Trypsin-Versene (Whitehead scientific). After detachment of the cells, 6 ml pre-warmed growth medium was added to the flask whilst agitating with a pipette to ensure a suspension consisting of single cells. The cell suspension was counted and subsequently diluted to a concentration of 20 000 cells/ml. Cells were seeded onto Transwell® plates with polycarbonate (PC) treated membranes (Corning Costar ® Corporation, Tewksbury, USA) with a pore size of 0.4 µm and a surface area of 4.67 cm^2. Seeding of the cells were performed in a laminar flow hood by pipetting 2.5 ml of the final cell suspension into each apical chamber of the wells of the Transwell® plates. The Caco-2 cells were grown for 21-24 days on these membrane filters to yield intact epithelial cell monolayers; and the growth medium was replaced every second day under sterile conditions. All transport experiments were conducted between passage 50 and 60. Figure 4.2 illustrates these monolayers prior to the commencing of bi-directional transport.

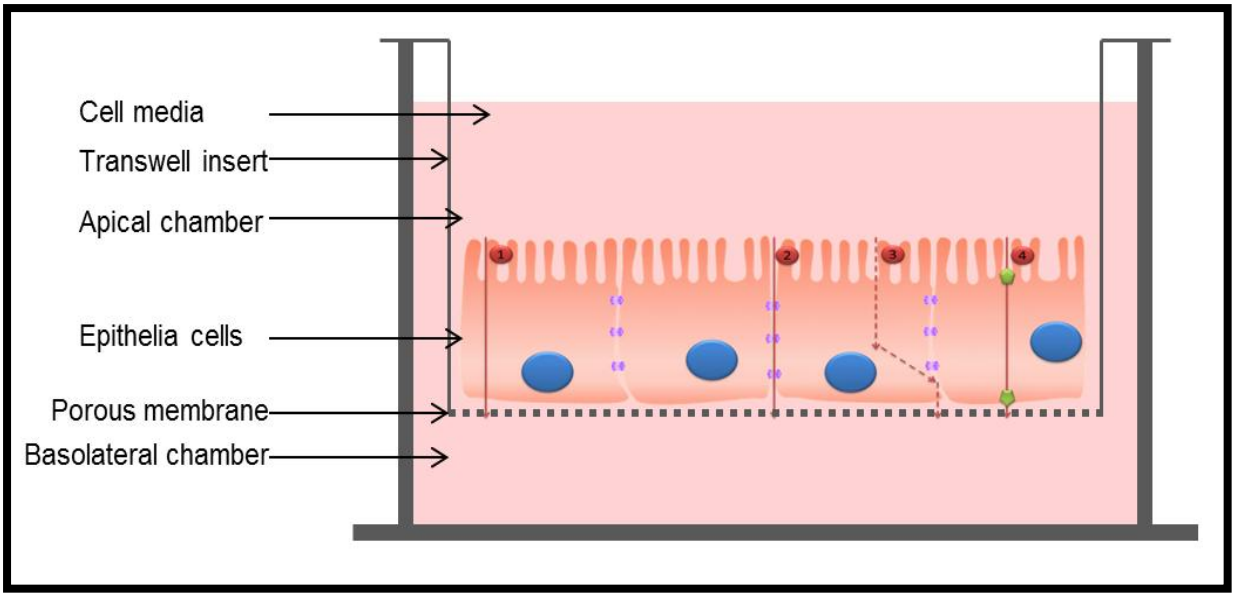


Figure 4.2: Diagram depicting a Caco-2 cell monolayer on a Transwell® membrane (Tavelin *et al*., 2002:238)

4.6 Bi-directional transport across Caco-2 cell monolayers

For transport studies in the AP-BL direction, the growth medium was removed from the basolateral chamber with an aspirator and replaced with 2.5 ml pre-warmed DMEM, buffered at pH 7.4 with 25 mM *n*-(2-hydroxymethyl) piperazine-*N*-(2-ethanesulfonic acid) or HEPES (The Scientific group, Biochrom, Randburg, South Africa). The TEER was measured using a Millicell ERS meter (Merck, Millipore, Darmstadt, Germany) connected to chopstick electrodes. All of the cells were incubated in a CO_2 incubator for 30 min at 37°C. Following incubation the growth medium in the apical chamber was replaced with 2.5 ml of each test solution. Samples (200 µl) were withdrawn from the basolateral chambers at pre-determined time intervals (20, 40, 60, 80, 100, 120 min) and replaced with pre-heated transport buffer after each withdrawal. The plates were incubated in a CO_2 incubator at 37°C between withdrawals. Subsequently, a final TEER measurement was taken after 120 min to confirm the monolayer integrity after exposure of the cells to the test solutions. All experiments were conducted in triplicate at 37ºC in a humidified atmosphere of 95% air and 5% CO_2. All samples were stored in HPLC vials at -80°C until these samples were analysed by means of LC/MS.

For permeability studies in the BL-AP direction, the TEER was measured after the growth medium was removed from the apical chamber, and replaced with 2.5 ml pre-heated DMEM. Following a 30 min incubation time, the growth medium was removed from the basolateral chamber and replaced with 2.5 ml test solutions. Samples (200 µl) were withdrawn from the

apical side at 20, 40, 60, 80, 100, and 120 min time intervals; and replaced with 200 µl pre-heated buffered DMEM (37°C). The plates were incubated in a CO_2 incubator at 37°C between withdrawals. A final TEER measurement was made at 120 min to confirm the monolayer integrity after exposure of the cells to the test solutions. All samples were stored in HPLC vials at -80°C pending analysis of the samples by means of LC/MS.

4.7 Liquid chromatography coupled to mass spectrometry (LC/MS) analysis of *Siphonochilus aethiopicus* transport samples

Liquid chromatography (LC) analyses were performed on a Waters Acquity Ultra Performance Liquid Chromatographic system with a PDA detector (Waters, Milford, MA, USA). LC separation was achieved on a Waters Acquity UPLC BEH C_{18} column (150 mm × 2.1 mm, i.d. 1.7 µm particle size), maintained at 40°C. The mobile phase consisted of 0.1% formic acid in water (solvent A) and acetonitrile (solvent B), which was set at a flow rate of 0.3 ml/min. The gradient elution was as follows: 80% A:20% B and maintained for 1 min; the mobile phase was changed to 65% A:35% B which was conserved for 9 min; the mobile phase was changed again to 50% A:50% B and maintained for 1 min; subsequently, it was altered to 10% A:90% B for 1 min and changed back to the initial ratio in 0.5 min. The running time was 18 min. All of the samples were injected into the mobile phase with an injection volume of 1.0 µl (full-loop injection). Mass spectrometry was operated in a positive ion electro spray mode. Nitrogen (N_2) was used as the desolvation gas. The desolvation temperature was set to 450°C at a flow rate of 550 L/Hr and the source temperature was set at 100°C. Capillary and cone voltages were set to 3 500 and 45 V, respectively. Data were collected between 100 and 1000 *m/z*.

4.8 Data analysis

The apparent permeability coefficient (P_{app} x 10^{-6} cm/s) values of each marker molecule in the (AP-BL) direction was calculated for the porcine buccal and sublingual tissue and in each direction (AP-BL and BL-AP) across the Caco-2 cell monolayers using Equation 1 (Tarirai *et al*., 2012:257). The P_{app} values represent the diffusion rate of the compound normalised for surface area of the cell monolayers and drug concentration on the donor side.

$$P_{app} = \frac{dQ}{dt}\left(\frac{1}{A.C_0.60}\right) \quad \textbf{(4.1)}$$

Where dQ/dt represents the increase in the amount of drug present in the acceptor compartment over time (i.e. the permeation rate as µg/s); A denotes the effective surface

area (cm^2) of the cell monolayer that is exposed to the test solutions, and C_0 (µg/ml) is the initial concentration of the compound present in the donor chamber.

Efflux ratio (ER) is an indication of asymmetry in the directional transport of a given compound. The ER of marker molecules in *S. aethiopicus* crude extract was calculated by means of Equation 2 (Hansen & Nilsen, 2009:88; Hellum & Nilsen, 2008:468; Tarirai *et al*., 2012:257).

$$ER = \frac{P_{app}(BL - AP)}{P_{app}(AP - BL)} \quad \textbf{(4.2)}$$

Where AP-BL is the transport in the apical to basolateral direction and BL-AP indicates the transport in the basolateral to apical direction.

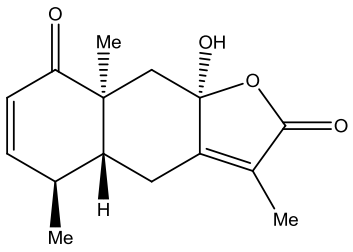
4.9 Introduction to results and discussion

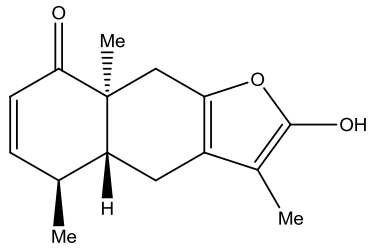
To establish if the isolated marker molecules of *S. aethiopicus* are absorbed, at which absorption site and in to what extent; transport across porcine buccal and sublingual tissues as well as across Caco-2 cell monolayers were employed. Due to the fact that the rhizomes of this medicinal plant are traditionally chewed, the assumption was made that the active compounds might possibly be absorbed in the mouth cavity. Transport across mucosal membranes of the oral cavity was therefore the main focus, but bi-directional transport across Caco-2 cell monolayers was used as an additional *in vitro* model to determine if there would be any absorption of the marker compounds when swallowed into the gastro-intestinal tract.

The concentration (40 mg/ml) of the crude extract employed in the study was selected based on a similar study by Shikanga *et al*. (2011:261); they also used medicinal plant material that is traditionally chewed. A commercial product was used to determine a concentration that would be expected after administration of the recommended dose (1.9 mg/ml). To ensure the membrane integrity of the porcine tissue (buccal, sublingual) and Caco-2 cell monolayers, TEER measurements were conducted. Both methanol and water extracts of *S. aethiopicus* were utilised in the permeability experiments.

A validated LC/MS method was used to analyse the concentration of the two marker molecules (AG 1 and AG 2) present in *S. aethiopicus* crude extract, which were previously described in Chapter 3 (shown in Table 4.1), in the transport samples. For the bi-directional transport across the Caco-2 cell monolayers the apparent permeability coefficient (P_{app}) and efflux ratio values were calculated.

Table 4.1: Isolated marker molecule structures and named as determined by NMR

Marker molecule	Structure	Name
AG 1		(4aR,5S,8aS,9aR)-9a-hydroxy-3,5,8a-trimethyl-4a,5,9,9a-tetrahydronaphtho[2,3-b]furan-2,8(4H,8aH)-dione

AG 2		(4aR,5S,8aS)-2-hydroxy-3,5,8a-trimethyl-4a,5,8a,9-tetrahydronaphtho[2,3-b]furan-8(4H)-one

4.10 Transport across porcine buccal and sublingual tissue

4.10.1 *Average transepithelial electrical resistance (TEER) values for the excised buccal and sublingual tissues before and after exposure to different Siphonochilus aethiopicus extracts*

The average TEER values for the buccal and sublingual tissues (Tables 4.2 & 4.3) indicate that the membrane integrity was not compromised during any of the permeation studies, and the transport data obtained from these experiments are therefore reliable. Furthermore, the average membrane thickness was measured before the membranes were mounted onto the chambers. As seen in Tables 4.2 and 4.3, both the buccal and sublingual tissues were well within the required parameters of 1.0 ±0.4 mm and 0.7 ±0.3 mm, respectively.

Table 4.2: Average TEER and thickness values for the excised buccal tissues before and after exposure to different *S. aethiopicus* extracts

***S. aethiopicus* crude extracts**	**Average TEER value (ohm/cm^2) (n=3)**		**Average membrane thickness, (n=3)**
	AP-BL		
	0 min	**120 min**	
Methanol extract (40 mg/ml)	417.67±90.66	509.83±123.35	0.788 mm
Water extract (40 mg/ml)	395.00±12.03	466.33±21.73	0.837 mm
Water extract (1.9 mg/ml)	344.00±16.52	442.67±14.57	0.825 mm

Table 4.3: Average TEER and thickness values for the excised sublingual tissues before and after exposure to different *S. aethiopicus* extracts

***S. aethiopicus* crude extracts**	**Average TEER value (ohm/cm^2) (n=3)**		**Average membrane thickness (n=3)**
	AP-BL		
	0 min	**120 min**	
Methanol extract (40 mg/ml)	86.83±69.25	104.00±106.49	0.670 mm
Water extract (40 mg/ml)	28.00±2.94	51.00±16.64	0.603 mm
Water extract (1.9 mg/ml)	28.33±4.72	47.33±6.02	0.602 mm

4.10.2 *Transport of marker molecules from Siphonochilus aethiopicus extracts across buccal tissue*

The percentage transport of the two selected marker molecules for the water extract (40 mg/ml) in the AP-BL direction across excised buccal tissue is presented as a function of time in Figure 4.3.

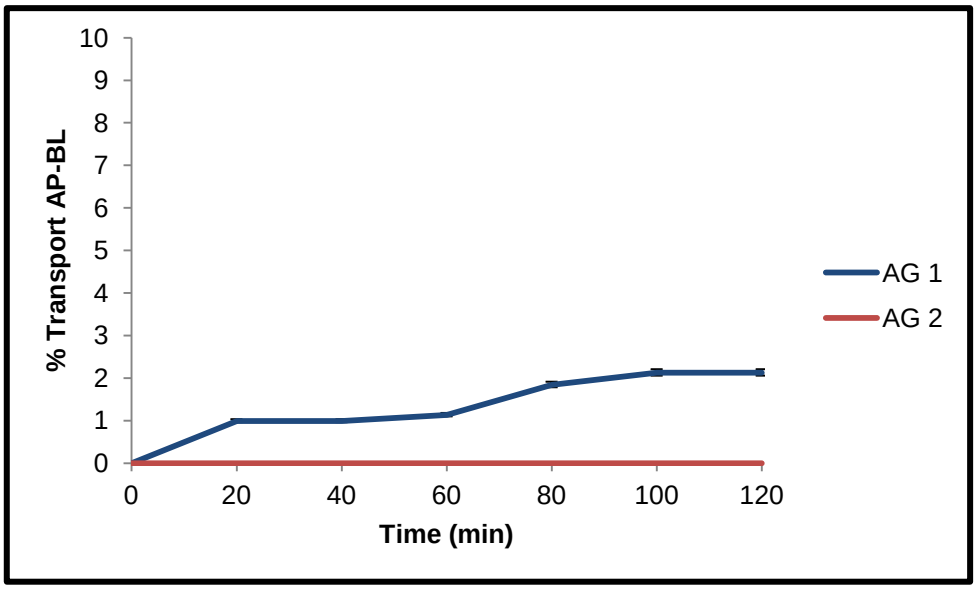


Figure 4.3: Percentage transport of marker molecules (AG 1 and AG 2) from *S. aethiopicus* water extract (40 mg/ml) across porcine buccal tissue

Exposure of the methanol extract (40 mg/ml) to buccal tissue did not result in any transport of any of the marker molecules (results not shown), whereas the water extract (40 mg/ml) showed transport for the marker molecule AG 1 only. From the data, it can be deduced that the compounds in the methanol extract was immiscible with the KRB used in the transport studies and improved solubility of the compounds was achieved with the water extract, resulting in the transport that is seen for the water extract. As indicated by the transport data of the water extract, no transport of the marker molecule AG 2 was observed.

Furthermore, the 1.9 mg/ml water extract resulted in no measurable transport of either of the marker molecules across the buccal tissue (results not shown). The commercial product that was used to determine this recommended dosage concentration is formulated as a tablet. It should be considered that the tablet would rather be swallowed than kept within the oral cavity, therefore the duration and exposure time in the buccal cavity is limited, and intestinal absorption would rather be expected. Because no buccal transport was observed, the results confirmed that no assumptions can be made for buccal absorption from this concentration as it is simply too low to be absorbed across the buccal mucosa, and alternative transport models should be considered to test whether this low concentration is being absorbed intestinally.

The average P_{app} values for the individual marker molecules across excised buccal tissue obtained when the 40 mg/ml water extract was applied can be seen in Table 4.4.

Table 4.4: Average P_{app} values for selected marker molecules of *S. aethiopicus* extract across porcine buccal tissues

***S. aethiopicus* crude extract**	**Average P_{app} value (x 10^{-6} cm/s) (n=3)**	
	AP-BL	
	AG 1	**AG 2**
Water extract (40 mg/ml) Buccal	0.61±0.02	-

The P_{app} value for the marker molecule; AG 1 is 0.61±0.02 x 10^{-6} cm/s, which suggests that incomplete absorption of the compound occurred as literature states that if a compound has a P_{app} value smaller than 1 x 10^{-6} cm/s, it is indicative of relatively poor absorption of the compound (Le Ferrec *et al*., 2001:659).

4.10.3 *Transport of marker molecules from Siphonochilus aethiopicus extracts across sublingual tissue*

The percentage transport of the two selected marker molecules in the AP-BL direction across sublingual tissue for the water extract (40 mg/ml) is presented as a function of time in Figure 4.4.

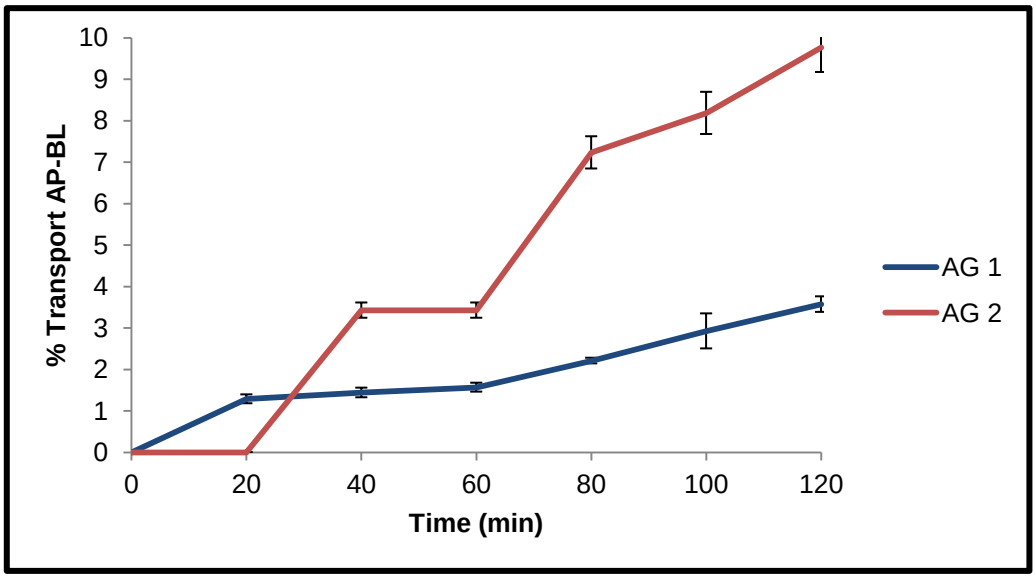


Figure 4.4: Percentage transport of marker molecules (AG 1 and AG 2) from *S. aethiopicus* water extract (40 mg/ml) across porcine sublingual tissue

Similar to what was previously observed for the buccal tissue, the marker molecules of the methanol extract (40 mg/ml) also revealed no transport across the sublingual tissue (results not shown), as discussed previously, this can probably be attributed to the insolubility of the methanol extract in the KRB.

However, both of the marker molecules (AG 1 and AG 2) from the water extract (40 mg/ml), indicated transport across the sublingual tissue. It was reasoned from the data that sublingual tissue showed an overall higher percentage transport when compared to the buccal tissue. This was expected as it is supported by literature that sublingual tissue has less keratinised cells compared to the buccal tissue; and therefore, it is more permeable compared to buccal tissue (Dodla & Vermulgen, 2013:40). Furthermore, the percentage transport of the marker molecule AG 2 (9.77%) is considerably higher than the percentage transport of the AG 1 (3.16%) marker molecule.

Transport of marker molecules from the water extract at the prescribed dosage concentration (1.9 mg/ml) again revealed no transport across the sublingual tissue; and this can probably be ascribed to similar reasons as discussed for the buccal tissue.

The average P_{app} values for the individual compounds obtained in the 40 mg/ml water extract were calculated. Results can be seen in Table 4.5.

Table 4.5: Average P_{app} values for selected marker molecules of *S. aethiopicus* extract across porcine sublingual tissues

***S. aethiopicus* crude extract**	**Average P_{app} value (x 10^{-6} cm/s) (n=3)**	
	AP-BL	
	AG 1	**AG 2**
Water extract (40 mg/ml) Sublingual	0.86±0.11	3.15±0.19

The P_{app} values are 0.86±0.11 x 10^{-6} cm/s and 3.15±0.19 x 10^{-6} cm/s for AG 1 and AG 2, respectively. For AG 1 the data points to incomplete absorption of the compound from the water extract. As previously stated, poor absorption is indicated if a compound has a P_{app} value smaller than 1 x 10^{-6} cm/s (Le Ferrec *et al*., 2001:659). The contrary is true if a compound has a P_{app} value higher than 1 x 10^{-6} cm/s, indicating that it was completely absorbed. This result was seen for the marker molecule AG 2, and can possibly be due to the more lipophilic nature of compound AG 2.

4.10.4 *A comparison of the mucus membrane permeability for Siphonochilus aethiopicus water extracts across the buccal and sublingual tissues.*

As discussed in Chapter 2, the literature indicates that sublingual tissue is more permeable than buccal tissue (Bhati & Nagrajan, 2012:661; Dodla & Vermulgen, 2013:40), which likely contributed to the differences observed in the level of transport between these two tissues (i.e. buccal: AG 1 (2.13%), AG 2 (0%) and sublingual: AG 1 (3.16%), AG 2 (9.77%)). The higher resistance to permeation of the buccal membrane compared to the sublingual membrane may have been a result of either the membrane thickness (buccal- 0.817 µm; sublingual- 0.625 µm), or the more hydrophilic nature of the connective tissue found in the buccal membrane which acts as a superior barrier to lipophilic compounds (Nicolazzo *et al*., 2003:2407). Furthermore, there are two permeation pathways for passive drug absorption through oral mucosa, namely paracellular and transcellular transport. Drugs can

simultaneously transverse through both of these pathways, although one is usually more effective than the other, depending on the chemical nature of the compounds (Dodla & Vermulgen, 2013:41; Shojaei *et al*., 2001:71). The cell membranes are relatively lipophilic and can cause severe difficulty for transport of hydrophilic compounds. In contrast with the previous statement, the hydrophilic nature of the intercellular spaces between the cells poses a significant barrier for lipophilic molecules (Shojaei *et al*., 2001:71). Thus, it is suggested in literature that hydrophilic compounds will tend to permeate through the intercellular spaces, i.e. the paracellular route, whereas lipophilic drugs would rather follow the transcellular route as it would be easier for a lipophilic compound to partition into the cell membranes (Bhati & Nagrajan, 2012:663; Dodla & Vermulgen, 2013:41). The connective tissue of the buccal membrane also alters the diffusion characteristics of the compounds, in addition to increasing the length of the diffusional pathway (Nicolazzo *et al*., 2003:2407). The results obtained in this study indicated that marker molecule AG 1 is more polar and, thus, more hydrophilic; therefore it can be posited that this compound is mainly absorbed through paracellular diffusion.

The results obtained for the marker molecule AG 2 indicated that this is the less polar, thus more lipophilic compound, and it is suggested that this compound will mainly be absorbed utilising the transcellular route. The lack of transport of AG 2 across the buccal membrane can possibly be explained by the presence of connective tissue in the buccal membrane that has a more hydrophilic nature; and this would have posed a major barrier to this compound.

The P_{app} values for both the buccal and sublingual transport of marker molecule AG 1 were lower than 1 $x10^{-6}$ cm/s, indicating that in both of these tissues incomplete absorption of the compound occurred. The data revealed that the P_{app} value for marker molecule AG 2 was higher than 1 x 10^{-6} cm/s across the sublingual tissue, which indicated that this compound was probably completely absorbed across this excised tissue (Le Ferrec *et al*., 2001:659).

4.11 Transport across Caco-2 cell monolayers

4.11.1 *Average transepithelial electrical resistance (TEER) for bi-directional transport across Caco-2 cell monolayers*

For the Caco-2 cell monolayers, a minimum TEER value of 150 ohm/cm^2 is required as indicative of an intact monolayer (Alqahtani *et al.,* 2013:2). Results showing the average TEER values obtained for the Caco-2 cell monolayers used in the transport experiments are provided in Table 4.6.

Table 4.6: Average TEER values for Caco-2 cell monolayers before and after exposure to the various *S. aethiopicus* extracts

***S. aethiopicus* crude extracts**	**Average TEER value (ohm/cm^2) (n=3)**			
	AP-BL		**BL-AP**	
	0 min	**120 min**	**0 min**	**120 min**
Methanol extract (NWU: 40 mg/ml)	344.33±12.39	177.00±4.36	346.67±16.77	144.67±16.65
Methanol extract (TUT: 40 mg/ml), 5% Methanol	287.00±61.58	134.00±13.08	-	-
Methanol extract (TUT: 40 mg/ml)	344.33±20.21	90.33±4.93	-	-

From Table 4.6 it can be seen that all of the Caco-2 cell monolayers remained intact, even after exposure to the different extracts, with the exception of the membrane exposed to the methanol extract prepared from the TUT plant material. The relatively low TEER value after 120 min indicates that the integrity of the monolayer was compromised during the exposure to the methanol extract.

The water extracts of *S. aethiopicus* was not tested for bi-directional transport across Caco-2 cells as intestinal absorption of the compounds was achieved with the methanol extracts.

4.11.2 *Bi-directional transport across Caco-2 cell monolayers*

During the study, an experiment was conducted to see whether there is any transport of the two marker molecules across the cell monolayers using the LC/MS detection method. All samples were frozen at -80°C prior to the analyses. During this study, however, difficulties regarding the solubility of the prepared methanol extract in the DMEM buffer was observed, because the extract has a syrup-like consistency and formed a glomerulus in the DMEM buffer. Therefore, a second study was conducted in an attempt to overcome the solubility problem. The extracts in the second study was therefore first dissolved in 5% methanol, prior to the dilution in DMEM buffer.

The results from the first transport study are given in Figure 4.5 for the AP-BL direction and Figure 4.6 for the BL-AP direction.

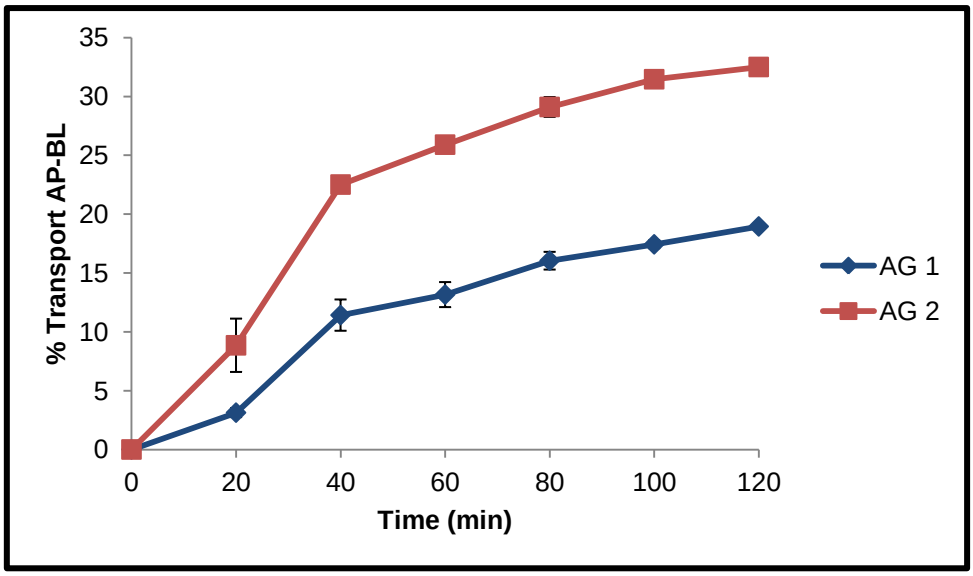


Figure 4.5: Percentage transport of *S. aethiopicus* methanol extract (40 mg/ml) across Caco-2 cell monolayers, in the AP-BL direction

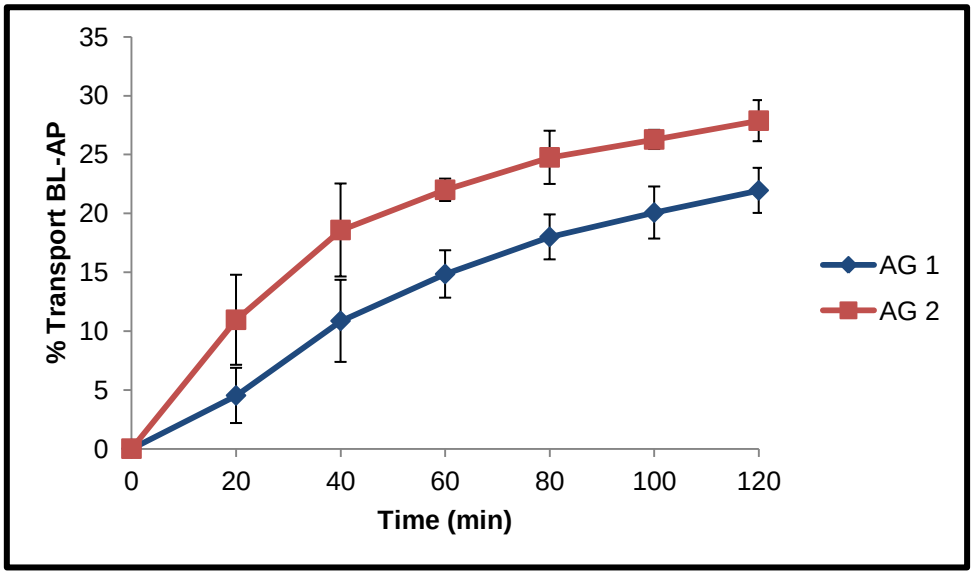


Figure 4.6: Percentage transport of *S. aethiopicus* methanol extract (40 mg/ml) across Caco-2 cell monolayers, in the BL-AP direction

From Figures 4.5 and 4.6 it is clear that both AG1 and AG2 were transported. The results obtained from this study indicates that intestinal absorption of the marker molecules tested was achieved, even though the extract was not dissolved completely in the DMEM buffer.

The second experiment was only performed in an AP-BL direction, and the average transport of AG 1 and AG 2 achieved are given in Figure 4.7 and 4.8 respectively.

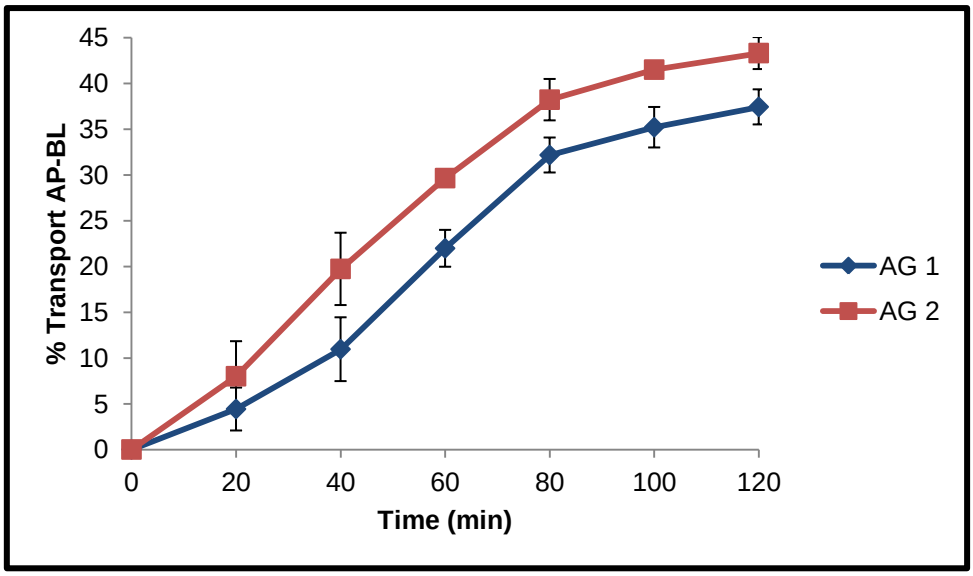


Figure 4.7: Percentage transport across Caco-2 cell monolayers of *S. aethiopicus* methanol extract (40 mg/ml), dissolved in 5% methanol prior to addition of DMEM, in the AP-BL direction

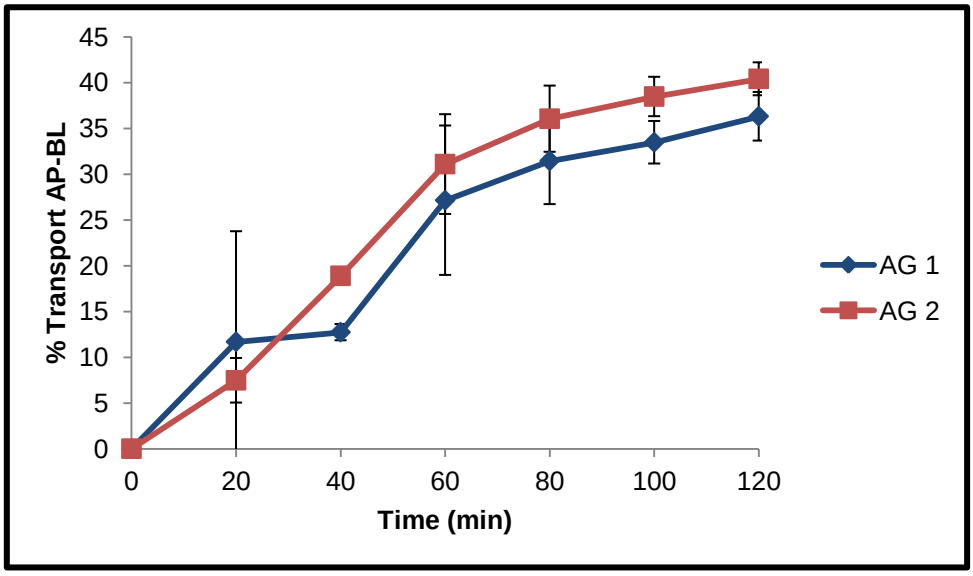


Figure 4.8: Percentage transport of *S. aethiopicus* methanol extract (40 mg/ml) across Caco-2 cell monolayers, transport in the AP-BL direction

A slight increase were observed after the extract was dissolved in methanol prior to dilution in DMEM (Figure 4.7), when compared to the extracts that was directly diluted in the DMEM buffer (Figures 4.5, 4.6 & 4.8). The higher percentage transport observed for these marker molecules was probably due to the improved solubility of the extract in the 5% methanol prior to the addition of DMEM.

In Table 4.7, the average P_{app} values and efflux ratio values of the two marker molecules are shown.

Table 4.7: Average P_{app} values for the different *S. aethiopicus* extracts during transport across Caco-2 cell monolayers

***S. aethiopicus* crude extracts**	**Average P_{app} (x 10^{-6} cm/s) value (n=3)**					
	AP-BL		**BL-AP**		**Efflux**	
	AG 1	**AG 2**	**AG 1**	**AG 2**	**AG 1**	**AG 2**
Methanol extract (NWU: 40 mg/ml)	5.74±0.18	9.51±0.30	6.63±0.23	7.67±0.26	1.16±0.21	0.81±0.28
Methanol extract (TUT: 40 mg/ml), 5% Methanol	12.43±0.24	13.72±0.19	-	-	-	-
Methanol extract (TUT: 40 mg/ml)	10.90±0.57	12.76±1.08	-	-	-	-

Artursson *et al*. (2001:30) stated that a P_{app} value larger than 1 x 10^{-6} cm/s is an indication of complete drug absorption, and a P_{app} value smaller than 1 x 10^{-7} cm/s is an indication of incomplete drug absorption. Thus, all of the permeation experiments across the Caco-2 cell monolayers offered an indication of good drug absorption, since all of the P_{app} values were greater than 1 x 10^{-6} cm/s.

Similar results regarding the transport of marker molecule AG 2 were also observed in the Caco-2 cell model, and it was greater than that observed for the marker molecule AG 1. The percentage transport across the intestinal epithelia observed was considerably higher than for the buccal and sublingual membranes, supporting literature which stated that intestinal epithelia is more permeable in comparison to buccal and sublingual tissue (Dodla & Vermulgen, 2013:40). This is due to the multi-cellular nature of the buccal and sublingual tissues, whereas the Caco-2 model consists of a monolayer. Compounds with an efflux ratio of 2 to 3 are normally classified as P-gp substrates (Balimane *et al*., 2006:8). Thus, from the results it is clear that neither marker molecules AG 1, nor AG 2, can be classified as P-gp substrates. In a study conducted by Balimane *et al.* (2006:9), they found that performing bi-directional transport studies at high concentrations (50 µM or more) often leads to saturation of the efflux transporter, and at these high concentrations, the P-gp is functionally knocked

out leading to false negative data. The relatively high concentration used in this study could have been responsible for saturation of the P-gp transporters, potentially increasing the permeation in both directions (Balimane *et al*., 2006:10). Results obtained suggested the possibility of false negative results, and the use of lower concentrations should be considered in the future.

The decrease seen in the TEER values when comparing the before and after values (Table 4.6), indicate the compounds found in the extract either opens the tight junctions between the epithelial cells, or may damage the monolayer. The majority of the tight junctions located between the epithelial cells may have lost their integrity when they were incubated with the methanol extract which may have caused damaged to the cell monolayer, resulting in a leakier cell membrane. The extent of the damage to the cell monolayer is normally concentration dependant and therefore the high concentration was possibly responsible for the damage observed (Balimane *et al*., 2006:10). Therefore, the transport observed is relatively high. However, as mentioned previously, this can be a false negative result due to the high concentration (40 mg/ml) that was applied to the cells.

If there is a linear relationship between the AP-BL and BL-AP flux (ER close to unity), the mechanism of transport is most likely passive diffusion (Le Ferrec *et al*., 2001:659). Considering all of the above mentioned data, it concludes that the marker molecules AG 1 and AG 2 will most likely be transported via passive diffusion through transcellular absorption.

4.12 Conclusion

In conclusion, transport of the selected marker molecules, AG 1 and AG 2, across buccal and sublingual membranes was only obtained for the 40 mg/ml water extract, while no measurable transport was observed for either the 40 mg/ml methanol extract or the 1.9 mg/ml water extract. Observing the two marker molecules; transport across sublingual mucosal membranes was achieved for both compounds compared to the transport of only marker molecule, AG 1, in the buccal mucosal membranes. The P_{app} values were low for the marker molecule AG 1 and high for AG 2, indicating that AG 2 depicted increased absorption compared to AG 1, which was possibly due to its more lipophilic nature. The Caco-2 cell model showed a relatively high level of transport, as well as high P_{app} values for both marker molecules AG 1 and AG 2 from the 40 mg/ml methanol extract.

CHAPTER 5 - CONCLUSION AND FUTURE PROSPECTS

5.1 Conclusion

The initial aim and objective of the study was to create adequate quality control protocols for the analysis of the indigenous plant; *S. aethiopicus*. This was achieved by employing several analytical techniques to investigate the chemical properties of various different S*. aethiopicus* methanol extracts. The chemical composition of the oil was previously established by (Viljoen *et al*., 2000:) and similar results were observed in this study, however one of the main constituents previously identified (sesquiterpenoid) was not present in either one of the oil samples analysed.

Four marker molecules (most abundant) were selected over the LC/MS spectra of *S. aethiopicus* and isolated by means of prep-HPLC. These compounds were then identified and the structures were determined using NMR. The four marker molecules have not been isolated or identified previously, although the structures revealed similarities to already known compounds. Moreover, these marker molecules were employed as reference standards in the different analytical techniques to identify the chemical composition of different samples. All of the different samples revealed the same chemical composition, but variation regarding the quantity of these chemical compounds among the different samples, was however observed.

The volatiles of *S. aethiopicus* were identified for the first time utilising GCxGC-TOF/MS; this contributes to the future identification and authentication of the plant. The HPTLC fingerprint that was developed during the study can also be used in future to identify, authenticate or even characterise the *S. aethiopicus* plant material, which offers a relatively inexpensive, high-throughput screening test for large quantities of plant material.

The second aim was to determine whether any of the selected marker molecules are absorbed; and where absorption of these compounds mainly occurs. This was achieved by transport studies using porcine buccal and sublingual tissue as a system investigating oral absorption; and Caco-2 cell monolayers as a model to investigate intestinal absorption. These models were used as a starting point, as transport studies have never been done previously, and the assumption was made that the compounds might be absorbed in the oral cavity because the plant is traditionally chewed. From the results obtained it is clear that

transport across the buccal and sublingual cavity of the selected marker molecules is poor and the conclusion can be made that these compounds are most properly being absorbed intestinally. The marker molecules AG 1 and AG 2 were absorbed over the Caco-2 cell monolayer membrane, and thus confirm that these molecules are absorbed by the intestine.

5.2 Future prospects and recommendations

The four marker molecules were not tested for biological activity and it would be recommended for the future to employ these molecules in assays testing the biological activity of *S. aethiopicus*. Known compounds previously isolated and identified by scientist, were all tested for biological activity, however not one of these compounds have previously been tested for absorption, and even though they reveal biological activity, these cannot be accepted until the absorption of these biological active compounds is achieved. It is recommended that these compounds should be tested for intestinal absorption as this might be the main possible site of absorption.

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ANNEXURES

ANNEXURE A

Table A-1: Individual sample preparation of wet plant material

Sample Name	Wet weight (g)	Dried weight (g)	Oven temperature (°C)	Total hours in oven
NW1	165.790	25.330	50-60	22
NW2	1478.067	212.000	50-60	24
NW (Cultivate)	935.404	149.940	50-60	24
FC1	25.482	3.822	50-60	48
FC2	27.813	4.171	50-60	48
FC3	23.126	3.469	50-60	48
FC4	26.112	3.916	50-60	48
FC5	25.913	3.887	50-60	48
FC6	26.453	3.967	50-60	48
FC7	26.443	3.967	50-60	48

Figure A-1: Fresh *S. aethiopicus* rhizomes (Personal photo)

Figure A-2: Individual fresh rhizome of *S. aethiopicus*

Figure A-3: *S. aethiopicus* rhizomes cut into small pieces (Personal photo)

Figure A-4: Dried *S. aethiopicus* rhizomes (Personal photo)

Table A-2: Methanol (MeOH) extract yield

Sample Name	Plant material weight (g)	Plant extract weight (g)	Percentage yield of MeOH extract
NW1	1	0.1230	12.30%
NW2	1	0.1126	11.26%
NW (Cultivate)	1	0.1490	14.90%
FC1	1	0.2150	21.50%
FC2	1	0.2404	24.04%
FC3	1	0.2239	22.39%
FC4	1	0.1812	18.12%
FC5	1	0.2859	28.59%
FC6	1	0.2808	28.08%
FC7	1	0.2648	26.48%
TUT	1	0.0873	8.73%

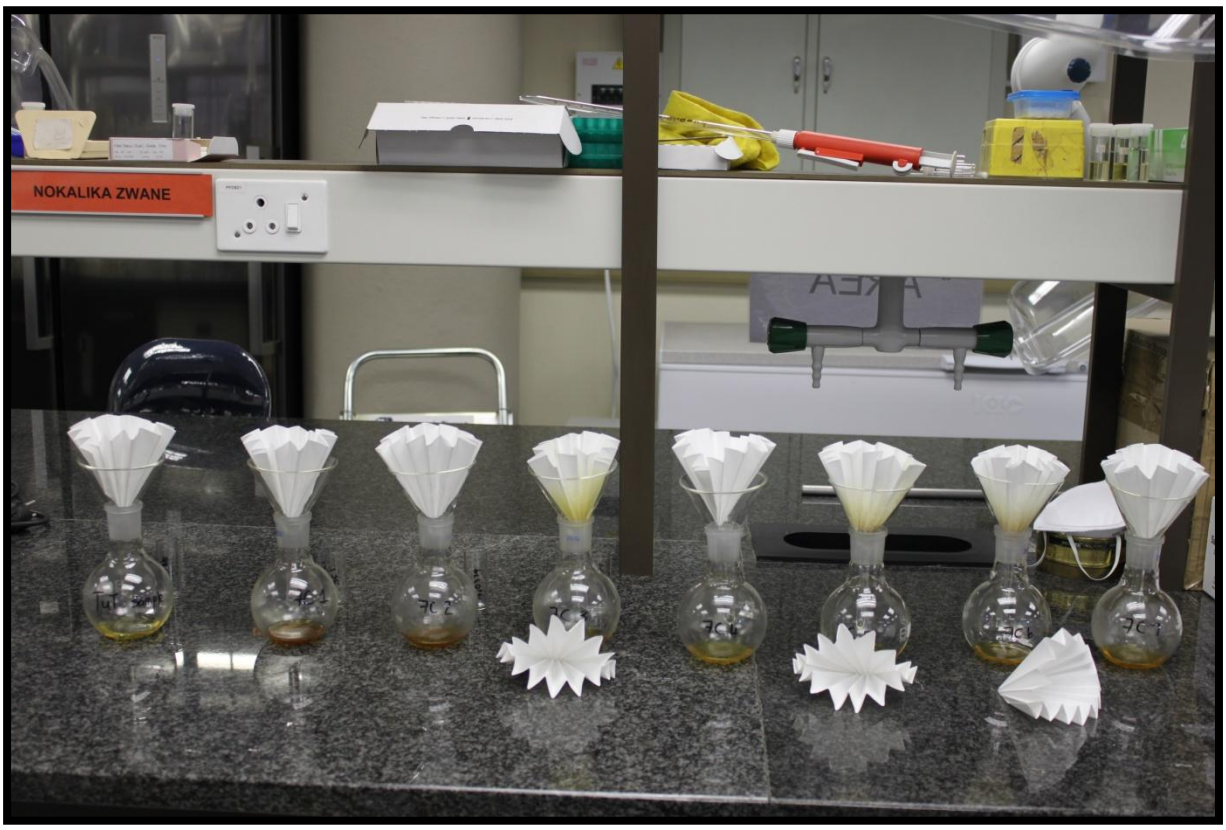

Figure A-5: Methanol: Water extraction of individual *S. aethiopicus* samples

Table A-3: Aqueous extract yield

Sample Name	Plant material weight (g)	Plant extract weight (g)	Percentage yield of aqueous extract
NW2	108.430	7.201	6.64%

ANNEXURE B

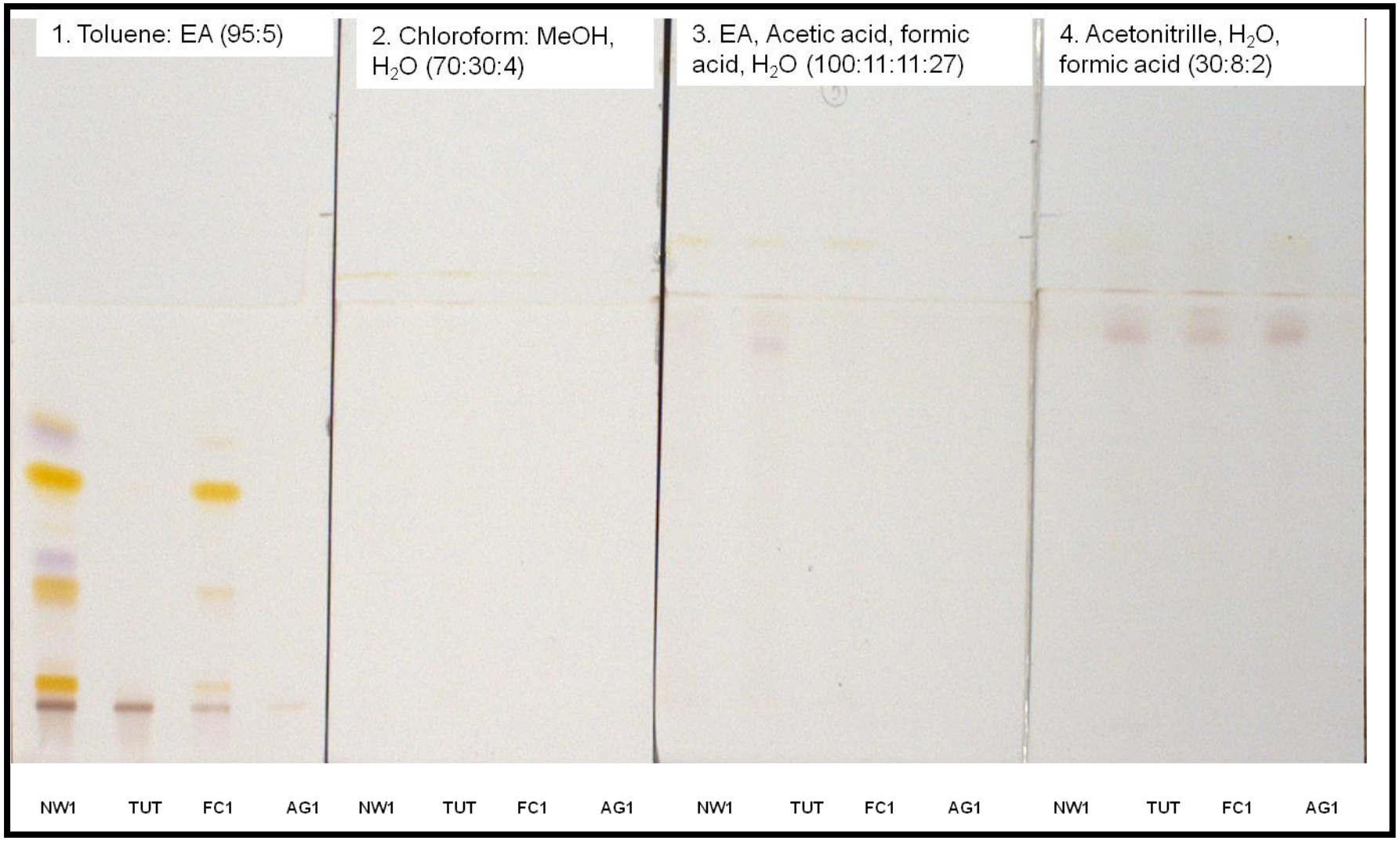

Figure B-1: *Siphonochilus aethiopicus* methanol extract, 5 mg/ml, Anisaldehyde- sulphuric acid dipping reagent, viewing: White R

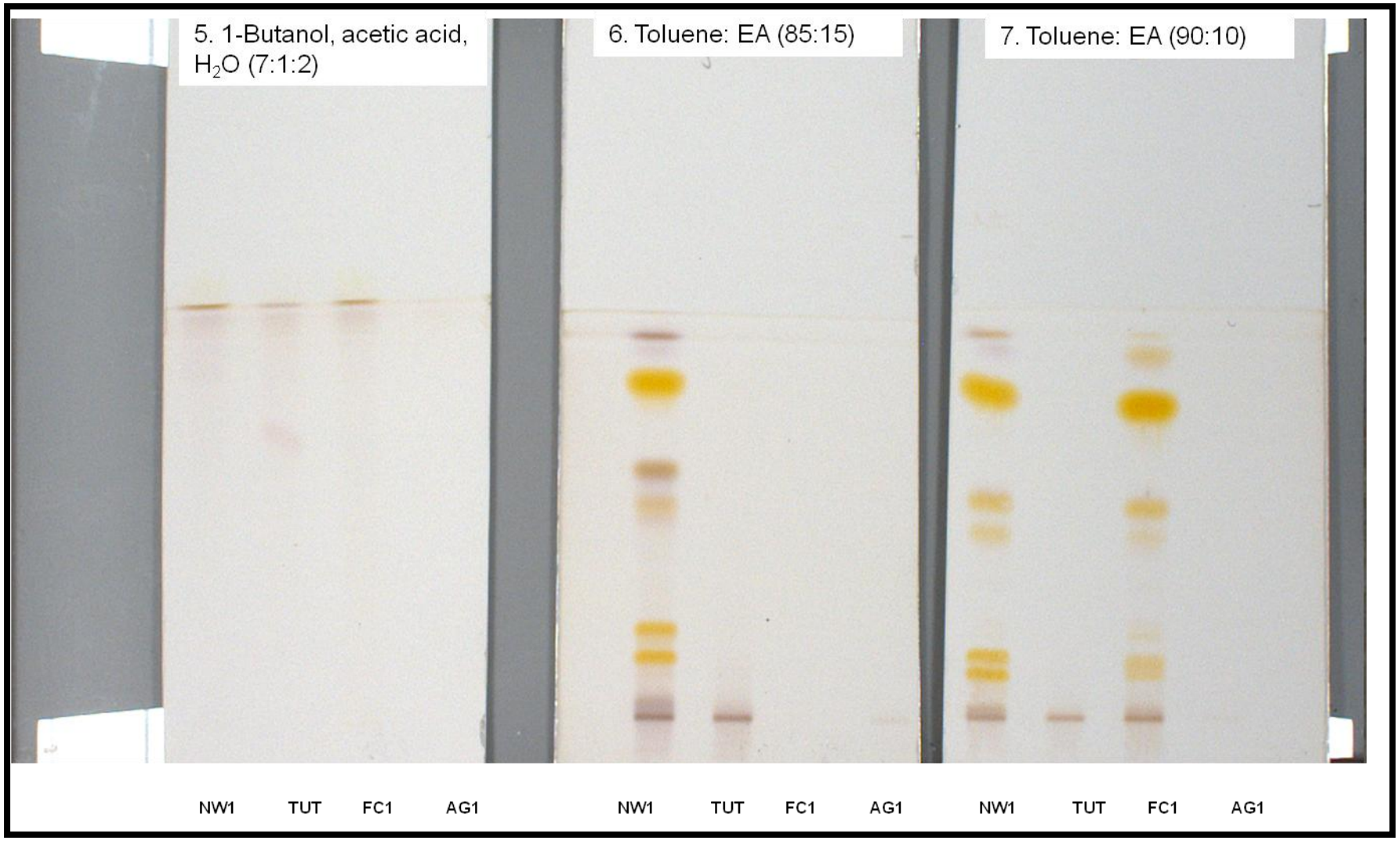

Figure B-2: *Siphonochilus aethiopicus* methanol extract, 5 mg/ml, Anisaldehyde- sulphuric acid dipping reagent, viewing: White R

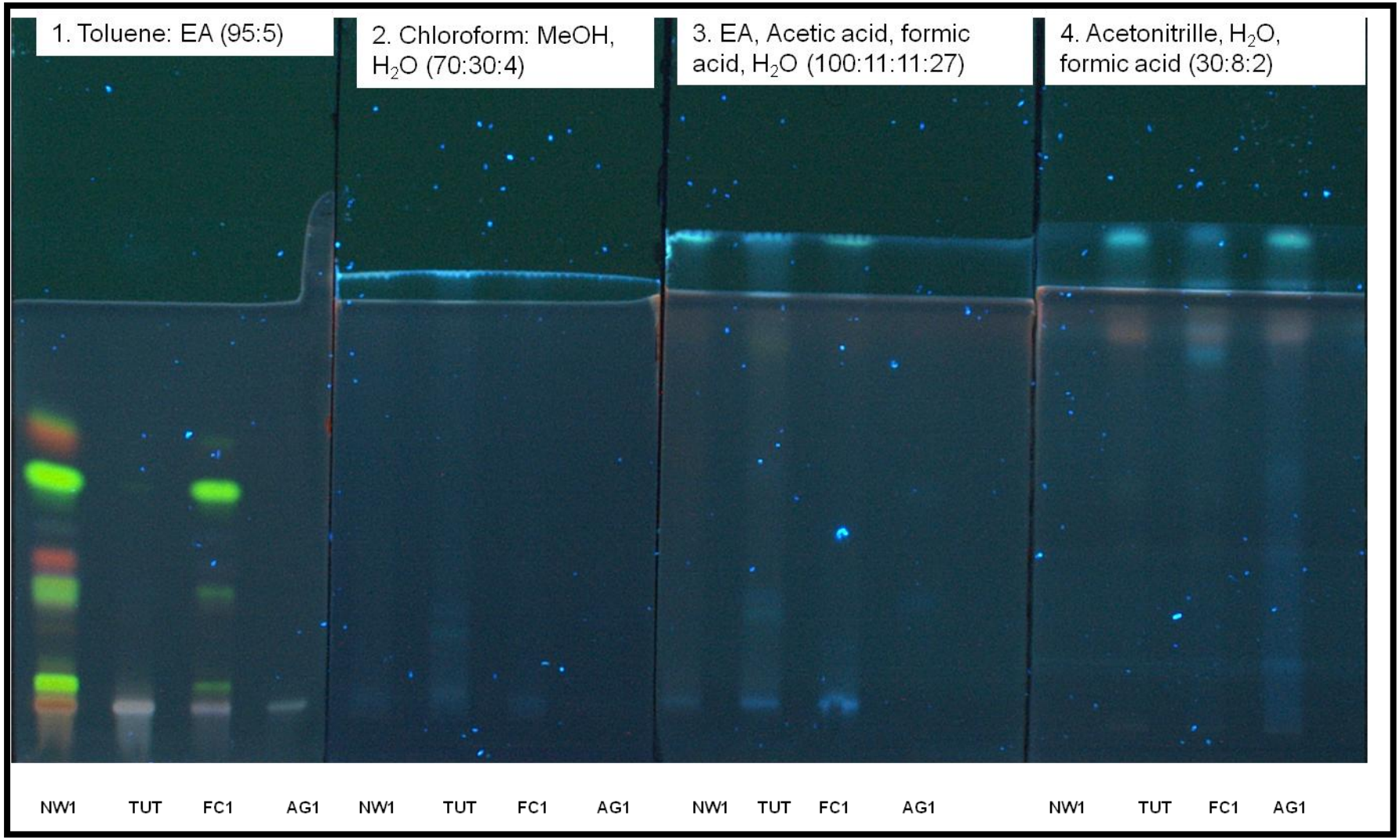

Figure B-3: *Siphonochilus aethiopicus* methanol extract, 5 mg/ml, Anisaldehyde- sulphuric acid dipping reagent, viewing: 366 nm

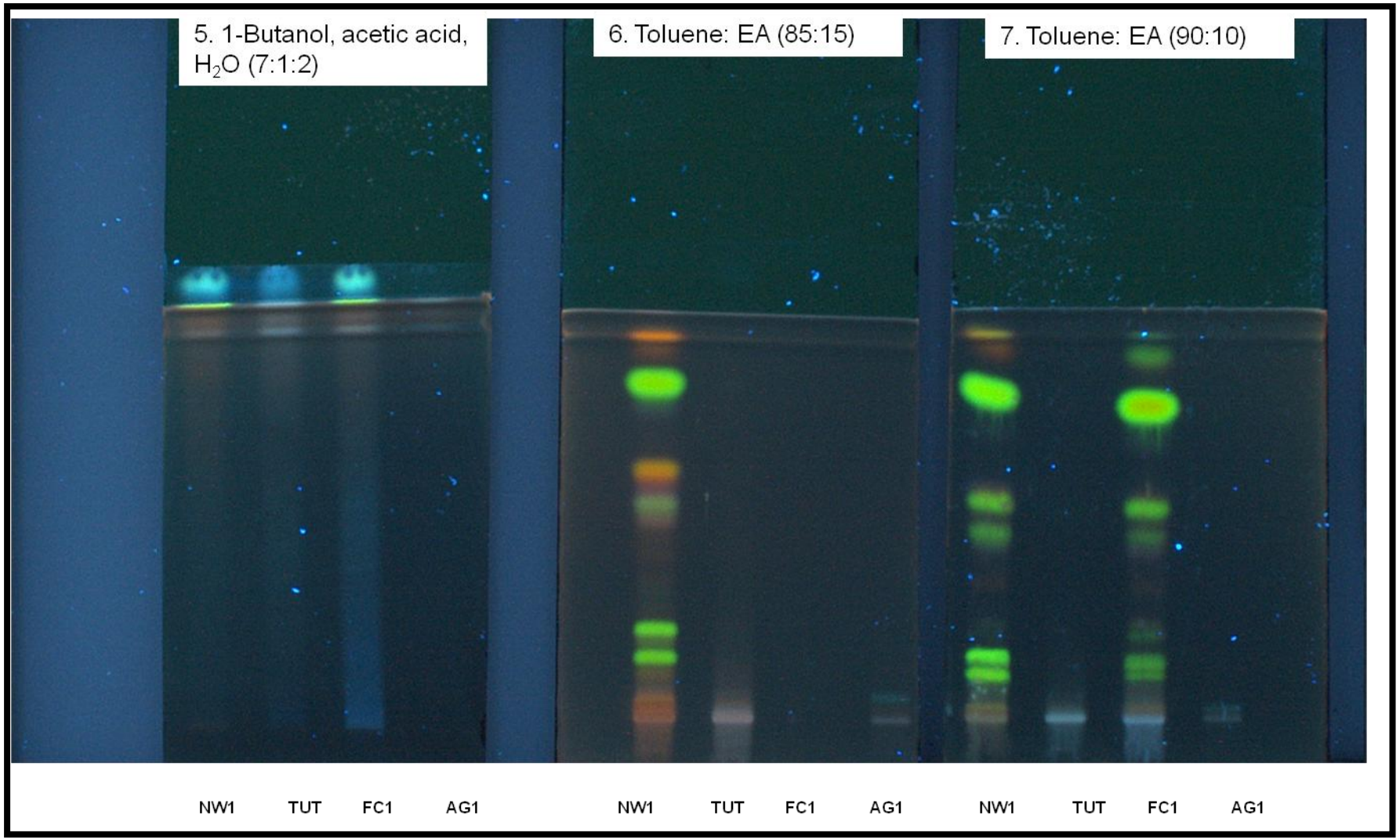

Figure B-4: *Siphonochilus aethiopicus* methanol extract, 5 mg/ml, Anisaldehyde- sulphuric acid dipping reagent, viewing: 366 nm

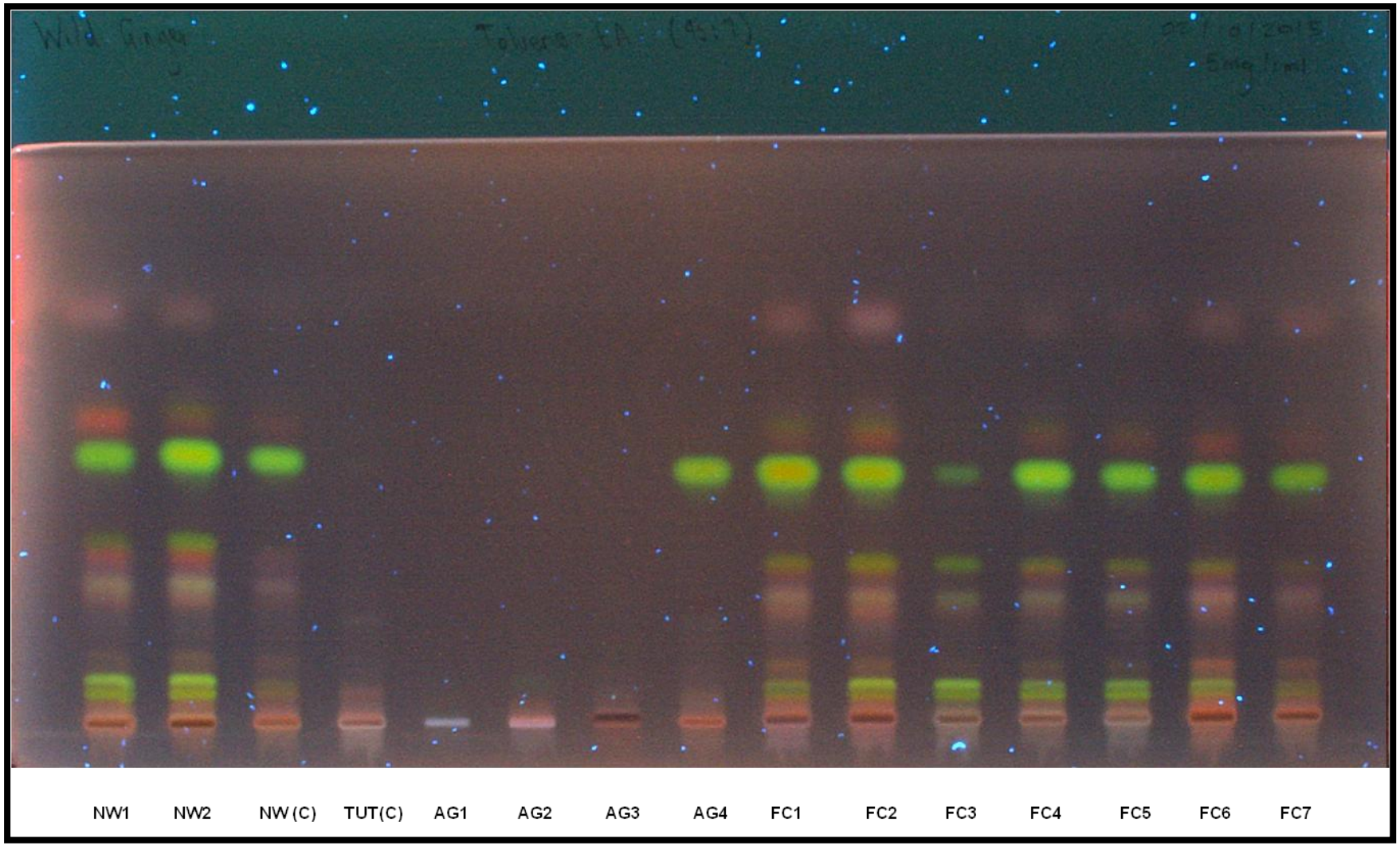

Figure B-5: *Siphonochilus aethiopicus* methanol extract, 5 mg/ml, Toluene: Ethyl acetate (93:7), Anisaldehyde- sulphuric acid dipping reagent, viewing: 366 nm

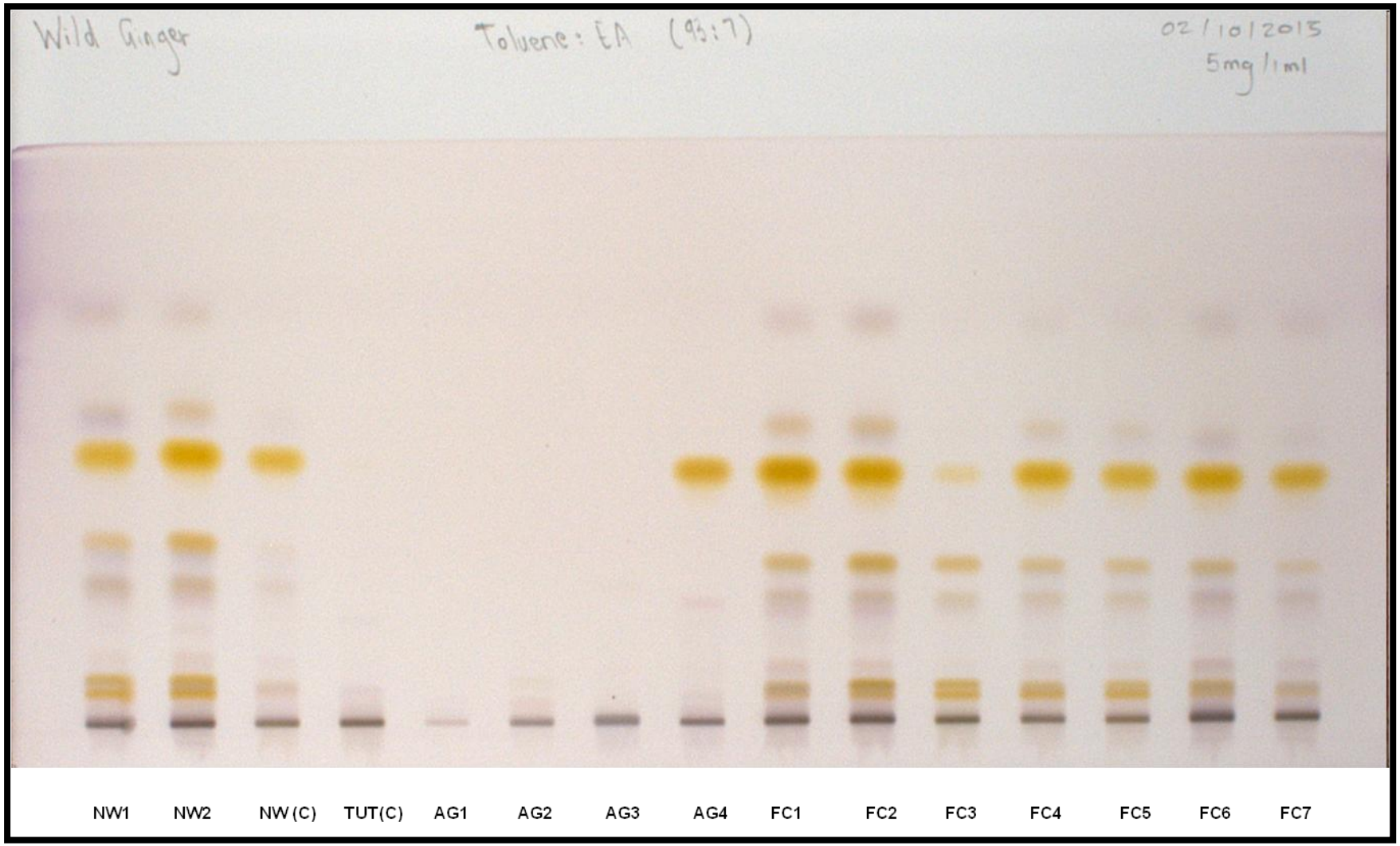

Figure B-6: *Siphonochilus aethiopicus* methanol extract, 5 mg/ml, Toluene: Ethyl acetate (93:7), Anisaldehyde- sulphuric acid dipping reagent, viewing: White R

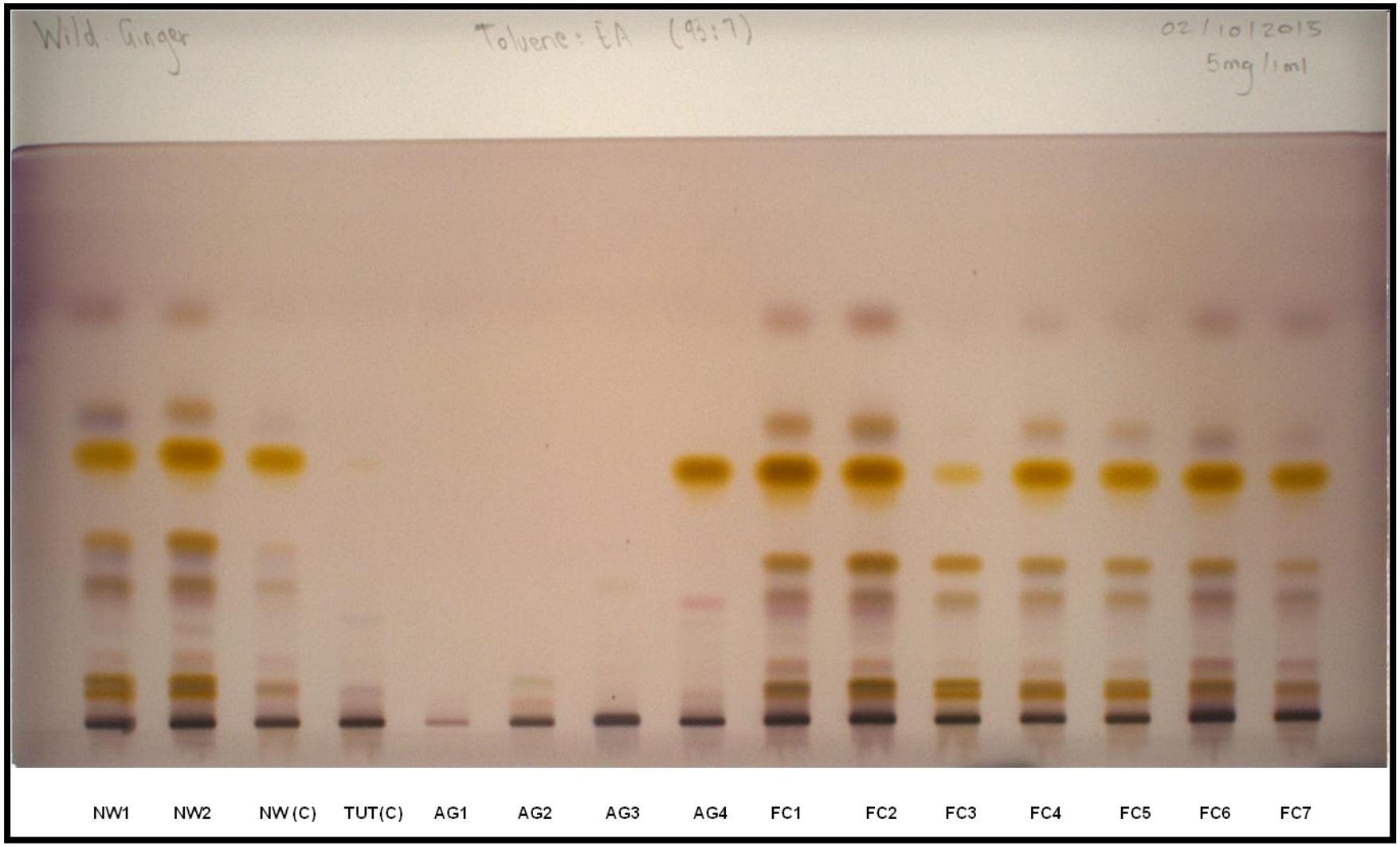

Figure B-7: *Siphonochilus aethiopicus* methanol extract, 5 mg/ml, Toluene: Ethyl acetate (93:7), Anisaldehyde- sulphuric acid dipping reagent, viewing: White RT

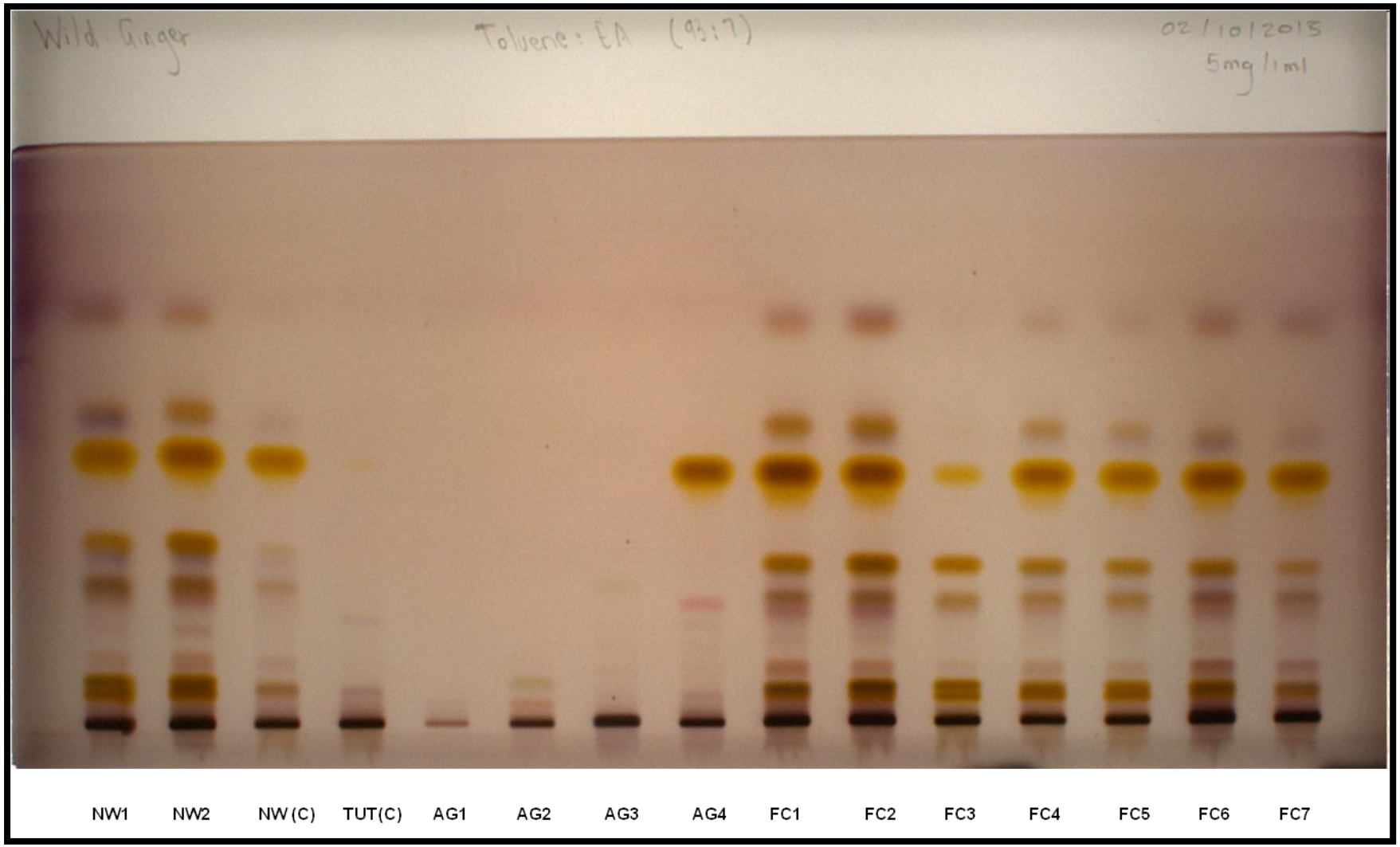

Figure B-8: *Siphonochilus aethiopicus* methanol extract, 5 mg/ml, Toluene: Ethyl acetate (93:7), Anisaldehyde- sulphuric acid dipping reagent, viewing: White T

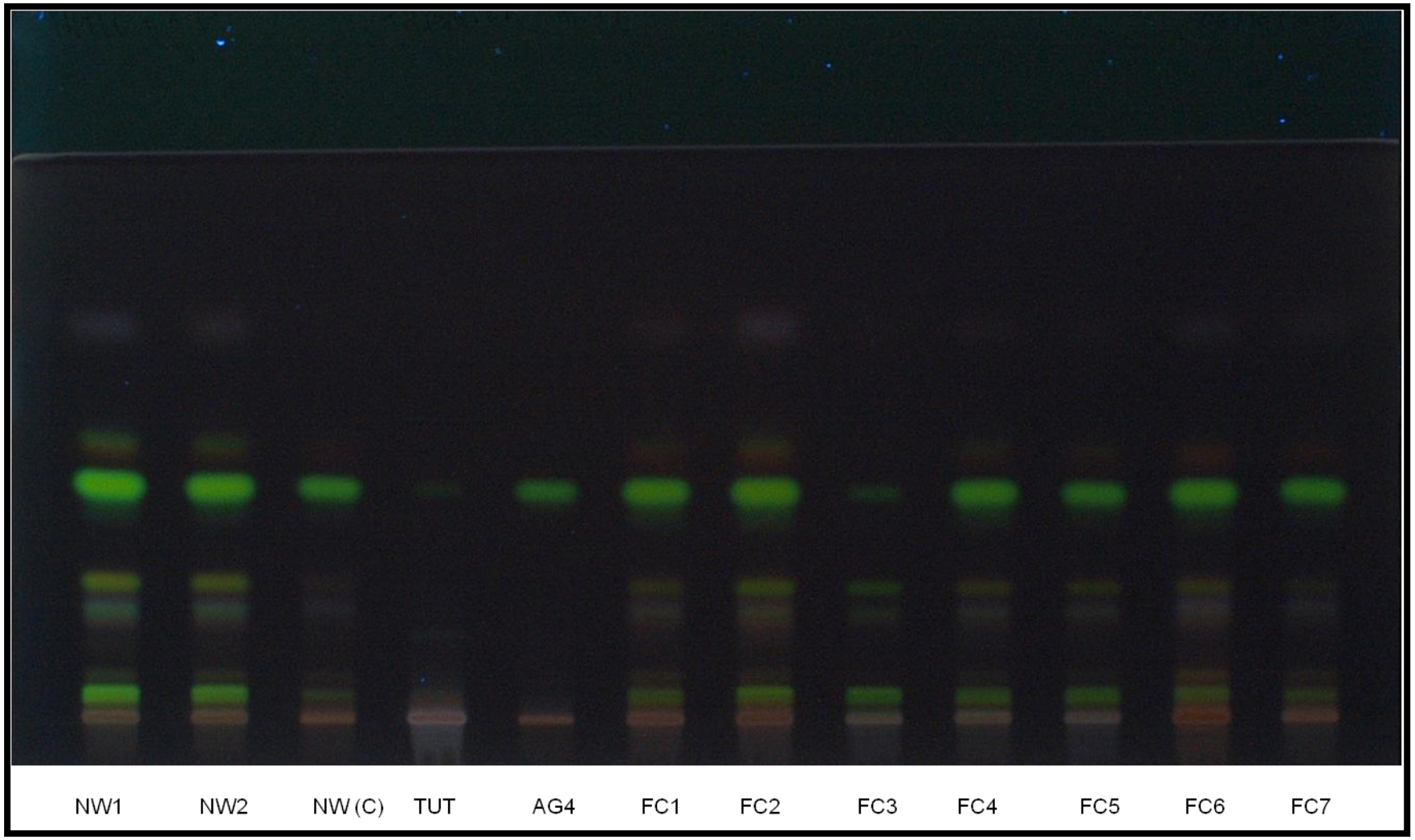

Figure B-9: *Siphonochilus aethiopicus* methanol extract, 5 mg/ml, Toluene: Ethyl acetate (93:7), Anisaldehyde- sulphuric acid dipping reagent, viewing: 366 nm

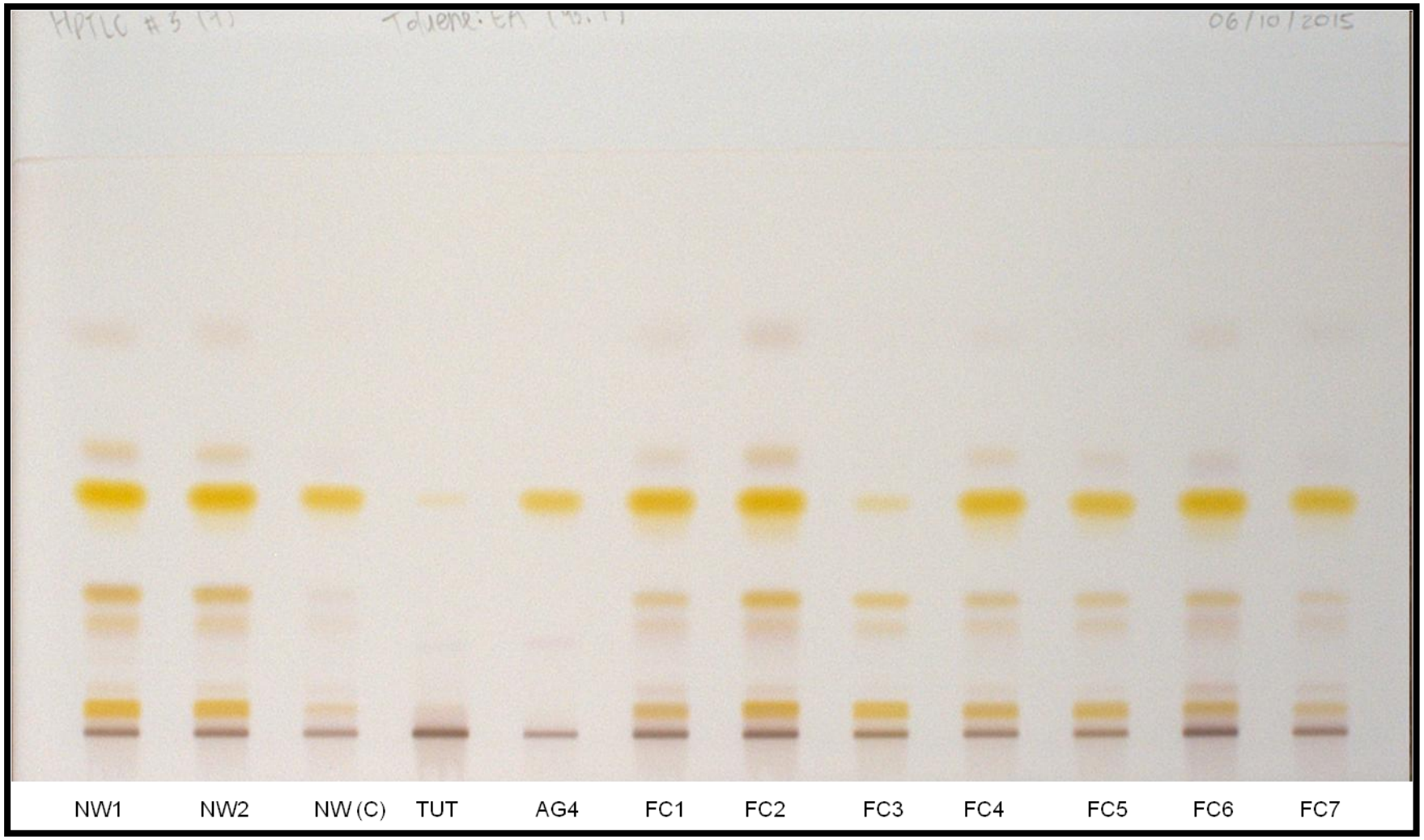

Figure B-10: *Siphonochilus aethiopicus* methanol extract, 5 mg/ml, Toluene: Ethyl acetate (93:7), Anisaldehyde- sulphuric acid dipping reagent, viewing: White R

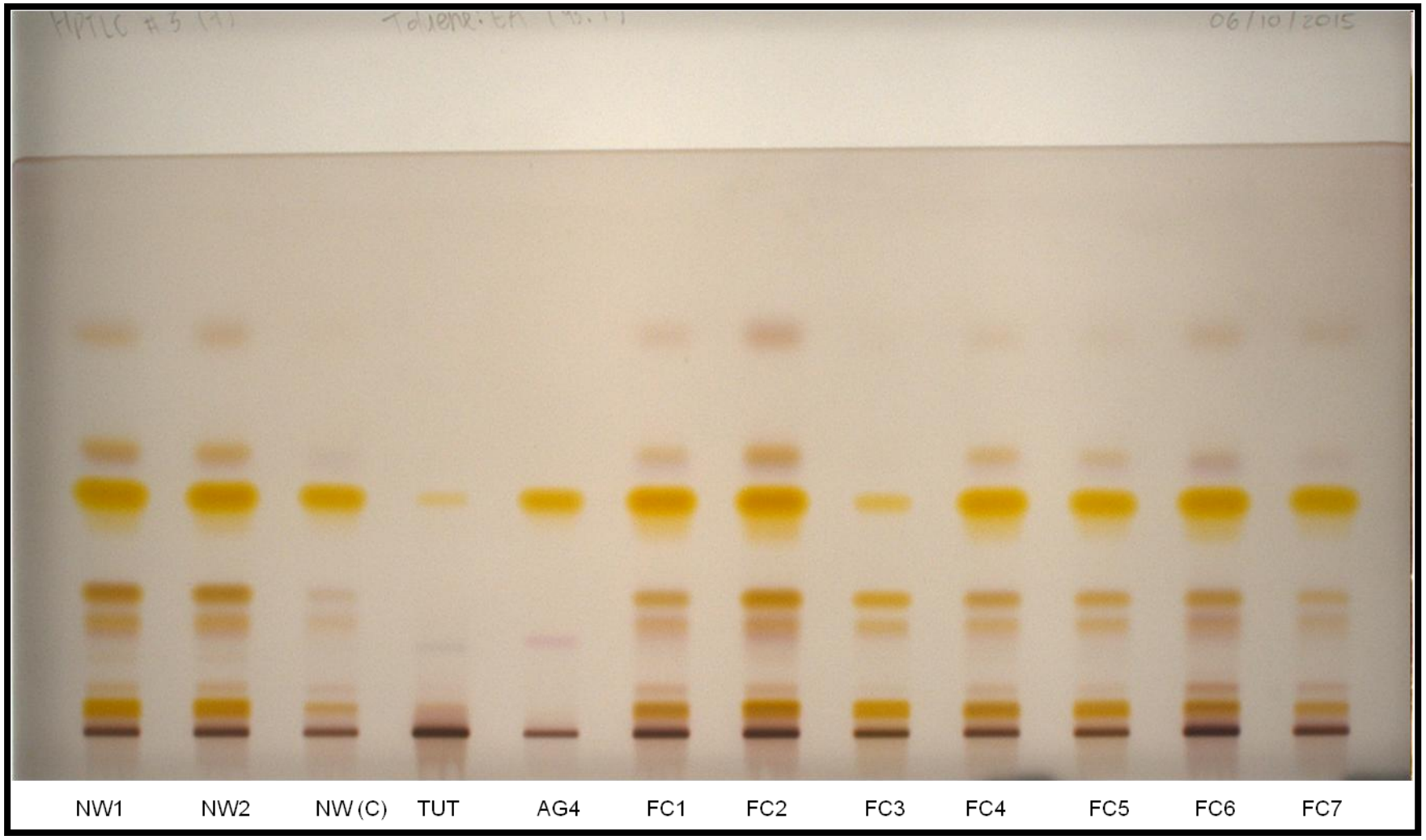

Figure B-11: *Siphonochilus aethiopicus* methanol extract, 5 mg/ml, Toluene: Ethyl acetate (93:7), Anisaldehyde- sulphuric acid dipping reagent, viewing: White RT

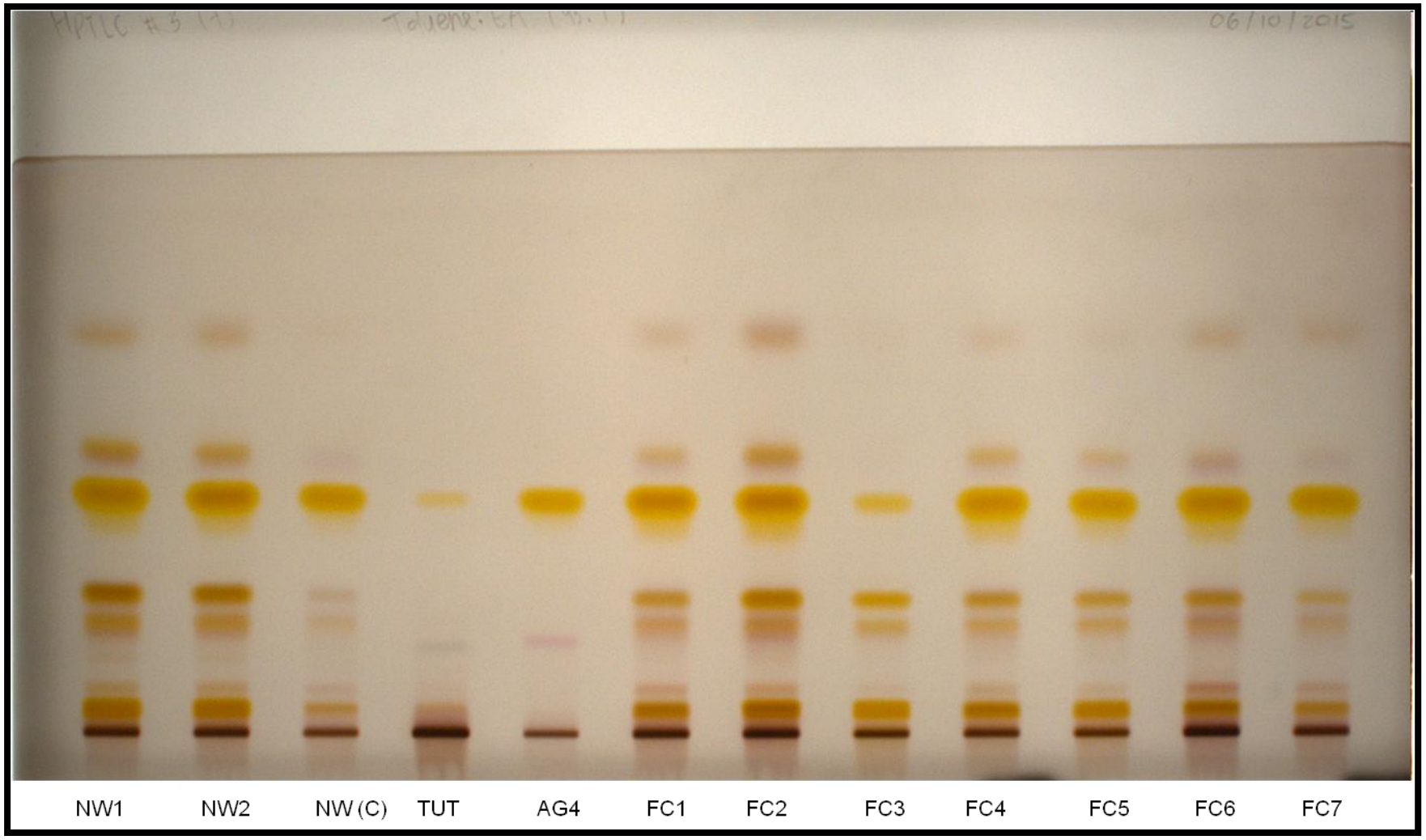

Figure B-12: *Siphonochilus aethiopicus* methanol extract, 5 mg/ml, Toluene: Ethyl acetate (93:7), Anisaldehyde- sulphuric acid dipping reagent, viewing: White T

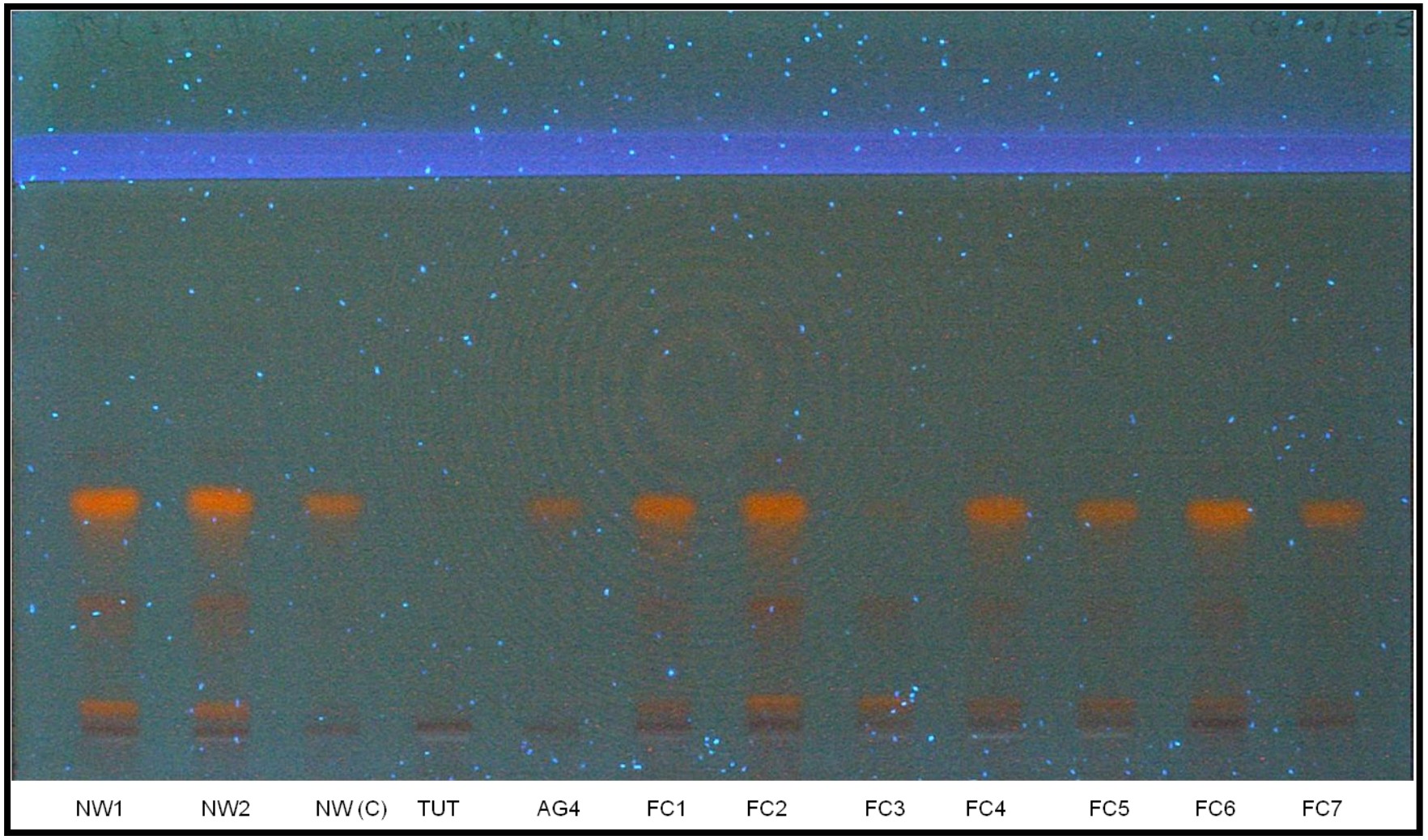

Figure B-13: *Siphonochilus aethiopicus* methanol extract, 5 mg/ml, Toluene: Ethyl acetate (93:7), Vanillin sulphuric acid dipping agent, viewing: 366 nm

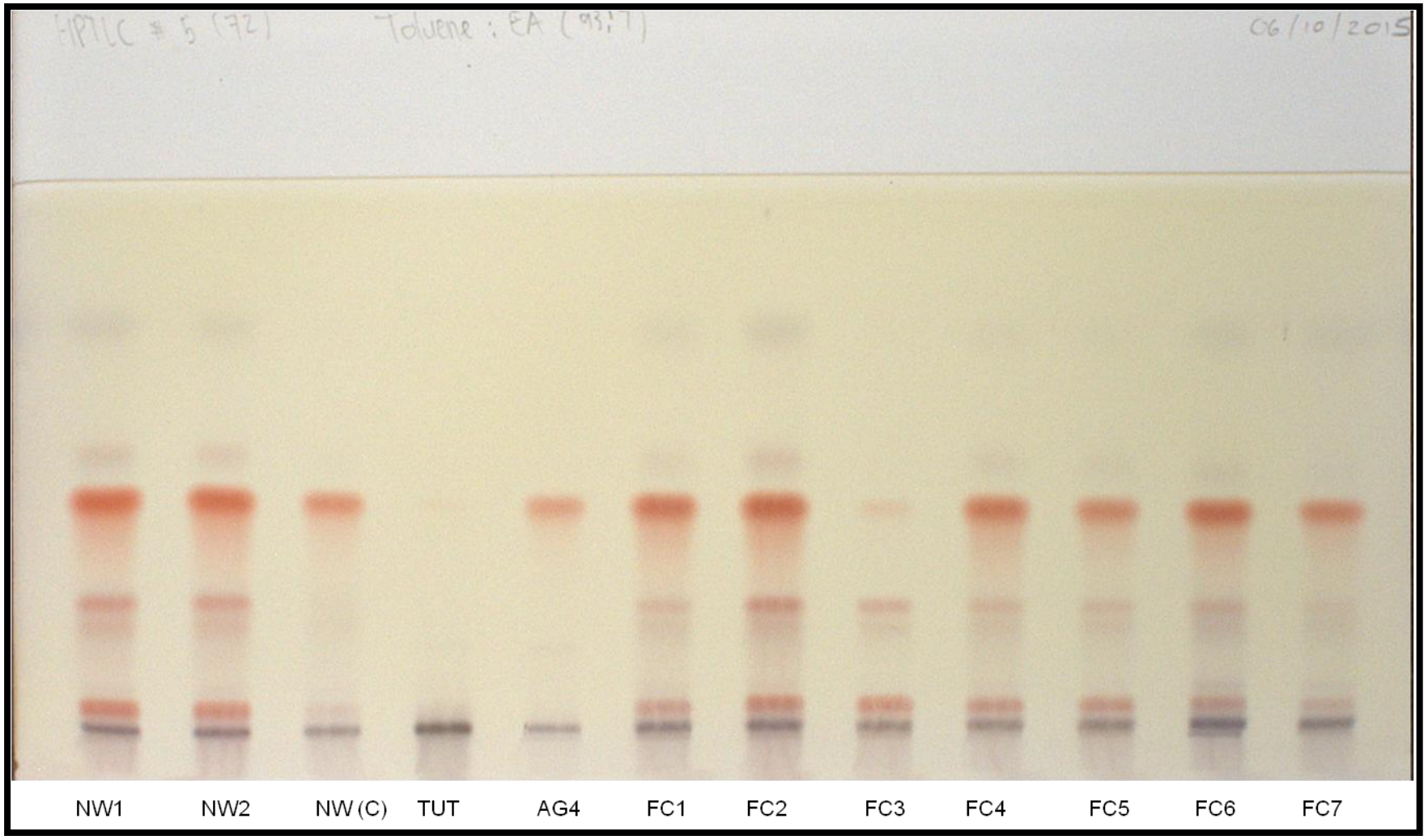

Figure B-14: *Siphonochilus aethiopicus* methanol extract, 5 mg/ml, Toluene: Ethyl acetate (93:7), Vanillin sulphuric acid dipping agent, viewing: White R

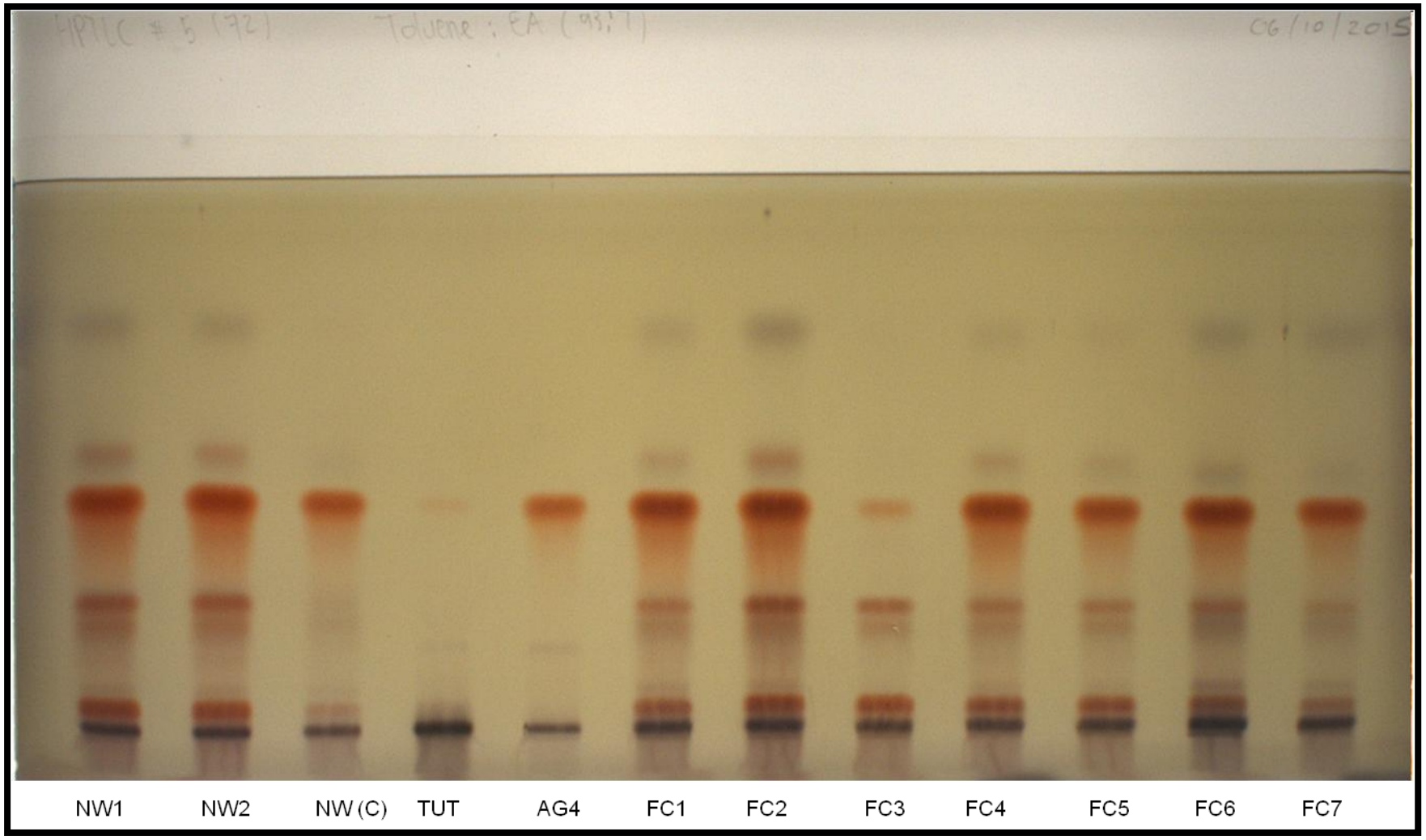

Figure B-15: *Siphonochilus aethiopicus* methanol extract, 5 mg/ml, Toluene: Ethyl acetate (93:7), Vanillin sulphuric acid dipping agent, viewing: White RT

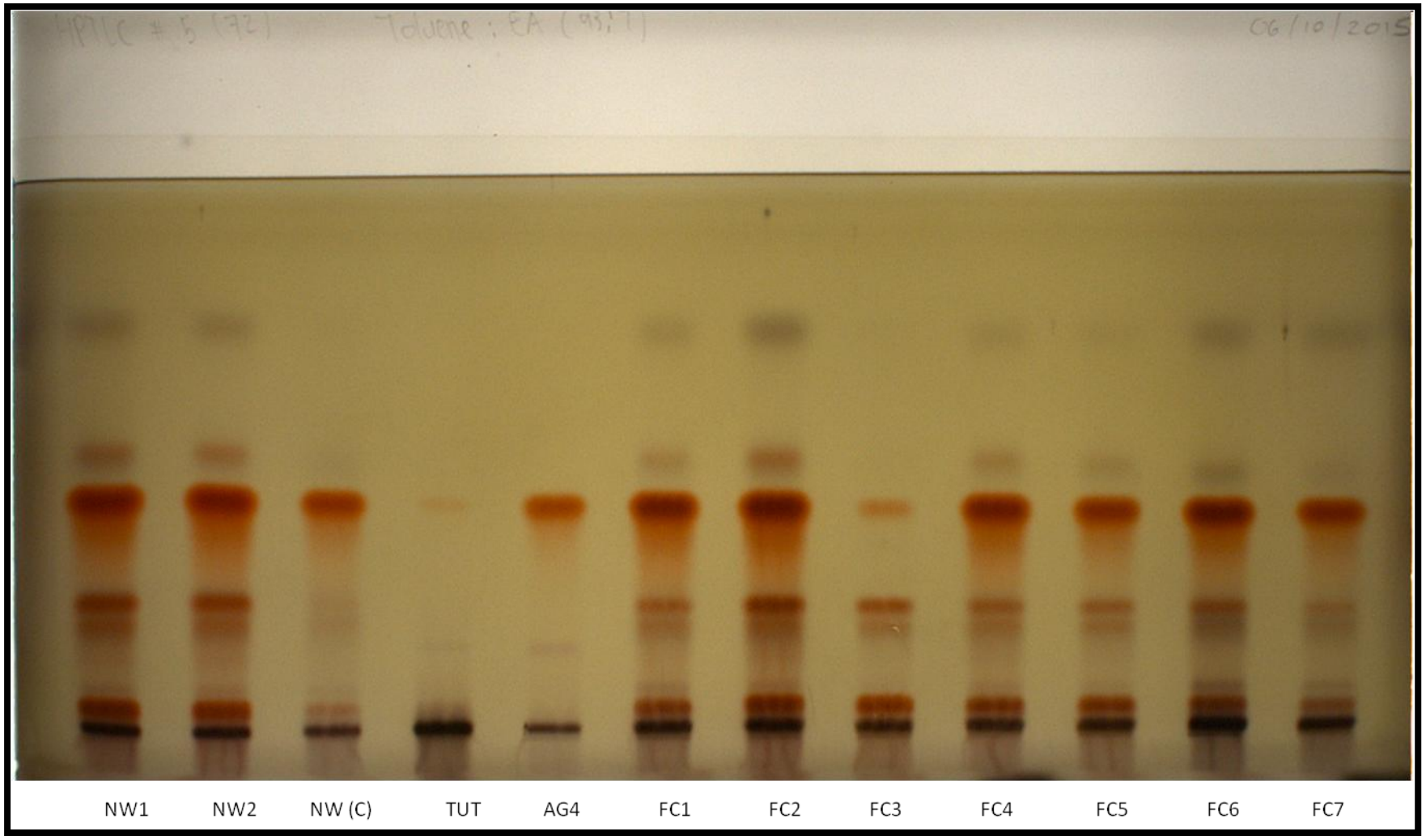

Figure B-16: *Siphonochilus aethiopicus* methanol extract, 5 mg/ml, Toluene: Ethyl acetate (93:7), Vanillin sulphuric acid dipping agent, viewing: White T

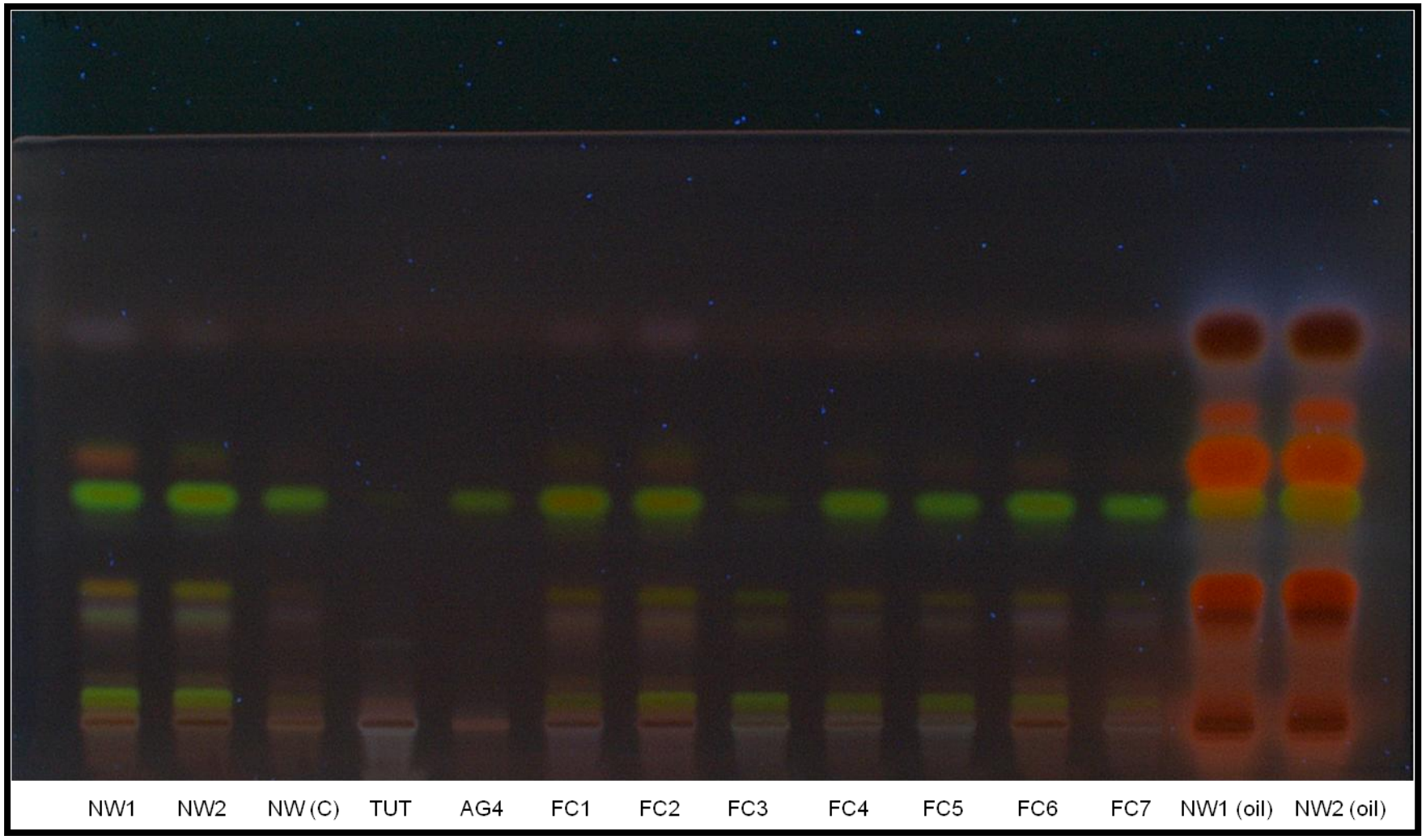

Figure B-17: *Siphonochilus aethiopicus* methanol extract, 5mg/ml, Toluene: Ethyl acetate (93:7), Anisaldehyde- sulphuric acid dipping reagent, viewing: 366nm

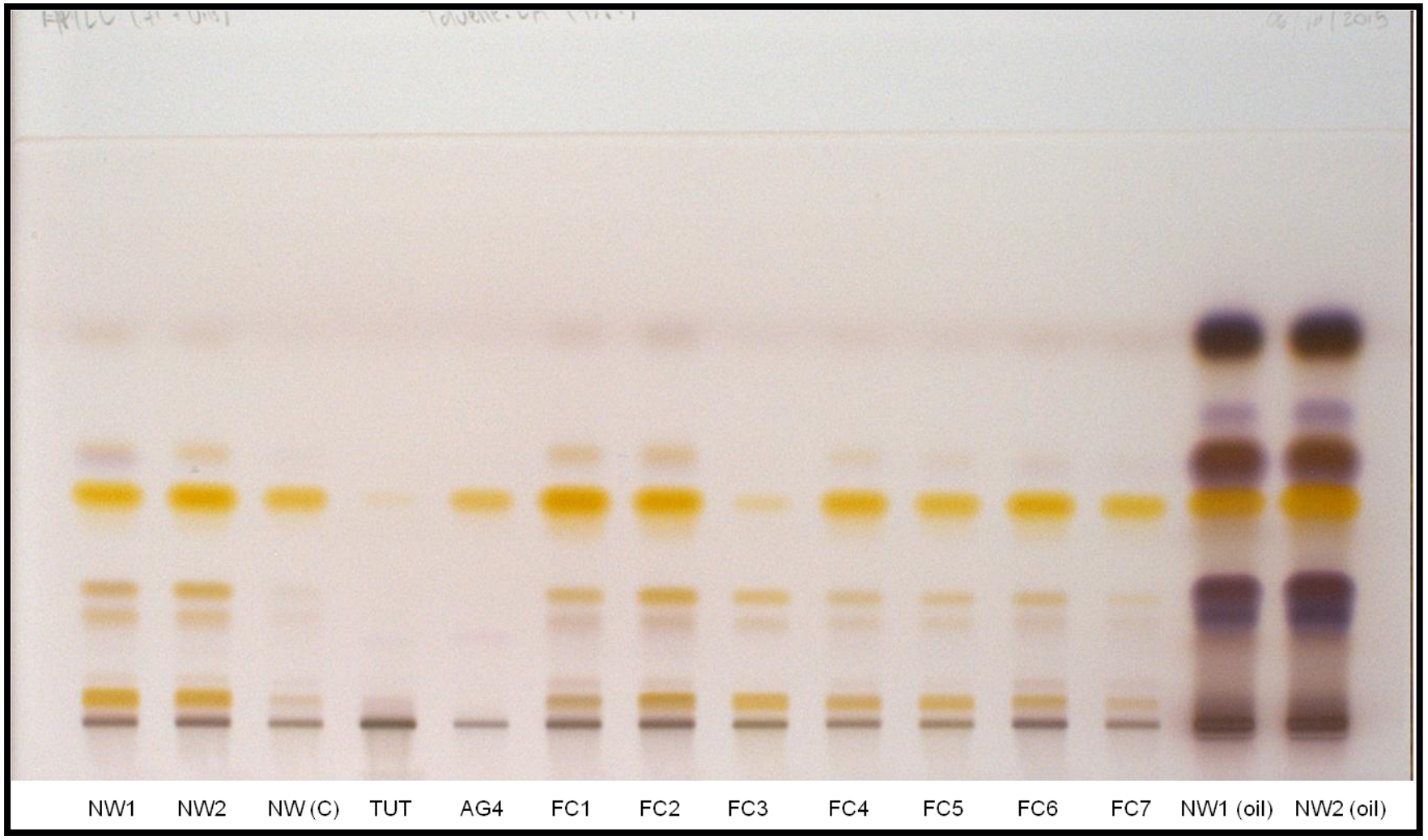

Figure B-18: *Siphonochilus aethiopicus* methanol extract, 5mg/ml, Toluene: Ethyl acetate (93:7), Anisaldehyde- sulphuric acid dipping reagent, viewing: White R

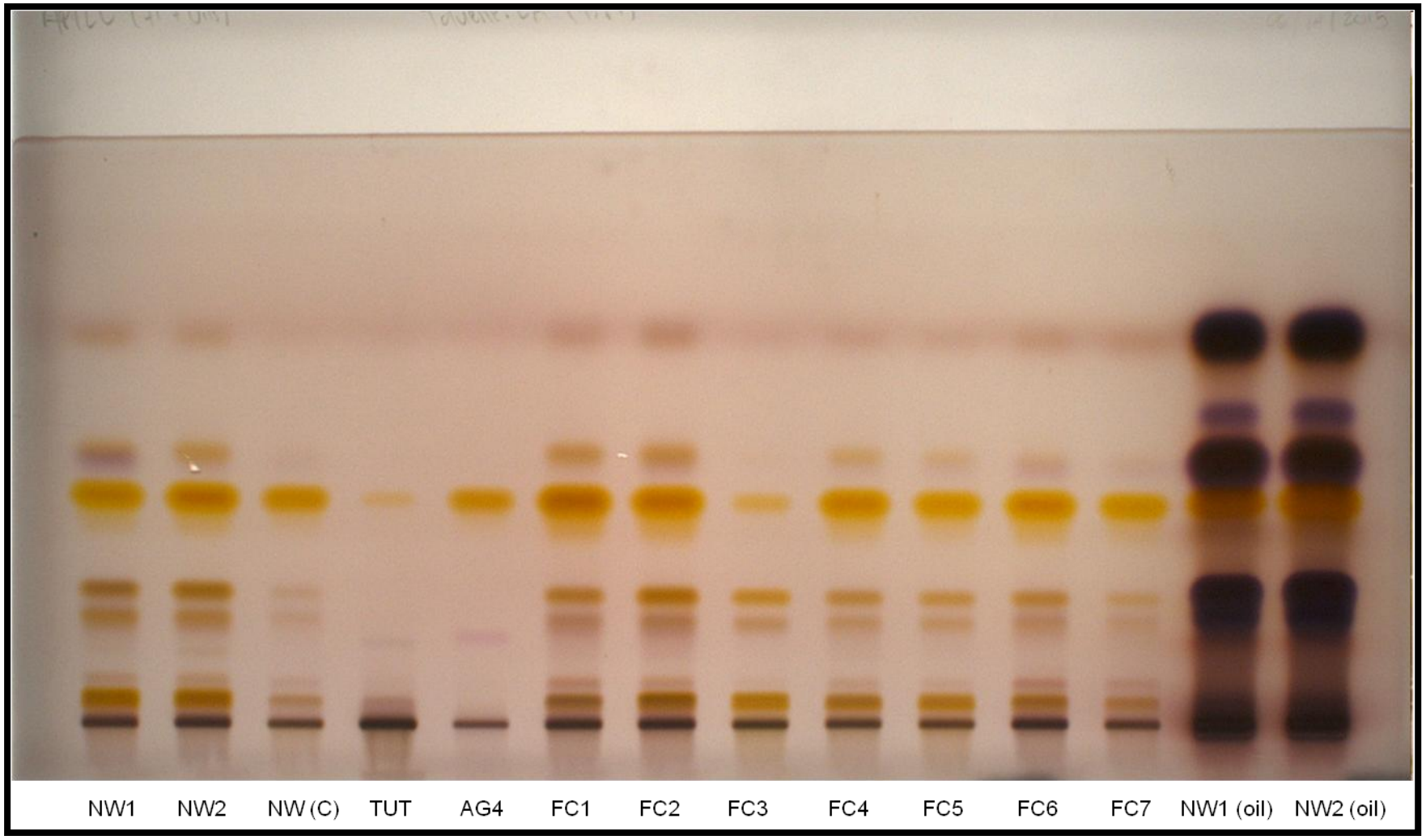

Figure B-19: *Siphonochilus aethiopicus* methanol extract, 5 mg/ml, Toluene: Ethyl acetate (93:7), Anisaldehyde- sulphuric acid dipping reagent, viewing: White RT

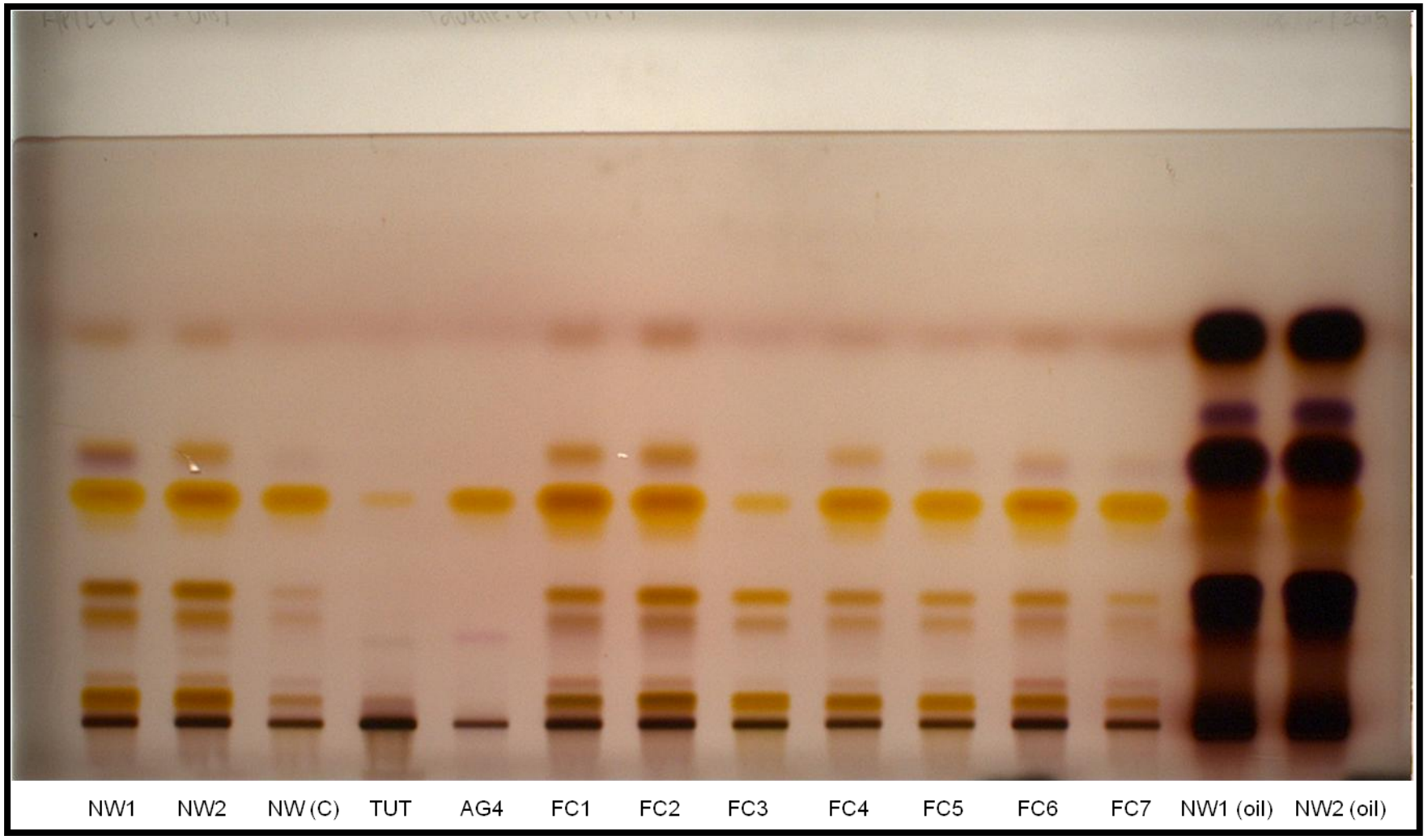

Figure B-20: *Siphonochilus aethiopicus* methanol extract, 5 mg/ml, Toluene: Ethyl acetate (93:7), Anisaldehyde- sulphuric acid dipping reagent, viewing: White T

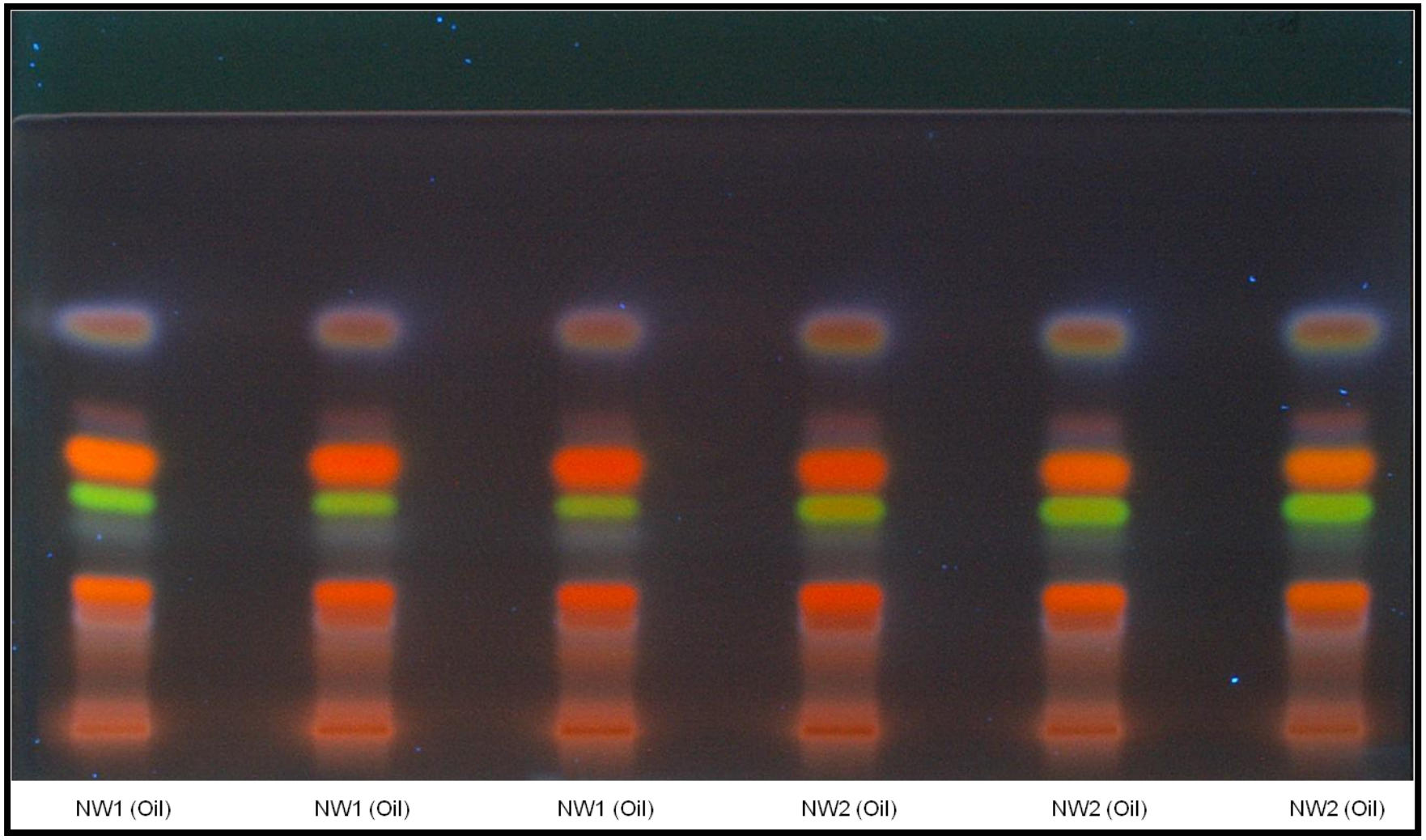

Figure B-21: *Siphonochilus aethiopicus* methanol extract, 1 mg/ml, Toluene: Ethyl acetate (93:7), Anisaldehyde- sulphuric acid dipping reagent, viewing: 366 nm

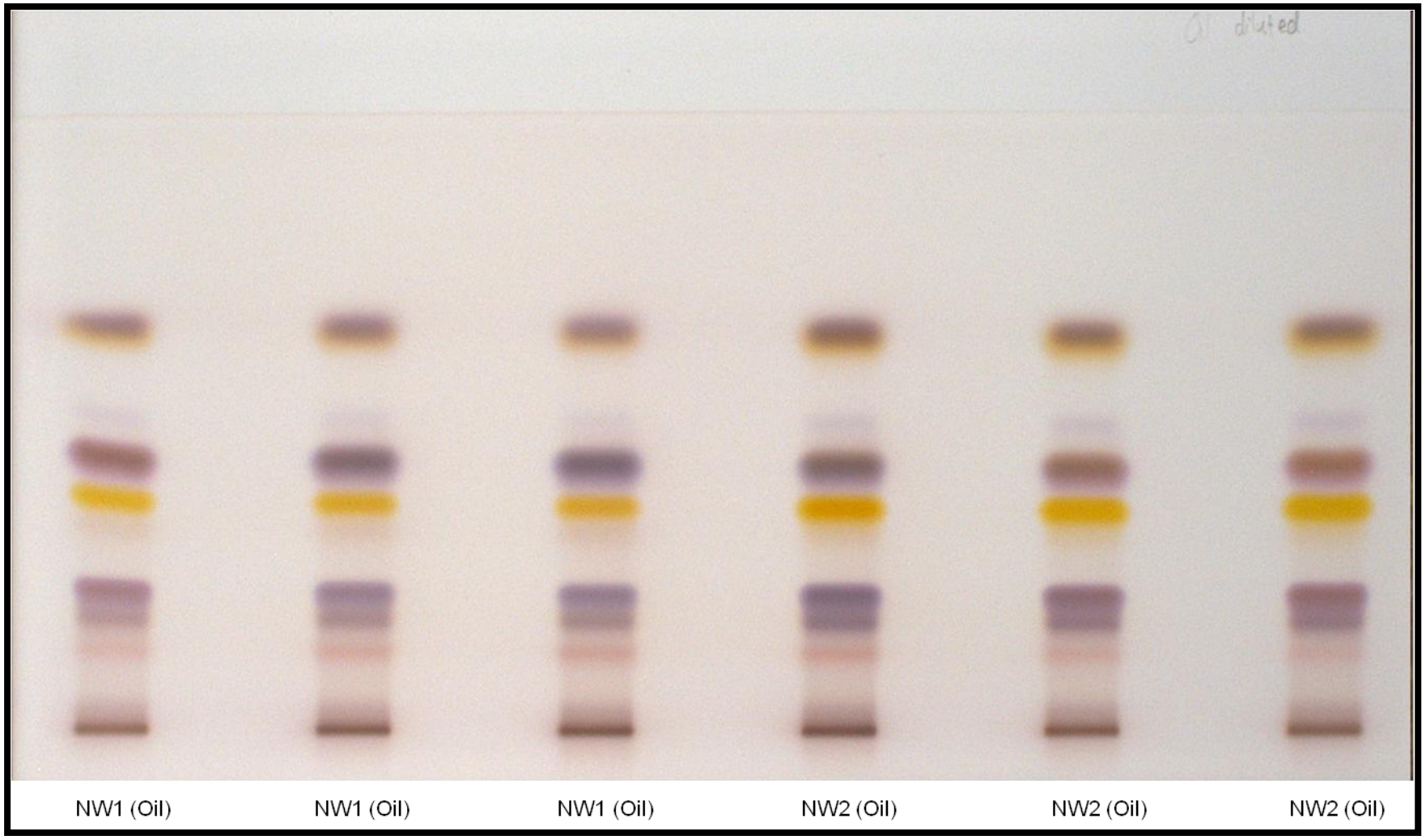

Figure B-22: *Siphonochilus aethiopicus* methanol extract, 1 mg/ml, Toluene: Ethyl acetate (93:7), Anisaldehyde- sulphuric acid dipping reagent, viewing: White R

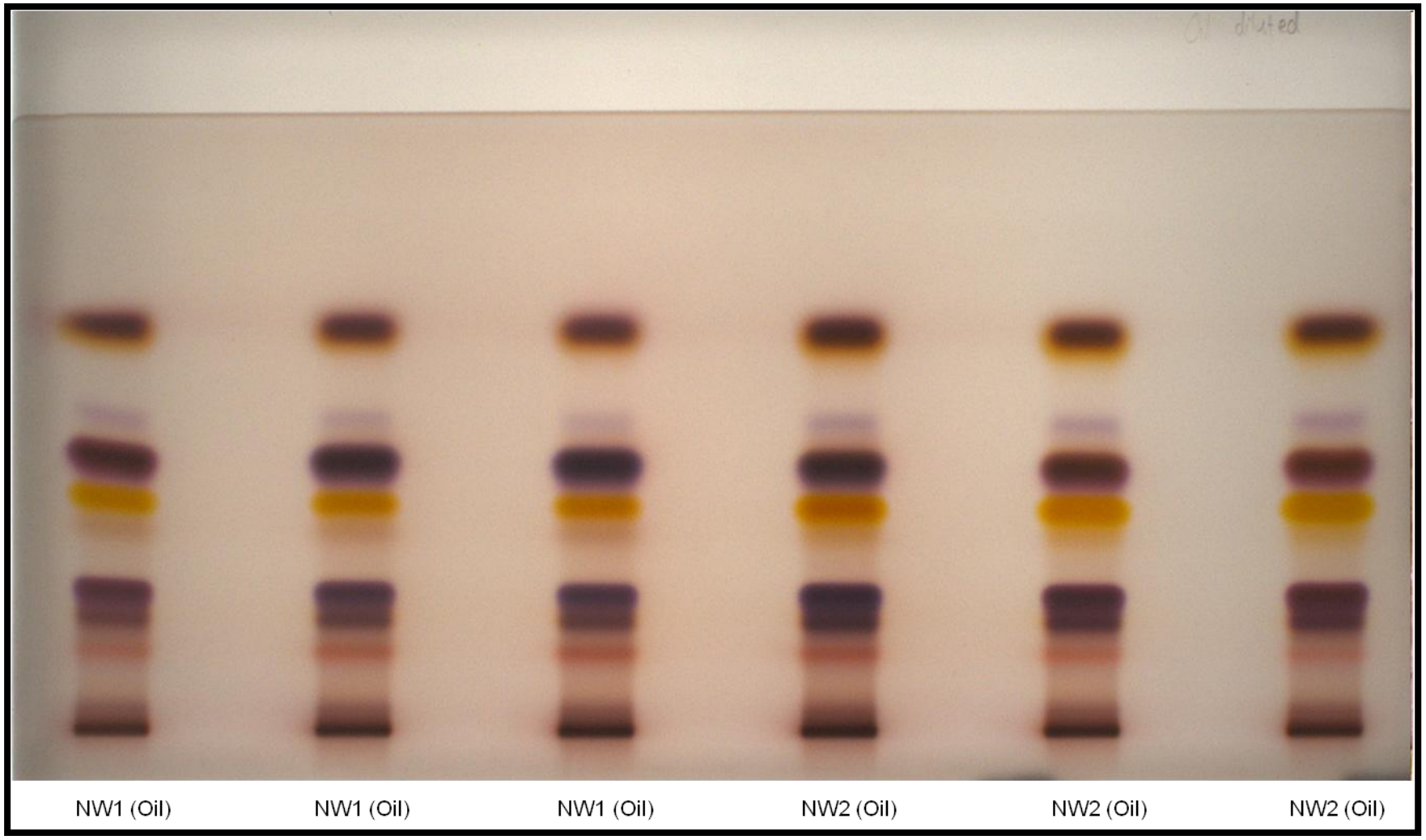

Figure B-23: *Siphonochilus aethiopicus* methanol extract, 1 mg/ml, Toluene: Ethyl acetate (93:7), Anisaldehyde- sulphuric acid dipping reagent, viewing: White RT

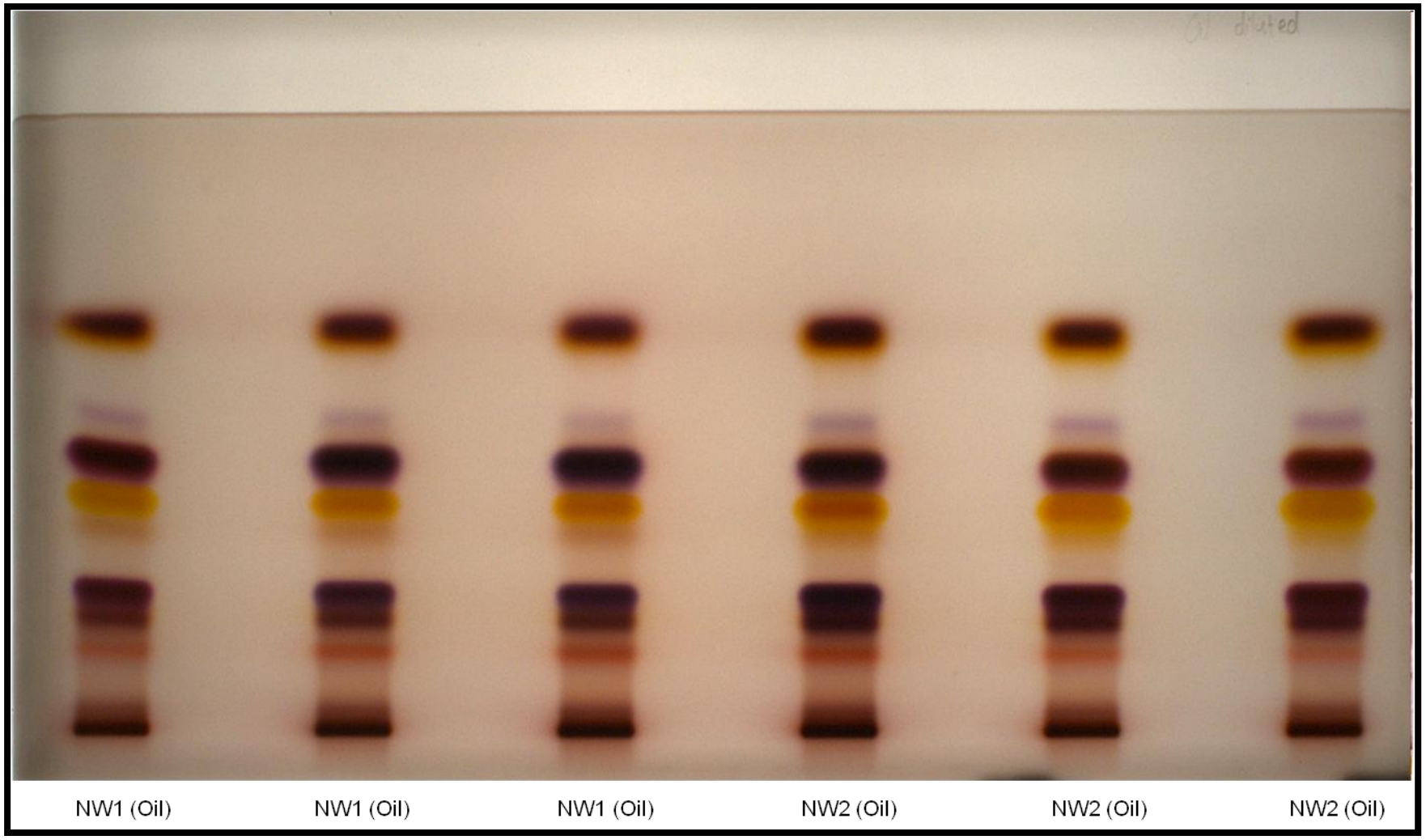

Figure B-24: *Siphonochilus aethiopicus* methanol extract, 1 mg/ml, Toluene: Ethyl acetate (93:7), Anisaldehyde- sulphuric acid dipping reagent, viewing: White T

ANNEXURE C

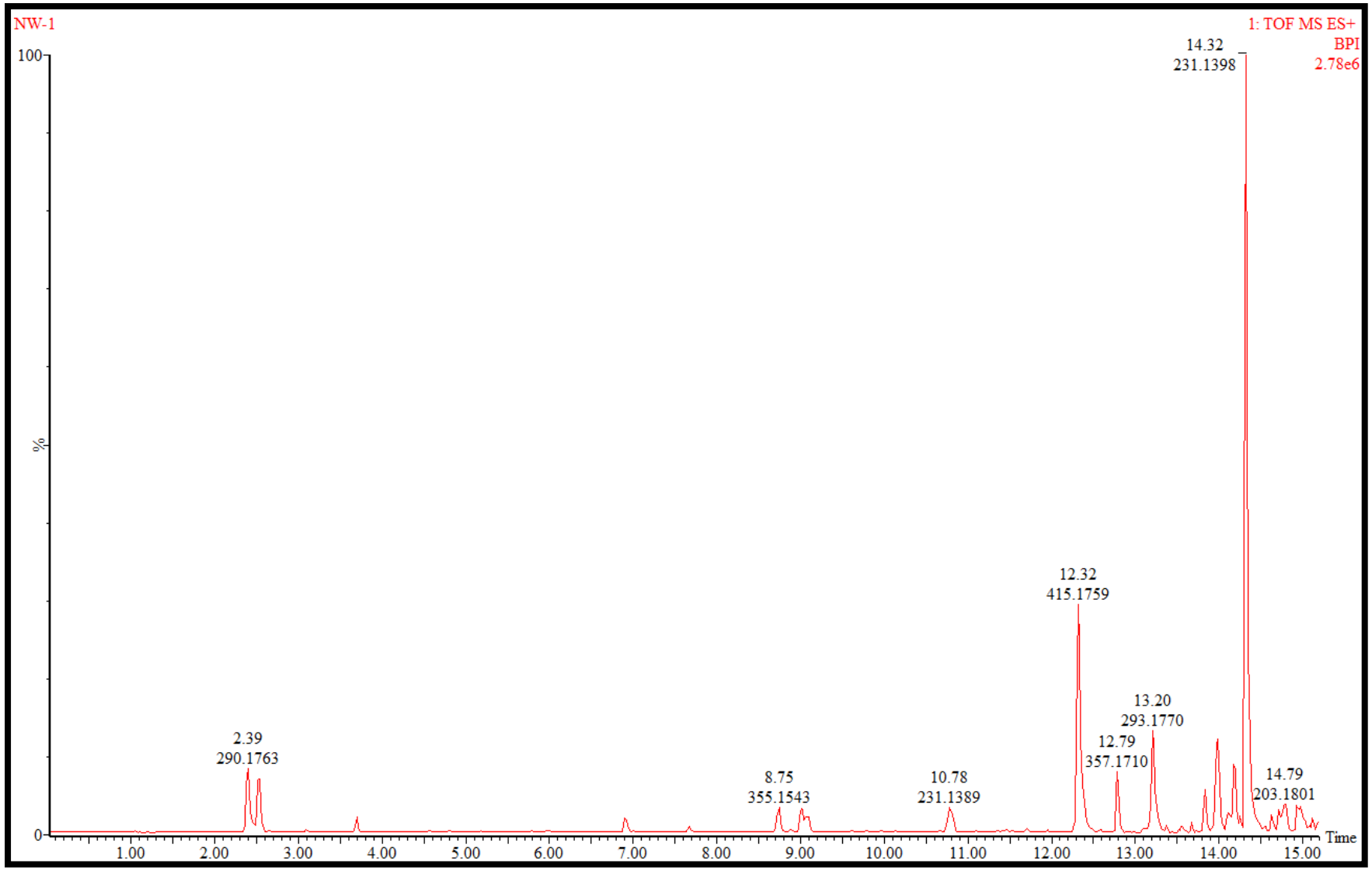

Figure C-1: BPI chromatogram of NW 1

Table C-1: Retention times and area percentage of individual compounds in NW 1

Retention (min)	Peak area	Area %
3.70	1930	0.72
6.90	**2349**	**0.88**
8.75	4563	1.70
9.01	**8135**	**3.04**
10.78	6159	2.30
12.32	**46304**	**17.30**
12.79	8881	3.32
13.20	21907	8.18
13.83	6034	2.25
13.99	19250	7.19
14.18	15201	5.68
14.32	**127019**	**47.44**
Total	**267732**	**100**

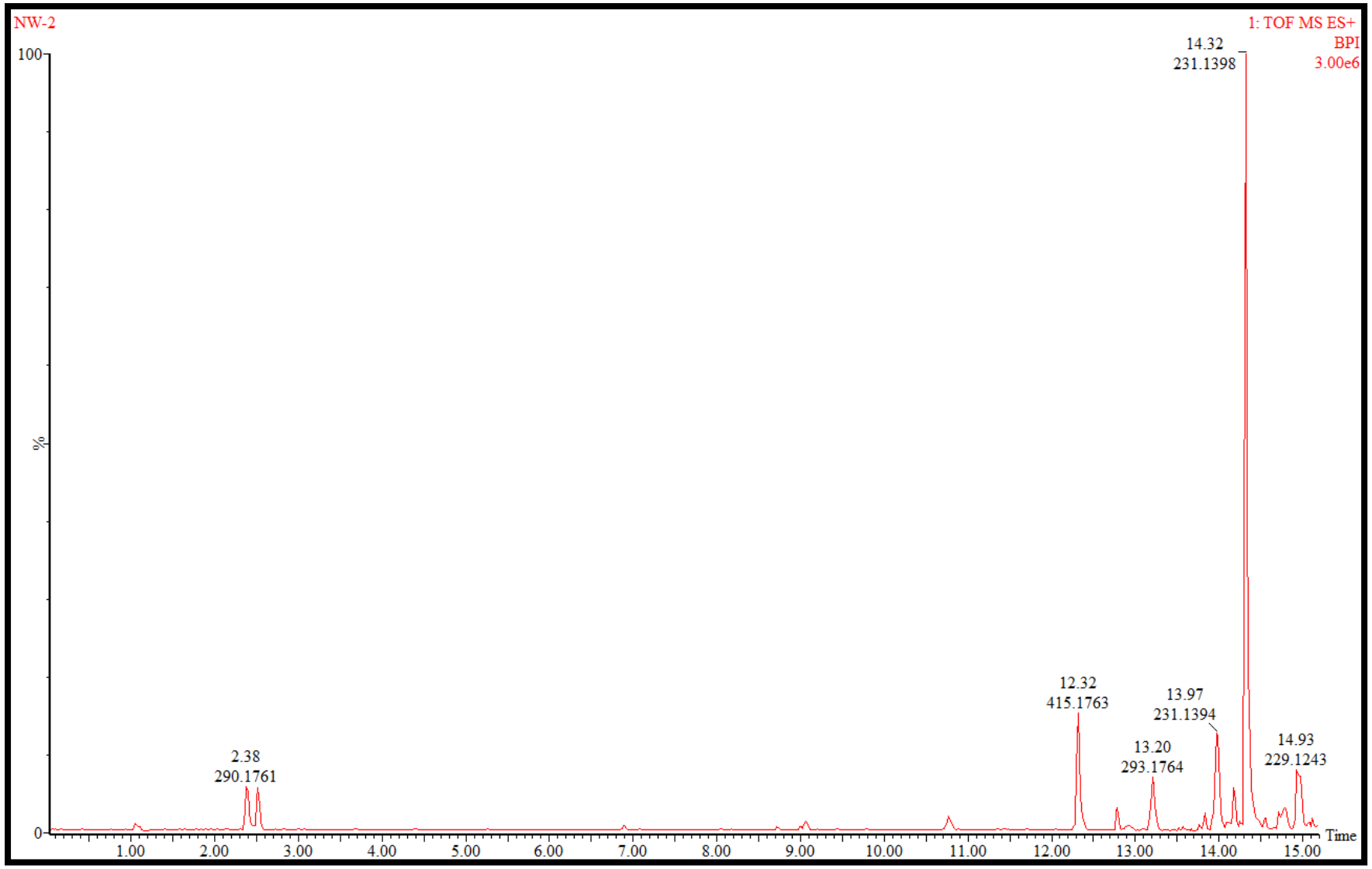

Figure C-2: BPI chromatogram of NW 2

Table C-2: Retention times and area percentage of individual compounds in NW 2

Retention (min)	Peak area	Area %
6.90	**859**	**0.42**
9.06	**1707**	**0.83**
10.78	3524	1.72
12.32	**21562**	**10.51**
12.79	3475	1.70
13.20	11915	5.81
13.83	3104	1.51
13.99	23800	11.60
14.18	7776	3.79
14.32	**127383**	**62.11**
Total	**205105**	**100**

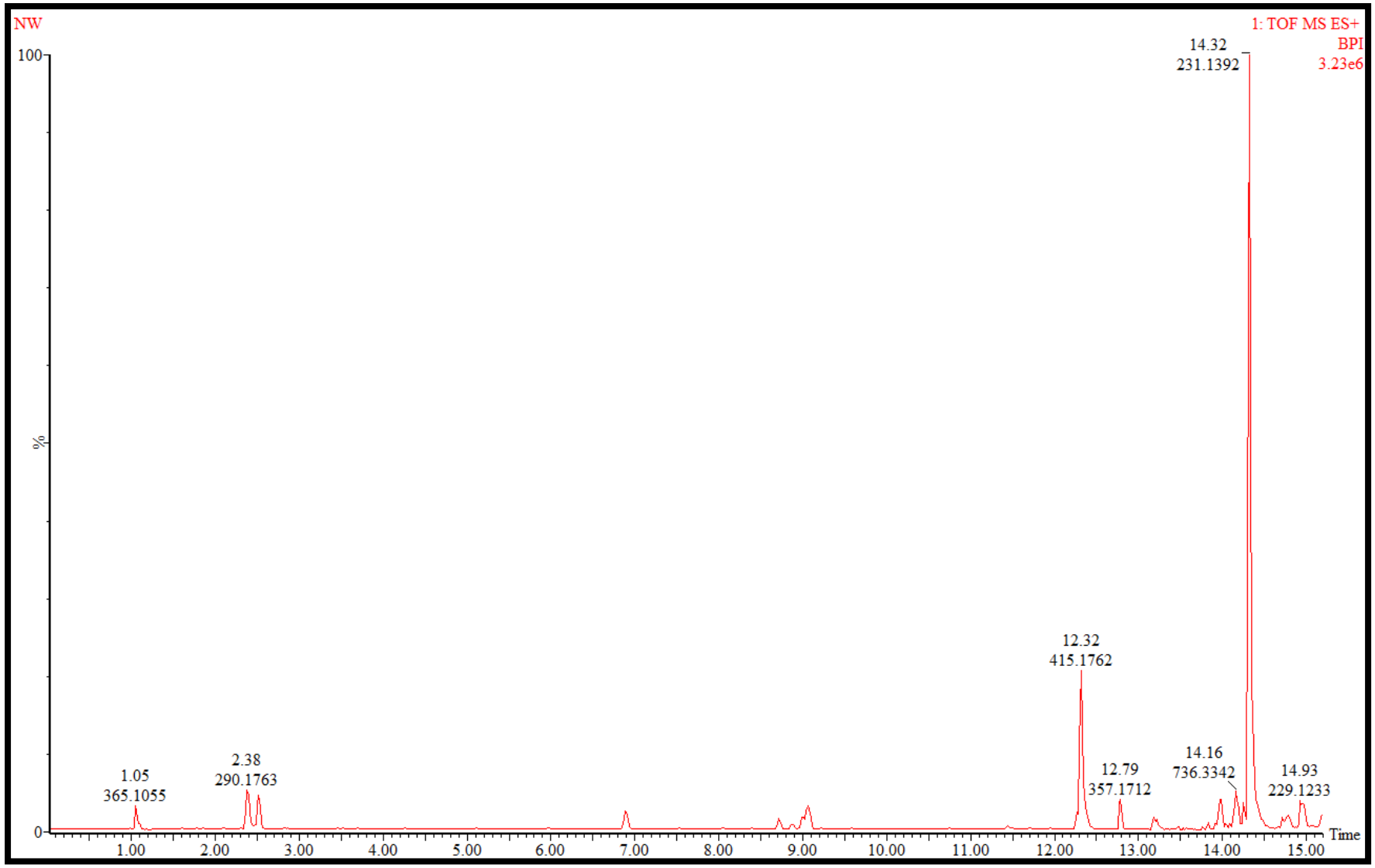

Figure C-3: BPI chromatogram of NW (C)

Table C-3: Retention times and area percentage of individual compounds in NW(C)

Retention (min)	Peak area	Area %
6.90	**4170**	**1.99**
8.71	1853	0.89
9.06	**8092**	**3.87**
12.32	**34340**	**16.42**
12.79	5062	2.42
14.16	9482	4.53
14.32	**146165**	**69.88**
Total	**209164**	**100**

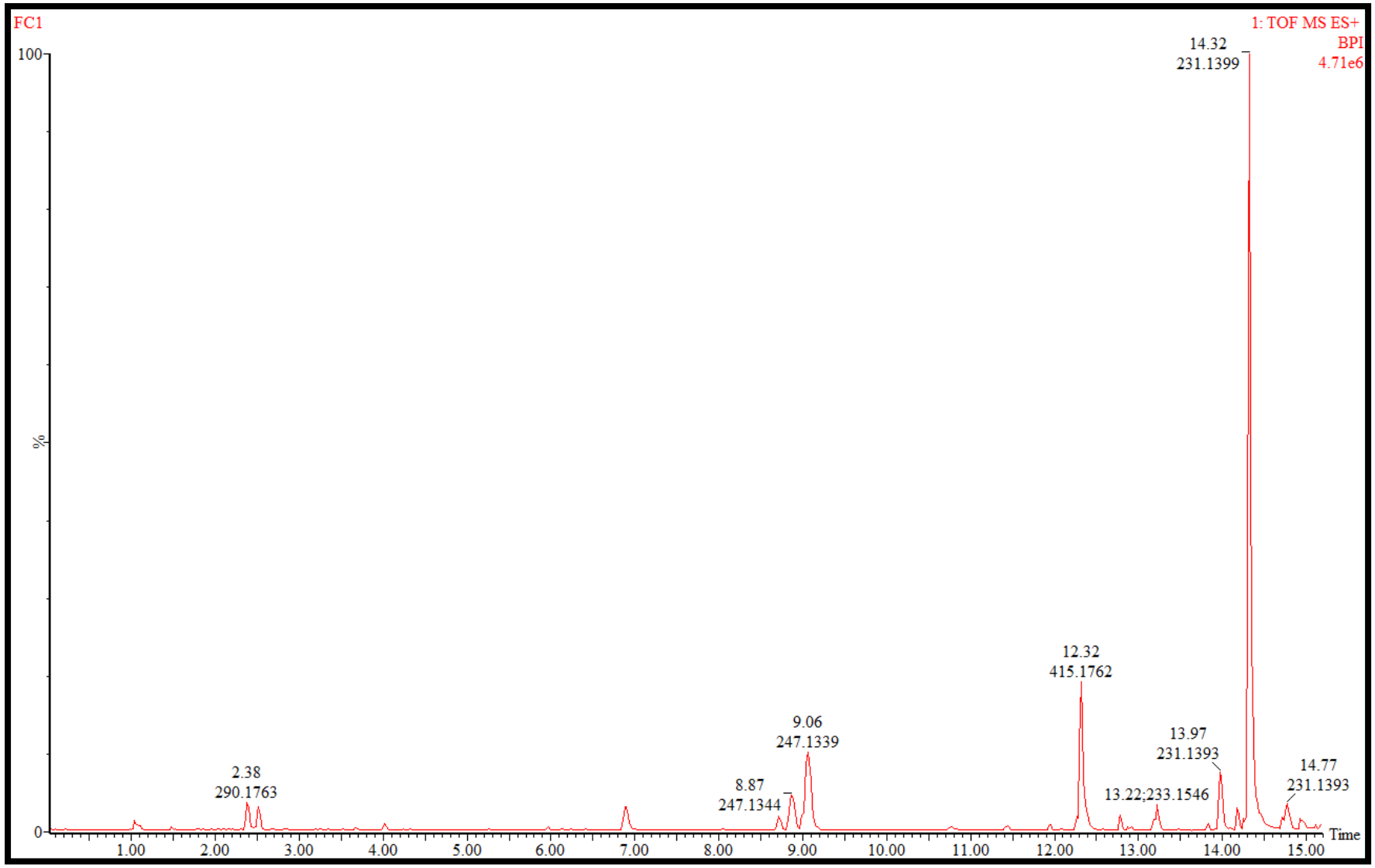

Figure C-4: BPI chromatogram of FC 1

Table C-4: Retention times and area percentage of individual compounds in FC 1

Retention (min)	Peak area	Area %
4.01	1387	0.41
6.89	**9403**	**2.77**
9.06	**41050**	**12.08**
12.32	**50614**	**14.90**
12.79	3626	1.07
13.97	19643	5.78
14.32	**214193**	**63.00**
Total	**339916**	**100**

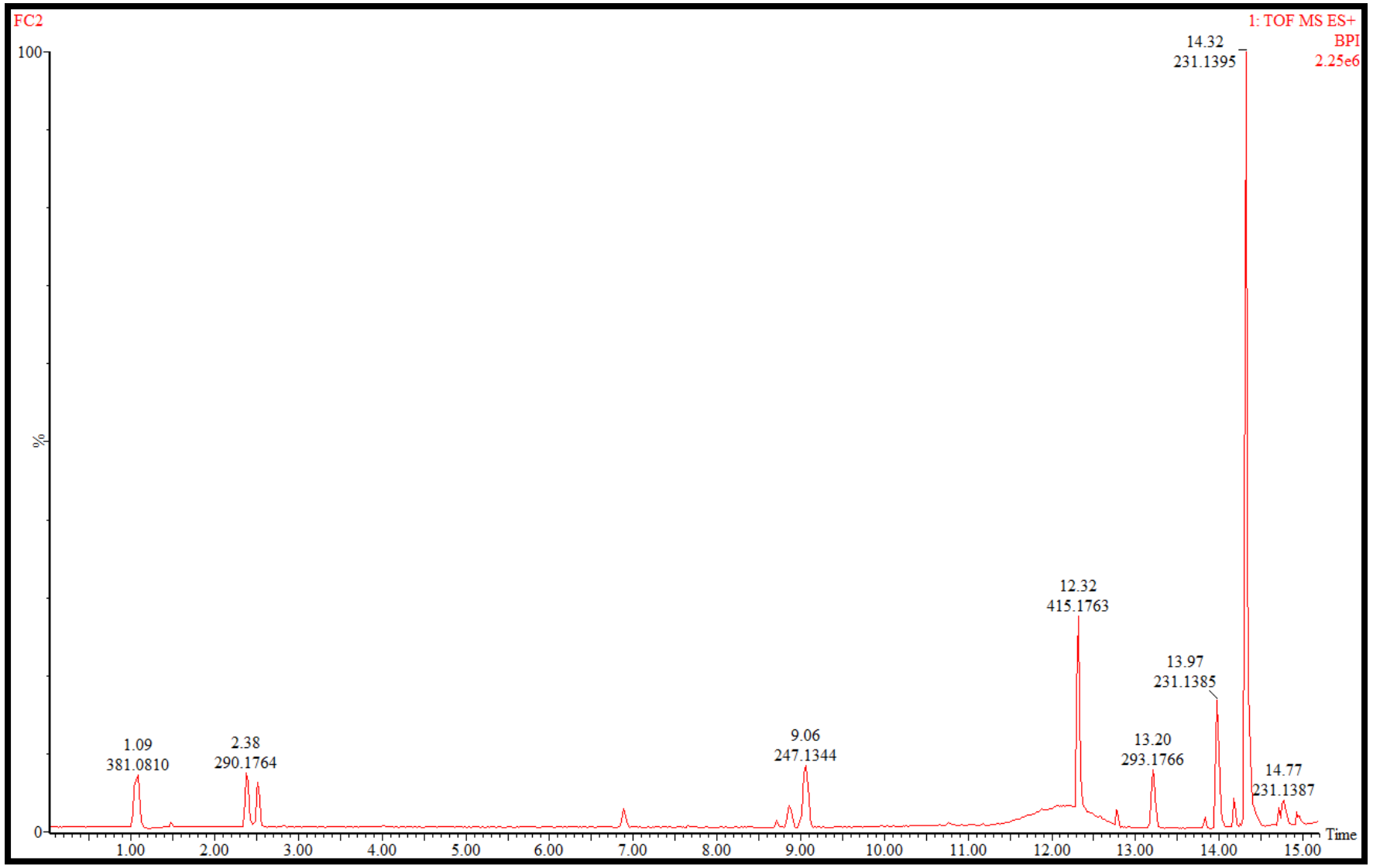

Figure C-5: BPI chromatogram of FC 2

Table C-5: Retention times and area percentage of individual compounds in FC 2

Retention (min)	Peak area	Area %
6.90	**3109**	**1.612393**
9.06	**13323**	**6.909589**
12.32	**49333**	**25.58513**
12.79	2200	1.140966
13.20	9649	5.004175
13.83	1038	0.538329
13.99	18892	9.79779
14.18	2712	1.4065
14.32	**92563**	**48.00512**
Total	**192819**	**100**

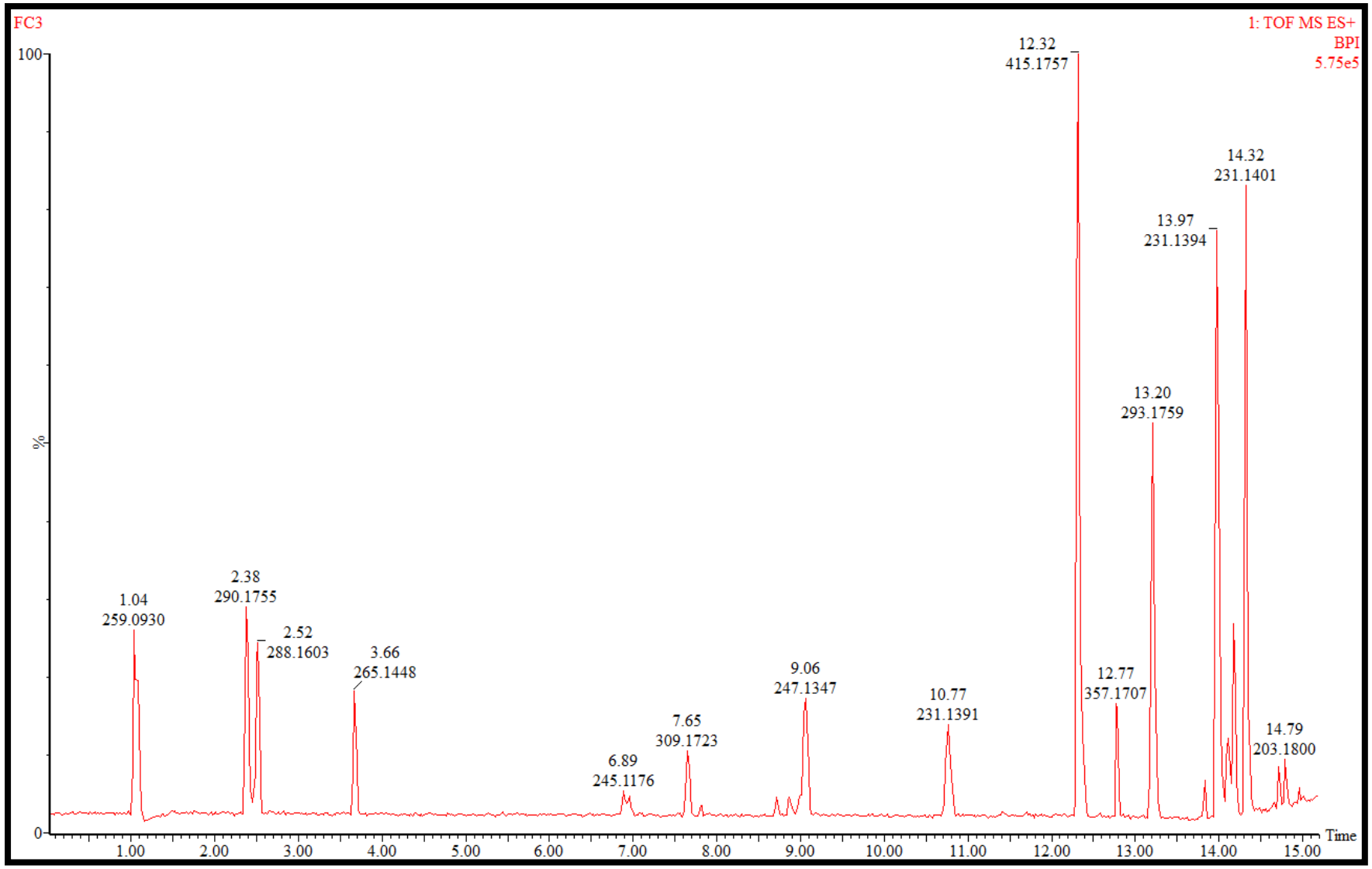

Figure C-6: BPI chromatogram of FC 3

Table C-6: Retention times and area percentage of individual compounds in FC 3

Retention (min)	Peak area	Area %
3.66	4102	3.615403
6.90	**937**	**0.825849**
7.65	2436	2.147031
9.06	**6111**	**5.386087**
10.78	4723	4.162737
12.32	**26077**	**22.98363**
12.77	3271	2.88298
13.20	14706	12.96151
13.97	22468	19.80275
14.18	8500	7.491693
14.32	**19196**	**16.91889**
Total	**113459**	**100**

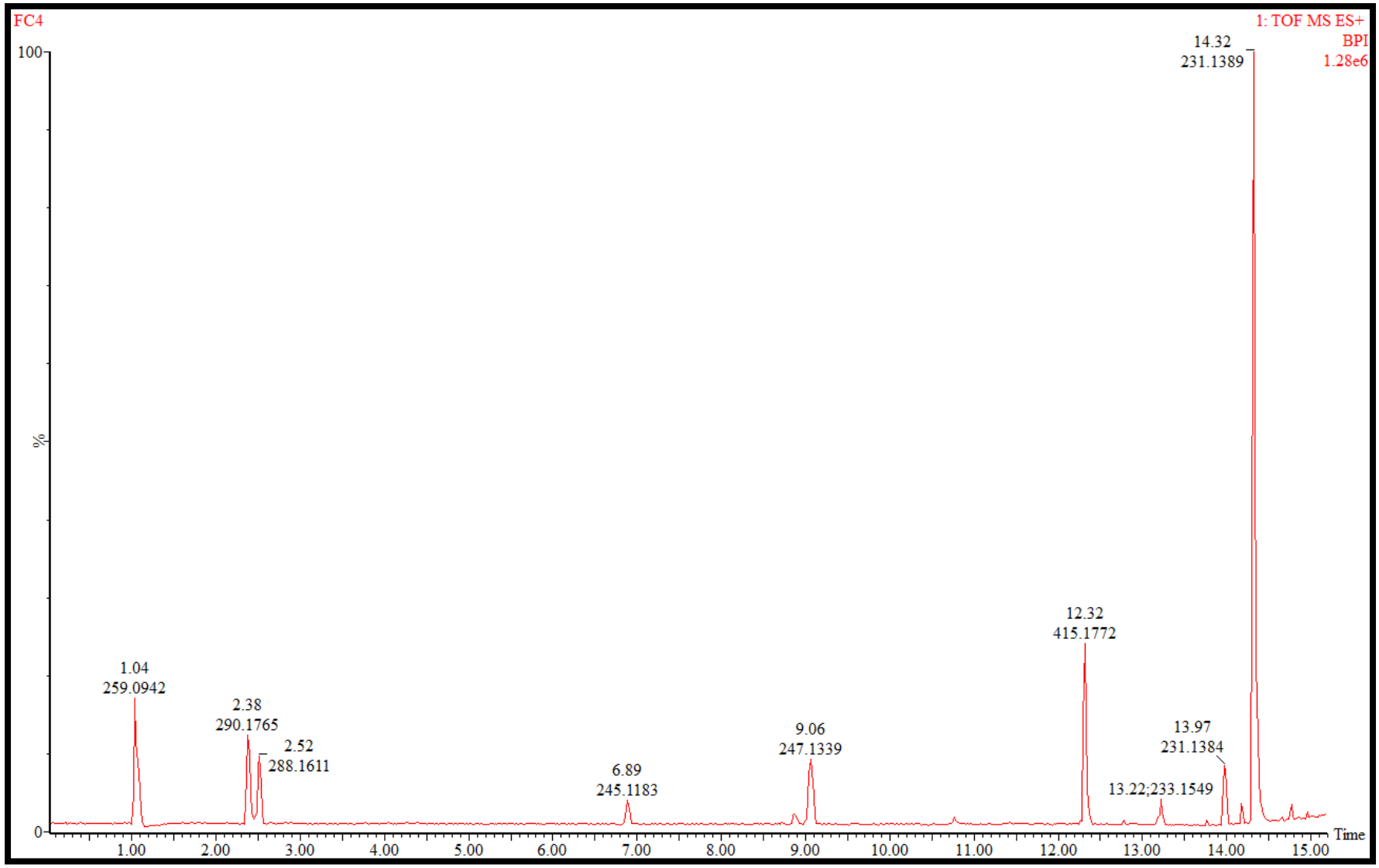

Figure C-7: BPI chromatogram of FC 4

Table C-7: Retention times and area percentage of individual compounds in FC 4

Retention (min)	Peak area	Area %
6.90	**2275**	**2.681645**
9.06	**6810**	**8.027253**
10.78	635	0.748503
12.32	**12669**	**14.93352**
13.20	2018	2.378707
13.97	4719	5.562497
14.18	823	0.970107
14.32	**54887**	**64.69777**
Total	**84836**	**100**

Figure C-8: BPI chromatogram of FC 5

Table C-8: Retention times and area percentage of individual compounds in FC 5

Retention (min)	Peak area	Area %
3.66	950	0.411706
6.90	**3774**	**1.635558**
8.71	3404	1.475209
9.06	**14031**	**6.080686**
10.78	5687	2.464604
12.32	**35207**	**15.25784**
12.79	5126	2.221481
13.20	11424	4.950877
13.83	4949	2.144773
13.99	18112	7.849289
14.18	8385	3.63385
14.32	**119698**	**51.87413**
Total	**230747**	**100**

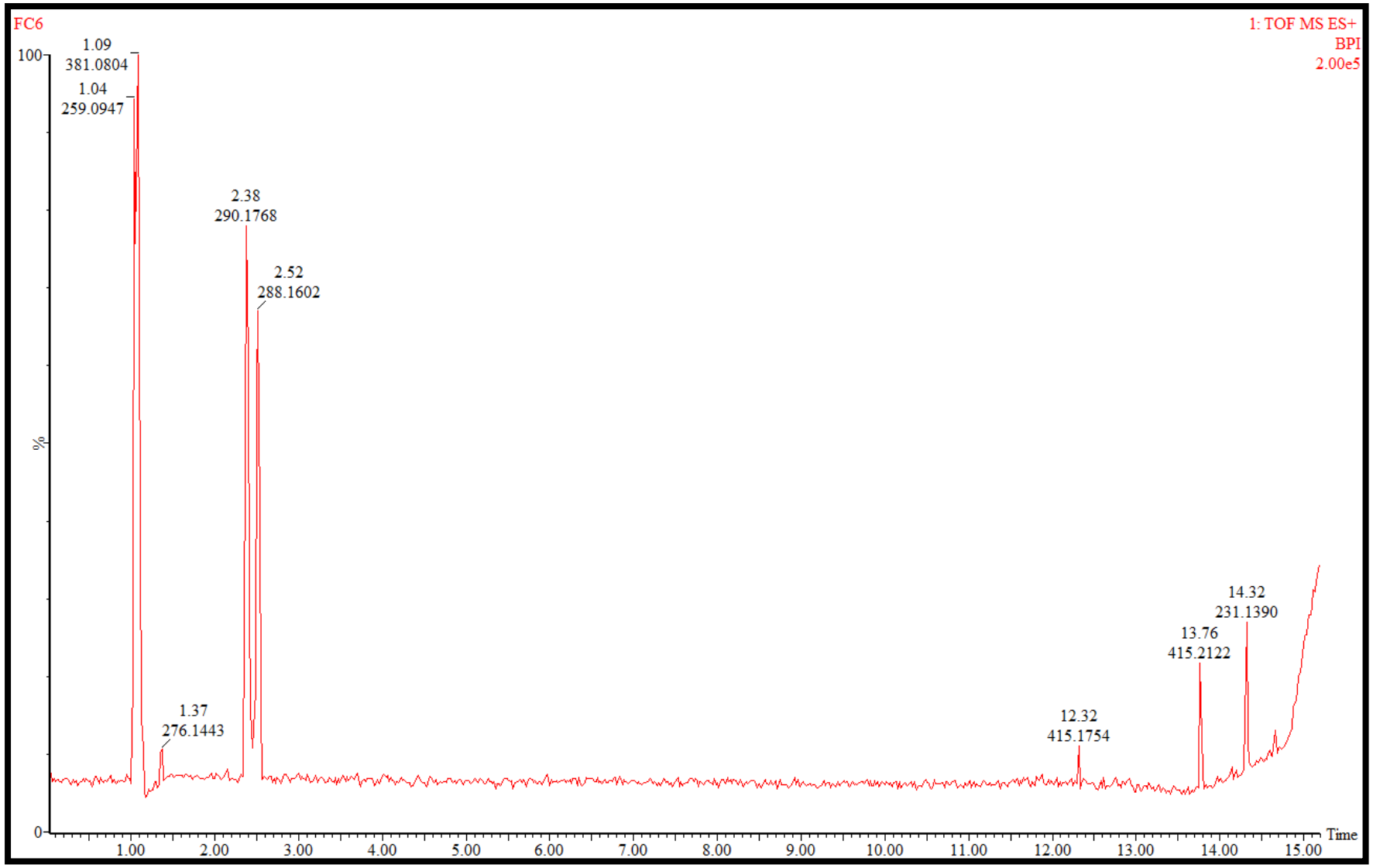

Figure C-9: BPI chromatogram of FC 6

Table C-9: Retention times and area percentage of individual compounds in FC 6

Retention (min)	Peak area	Area %
12.32	**183**	**7.436002**
13.76	1053	42.78748
14.32	**1225**	**49.77651**
Total	**2461**	**100**

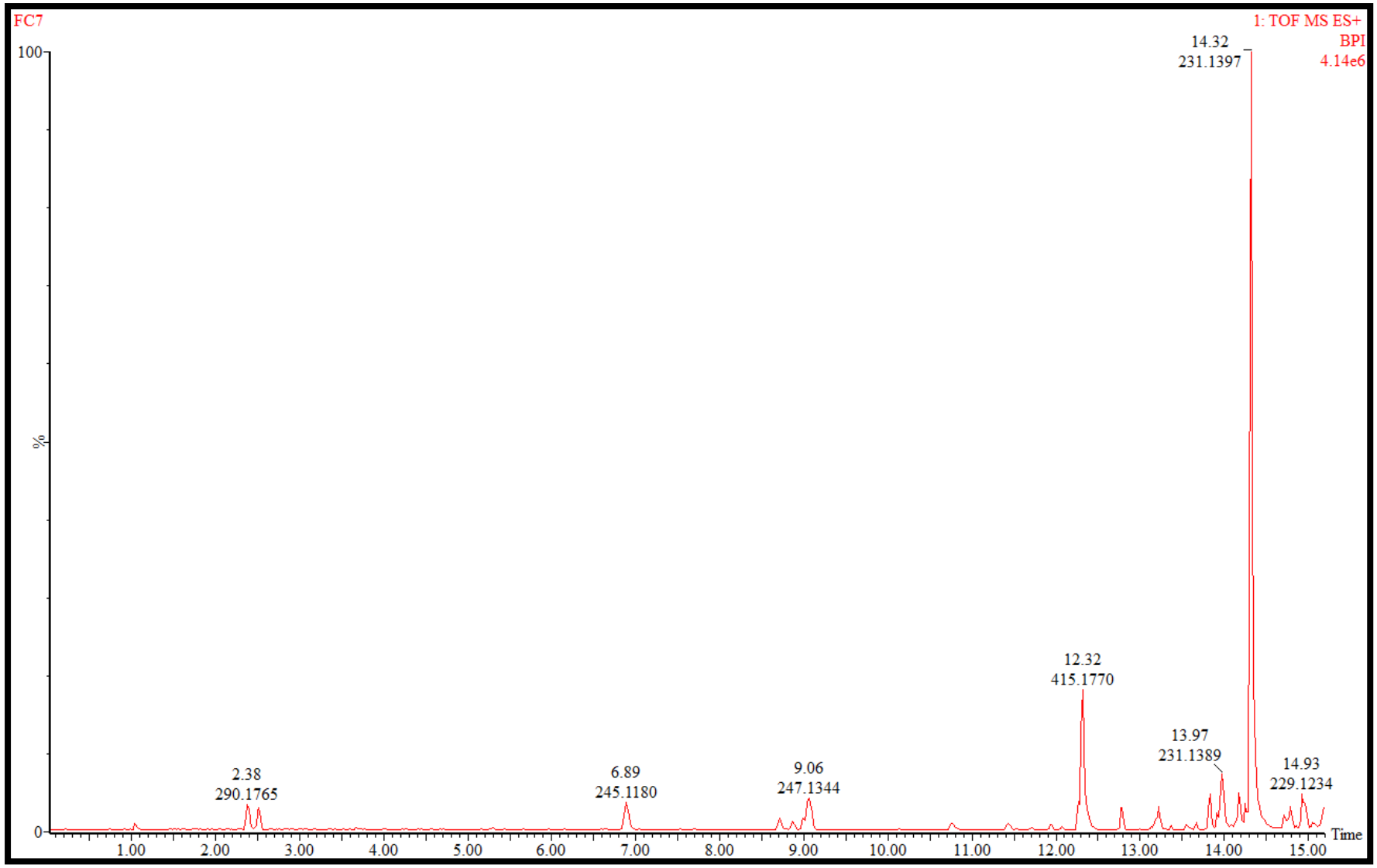

Figure C-10: BPI chromatogram of FC 7

Table C-10: Retention times and area percentage of individual compounds in FC 7

Retention (min)	Peak area	Area %
6.90	**9341**	**3.069315**
8.71	2909	0.955855
9.06	**14262**	**4.686283**
10.78	2306	0.757718
11.43	2015	0.662099
12.32	**43832**	**14.40255**
12.79	5516	1.812476
13.20	7988	2.624739
13.83	7346	2.413787
13.99	18166	5.96908
14.18	7238	2.3783
14.32	**180731**	**59.38555**
Total	**304335**	**100**

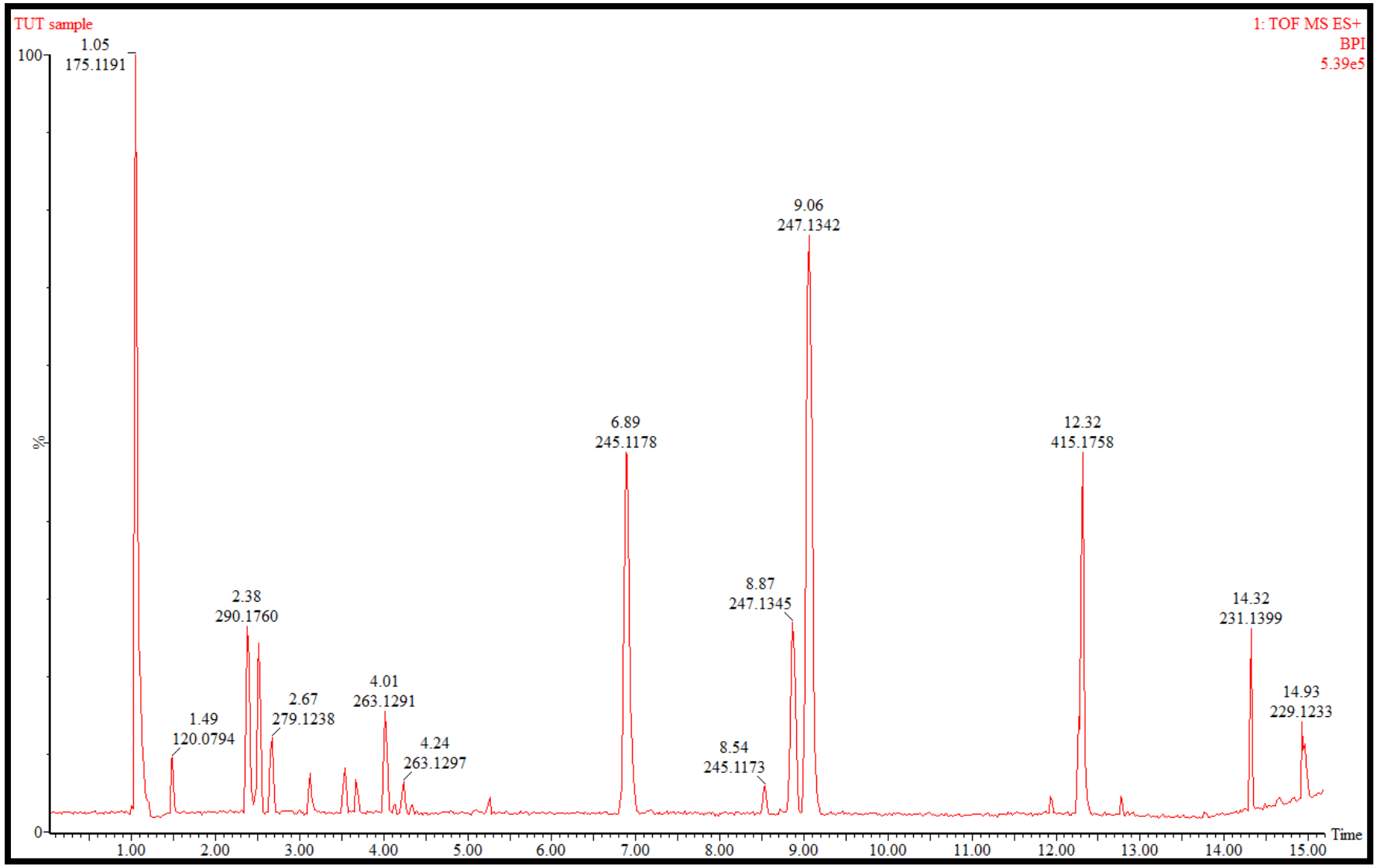

Figure C-11: BPI chromatogram of TUT (C)

Table C-11: Retention times and area percentage of individual compounds in TUT(C)

Retention (min)	Peak area	Area %
4.01	3164	3.796086
6.90	**19530**	**23.43159**
8.54	953	1.143385
8.87	8842	10.60841
9.06	**32789**	**39.3394**
11.93	684	0.820646
12.32	**12610**	**15.12916**
12.79	516	0.619084
14.32	**4261**	**5.112239**
Total	**83349**	**100**

ANNEXURE D

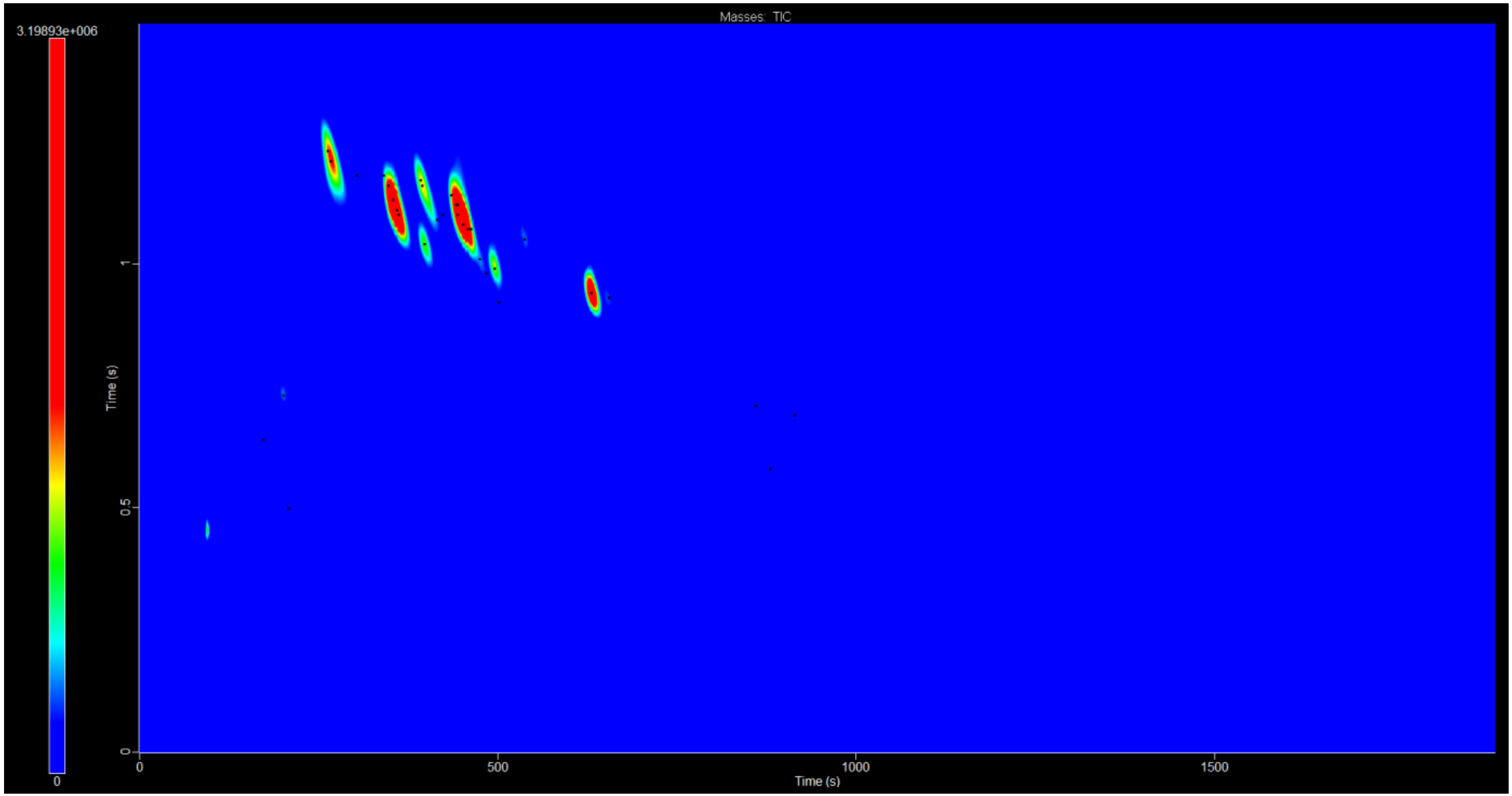

Figure D-1: North-West sample 1 (Muti)

Table D-1: North-West sample 1

Peak #	Name	Similarity	Area %	R.T. (s)
4	α-Pinene	939	11.281	267 , 1.210
5	Camphene	945	0.70827	303 , 1.180
6	Geranyl formate	685	0.21669	340.5 , 1.180
8	Sabinene	955	21.27	354 , 1.130
9	β-Pinene	912	7.5857	394.5 , 1.160
10	Myrcene	935	3.2326	397.5 , 1.040
12	1,3-Cyclohexadiene, 1,3,5,5-tetramethyl-	765	0.22769	423 , 1.100
13	Cyclobutane, 1,3-diisopropenyl-, trans	854	0.45757	435 , 1.140
14	Sylvestrene	901	4.5412	444 , 1.100
15	Eucalyptol	862	15.906	444 , 1.120
16	Sabinene hydrate<cis->(IPP vs OH)	809	6.4879	451.5 , 1.080
17	β-Phellandrene<beta->	843	9.2735	457.5 , 1.070
18	Eucalyptol	833	0.62445	462 , 1.070
19	à-Pinene	906	0.68722	474 , 1.010
20	Hypotaurine	956	0.10568	483 , 0.980
21	β-Ocimene	941	4.1059	495 , 0.990
22	o-Cymene	956	0.45738	501 , 0.920
23	terpinolene	920	1.0556	537 , 1.050
24	Bicyclo[3.1.0]hex-2-ene, 4,4,6,6-tetramethyl-	912	8.575	630 , 0.940
25	neo allo Ocimene	899	0.76248	654 , 0.930
26	4-Ethyl-2-hexynal	778	0.27374	859.5 , 0.710
27	Furan, 3-methyl-	781	0.066848	879 , 0.580
28	4-Ethyl-2-hexynal	786	0.2608	913.5 , 0.690

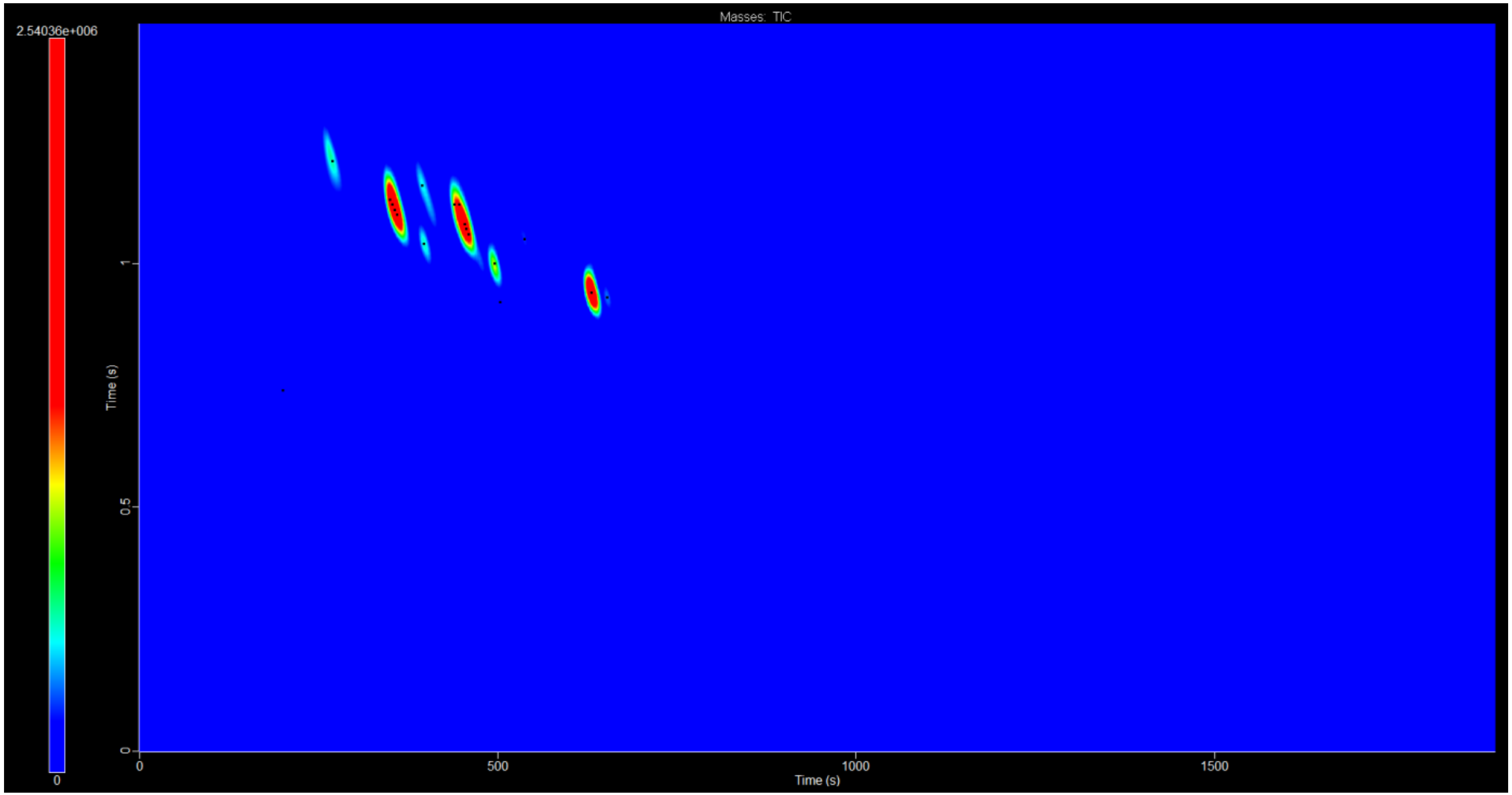

Figure D-2: North-West sample 2 (Muti)

Table D-2: North-West sample 2

Peak #	Name	Similarity	Area %	R.T. (s)
2	α-Pinene	822	4.8111	273 , 1.140
3	á-Thujene	911	1.0718	292.5 , 1.110
4	Camphene	954	1.9768	318 , 1.130
5	Sabinene	954	17.05875	345 , 1.150
7	á-Phellandrene	786	1.9942	355.5 , 1.130
8	2-Methoxybenzyl alcohol	745	0.2723	358.5 , 1.070
12	α-Phellandrene	770	4.4575	373.5 , 1.060
13	Myrcene	915	5.1597	405 , 1.020
14	Phellandrene<alpha->	880	0.79276	417 , 1.070
16	2,4,6-Octatriene, 2,6-dimethyl-	822	3.9574	429 , 1.080
17	β-Phellandrene	923	1.0807	430.5 , 1.040
18	α-Terpinene	906	0.61093	435 , 1.070
20	Eucalyptol	895	19.839	448.5 , 1.110
24	ç-Terpinene	893	0.51169	493.5 , 1.060
26	α-Ocimene	912	6.626	499.5 , 0.990
27	γ-Terpinene	844	1.8305	499.5 , 1.050
29	o-Cymene	923	1.0609	507 , 0.910
30	á-Ocimene	901	2.5439	507 , 0.970
31	(+)-4-Carene	817	0.59296	508.5 , 1.020
32	o-Cymene	943	0.57383	513 , 0.900
35	Allo-Ocimene	854	1.4068	624 , 0.960
37	neo allo Ocimene	915	3.2157	648 , 0.910
38	2,4,6-Octatriene, 3,4-dimethyl-	923	3.5077	654 , 0.930
39	2,4,6-Octatriene, 2,6-dimethyl-	868	1.4062	657 , 0.930
42	Terpinen-4-ol	897	0.36506	837 , 0.730

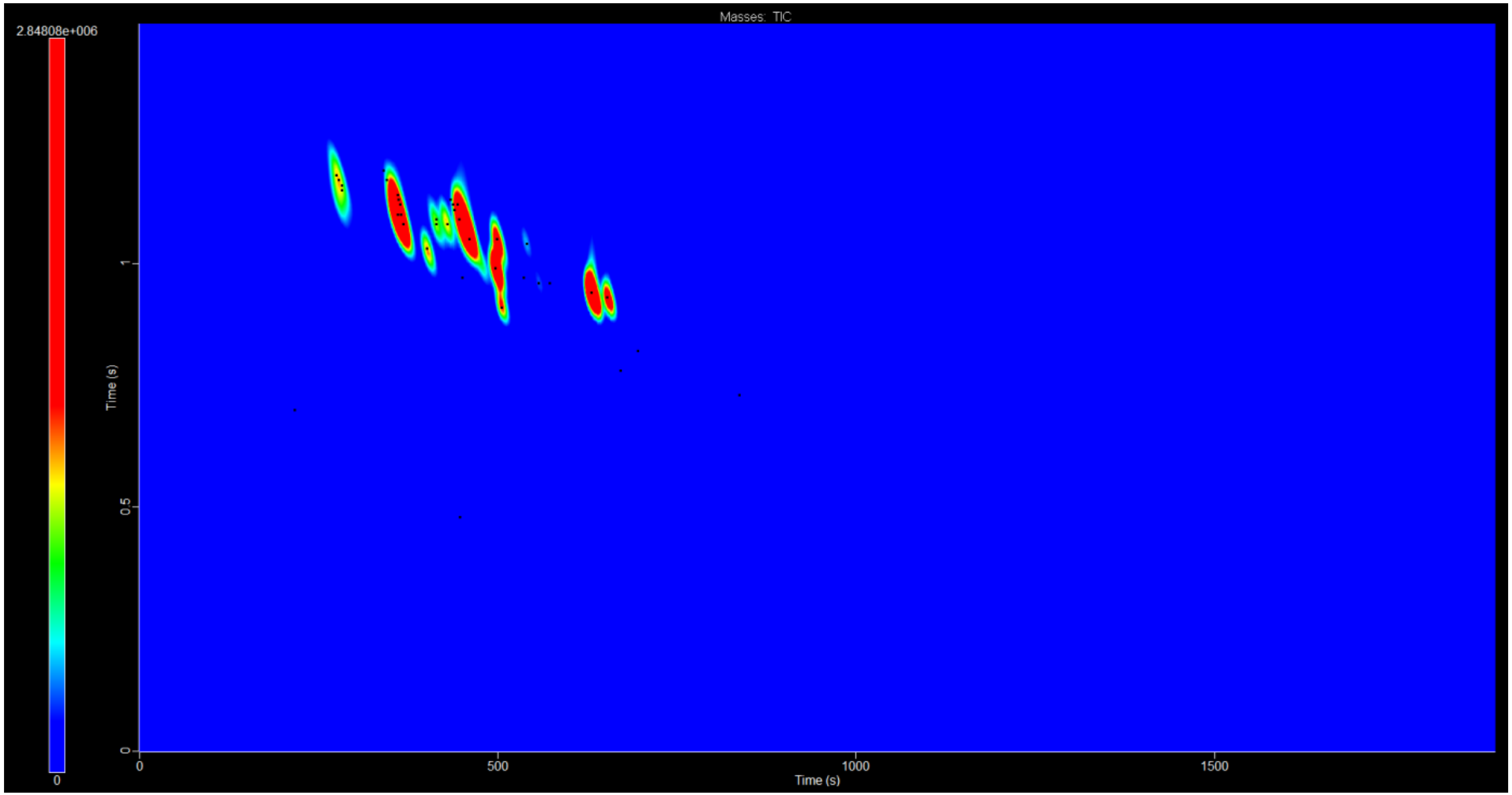

Figure D-3: Fresh Cone 1

Table D-3: Fresh cone 1

Peak #	Name	Similarity	Area %	R.T. (s)
2	α-Pinene	938	3.3183	274.5 , 1.180
7	β-Pinene	840	0.24207	345 , 1.170
8	Sabinene	949	14.056	360 , 1.100
10	Myrcene	695	0.75369	361.5 , 1.130
13	α-Phellandrene	914	4.702	367.5 , 1.080
14	Myrcene	942	2.3971	400.5 , 1.030
15	2-Thujene	909	2.878	414 , 1.080
16	o-Cymene	823	0.7638	414 , 1.090
17	delta-2 Carene	864	2.8967	429 , 1.080
19	1,5-Cyclooctadiene, 3,4-dimethyl-	814	0.19536	436.5 , 1.120
20	Limonene	902	0.4604	439.5 , 1.110
21	Eucalyptol	922	13.88	444 , 1.120
23	Isopropyl Alcohol	830	0.04575	447 , 0.480
24	1,3,8-p-Menthatriene	823	0.06602	450 , 0.970
25	β-Phellandrene<beta->	901	20.673	460.5 , 1.050
26	á-Ocimene	948	5.6708	496.5 , 0.990
27	ç-Terpinene	896	4.1447	498 , 1.050
28	o-Cymene	953	2.0719	505.5 , 0.910
29	1,3-Cyclopentadiene, 5,5-dimethyl-2-propyl-	869	0.13371	535.5 , 0.970
30	Terpinolene	920	0.69121	540 , 1.040
31	7.84 Carene<delta-2->	886	0.47349	556.5 , 0.960
32	1,3-Cyclopentadiene, 5,5-dimethyl-2-propyl-	864	0.33941	571.5 , 0.960
33	Neo-allo-ocimene	920	8.0857	630 , 0.940
34	Bicyclo[3.1.0]hex-2-ene, 4,4,6,6-tetramethyl-	912	3.2747	652.5 , 0.930
35	p-Cymenene	946	0.11777	670.5 , 0.780
36	2,6-Dimethyl-1,3,5,7-octatetraene, E,E-	910	0.22946	694.5 , 0.820
37	(-)-Terpinen-4-ol	886	0.0497	837 , 0.730

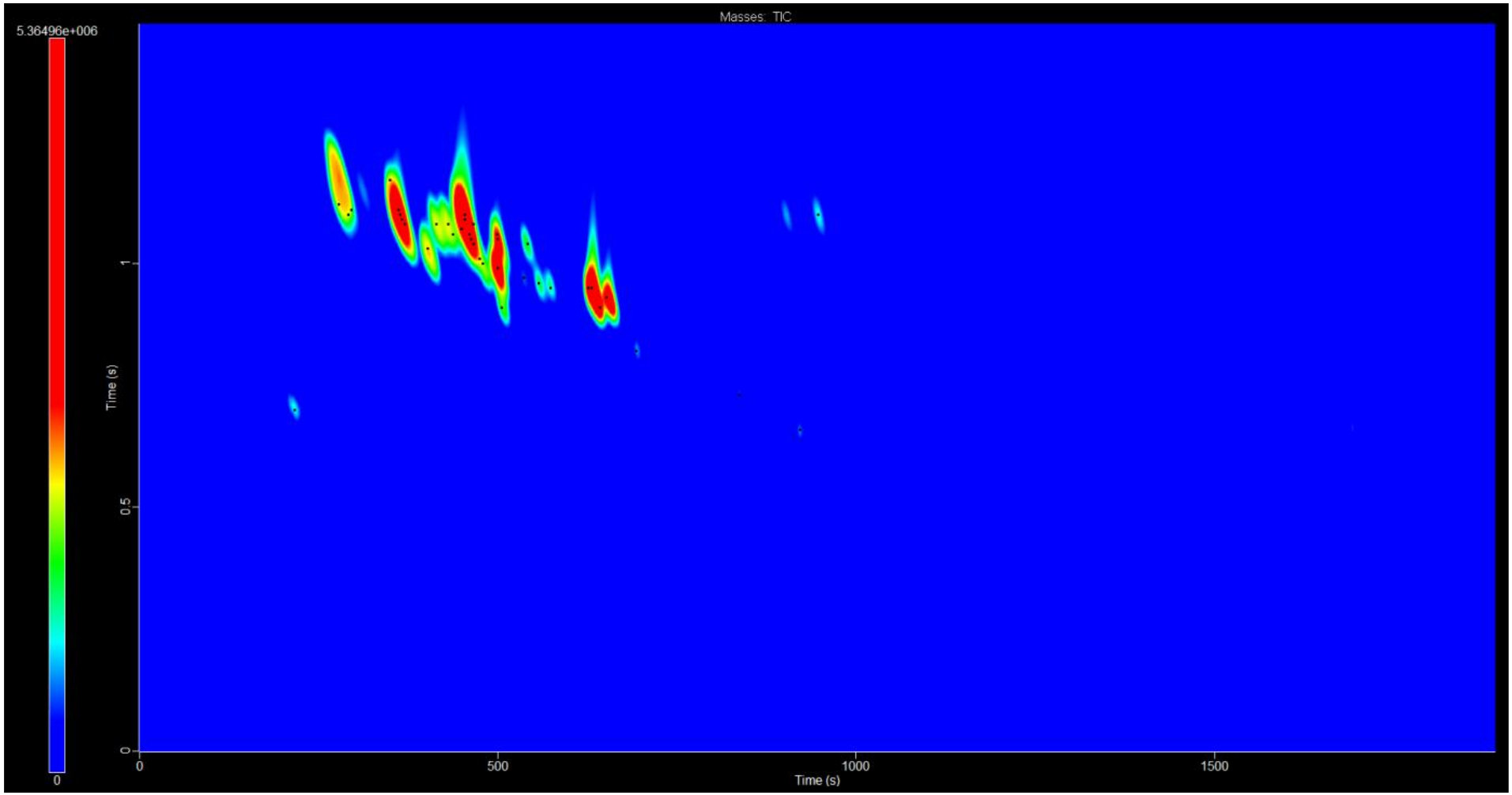

Figure D-4: Fresh Cone 2

Table D-4: Fresh cone 2

Peak #	Name	Similarity	Area %	R.T. (s)
3	α-Thujene	908	6.5106	291 , 1.100
4	α-Pinene	943	1.0259	295.5 , 1.110
5	β-Pinene	830	1.5341	349.5 , 1.170
6	Sabinene	864	11.248	361.5 , 1.110
7	α-Phellandrene	782	6.4748	363 , 1.100
10	Myrcene	945	3.35	402 , 1.030
12	Allo-Ocimene	864	2.6129	430.5 , 1.080
13	Terpinolene	906	0.8546	436.5 , 1.060
14	Limonene	819	4.9222	448.5 , 1.070
16	Eucalyptol	888	14.807	453 , 1.100
17	β-Phellandrene	697	4.1988	460.5 , 1.060
23	Bicyclo[4.2.0]oct-1-ene, 7-exo-ethenyl-	782	0.53511	498 , 1.060
24	α-Ocimene	914	7.3844	499.5 , 0.990
25	γ-Terpinene	886	3.6137	499.5 , 1.050
26	o-Cymene	942	1.0893	505.5 , 0.910
27	1,3-Cyclopentadiene, 5,5-dimethyl-2-propyl-	876	0.31324	535.5 , 0.970
28	Terpinolene	936	1.2097	541.5 , 1.040
29	Cyclohexene, 4-methyl-3-(1-methylethylidene)-	894	1.1956	556.5 , 0.960
30	1,3-Cyclopentadiene, 5,5-dimethyl-2-propyl-	890	0.78121	573 , 0.950
31	2,4,6-Octatriene, 2,6-dimethyl-, (E,Z)-	911	1.8223	625.5 , 0.950
32	3-Octen-5-yne, 2,7-dimethyl-, (E)-	877	5.3908	630 , 0.950
33	Bicyclo[3.1.0]hex-2-ene, 4,4,6,6-tetramethyl-	913	1.8062	642 , 0.910
34	allo-Ocimene	913	5.9132	651 , 0.930
35	2,6-Dimethyl-1,3,5,7-octatetraene, E,E-	931	0.31249	693 , 0.820
36	Terpinen-4-ol	892	0.16239	837 , 0.730
37	α-Terpineol	931	0.19544	921 , 0.660
38	ç-Elemene	919	0.98642	946.5 , 1.100

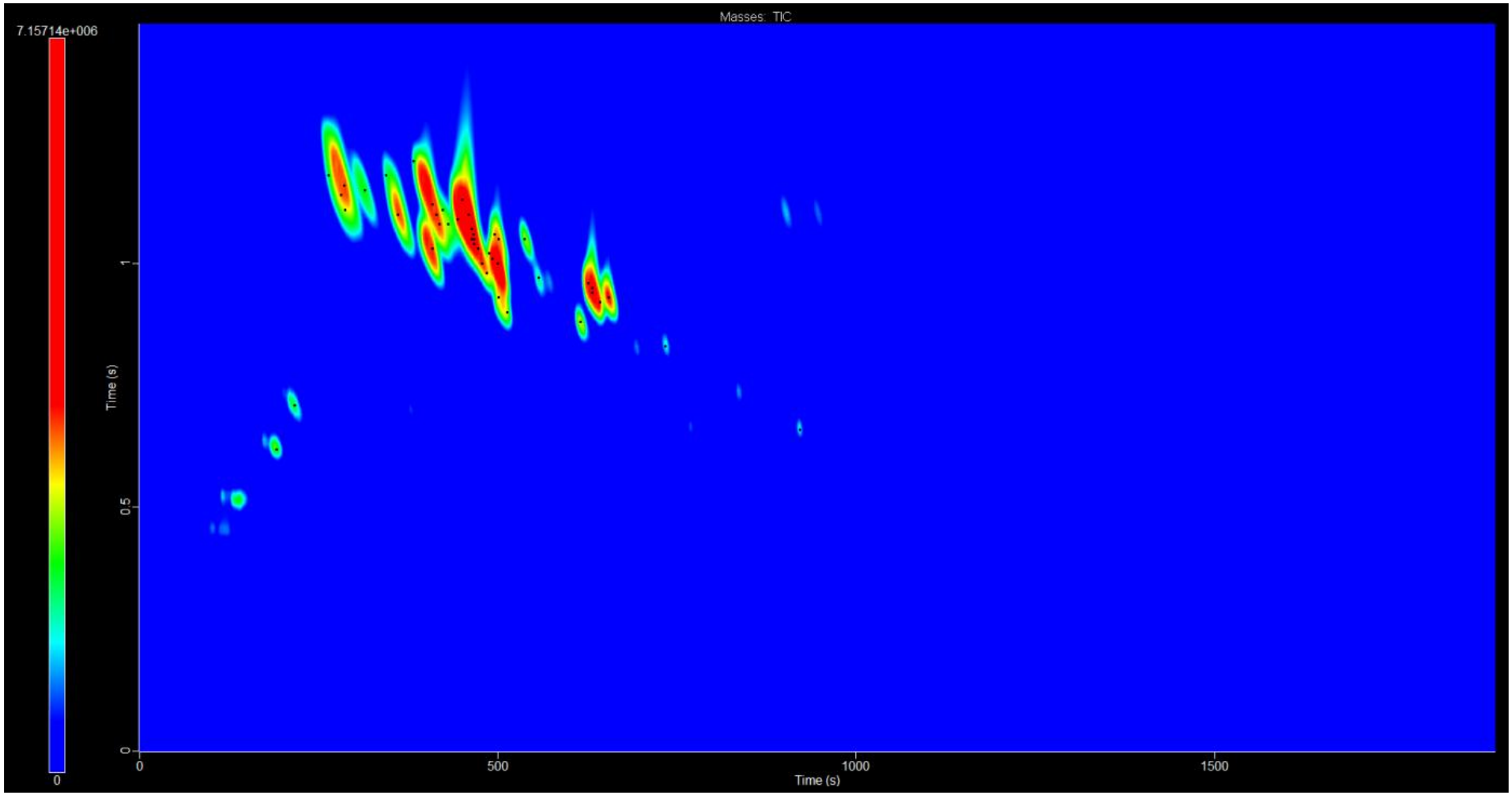

Figure D-5: Fresh Cone 3

Table D-5: Fresh cone 3

Peak #	Name	Similarity	Area %	R.T. (s)
7	Camphene	963	1.9829	313.5 , 1.150
8	α-Pinene	936	2.74	343.5 , 1.180
9	Sabinene	865	5.2018	360 , 1.100
10	3-Carene	930	0.86958	382.5 , 1.210
11	α-Myrcene	920	5.98	408 , 1.030
12	α-Phellandrene	871	1.0434	408 , 1.120
14	α-Thujene	872	1.2176	418.5 , 1.080
17	Limonene	846	3.1633	444 , 1.090
18	Eucalyptol	904	15.042	450 , 1.130
20	β-Phellandrene	711	5.42	463.5 , 1.050
25	β-Phellandrene	769	2.8291	472.5 , 1.030
26	trans-á-Ocimene	904	0.71207	477 , 1.000
28	17-Octadecen-14-yn-1-ol	664	0.58079	487.5 , 1.020
29	1,3,7-Octatriene, 3,7-dimethyl-	796	1.0755	492 , 1.010
30	γ-Terpinene<gamma->	898	1.3339	495 , 1.060
31	α-Ocimene	891	10.435	499.5 , 1.000
32	o-Cymene	922	0.15098	501 , 0.930
33	1,3-Cyclohexadiene, 1-methyl-4-(1-methylethyl)-	808	1.9729	501 , 1.050
34	o-Cymene	950	1.1092	513 , 0.900
35	Terpinolene	936	1.7386	537 , 1.050
36	Cyclohexene, 1,5,5-trimethyl-3-methylene-	895	1.179	556.5 , 0.970
38	Ocimene<allo->	894	7.2252	625.5 , 0.960
40	4-Decen-6-yne, (E)-	822	3.4546	631.5 , 0.950
41	Bicyclo[3.1.0]hex-2-ene, 4,4,6,6-tetramethyl-	918	1.7979	642 , 0.920
42	4-trans,6-cis-Allocimene	915	4.802	654 , 0.930
43	Piperitenone	812	0.45962	733.5 , 0.830
44	α-Terpineol<alpha->	935	0.35709	921 , 0.660

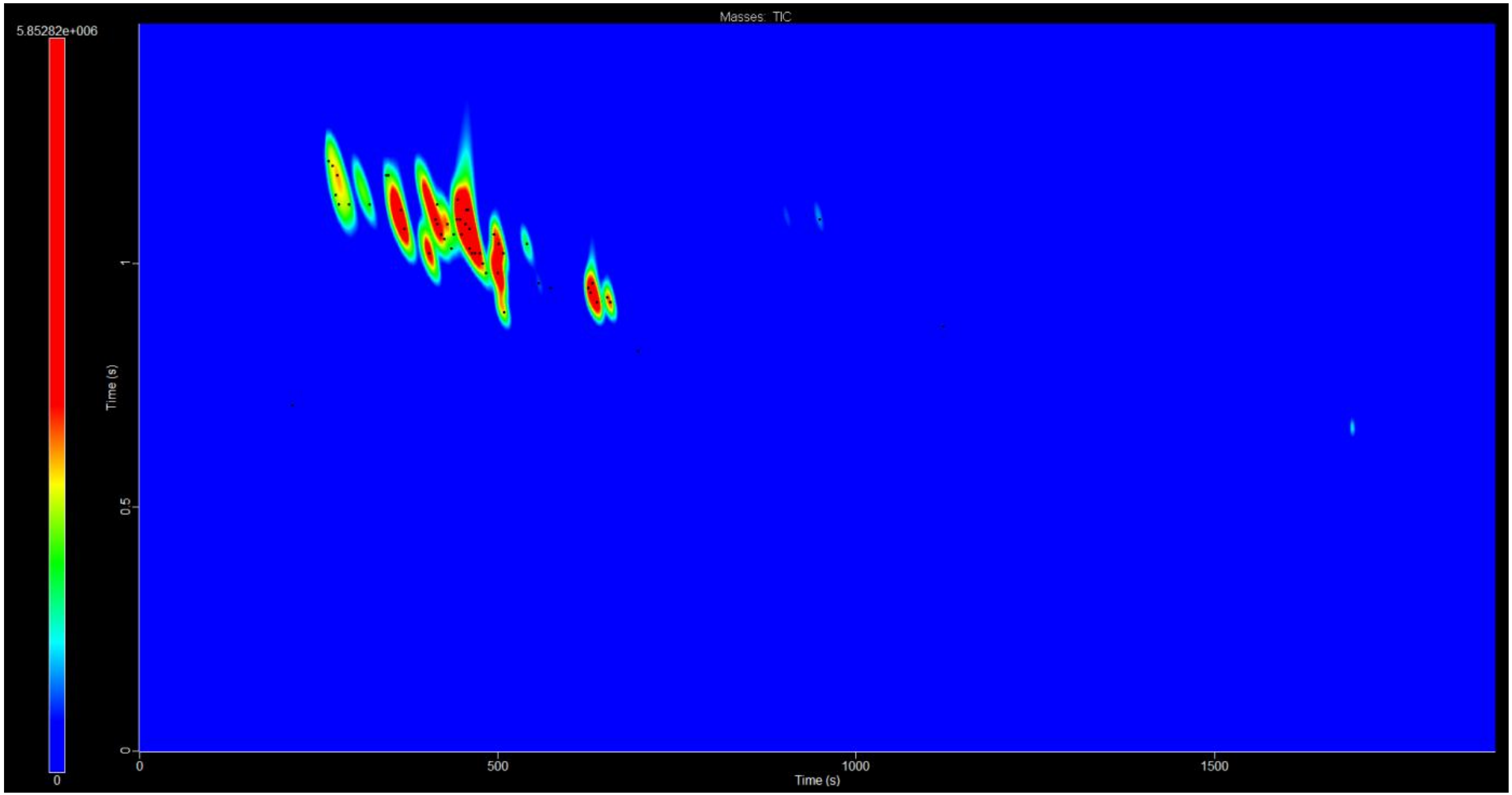

Figure D-6: Fresh Cone 4

Table D-6: Fresh cone 4

Peak #	Name	Similarity	Area %	R.T. (s)
7	α-Pinene	945	1.5129	292.5 , 1.120
8	Camphene	954	2.9395	321 , 1.120
10	β-Pinene	854	4.6584	346.5 , 1.180
12	Sabinene	941	10.6575	369 , 1.070
13	Myrcene	938	3.6396	403.5 , 1.020
14	á-Thujene	900	1.8041	412.5 , 1.090
17	α-Phellandrene	907	3.8294	420 , 1.060
19	1,3-Cyclohexadiene, 1-methyl-4-(1-methylethyl)-	918	3.4805	429 , 1.080
20	α-Phellandrene<alpha->	908	0.21904	435 , 1.030
22	Sylvestrene	898	2.7356	442.5 , 1.090
23	Eucalyptol	836	7.928	444 , 1.130
30	β-Phellandrene	759	9.2336	460.5 , 1.070
36	γ-Terpinene	906	1.61584	493.5 , 1.060
37	α-Ocimene	936	6.2365	499.5 , 0.980
38	(+)-4-Carene	786	1.2261	501 , 1.040
40	o-Cymene	948	1.6435	508.5 , 0.900
41	Terpinolene	936	1.0616	540 , 1.040
42	Cyclohexene, 4-methyl-3-(1-methylethylidene)-	888	0.40493	556.5 , 0.960
43	1,3-Cyclopentadiene, 5,5-dimethyl-2-propyl-	879	0.27737	573 , 0.950
44	(4E,6Z)-allo-Ocimene	910	3.1238	625.5 , 0.950
46	Tricyclo[3.1.0.0(2,4)]hexane, 3,3,6,6-tetramethyl-, (1à,2á,4á,5à)-	854	1.4235	631.5 , 0.960
47	Neo-allo-ocimene	916	1.8989	637.5 , 0.920
49	Bicyclo[3.1.0]hex-2-ene, 4,4,6,6-tetramethyl-	913	1.0292	657 , 0.920
50	2,6-Dimethyl-1,3,5,7-octatetraene, E,E-	924	0.16235	694.5 , 0.820
51	ç-Elemene	918	0.64234	948 , 1.090
52	Curzerene	931	0.19836	1120.5 , 0.870

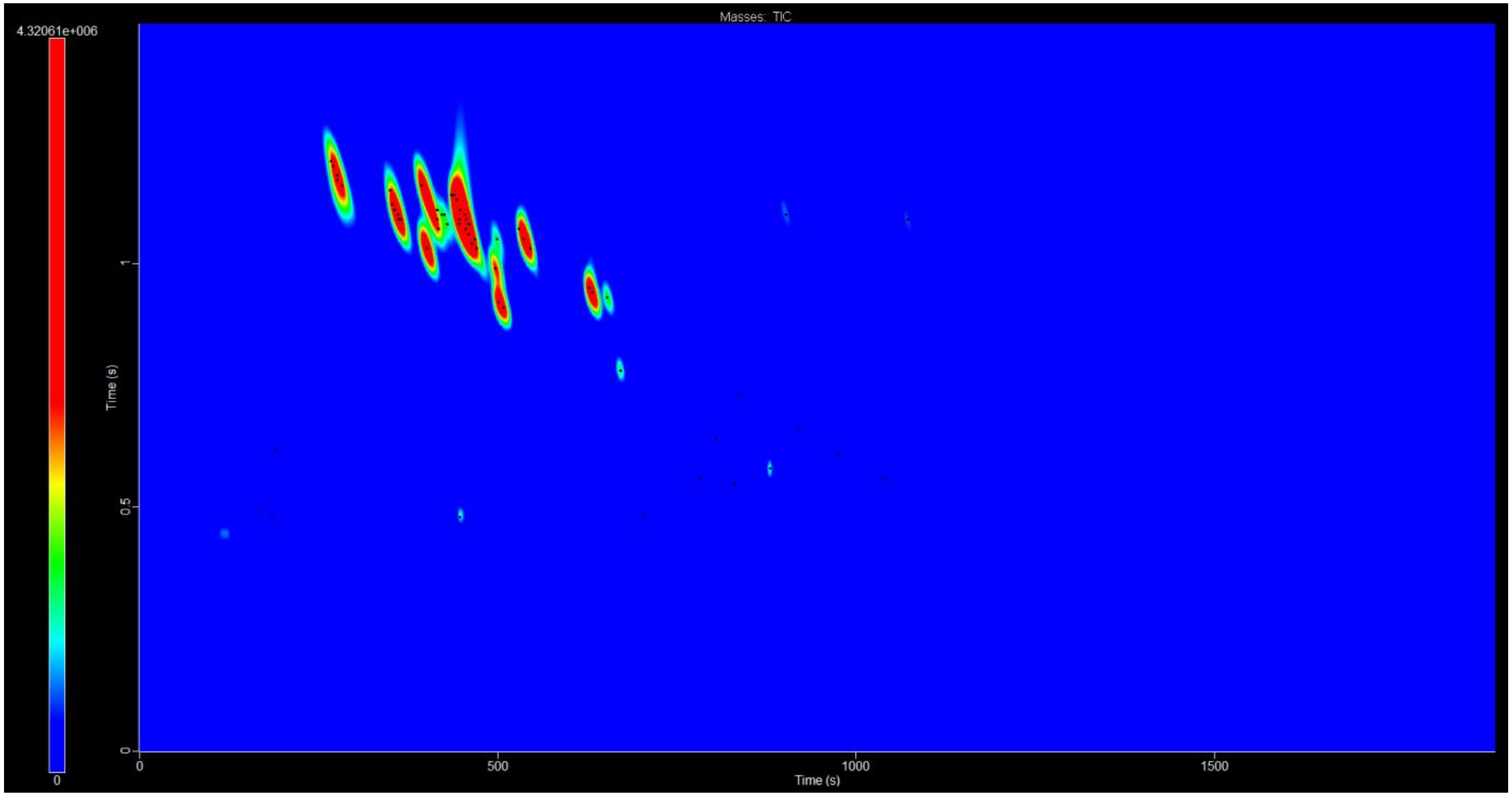

Figure D-7: Fresh Cone 5

Table D-7: Fresh cone 5

Peak #	Name	Similarity	Area %	R.T. (s)
1	α-Pinene	946	5.3402	277.5 , 1.170
2	Sabinene	952	4.3957	363 , 1.090
3	3-Carene	926	2.1346	400.5 , 1.140
4	Myrcene	940	3.2134	403.5 , 1.020
5	p-(Pentyloxy)acetophenone	690	0.53548	420 , 1.110
6	1,3-Cyclohexadiene, 1-methyl-4-(1-methylethyl)-	908	4.8556	429 , 1.080
7	Sylvestrene	812	0.22429	438 , 1.120
8	Acetoin	865	0.11141	448.5 , 0.480
9	Eucalyptol	849	15.121	448.5 , 1.110
11	Cyclobutane, 1,2-bis(1-methylethenyl)-, trans-	828	1.8736	456 , 1.030
13	Bicyclo[2.2.1]heptane, 2,2-dimethyl-3-methylene-, (1R)-	846	1.072	462 , 1.020
14	α-Phellandrene	893	3.1587	463.5 , 1.040
15	cis-Sabinene hydrate	766	0.88047	463.5 , 1.080
17	β-Phellandrene	890	7.5994	466.5 , 1.040
19	Hypotaurine	890	0.15239	487.5 , 0.970
20	α-Ocimene	951	5.7442	496.5 , 0.990
22	γ-Terpinene	908	0.99655	501 , 1.040
23	o-Cymene	949	1.2569	507 , 0.910
24	(+)-2-Carene	932	2.3099	534 , 1.070
25	1,3-Cyclopentadiene, 5,5-dimethyl-2-propyl-	849	0.22733	537 , 0.970
26	Terpinolene	937	3.1814	540 , 1.050
27	1,3-Cyclopentadiene, 5-(1,1-dimethylethyl)-	726	0.087271	541.5 , 0.960
28	E-βOcimene	782	0.87893	541.5 , 1.070
29	trans-α-Ocimene	850	1.4888	546 , 1.010
30	3-Cyclopentylpropionic acid, 4-isopropylphenyl ester	800	2.1113	546 , 1.030
31	1,3,6-Heptatriene, 2,5,5-trimethyl-	855	0.97736	552 , 0.990
32	Cyclohexene, 1-methyl-4-(1-methylethylidene)-	916	1.9328	553.5 , 1.000
33	1,3-Cyclopentadiene, 5,5-dimethyl-2-propyl-	897	1.1503	573 , 0.950

34	allo Ocimene	900	1.7267	621 , 0.960
37	neo allo Ocimene<E,Z)->	894	7.6393	634.5 , 0.940
41	2,4,6-Octatriene, 2,6-dimethyl-	853	0.8863	651 , 0.930
42	Tricyclo[3.1.0.0(2,4)]hexane, 3,3,6,6-tetramethyl-, (1à,2á,4á,5à)-	852	2.1531	652.5 , 0.950
44	Bicyclo[3.1.0]hex-2-ene, 4,4,6,6-tetramethyl-	916	1.3143	663 , 0.910
45	p-Cymenene	958	0.35081	672 , 0.780
46	2,6-Dimethyl-1,3,5,7-octatetraene, E,E-	920	0.28793	694.5 , 0.820
47	β-Elemene	908	0.7085	901.5 , 1.100
48	Curzerene	928	0.15142	1120.5 , 0.870

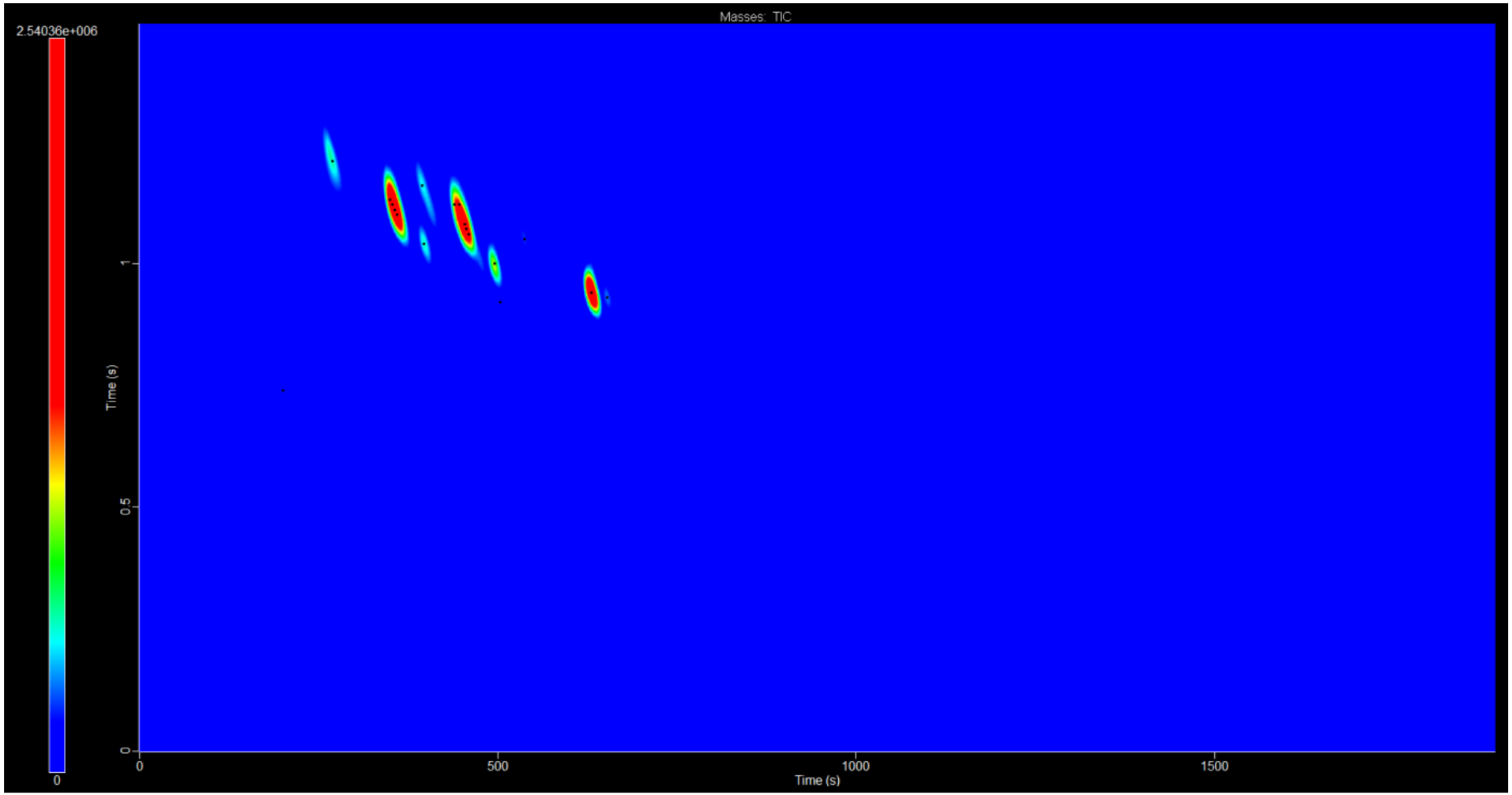

Figure D-8: Fresh Cone 6

Table D-8: Fresh cone 6

Peak #	Name	Similarity	Area %	R.T. (s)
1	O-(Carboxymethyl)hydroxylamine	936	0.090694	159 , 0.440
2	α-Pinene	916	1.5667	273 , 1.180
3	2-Thujene	915	3.8941	279 , 1.160
4	β-Pinene	860	1.1295	357 , 1.140
6	α-Phellandrene	841	2.7085	361.5 , 1.090
7	Sabinene	946	14.017	366 , 1.080
8	3-Carene	925	1.9466	400.5 , 1.140
9	Myrcene	935	3.3869	403.5 , 1.020
11	Phellandrene	763	1.439	423 , 1.060
12	α-Terpinene	904	4.4291	426 , 1.090
15	Eucalyptol	820	9.9606	445.5 , 1.140
17	Phellandrene<beta->	895	8.978	466.5 , 1.030
19	2-Thujene	787	0.99555	493.5 , 0.990
20	à-Pinene	828	2.7988	498 , 1.040
21	α-Ocimene	853	4.6749	499.5 , 0.980
22	(+)-4-Carene	819	1.3137	499.5 , 1.040
23	ç-Terpinene	872	2.36458	502.5 , 1.040
25	o-Cymene	932	1.665	505.5 , 0.910
27	ç-Terpinene	905	1.0967	508.5 , 1.020
28	Terpinolene	938	1.5671	541.5 , 1.030
29	Carene<delta-2->	889	0.98721	559.5 , 0.950
30	1,3-Cyclopentadiene, 5,5-dimethyl-2-propyl-	881	0.78859	571.5 , 0.960
32	p-Ethylanisole	785	1.5543	631.5 , 0.930
34	Bicyclo[3.1.0]hex-2-ene, 4,4,6,6-tetramethyl-	920	7.1413	636 , 0.920
35	Terpinen-4-ol	888	0.19239	835.5 , 0.730
36	ç-Elemene	931	2.2416	946.5 , 1.100
37	Curzerene	939	0.21276	1119 , 0.870

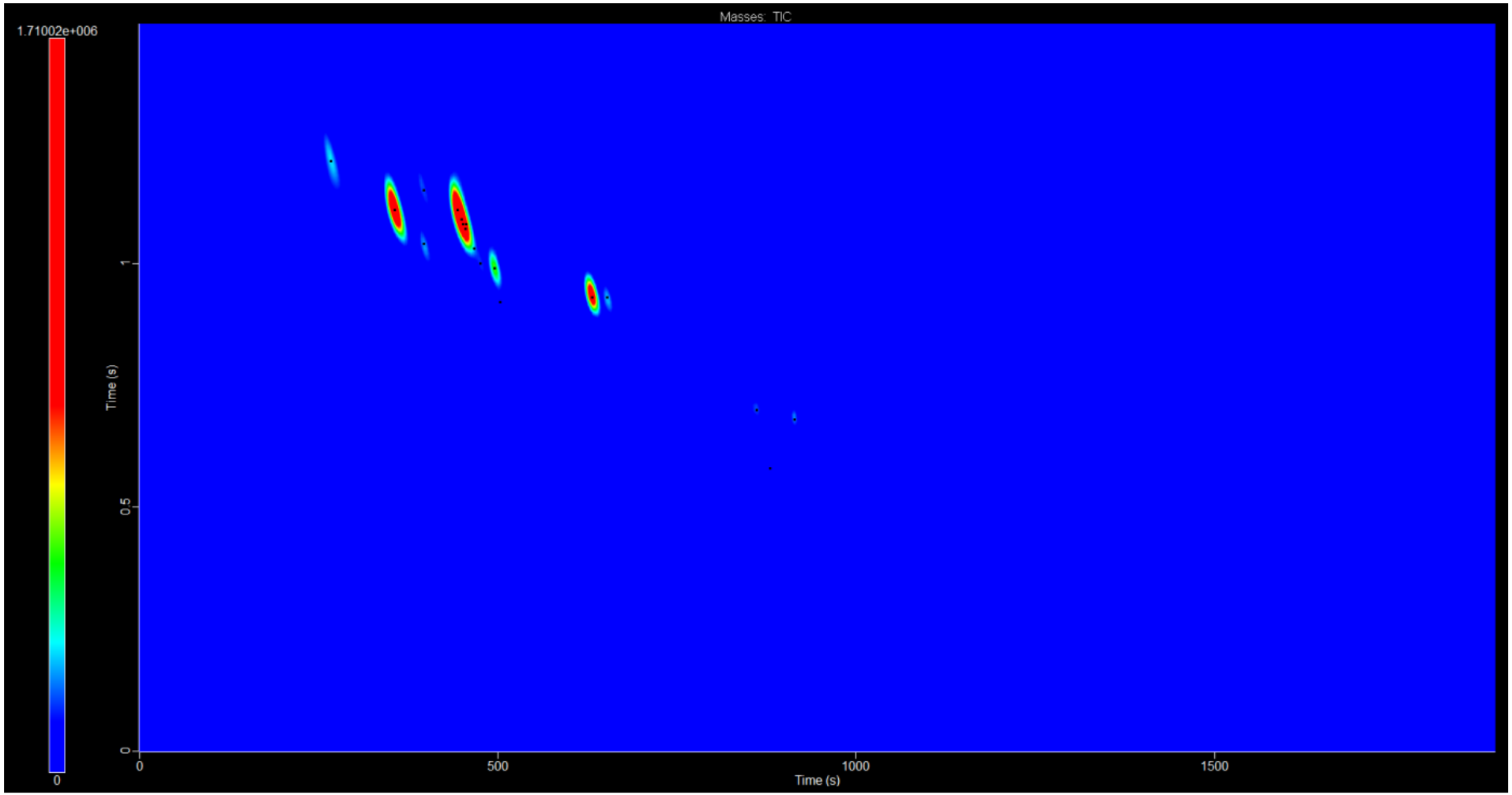

Figure D-9: Fresh Cone 7

Table D-9: Fresh cone 7

Peak #	Name	Similarity	Area %	R.T. (s)
1	à-Pinene	932	6.9197	267 , 1.210
2	1Sabinene	945	22.866	355.5 , 1.110
3	Myrcene	928	2.6962	396 , 1.040
4	β-Pinene	886	3.3945	396 , 1.150
5	Eucalyptol	858	21.981	448.5 , 1.090
6	Sabinene hydrate<cis->(IPP vs OH)	835	6.4684	451.5 , 1.080
7	Sabinene hydrate<trans->(IPP vs OH)	828	10.238	454.5 , 1.070
8	β-Phellandrene	843	1.2596	466.5 , 1.030
9	à-Pinene	912	0.82066	475.5 , 1.000
10	α-Ocimene	930	6.7784	495 , 0.990
11	o-Cymene	939	0.65452	502.5 , 0.920
12	Neo allo Ocimene	891	13.386	631.5 , 0.930
13	4-Ethyl-2-hexynal	760	1.2325	861 , 0.700
14	Furan, 3-methyl-	768	0.16537	879 , 0.580
15	4-Ethyl-2-hexynal	764	1.1392	913.5 , 0.680

ANNEXURE E

Table E-1: Transport AP-BL: Buccal 40 mg/ml water extract

Begin		**End**	
Well 1	386	Well 1	443
Well 2	387	Well 2	486
Well 3	412	Well 3	470
Average	**395**	**Average**	**466.3333333**
SD	**12.0277457**	**SD**	**21.73323108**

n= 3
SD = standard deviation

Table E-2: Transport: Buccal AP-BL: Water extract 40mg/ml, Compound 1: AG 1

Time	**Concentration (µg/ml)**	**% Transport**	**Average**	**STDEV**
M1	**68.2**	**100**	70.6	
0	0	0	0	0
20	0.7	1.026392962	0.99233	0.0351
40	0.7	1.026392962	0.99233	0.0351
60	0.8	1.173020528	1.13409	0.04011
80	1.3	1.906158358	1.8429	0.06518
100	1.5	2.19941349	2.12643	0.07521
120	1.5	2.19941349	2.12643	0.07521
M2	**70.4**	**100**		
0	0	0		
20	0.7	0.994318182		
40	0.7	0.994318182		
60	0.8	1.136363636		
80	1.3	1.846590909		
100	1.5	2.130681818		
120	1.5	2.130681818		
M3	**73.2**	**100**		
0	0	0		
20	0.7	0.956284153		
40	0.7	0.956284153		
60	0.8	1.092896175		
80	1.3	1.775956284		
100	1.5	2.049180328		
120	1.5	2.049180328		

Table E-3: Transport AP-BL: Sublingual 40 mg/ml water extract

Begin		**End**	
Well 1	29	Well 1	39
Well 2	31	Well 2	44
Well 3	24	Well 3	70
Average	**28**	**Average**	**51**
SD	**2.943920289**	**SD**	**16.64331698**

n= 3
SD = standard deviation

Table E-4: Transport: Sublingual AP-BL Water extract 40mg/ml, Compound 1: AG-1

Time	**Concentration (µg/ml)**	**% Transport**	**Average**	**STDEV**
M1	**63.9**	**100**	**64.9**	
0	0	0	0	0
20	0.9	1.408450704	1.285	0.10706
40	0.95	1.486697966	1.43815	0.11608
60	1	1.564945227	1.56617	0.1075
80	1.4	2.190923318	2.20819	0.06795
100	1.6	2.503912363	2.92368	0.42234
120	1.9	2.973395931	3.15698	0.18771
M2	**65.7**	**100**		
0	0	0		
20	0.8	1.217656012		
40	1	1.522070015		
60	1.1	1.674277017		
80	1.5	2.283105023		
100	2.2	3.348554033		
120	2.2	3.348554033		
M3	**65.1**	**100**		
0	0	0		
20	0.8	1.228878648		
40	0.85	1.305683564		
60	0.95	1.459293395		
80	1.4	2.150537634		
100	1.9	2.91858679		
120	2.05	3.149001536		

Table E-5: Transport: Sublingual AP-BL water extract 40mg/ml, Compound 2: AG-2

Time	Concentration (µg/ml)	% Transport	Average	STDEV
M1	**25.7**	**100**		
0	0	0	0	0
20	0	0	0	0
40	0.9	3.501945525	3.42451	0.18416
60	0.9	3.501945525	3.42451	0.18416
80	1.9	7.392996109	7.22953	0.38877
100	2.1	8.171206226	8.18181	0.50862
120	2.55	9.922178988	9.76867	0.59969
M2	**25.3**	**100**		
0	0	0		
20	0	0		
40	0.9	3.557312253		
60	0.9	3.557312253		
80	1.9	7.509881423		
100	2.2	8.695652174		
120	2.6	10.27667984		
M3	**28**	**100**		
0	0	0		
20	0	0		
40	0.9	3.214285714		
60	0.9	3.214285714		
80	1.9	6.785714286		
100	2.15	7.678571429		
120	2.55	9.107142857		

ANNEXURE F

Table F-1: TEER values for Plate 1: Study 1

Begin		**End**	
Well 1	347	Well 1	182
Well 2	328	Well 2	174
Well 3	358	Well 3	175
Average	**344.3333333**	**Average**	**177**
SD	**12.39175353**	**SD**	**4.358898944**
Begin		**End**	
Well 4	336	Well 4	150
Well 5	338	Well 5	126
Well 6	366	Well 6	158
Average	**346.6666667**	**Average**	**144.6666667**
SD	**16.77299417**	**SD**	**16.653328**

n= 3
SD = standard deviation

Table F-2: Transport: Study 1: DMEM, AP-BL, Compound 1: AG-1

Time	Concentration (µg/ml)	% Transport		
M1	**105.4**	**100**	**Average**	**STDEV**
0	0	0	0	0
20	2.9	2.75	3.13	0.38
40	11.9	11.29	11.4133	1.3293
60	12.6	11.95	13.1567	1.06214
80	17.5	16.6	16.0333	0.75215
100	18	17.08	17.4267	0.33126
120	20	18.98	18.9467	0.24173
M2	**105.4**	**100**		
0	0	0		
20	3.7	3.51		
40	13.5	12.8		
60	14.7	13.95		
80	16	15.18		
100	18.7	17.74		
120	19.7	18.69		
M3	**105.4**	**100**		
0	0	0		
20	3.3	3.13		
40	10.7	10.15		
60	14.3	13.57		
80	17.2	16.32		
100	18.4	17.46		
120	20.2	19.17		

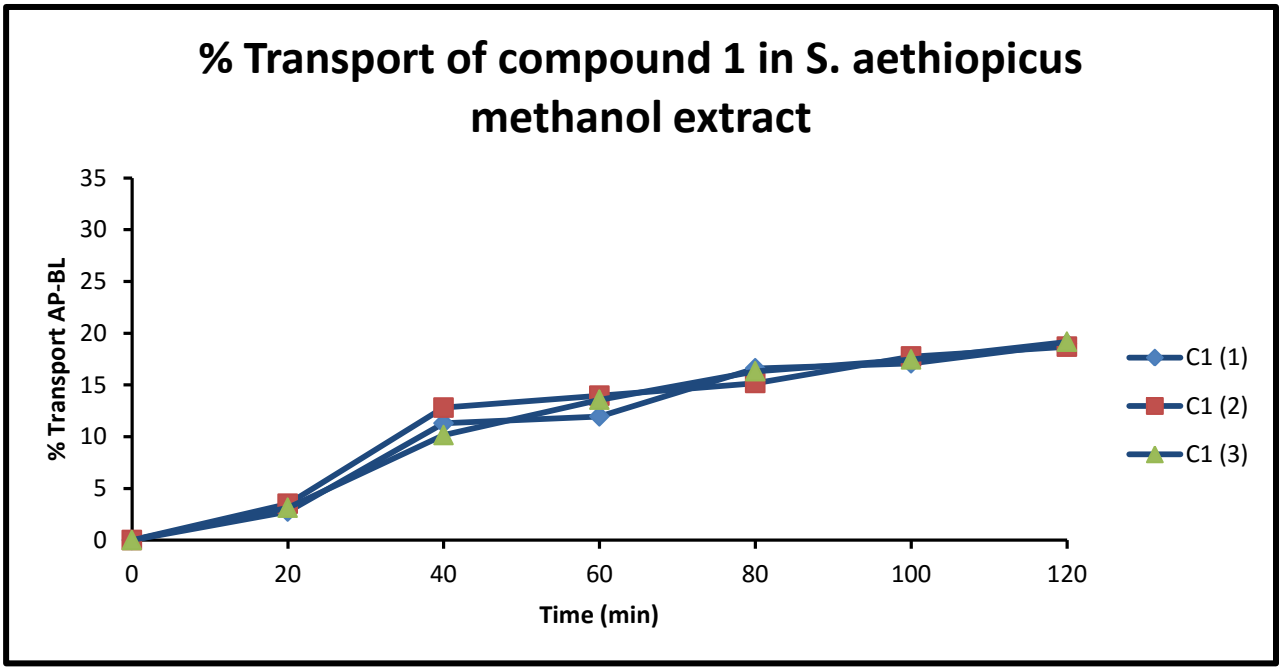

Figure F-1: Percentage transport of compound 1 in triplicate (AP-BL)

Table F-3: Transport: Study 1: DMEM, AP-BL, Compound 2: AG-2

Time	Concentration (µg/ml)	% Transport		
M1	**35.4**	**100**	**Average**	**STDEV**
0	0	0	0	0
20	3.2	9.03	8.85	2.26537
40	8.1	22.88	22.5033	0.43317
60	9.2	25.99	25.8933	0.70995
80	10.6	29.94	29.0933	0.845
100	11	31.07	31.45	0.43209
120	11.5	32.49	32.49	0
M2	**35.4**	**100**		
0	0	0		
20	3.9	11.02		
40	8	22.6		
60	8.9	25.14		
80	10	28.25		
100	11.3	31.92		
120	11.5	32.49		
M3	**35.4**	**100**		
0	0	0		
20	2.3	6.5		
40	7.8	22.03		
60	9.4	26.55		
80	10.3	29.09		
100	11.1	31.36		
120	11.5	32.49		

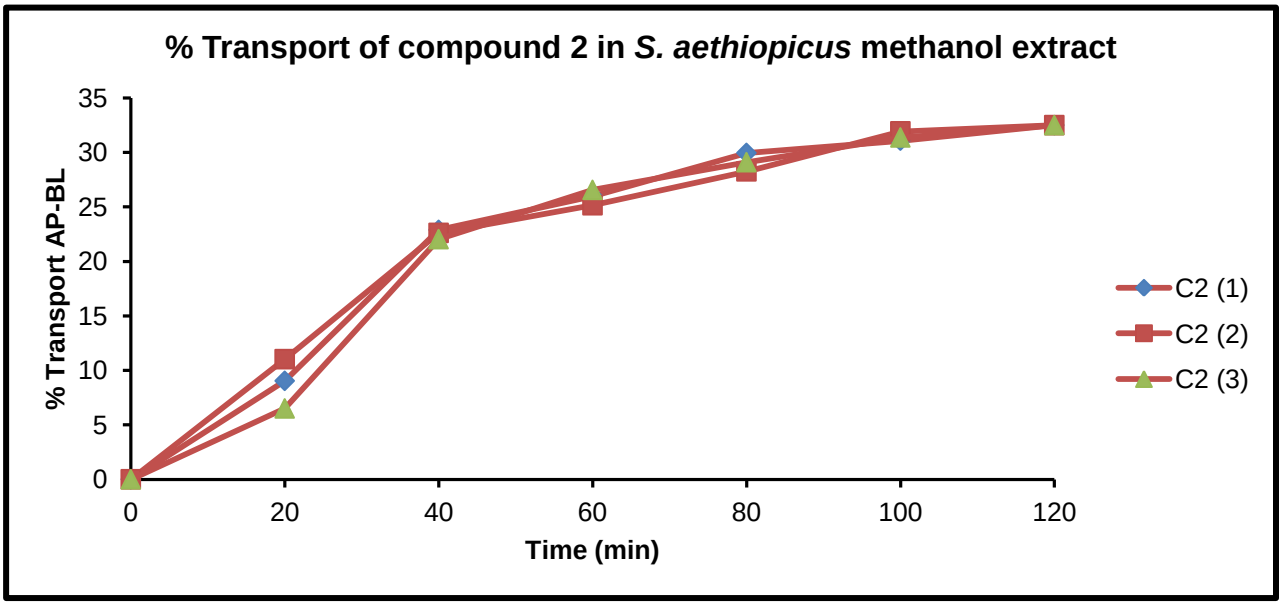

Figure F-2: Percentage transport of compound 2 in triplicate (AP-BL)

Table F-4: Transport: Study 1: DMEM, BL-AP, Compound 1: AG-1

Time	Concentration (µg/ml)	% Transport		
M1	**104.7**	**100**	**Average**	**STDEV**
0	0	0	0	0
20	4.5	4.3	4.52	1.30399
40	12	11.46	10.8533	1.57049
60	17.1	16.33	14.8367	1.52599
80	18.2	17.38	17.9867	1.93278
100	20.7	19.77	20.0567	0.28501
120	22	21.01	21.9367	0.91046
M2	**104.7**	**100**		
0	0	0		
20	3.5	3.34		
40	9.5	9.07		
60	13.9	13.28		
80	17.2	16.43		
100	21	20.06		
120	23	21.97		
M3	**104.7**	**100**		
0	0	0		
20	6.2	5.92		
40	12.6	12.03		
60	15.6	14.9		
80	21.1	20.15		
100	21.3	20.34		
120	23.9	22.83		

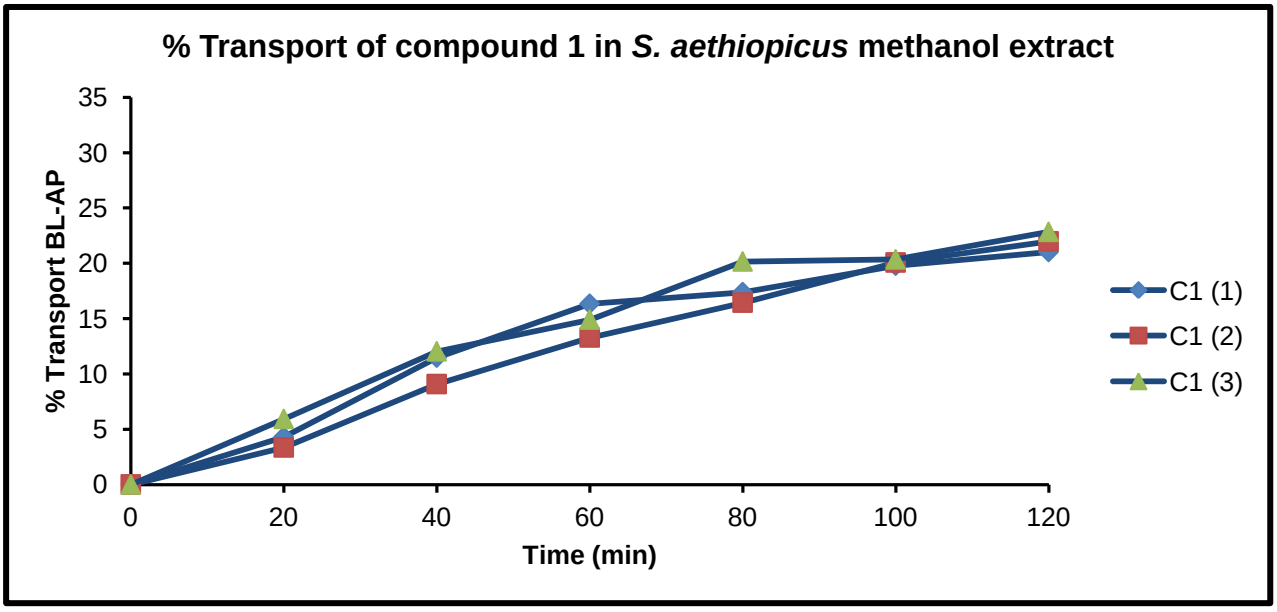

Figure F-3: Percentage transport of compound 1 in triplicate (BL-AP)

Table F-5: Transport: Study 1: DMEM, BL-AP, Compound 2: AG-2

Time	Concentration (µg/ml)	% Transport		
M1	**48.1**	**100**	**Average**	**STDEV**
0	0	0	0	0
20	5.1	10.6	10.9467	2.40878
40	9.2	19.13	18.5733	1.93598
60	11.6	24.17	21.9867	2.02505
80	12.1	25.16	24.7433	1.70365
100	12.5	25.99	26.2633	0.47343
120	13.4	27.86	27.86	0
M2	**48.1**	**100**		
0	0	0		
20	4.2	8.73		
40	7.9	16.42		
60	9.7	20.17		
80	11	22.87		
100	12.5	25.99		
120	13.4	27.86		
M3	**48.1**	**100**		
0	0	0		
20	6.5	13.51		
40	9.7	20.17		
60	10.4	21.62		
80	12.6	26.2		
100	12.9	26.81		
120	13.4	27.86		

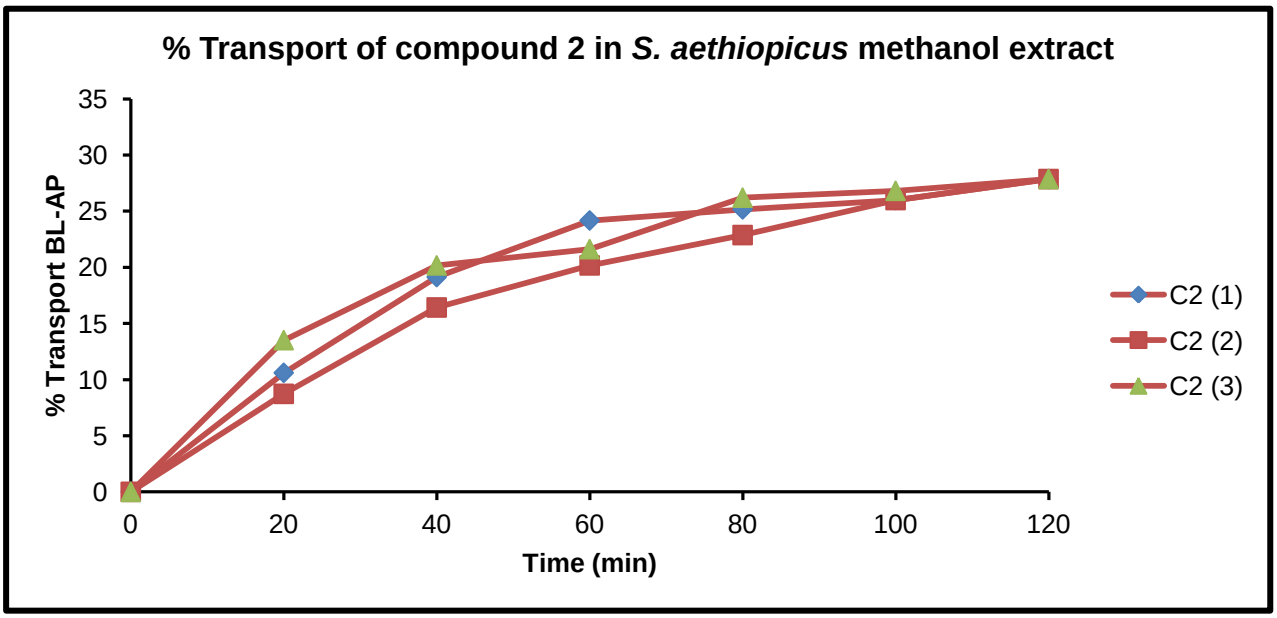

Figure F-4: Percentage transport of compound 2 in triplicate (BL-AP)

Table F-6: Permeability coefficient for transport study 1

	Slope	1/A.60.C0	Papp	
C1 (1)	0.16234	3.56888E-05	5.79369E-06	5.79
C1 (2)	0.1552	3.56888E-05	5.53877E-06	5.54
C1 (3)	0.16489	3.56888E-05	5.88483E-06	5.88
			Average	**5.736666667**
			SD	**0.176162803**
	Slope	**1/A.60.C0**	**Papp**	
C2 (1)	0.26538	3.56888E-05	9.47091E-06	9.47
C2 (2)	0.25879	3.56888E-05	9.23575E-06	9.24
C2 (3)	0.27545	3.56888E-05	9.83035E-06	9.83
			Average	**9.513333333**
			SD	**0.297377426**
	Slope	**1/A.60.C0**	**Papp**	
C1 (1)	0.17838	3.56888E-05	6.36599E-06	6.37
C1 (2)	0.19055	3.56888E-05	6.80063E-06	6.8
C1 (3)	0.1883	3.56888E-05	6.72033E-06	6.72
			Average	**6.63**
			SD	**0.228691933**
	Slope	**1/A.60.C0**	**Papp**	
C2 (1)	0.21498	3.56888E-05	7.67245E-06	7.67
C2 (2)	0.22241	3.56888E-05	7.93757E-06	7.94
C2 (3)	0.20752	3.56888E-05	7.40606E-06	7.41
			Average	**7.673333333**
			SD	**0.265015723**

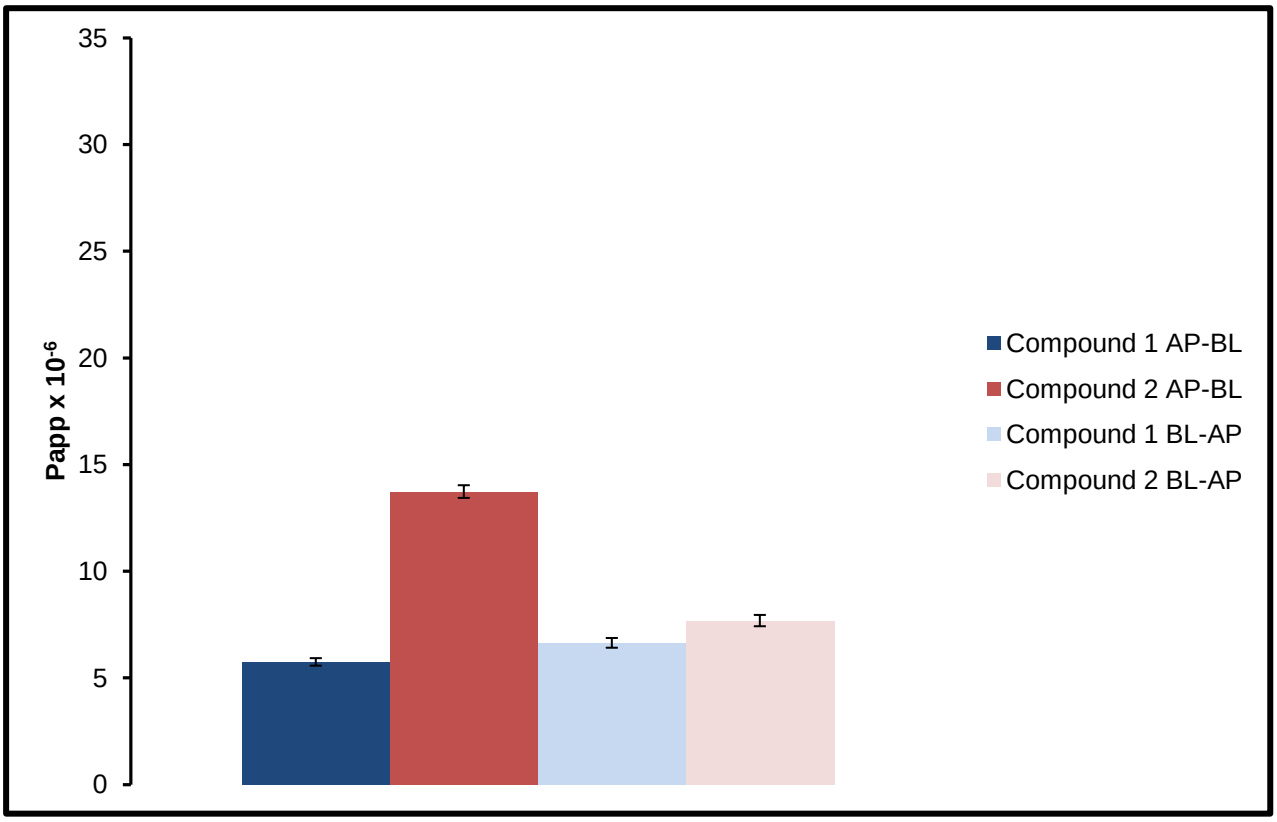

Figure F-5: P_{app} values for compound 1 and 2 in the AP-BL and BL-AP direction

Table F-7: TEER values for Plate 2: Study 2

Begin		**End**	
Well 1	200	Well 1	140
Well 2	327	Well 2	143
Well 3	334	Well 3	119
Average	**287**	**Average**	**134**
SD	**61.58463012**	**SD**	**13.07669683**
Begin		**End**	
Well 4	366	Well 4	87
Well 5	326	Well 5	88
Well 6	341	Well 6	96
Average	**344.3333333**	**Average**	**90.33333333**
SD	**20.20725942**	**SD**	**4.932882862**

n= 3
SD = standard deviation

Table F-8: Transport: Study 2: DMEM+ 5% MeOH, AP-BL, Compound 1: AG-1

Time	Concentration (µg/ml)	% Transport		
W1	**676.1**	**100**	**Average**	**STDEV**
0	0	0	0	0
20	14.6	2.16	4.41991	2.34411
40	53.4	7.9	10.9519	3.48544
60	135.7	20.07	21.9698	2.01285
80	210.7	31.16	32.159	1.90884
100	224.1	33.15	35.1938	2.21058
120	238.2	35.23	37.4153	1.91436
W2	**676.1**	**100**		
0	0	0		
20	46.3	6.84		
40	99.7	14.75		
60	147.1	21.76		
80	232.3	34.36		
100	253.8	37.54		
120	258.4	38.22		
W3	**676.1**	**100**		
0	0	0		
20	28.8	4.259724893		
40	69	10.20559089		
60	162.8	24.07927821		
80	209.3	30.95695903		
100	235.9	34.89128827		
120	262.3	38.79603609		

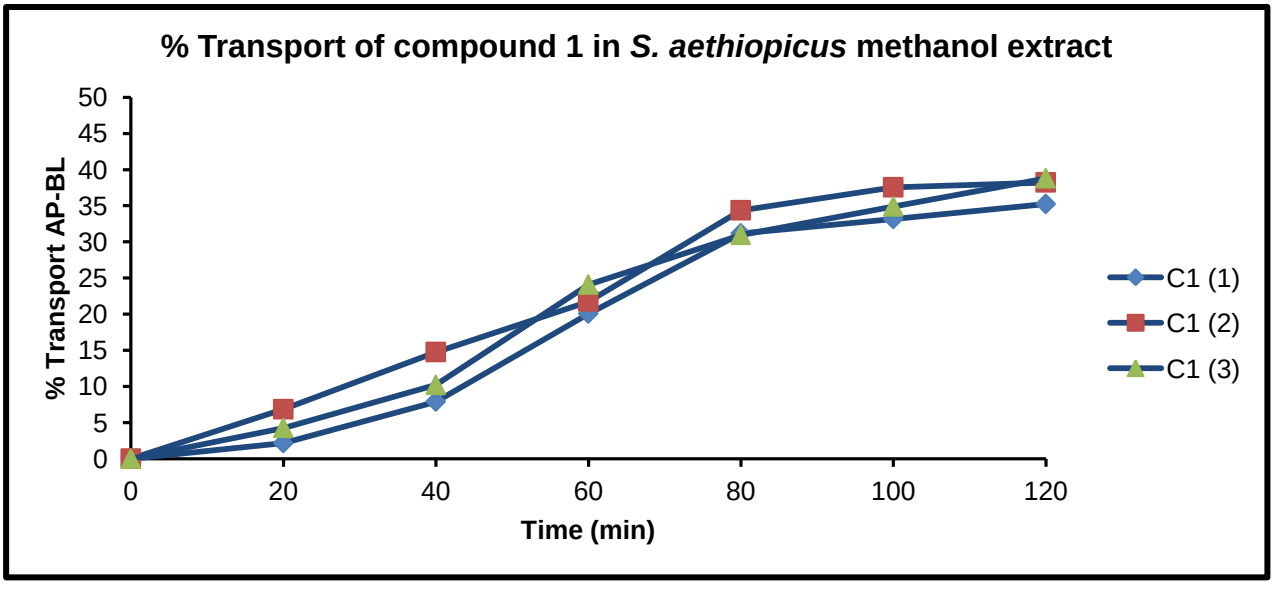

Figure F-6: Percentage transport of compound 1 in triplicate (AP-BL)

Table F-9: Transport: Study 2: DMEM+ 5% MeOH, AP-BL, Compound 2: AG-2

Time	Concentration (µg/ml)	% Transport		
W1	**153.3**	**100**	**Average**	**STDEV**
0	0	0	0	0
20	6.2	4.044357469	8.00174	3.82389
40	24.7	16.1121983	19.7217	3.94925
60	43.8	28.57142857	29.6369	0.95053
80	55.3	36.07305936	38.204	2.26
100	63.2	41.22635356	41.4873	0.81474
120	63.5	41.42204827	43.292	1.74588
W2	**153.3**	**100**		
0	0	0		
20	17.9	11.6764514		
40	36.7	23.93998695		
60	46.6	30.39791259		
80	62.2	40.57403783		
100	65	42.40052185		
120	68.8	44.87932159		
W3	**153.3**	**100**		
0	0	0		
20	12.7	8.284409654		
40	29.3	19.11285062		
60	45.9	29.94129159		
80	58.2	37.96477495		
100	62.6	40.83496412		
120	66.8	43.57469015		

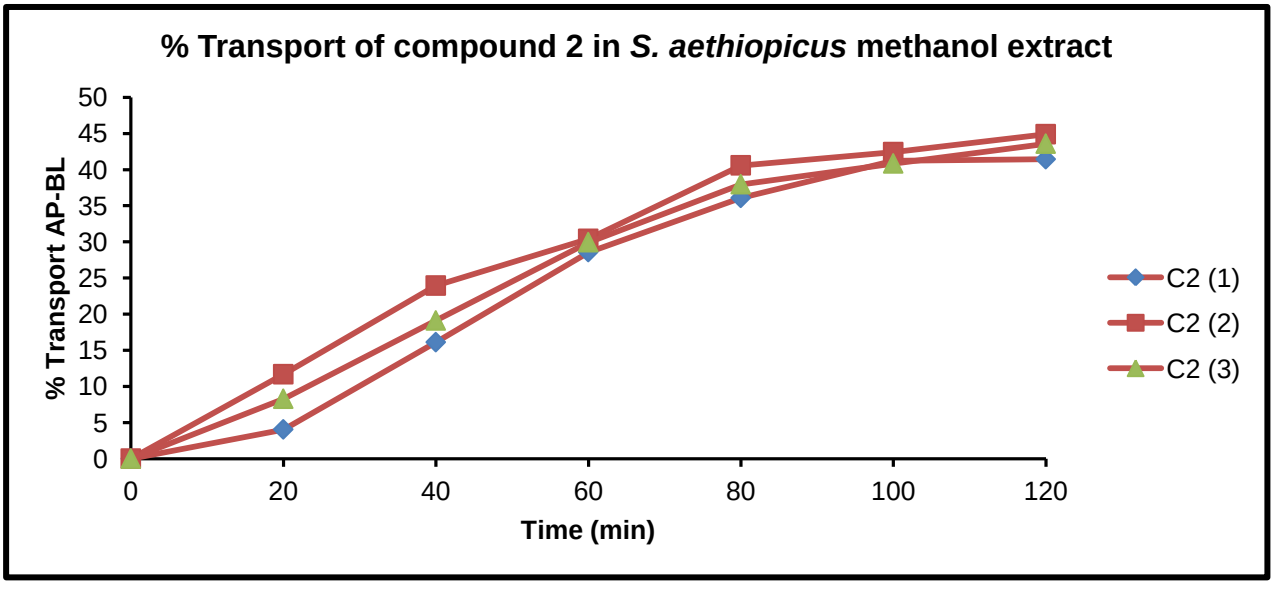

Figure F-7: Percentage transport of compound 2 in triplicate (AP-BL)

Table F-10: Transport: Study 2: DMEM, AP-BL, Compound 1: AG-1

Time	Concentration (µg/ml)	% Transport		
W1	**881.5**	**100**	**Average**	**STDEV**
0	0	0	0	0
20	53.8	6.103233125	11.6771	12.0681
40	117.6	13.3408962	12.7207	0.88495
60	197.6	22.41633579	27.1318	8.15757
80	277.1	31.43505389	31.4351	4.73057
100	304.1	34.49801475	33.4619	2.32426
120	306.7	34.79296653	36.298	2.65600
W2	**881.5**	**100**		
0	0	0		
20	30	3.403289847		
40	115.6	13.11401021		
60	197.7	22.42768009		
80	235.4	26.704481		
100	271.5	30.79977311		
120	306.2	34.73624504		
W3	**881.5**	**100**		
0	0	0		
20	225	25.52467385		
40	103.2	11.70731707		
60	322.2	36.55133296		
80	318.8	36.16562677		
100	309.3	35.08791832		
120	347	39.36471923		

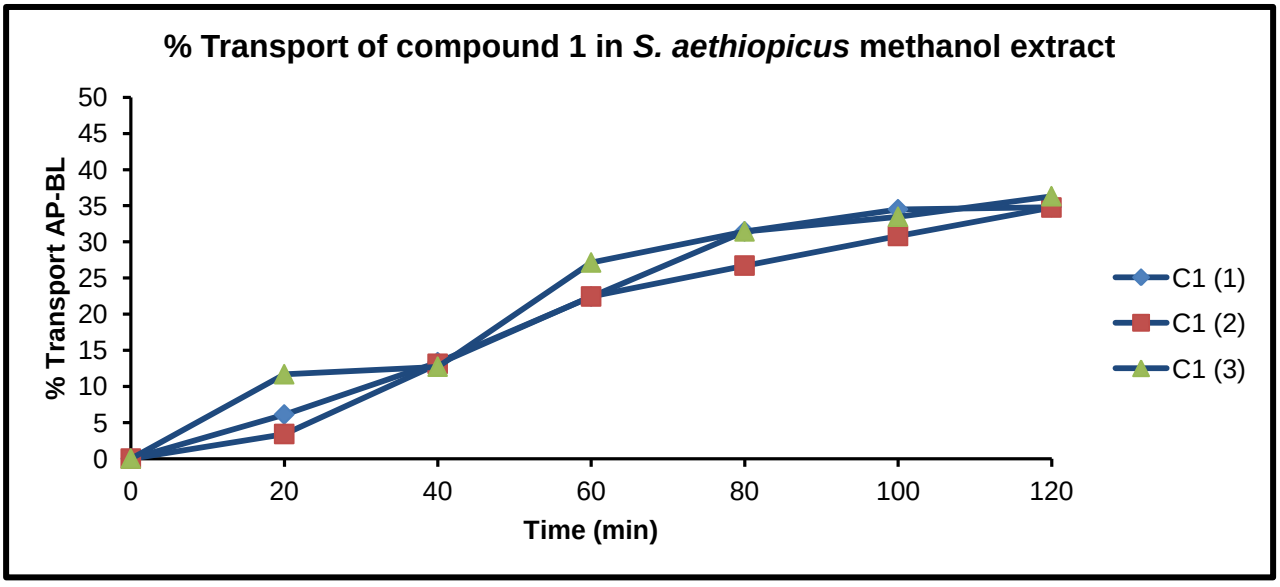

Figure F-8: Percentage transport of compound 1 in triplicate (AP-BL)

Table F-11: Transport: Study 2: DMEM, AP-BL, Compound 2: AG-2

Time	Concentration (µg/ml)	% Transport		
W1	**195.1**	**100**	**Average**	**STDEV**
0	0	0	0	0
20	19.9	10.19989749	7.46626	2.43361
40	38.5	19.73347002	18.8792	0.93017
60	54.2	27.78062532	31.0781	5.4472
80	70	35.87903639	36.0328	3.61598
100	71.9	36.85289595	38.4589	2.1505
120	77.8	39.87698616	40.3895	1.79834
W2	**195.1**	**100**		
0	0	0		
20	13	6.663249616		
40	37.1	19.01588929		
60	54.8	28.08815992		
80	63.4	32.49615582		
100	73.4	37.62173244		
120	75.9	38.9031266		
W3	**195.1**	**100**		
0	0	0		
20	10.8	5.535622758		
40	34.9	17.88826243		
60	72.9	37.36545361		
80	77.5	39.72321886		
100	79.8	40.90210149		
120	82.7	42.38851871		

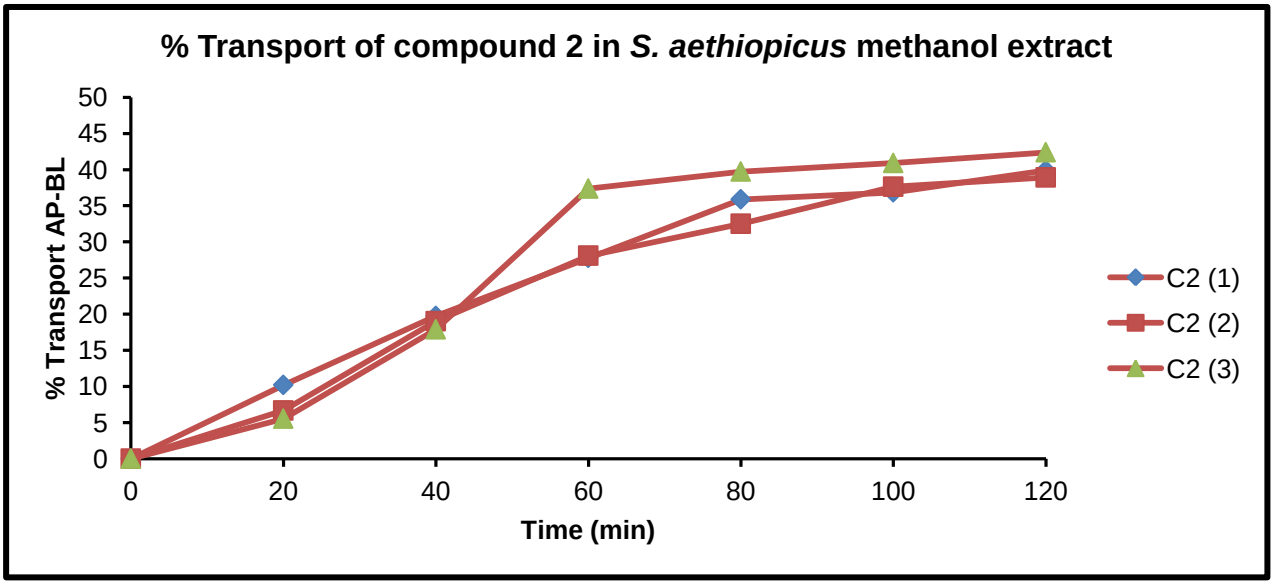

Figure F-9: Percentage transport of compound 2 in triplicate (AP-BL)

Table F-12: Permeability coefficient for transport study 2

	Slope	1/A.60.C0	Papp	
C1 (1)	0.34095	3.56888E-05	1.2168E-05	12.17
C1 (2)	0.34941	3.56888E-05	1.247E-05	12.47
C1 (3)	0.35429	3.56888E-05	1.26442E-05	12.64
			Average	**12.42666667**
			SD	**0.23797759**
	Slope	1/A.60.C0	Papp	
C2 (1)	0.39034	3.56888E-05	1.39308E-05	13.93
C2 (2)	0.37986	3.56888E-05	1.35567E-05	13.55
C2 (3)	0.38335	3.56888E-05	1.36814E-05	13.68
			Average	**13.72**
			SD	**0.193132079**
	Slope	**1/A.60.C0**	**Papp**	
C1 (1)	0.32011	3.56888E-05	1.14244E-05	11.42
C1 (2)	0.3082	3.56888E-05	1.09993E-05	10.99
C1 (3)	0.28871	3.56888E-05	1.03038E-05	10.3
			Average	**10.90333333**
			SD	**0.565007375**
	Slope	**1/A.60.C0**	**Papp**	
C2 (1)	0.33765	3.56888E-05	1.20502E-05	12.05
C2 (2)	0.34305	3.56888E-05	1.2243E-05	12.24
C2 (3)	0.39238	3.56888E-05	1.40036E-05	14
			Average	**12.76333333**

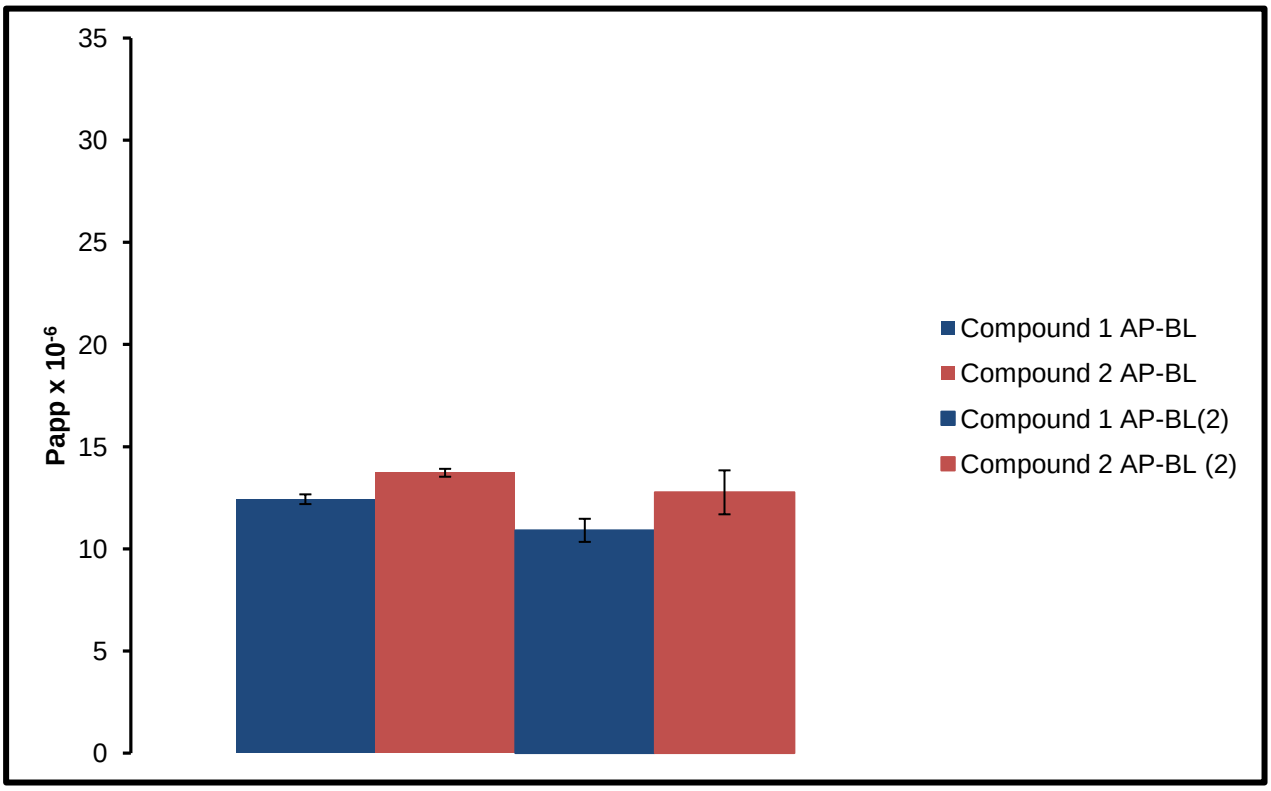

Figure F-10: P_{app} values for compound 1 and 2 in the AP-BL direction, with and without 5% methanol